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THE PARASITE BROOMRAPE (*OROBANCHE CUMANA*) IN SUNFLOWER – IDENTIFYING SOURCES FOR GENETIC RESISTANCE

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Abstract

Sunflower broomrape (Orobanche cumana Wallr.) is causing a great deal of damage to sunflower production in many countries in Europe as well as in countries from Asia and in Australia.
Extensive research on sunflower resistance to broomrape has been conducted in Russia, Romania, Bulgaria, Turkey, Serbia and Spain. New races of the parasite appear frequently. Sunflower breeders have been trying to develop sunflower genotypes resistant to all known races of this parasite.
In our research work regarding resistance to broomrape we have used different sources of resistance, the best ones being the sunflower wild species.
For obtaining results presented in this paper there have been used six cultivated sunflower genotypes which were crossed with two sunflower wild species, in order to obtain some populations. These populations are used for obtaining sunflower inbred lines which can to be used as sources of resistance (donor of genes) or directly to obtain hybrids.
There have been studied the interspecific hybrids as well as the parental forms, for the number of released seeds, for oil content, one thousand seed weight and resistance to different populations of broomrape parasite, in the artificial and natural infestation conditions.
We identified some populations full resistant to the most virulent races of broomrape in sunflower crop in Romania.

Key words*: sunflower, broomrape, genetic resources, wild species, interspecific hybrids.*

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) is a parasitic angiosperm that has been causing a great deal of damage to sunflower production for more than a century. According to Morozov (1947), the first reports of broomrape in sunflower came from Saratov Oblast in Russia and date back to the 1890s. The same author mentions that the first sunflower varieties resistant to race A of *Orobanche* were developed by Plachek (1918) at the Saratov breeding station. Morozov (1947) and Pustovoit (1966) both note that Ždanov (1926) identified a new broomrape race (B) in Rostov Oblast and soon after the discovery developed a number of sunflower varieties resistant to it. In the period that followed, according to Pustovoit (1966), a number of high-oil varieties resistant to race B were developed at the VNIIMK institute in Krasnodar, Russia that thereafter played an important role in the spread of sunflower around the world. Later on, a new race that could not be controlled by the genes for resistance to races A and B was discovered in Moldova by Sharova (1968) and in Bulgaria by Petrov (1970). Through genetic research, Vrânceanu et al. (1980) established that five broomrape races (A, B, C, D, E) were detected in Romania and the dominant genes controlling resistance to them were identified. Race F was detected for the first time in Romania in 1995 (Pacureanu – Joita et al., 1998). Alonso et al. (1996) found the race (F) of the pathogen in 1996 in Spain. Papers by Alonso et al. (1996), Škorić and Jocić (2005), Fernandez-Martinez et al. (2007), Imerovski I. et al. (2015), each provide a detailed overview of the achievements of sunflower breeding for resistance to *Orobanche*.

Extensive research on broomrape resistance has been conducted in countries of the former USSR as well as in Romania, Bulgaria, Turkey, and Spain. In all these countries, broomrape causes great damage to sunflower production and new races of the pathogen appear frequently. In addition to Russia, Ukraine, Romania, Bulgaria , Turkey, and Spain, broomrape is also present in Serbia, Hungary, Moldova, Greece, Israel, Iran, Kazakhstan, China, Mongolia, and Australia (Antonova T., 2014, Batchvarova R., 2014, Pacureanu-Joita M., 2014, Pototskyi G., 2014, Molinero-Ruiz L.et al., 2015) and possibly in a few other countries as well. Sunflower breeders and geneticists have been trying to develop genotypes resistant to all known races of the parasite.

The objective of this paper was to identify new sources of sunflower resistance to *Orobanche*, useful in further breeding approaches.

MATERIALS AND METHODS

Six cultivated sunflower inbred lines belonging to NARDI Fundulea, Romania (LC 1029B, LC 991B, LC 1093B, LC1085C, LC 1095C, LC 1088C) and two sunflower wild species (*Helianthus tuberosus, Helianthus maximiliani*) have been introduced in crossing for obtaining interspecific hybrids, in order to create sunflower populations which will be used for releasing inbred lines with high resistance to broomrape (*Orobanche cumana*). These hybrids as well as the parental forms (cultivated and wild) have been studied for oil content, using the nuclear magnetic resonance (NMR) analyzer , one thousand seeds weight and resistance to the parasite broomrape.

There have been analyzed the number of sunflower heads and number of seeds/head, for each crossing.

The crossing between cultivated and wild sunflower was made by emasculation in cultivated inbred lines and making pollination with wild species pollen, as well as making emasculation in wild forms and pollination with pollen of cultivated ones (Jan and Seiler, 2008; Christov, 2008; Hristova-Cherbadzi, 2009).

For studying resistance to broomrape in different cultivated areas in Romania, there have been used some sunflower populations obtained from interspecific hybrids *(H. annuus x H. tuberosus*) after 5-6 generations of selfpollination. These populations are different regarding the level of resistance to broomrape, taking into consideration the races of the parasite which are present in each infested area. So, the populations with symbol POR, as well as some lines, having the symbol L, have been identified to be resistant to the races G or H and populations having the symbol PM and PT, as well as the differentials for these races (D1 and D2) are resistant to races F or G.

The resistance to broomrape parasite was made in natural and artificial infestation conditions. The testing in the artificial infestation conditions was made in glass house, in pots of 5 liters capacity, having inside a mixture of soil and sand (3/1) as well as broomrape seeds, races G and H, from Constanta and Braila areas (1g/pot). In natural infestation, the testing was made in four locations (Tulcea, Constanţa, Brăila, Ialomiţa) situated in different areas with different virulence of broomrape populations.

RESULTS AND DISCUSSIONS

Differences regarding the number of seeds/head obtained after the crosses between wild and cultivated sunflower were observed. When cultivated sunflower was used as pollen receptor, the number of seeds/head was higher, comparing with the case of using the wild sunflower as pollen receptor (Table 1). In this case (the second one) the number of heads was higher, taking into consideration that the wild sunflower is high branched, and so, there are many small heads.

The oil content determination for interspecific hybrids, as well as for the parental forms has shown the highest values for cultivated sunflower (fig. 1). In case of wild sunflower species, the highest level of oil content was observed in *H. maximiliani* seeds. The interspecific hybrids released by crossing between *H. annuus* and *H. tuberosus* have higher oil content, comparing with hybrids between *H. annuus* and *H. maximiliani*.

Table 1. Results regarding the number of heads and seeds obtained by hybridization of *H. maximiliani* or *H. tuberosus* with cultivated sunflower

Pollen donator / Pollen receptor	*H. tuberosus*	*H. maximiliani*	LC 1029 B	LC 991 B	LC 1093 B	LC 1085 C	LC 1095 C	LC 1088 C
	Hybridization: number of heads/number of seed							
LC 1029 B	3/640	3/250						
LC 991 B	3/520	3/120						
LC 1093 B	3/390	3/360						
LC 1085 C	3/150	3/150						
LC 1095 C	3/280	3/250						
LC 1088 C	3/130	2/110						
Helianthus tuberosus			25/15	25/7	25/5	25/14	25/23	25/12

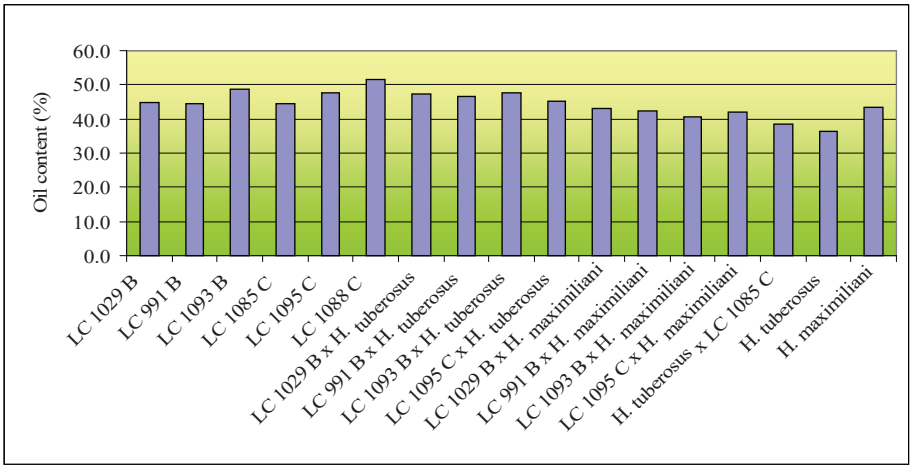

Fig. 1. The oil content for interspecific hybrids and their parental forms

Another characteristic examined for the interspecific hybrids, as well as wild parental forms and cultivated sunflower forms was the weight of one thousand seeds (fig. 2). The highest seed weight was obtained from cultivated parental forms, which have one single head. The lowest weights have the wild sunflower forms. There are some interspecific hybrids which have high seeds weight, in both cases (released with one or other wild species), this depending by the used cultivated sunflower. Some hybrids released by crossing between *H. annuus* and *H. tuberosus* have the lowest thousand seed weight.

The main objective of this paper was the selection of some interspecific hybrids of sunflower for their resistance to broomrape. In this respect, the hybrids were evaluated for the resistance to two *Orobanche* populations (race F and race G), in the artificial infestation conditions (table 2).

There are combinations released with both wild species which are resistant to both broomrape populations. The best combinations, resistant to both races are the ones obtained by crossing *H. tuberosus* with LC1085C cultivated and *H. maximiliani* with LC991B cultivated sunflower.

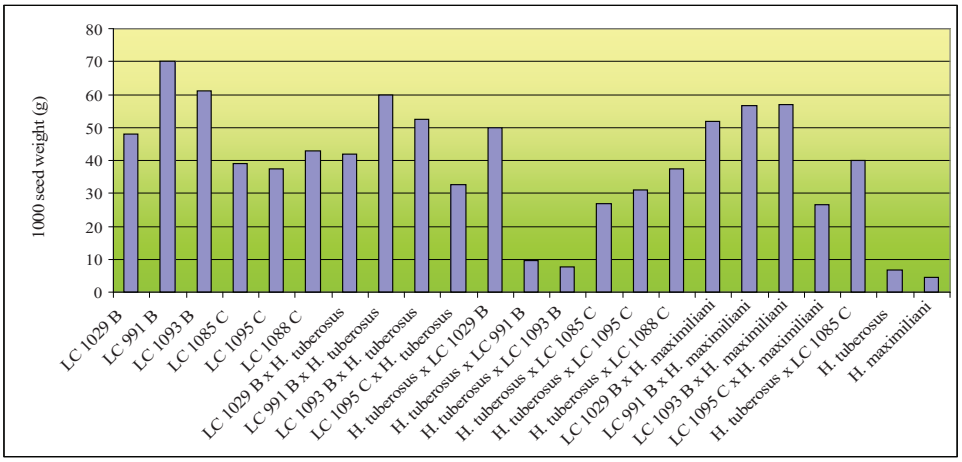

Fig. 2. One thousand seed weight for interspecific hybrids and their parental forms

Table 2. Results regarding testing of sunflower interspecific hybrids for resistance to broomrape

Combination	Variant	Race F (number of broomrapes/ sunflower plant)	Race G (number of broomrapes/ sunflower plant)
Check	1	15	25
	2	21	30
	3	17	28
LC1029 B x *H. tuberosus*	1	0	0
	2	3	2
	3	5	7
LC1029 B x *H. maximiliani*	1	2	16
	2	10	12
	3	5	24
LC991 B x *H. maximiliani*	1	0	2
	2	3	2
	3	0	0
LC1095C x *H. maximiliani*	1	2	5
	2	9	6
	3	10	4
H. tuberosus x LC1085C	1	0	0
	2	0	2
	3	0	0

Comparing the results regarding the resistance to broomrape of interspecific hybrids sunflower populations obtained in two locations situated in Tulcea and Constanta areas, differences among the populations were observed (fig. 3).

It was shown that some populations are full resistant in Tulcea area, while in Constanta area they presented a low attack degree. The sunflower differential line (LC1093B) for the race F of the parasite has a high infestation degree, in both locations.

This it means that in these locations the parasite has developed races more virulent than race F.

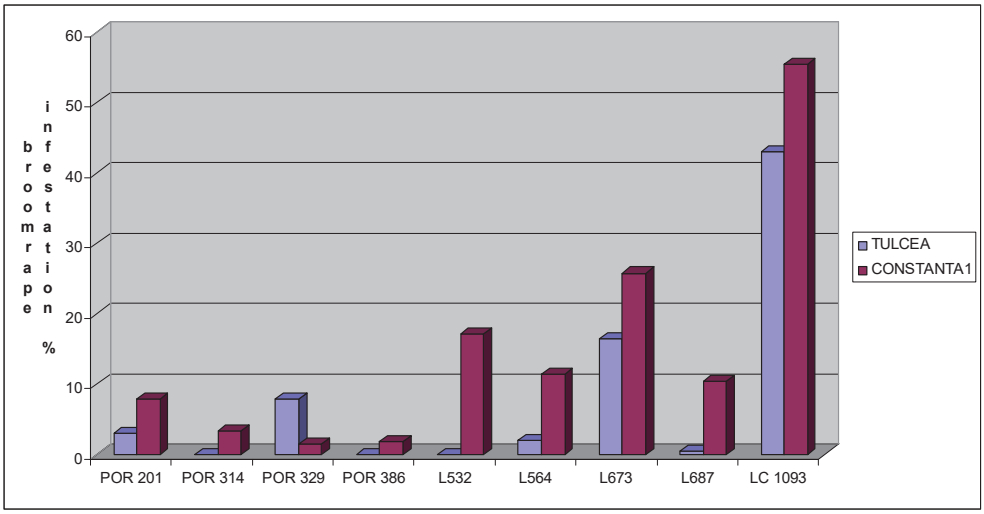

Fig. 3. Results regarding the resistance of some sunflower populations obtained from interspecific hybrids, to the broomrape parasite, in the natural infestation conditions, in two areas in Romania (average of two years, 2014 and 2015).

Among the hybrid populations of sunflower, best results were obtained with the interspecific hybrid designated as *H. tuberosus* x LC 1085 C. For this reason, the behavior of this sunflower population to the attack of broomrape parasite was examined in ten locations from five areas in Romania (Tulcea, Calaraşi, Brăila, Constanţa, and Alexandria), in two years, 2014 and 2015 (fig.4). The results are showing that in two locations from Braila and Calarasi areas, the sunflower population is full resistant in both years. In one location from Tulcea area there is a small difference regarding the resistance in two years, in other locations having a higher difference with higher infestation degree in 2015 year.

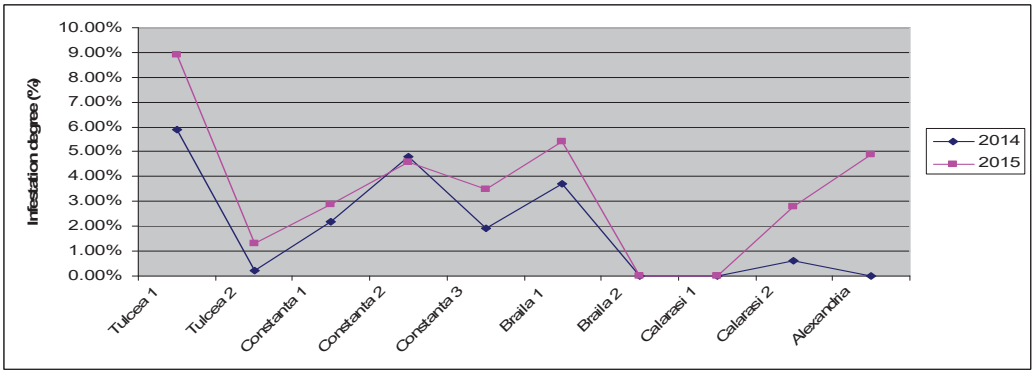

Fig. 4. Results regarding the behavior of one sunflower population obtained from an interspecific hybrid (*H. tuberosus* x LC1085C), to the attack of broomrape parasite, in ten locations from 5 areas in Romania

Moreover, the tests performed in Brăila area allowed the observation that some populations of sunflower are full resistant to broomrape, while others have a low infestation degree (fig.5). In this area, the new races of the parasite started to be present in the last years.

Similar experiments were realized in Ialomiţa County (2014-2015) the number of full *Orobanche* resistant sunflower populations

observed in this area was higher comparing with other areas (Braila, Tulcea, Constanta) (fig.6). This it means that, in this area, the broomrape parasite did not develop the new virulent races.

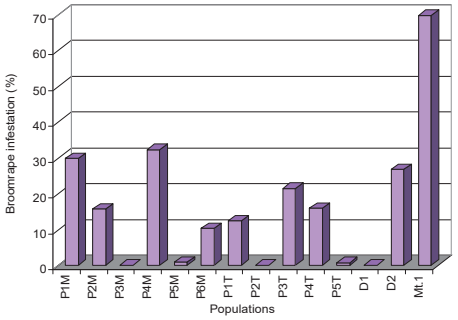

Fig. 5. Results regarding the behavior of sunflower populations obtained from interspecific hybrids, to the attack of broomrape parasite, in Braila area.

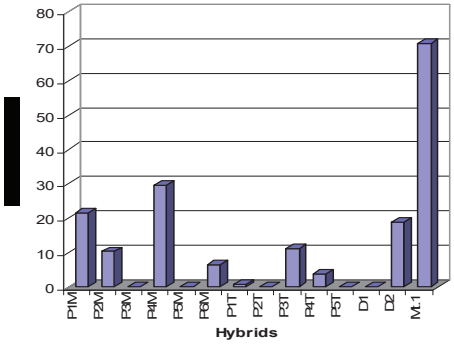

Fig. 6. Results regarding the behavior of sunflower populations obtained from interspecific hybrids to the broomrape parasite, in Ialomita area.

CONCLUSIONS

The broomrape parasite has become very dangerous for sunflower crop in almost all areas cultivated with sunflower in Europe as well as in Romania.

It is of a great importance to identify sources of resistance to the new races of broomrape. For this, the sunflower wild species are very important, they being the best source of genes for resistance.

The experiments allowed the selection of several sunflower populations obtained by crossing sunflower wild species with cultivated genotypes that have good resistance to the broomrape races which are spread in the most important areas cultivated with sunflower in Romania.

These hybrids presented also high oil contents and increased seeds weight being promising for further experiments.

REFERENCES

Alonso L.C., Fernandez-Escobar J., Lopez G., Rodriguez-Ojeda M., Sallago F., 1996. New highly virulent sunflower broomrape (Orobanche cernua Loefl.) pathotype in Spain. In: M. Moreno, J. Cubero, D. Berner, D. Joel, L. Musselman, and C. Parker (Eds), Advances in Parasitic Plant Research. Proc. 6th Int. Symp. Parasitic Weeds. Cordoba, Spain, 16-18 April 1996. pp. 639-644.

Antonova T. 2014. The history of interconnected evolution of *Orobanche Cumana* Wallr. and sunflower in the *Russian* Federation and Kazakhstan. In: Proceedings of the 3rd International Symposium on Broomrape (*Orobanche* spp.) in sunflower, Cordoba, 3–6 June 2014, pp. 57–64

Batchvarova R. 2014. Current situation of sunflower broomrape in Bulgaria. In: Proceedings of the 3rd International Symposium on Broomrape (*Orobanche* spp.) in sunflower, Cordoba, 3–6 June 2014, pp. 51–54

Christov M., 2008. *Helianthus* species in breeding research in sunflower. In: L. Velasco [ed.], Proc 17th Intl.Sunflower Conf., Cordoba, Spain, 8-12 June, 2008. Intl. Sunflower Assoc., Paris, France. pp. 709-714

Fernandez-Martinez J.M., Domingues J., Velasco L., Perez-Vich B., 2007. Update on breeding for resistance to sunflower broomrape. EUCARPIA-Oil and Protein Crops Section Meeting-„Present status and future needs in breeding oil and protein crops“.(Book of Abstracts). Budapest. October 7-10. 2007. Hungary. pp.32-34.

Hristova-Cherbadzi M. 2009. Characterization of hybrids, forms and lines obtained from interspecific hybridization of cultivated sunflower *Helianthus annuus* L. with wild species of the genus *Helianthus*. Biotechnol. &Biotechnol. Eq. 23(2):112-116.

Imerovski I., Dimitrijević A., Miladinović D., Dedić B., Jocić S., Kočiš Tubić N., Cvejić S. 2015. Mapping of a new *gene* for resistance to broomrape races higher than F. Euphytica. pp 1-9.

Jan C.C. and Seiler G.J., 2008. Sunflower germplasm development utilizing wild *Helianthus* species. In: L. Velasco [ed.], Proc 17th Intl. Sunflower Conf., Cordoba, Spain, 8-12 June, 2008. Intl. Sunflower Assoc., Paris, France. pp. 29-43.

Molinero-Ruiz L., Delavault P., Perez-Vich B., Păcureanu Joița M., Bulos M., Altieri E., Dominguez J. 2015. Review: Joint evolution of the race structure of *Orobanche Cumana* and the breeding of sunflower for resistance to the parasitic weed. Spanish Journal of Agricultural Research. Chp. Plant Protection (Vol. 13, Nr. 4, Decembrie, 2015).

Morozov V.K., 1947. Sunflower breeding in USSR. Pishchepromizdat. Moscow. (In Russian). pp. 1-274.

Pacureanu-Joita M. 2014. Current situation of sunflower broomrape (*Orobanche Cumana* Wallr.) in Romania. In: Proceedings of the 3rd International Symposium on Broomrape (*Orobanche* spp.) in sunflower, Cordoba, 3–6 June 2014, pp. 39–43.

Păcureanu-Joița M., Vrânceanu A.V., Marinescu G.A., Sandu I., 1998. The evaluation of the parasite-host interaction in the system Helianthus annuus L.-Orobanche cumana Wallr. In Romania. Proc. Second Balkan Symp. On Field Crops, Novi Sad, Yugoslavia, 16-20 June, 1998, Vol. I: 153-155

Petrov D., 1970. Nova fiziološka rasa Orobanche cumana Wallroth kod nas. Plant protection in service of agriculture. Sofia. (In Bulgarian). pp. 37-48.

Pototskyi G. 2014. Current situation of sunflower broomrape in Ukraine. In: Proceedings of the 3rd International Symposium on Broomrape (O*robanche* spp.) in sunflower, Cordoba, 3–6 June 2014

Pustovoit V.S., 1966. Breeding, seed production and sunflower crop management. Kolos, Moscow. (In Russian). pp. 1-368.

Škorić D. and Jocić S. . 2005. Broomrape (Orobanche cumana Wallr.) and its possible control by genetic and chemical means. Production and Processing of Oil seeds Proc. of the 46th Oil Industry Conference. pp. 9-21. Petrovac na moru. Juny 6-10.2005. (In Serbian).

Sharova P.G., 1968. Sunflower breeding for resistance to virulent races of broomrape (In Russian). Seljskoe#hozjajstvo Moldaviji. No.5.

Vranceanu A.V., Tudor V.A., Stoenescu F.M., and Pirvu N., 1980. Virulence groups of Orobanche cumana Wallr., differential hosts and resistance source genes in sunflower. In Proc. of the 9th Int. Sunflower Conf. Vol. 2:74-82.Torremolinos. Spain. Juny 8-9. ISA. Paris.

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THE INFLUENCE OF THE FEMALE PARENT ON THE INDUCING RATE WITH FIVE DIFFERENT INDUCER LINES IN MAIZE DH TECHNOLOGY

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Abstract

In the last decade, DH technology has been integrated by many maize breeding programs in Europe, North America and China due to the releasing of lines with inducing efficiency up to 15 %, developed for temperate and tropical areas as well as due to improved selection of the putative haploid kernels (PHK) on the basis of the expression of anthocyanin coloration conferred by R1-nj as a genetic marker. Even though the R1-nj marker system offers an efficient way to identify haploids, the expression given by this genetic marker could be influenced by several factors such as the genetic background of the female parent. According to the literature, haploids obtained from dent genotypes are more easily recognized and both anthocyanin coloration and intensity of the coloration are better expressed than in flint genotypes; anthocyanin coloration could be also affected by the moisture of kernels at time of harvest and may vary from a small patch to covering the entire aleuronezone, exception kernel basis. Intensityof anthocyanin can range from very poor to strong anthocyanin both in the embryo and aleurone.

In 2014 at NARDI Fundulea, 27 populations were used as female parent in crosses with five different inducers and inducing efficiency was determined for each cross to see the influence of the female parent on the inducing efficiency.

Key words: *doubled haploid technology, female parent, inducer line, anthocyanin coloration.*

INTRODUCTION

Releasing maize hybrids in the classic system is a process that takes 5-7 years only to obtain homozygous lines, to which at least 4-6 years are needed for testing and registration of the hybrids (Rotarenco et al., 2010).

Hence the need to approach new methods that make possible to obtain a greater number and more diverse hybrids in a short time corresponding to the current needs. In vivo maternal haploid induction offers undoubtedly great advantages to modernize maize programs by simplifying the protocol for obtaining homozygous lines in a period of 2 years but also by reducing the costs involved in this process. The procedure for the production of maternal haploids allows obtaining haploidsfrom different genotypes on a large scale (Deimling et al. 1997; Chalyk and Rotarenco1999; Eder and. Chalyk, 2002).

DH technology requires an inducer with a good induction rate (HIR) up to 15% and a marker system that can provide an easier recognition of the haploid forms at different stages of vegetation.

The most common marker system used in obtaining in vivo maternal haploid is based on *R1-nj* gene that is involved in the synthesis of anthocyanin(Nanda and Chase, 1966; Chase, 1969; Neuffer et al., 1997; Eder and Chalyk, 2002; Röber et al., 2005).

The main limitations of this system are the presence of the *C1-I* gene in the maternal germplasm, which inhibits the expression of anthocyanin coloration, and the influence of different maternal germplasm (dent and flint forms) on the size and intensity of anthocyanin

coloration. Consequently, several attempts were made to improve the marking system by incorporating other two marker genes as *B1* and *Pl-1* in the new inducer lines recently released. This improved system makes possible, the recognition of haploid forms, post doubling, in the first stages of vegetation (Prasanna et al., 2012).

The objective of the present study was assessing the influence of different female parents within each inducer on the inducing efficiency.

MATERIALS AND METHODS

The study was carried out at the National Institute of Research and Development Fundulea in 2014. A number of 27 F2 maize populations from different heterotic groups were used as female sources. Each female source was crossed with 5 inducer lines as MHI (Moldavian Haploid Inductor), Td RhA, Td RhR, Td RhAPM, Td RhRPM, in the field under controlled pollination. Grains resulted from crosses were divided in 3 categories based on the expression of the anthocyanin coloration coded by *R1-nj* gene on the kernel as follows: category 1, kernels with no coloration on both aleurone and embryo; category 2, kernels with coloration of both aleurone and embryo and category 3 considered as PHK (Putative haploid kernels) with purple coloration only on the aleurone and uncolored embryo. A scale 0-4 was used for visual assessment of the intensity of anthocyanin coloration on aleurone and embryo from category 2 (kernels with coloration in both aleurone and embryo): 4 = intense pigmentation, 3=normal pigmentation, 2 = poor pigmentation, 1 = very weak pigmentation and 0 = lack of pigmentation). Cytological analysis was performed to check visually selected PHK. Root tips were cut from a random sample of selected PHK from each of 12 populations. The chromosome complement of plantlets was established by means of chromosome counts on root-tips squashed, stained by Feulgen method.

RESULTS AND DISCUSSIONS

According to the results obtained by Eder and Chalyk (2002); Kebede et al. (2011), the female sources influence the haploid rate, and those obtained by Coe (1994) demonstrated the influence of the female on the expression of the marker gene*R1-nj.*

All 27 populations showed a high variability of the expression of anthocyanin coloration for both embryo and aleurone, appreciated on average with scores between 2 (identification of haploid is possible but errors could occurdue to very weak staining in the embryo) and 4 (level that allows easy identification of PHK), the variation in the size and intensity of the anthocyanin coloration it can be seen in table 1.

Table 1.Type of kernel and the expression of the anthocyanin coloration of each maternal form for the aleurone and embryo depending of the haploid inducer

Genotype	Td. RhR	Td. RhA	Td RhR P.M	Td. RhA P.M	MHI
*P 1	**D/3/4	D/3/4	D/3/3	D/3/3	D/4/4
P 2	D/2/3	D/3/3	D/3/3	D/3/3	D/3/3
P 3	D/3/3	D/4/4	D/3/4	D/3/4	D/3/3
P 4	D/4/4	D/3/4	D/3/4	D/3/3	D/4/4
P 5	D/2/3	D/2/3	D/2/2	D/2/2	D/3/3
P 6	D/3/4	D/3/3	D/4/4	D/3/4	D/3/3
P 7	D4/3	D/3/4	D/3/3	D/4/4	D/4/4
P 8	D/2/3	D/3/3	D/3/3	D/3/3	D/3/4
P 9	D/3/4	D/4/4	D/3/4	D/3/4	D/4/4
P 10	D/3/2	D/3/3	D/3/2	D/3/3	D/3/3
P 11	D/2/2	D/3/3	D/2/3	D/3/3	D/3/2
P 12	D/4/4	D/3/4	D/3/4	D/3/2	D/4/4
P 13	D/3/4	D/3/3	D/3/4	D/3/3	D/4/4
P 14	D/3/4	D/4/4	D/3/4	D/2/4	D/4/3
P 15	D/2/3	D3/4	D/3/4	D/3/3	D/2/3
P 16	D/2/2	D/3/3	D/3/3	D/3/3	D/2/4
P 17	D/3/3	D/3/4	D/3/3	D/4/4	D/3/4
P 18	D/3/2	D/3/3	D/2/3	D/3/3	D/3/3
P 19	D/3/4	D/3/3	D/3/3	D/4/4	D/3/3
P 20	D/3/3	D/3/4	D/3/3	D/3/4	D/3/3
P 21	D/3/4	D/3/4	D/3/3	D/3/4	D/3/3
P 22	D/3/4	D/3/4	D/4/4	D/3/4	D/4/4
P 23	D/3/3	D/3/3	D/3/3	D/3/3	D/3/3
P 24	D/3/4	D/3/4	D/2/3	D/4/4	D/3/3
P 25	D/3/2	D/2/2	D/2/2	D/2/3	D/3/3
P 26	D/3/4	D/3/3	D/3/4	D/3/4	D/3/3
P 27	D/3/3	D/2/3	D/3/3	D/4/4	D/4/4

*P = Population; **D=Dent type

Populations that received scores over 3 on the embryo, allowed easy identification of PHK, demonstrated also by cytological analysis (table 2). In populations 1, 14 and 15 correct recognition of PHK was 100%. For the variants with scores 2 for embryo coloration, the proportion of correct recognition was a little over 50% (populations 11 and 5). This might be explained by the fact that the score 2 for the embryo means a very weak intensity of the anthocyanin coloration so errors could occur, recognition of the haploid forms from the diploid one require an increased attention from staff.

Table 2. Cytological analysis for 12 random female parents with all 5 inducers

Genotype	Inducer	Total kernels analyzed	Number of confirmed real haploid kernels		Scores for the antocyanin coloration (aleurone /embryo)
			No.	%	
*P 1	MHI	12	12	100	**D/4/4
P 14	Td.RhR P.M.	12	12	100	D/3/4
P 15	Td.RhR	11	11	100	D/2/3
P 6	Td.RhR	9	8	88.8	D/3/4
P 4	Td.RhR	12	10	83.3	D/4/4
P 7	Td.RhA	12	10	83.3	D/3/4
P 9	MHI	12	10	83.3	D/4/4
P 13	MHI	12	10	83.3	D/4/4
P 10	Td.RhR	11	9	81.8	D/3/2
P 2	MHI	12	10	83.3	D3/3
P 11	MHI	12	7	58.3	D/3/2
P 5	Td.RhA P.M.	9	5	55.5	D/2/2
Total		136	114	Average 83.41	

*P = Population**D=Dent type

An evident interaction between inducers and populations is suggested by the data. Inducing capacity of each inducer is highlighted in table 3. On the average, the best inducing efficiency was registered for inducer MHI, recommended to be used intensively. The lowest inducing efficiency was obtained with the inducer Td. RhA. With regards to population, better inducing efficiencies (over 3%) were produced by populations 4, 17, 19 and 26 since populations 6, 7, 14, and 18 had efficiencies under 1%. The best percentage was obtained by the population 17 with the inducer MHI, 9.8% scored 4 for the embryo, level that allows easy identification of the PHK kernels.

Table 3. Inducing efficiency of the inducers over 27 female populations

Genotype	Inducer					Population Average
	MHI	Td. RhR	Td. RhA	Td.RhR P.M	Td.RhA P.M	
*P 1	5.51	0	0.8	3.81	1.22	2.26
P 2	2.36	3.11	0.19	3.27	0.65	1.91
P 3	3.15	3.03	0.5	4.44	0.89	2.40
P 4	3.04	0.4	3.43	5.39	4.43	3.33
P 5	2.1	***0.27***	3.15	0	1.15	1.33
P 6	0.67	1.14	1.16	0.84	0.56	0.87
P 7	1.54	0	0.62	1.29	0	0.69
P 8	2.46	2.03	1.09	2.18	1.26	1.80
P 9	2.33	1.04	2.36	0	2.65	1.67
P 10	2.36	2.36	2.63	2.31	2.52	2.43
P 11	3.05	1.73	3.25	1.97	2.82	2.56
P 12	1.91	0.28	1.06	2.56	0.81	1.32
P 13	0.56	1.78	1.22	5.25	2.8	2.32
P 14	1.21	0.41	1.74	0.78	0	0.82
P 15	2.18	2.27	2.02	2.5	2.86	2.36
P 16	1.49	0	0.63	0.8	2.32	1.04
P 17	9.8	3.42	5.73	0	0	3.79
P 18	3.35	0	0.65	0	0.91	0.98
P 19	4.57	5.22	1.25	3.45	2.78	3.45
P 20	1.93	1.03	1.91	0	1.55	1.28
P 21	3.92	1.39	0.69	1.17	4.1	2.25
P 22	2.26	1.31	2.09	2.33	3.07	2.21
P 23	1.66	0.33	5.14	1.37	0.74	1.84
P 24	3.63	0.98	2.7	2.01	1.5	2.16
P 25	3.5	4.44	0	2.3	0	2.04
P 26	6.76	4.29	6.09	5	0	4.42
P 27	5.72	0.37	0.19	0	0	1.25
Average	**3.07**	**1.58**	**1.94**	**2.1**	**1.6**	

*P = Population

Furthermore, the analyses of variance for inducing efficiency (table 4) confirmed that all the variance sources - inducers, female populations, as well as the interaction between inducers and female populations have significant effect on the efficiency of haploid induction.

Even when an inducer with a high average efficiency is used, the haploid induction rate

(HIR) is significantly influenced by the genetic background of the maternal forms.

Table 4. ANOVA for inducing efficiency

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Inducers (I)	4	41.68	10.42	12.41***
Populations (P)	26	112.66	4.33	5.16***
I x P	104	241.63	2.32	2.77**
Error	104	87.30	0.84	

, * - significant at P=0.01 and P=0.001, respectively

CONCLUSIONS

Haploid inducing efficiency is influenced by the genotype of the population submitted to the induction, haploid inducing capacity of the inducer and the inducing protocol. High efficiency of the inducer is essential insuchprotocol, but increasing the number of pollinated plants within crosses between maternal populations and inducer is highlyrecommended also to ensure the identification of sufficient PHK in the populations with genetic lower inducing capacity.

Introduction and assimilationof an improved marker system based on *R1-nj*, *B1* and *Pl-1*, capable of detecting and separation of haploid from diploid forms in different vegetation stages (field separation at plantlets or mature plants stages).

REFERENCES

Chalyk S.T., Rotarenco V., 1999. Using maternal haploid plants in recurrent selection in maize. Maize Genet Coop Newslett 73, p. 56–57.

Chase S.S., 1969. Monoploids and monoploidderivatives of maize (Zea mays L.). Bot Rev 135, p. 117–167

Coe E., 1994. Anthocyanin genetics. In: Freeling M, Walbot V (eds) The maize handbook. Springer-Verlag, New York, p. 279–281.

Deimling S, Röber F, Geiger H.H., 1997. Methodik und Genetik der in vivo-Haploiden induction bei Mais.Vortr Pflanzenzüch-tung 38:203–204.

Eder J., Chalyk S., 2002.In vivo haploid induction in maize.Theor. Appl. Genet, 104, p. 703-708.

Kebede A. Z., Dhillon B. S., Schipprack W., Araus J. L., Banziger M., Semagan K., Alvarado G., Melchinger A.E., 2011. Effect of sources germplasm and season on the in vivo haploid induction rate in tropical maize.Euphytica 180, p. 219-226.

Nanda D.K., Chase S.S., 1966. An embryo marker for detecting monoploids of maize (Zea mays L.). Crop Sci 6, p. 213–215.

Neuffer M.G., Coe E., Wessler S.R. , 1997. Mutants of maize.CSHL Press, New York, USA.

Prasanna B.M., Chaikan V., Mahuku G., 2012. Doubled Haploid Technology in Maize Breeding: Theory and Practice, D.F.: CIMMYT, Mexico.

Rotarenco V., Dicu G., State D. and Fuia S., 2010. New inducers of maternal haploids in maize. Maize Genetics Cooperation Newsletter vol. 84, p. 1-7.

Röber F.K., Gordillo G. A., Geiger H.H., 2005. *In vivo* haploid inductionin maize – Performance of new inducers and significance of doubled haploid lines in population breeding. Maydica 50, p. 275-283.

Sarmaniuc M., Mihailov M., Rusu G., 2013. Eficiența noilor inductor în obținerea haploizilor materni la porumb (ZeamaysL.).Buletinul Acad. De Stiințe a Moldovei. Științele vieții, nr.2, p. 105-110.

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PRELIMINARY RESULTS OF THE WINTER PEAS BREEDING PROGRAM

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Abstract

The development of the winter pea crop represent a major challenge to expand plant protein production in temperate areas. Breeding winter cultivars requires the combination of freezing tolerance as well as high seed productivity and quality.
Winter peas have some advantages over spring peas like: better establishment and more efficient use of humidity during the winter season, which makes it less vulnerable to drought over the spring, frequently in Romania in the last years; winter peas can be sown in mixture with some cereal (barley, triticale, grasses) for obtaining high nutritive green forage; earlier harvest; has a longer vegetation period and get higher productivity and more stable yield than spring peas type.
In this paper we present data obtained from the first F3 lines of winter peas obtained in the NARDI-Fundulea program with the germplasm of winter peas from USA and Austria. A number of 176 lines, selected from winter/winter and winter/spring crosses pea genotypes, have been tested in preliminary trials in 2015. Data for yield showed a large variation, but some lines over yield significantly the winter control (Specter, Checo and Windham).
The conclusion of this preliminary study is that will be possible to realize the genetic progress in breeding in winter peas, to select the new varieties with good enough winter hardiness and being with high yield, different earliness or plant height.
Of course, the breeding program just started, from a short time, and it is needed to improve the genetic bases of the germplasm use for all traits, but mainly for winter hardiness.

Key words*: winter pea, breeding, witer hardiness, yield.*

INTRODUCTION

Field pea (*Pisum sativum* L.) is an economically important grain legume crop cultivated worldwide. Its agricultural benefits include improved soil nitrogen, better weed management and reduced disease for other crops with which it is grown in rotation. It is high in protein and is extensively used for animal feed and is increasingly used for the production of food products. The growing market for this crop demands adapted varieties with high grain yield and potential to tolerate the stresses prevalent in a range of production environments (Shafiq et al. 2012)
Mechanisms of resistance or tolerance to freezing temperatures in cool season of grain legumes have already been reviewed. The plants can avoid or resist ice crystal formation in the cells. They can also escape freezing stress by delaying sensitive phonological stages, particularly floral initiation and flowering, until after winter freezing periods have passed.
Forage peas have been used in all winter pea breeding programs in Europe. Winter peas have a good level of frost resistance, and are also characterized by a large foliage development in spring which favors lodging and fungal diseases in humid conditions (Lejeune-Heaut et al., 1999).
Development of winter pea cultivars should be a way to improve yield potential and stability through a longer life cycle and hence increase crop competitiveness. Autumn sowing allows for a higher biomass production as well as the

avoidance of drought and heat stresses of late spring (Stoddard et al., 2006).
Winter hardiness is a complex quantitative trait conditioned by the plant genotype and the environment in which is grown. Current varieties grown in world are considered as cold tolerant. Winter hardiness has been sought by North American and French scientists and although some level tolerance to cold has been obtained it remains a challenge (Murray and Swensen, 1991).
Most winter pea breeding programs have used the wild winter *Pisum fulvum* pea because it has good resistance to freezing: such genotypes can survive temperatures of -6°C (no damage to leaves; 100% survival). Although several genetic studies have been conducted, there is not a general consensus on the gene action controlling the expression of cold hardiness in pea. Genetic studies on winter hardiness in pea have rivaled that it is quantitative in nature and governed by intermediate dominance and additive genes (Ercan, 2006).
Quantitative trait loci (QTL) for frost tolerance were detected on linkage groups III, V and VI. A major QTL of pea frost tolerance on LGIII was located in vicinity of the Hr locus (Weller et al., 2012).
Lejeune-Henaut et al. (1999; 2008) proposed that reproductive frost damage might be avoided by developing winter varieties with the Hr (gene) flowering phenotype, in which floral initiation is delayed under short days. In European production environments, Hr plants may be able to escape frost stress by delaying flowering until after freezing periods have passed.
Breeding winter forage pea emphasizes the development of the lines with satisfying tolerance to low temperatures and more prominent earliness, with great potential for both forage and grain yields.
Breeding and the cultivation of fall-sown pea confirm that it could be one of the least expensive and most efficient ways to decrease the unpredictable and destroying effects of spring droughts and other manifestations of climatic changes on protein-rich crops such as pea. They also establish a solid basis for the anticipation that the existence of high-yielding, early and winter hardy fall-sown dry pea cultivars will increase the total area under grain legumes, especially in Europe, and thus contribute to a significant increase of the protein needed for ever demanding animal husbandry (Crîngaşu, 2015).
The aim of this work was to appreciate the yield performance and other traits and mainly the winter hardiness of several winter pea genotypes in the climatic conditions from NARDI-Fundulea.

MATERIALS AND METHODS

The winter peas breeding program started at NARDI Fundulea in 2010, using a germplasm originated from USA (Specter and Windham) and Austria, (Checo). Beside this winter type germplasm was added several spring Romanian genotypes with some tolerance to winter hardiness after autumn planted test. All the experiments were performed on experimental fields at NARDI Fundulea.
Yield performance and winter hardiness level were studied in a trial with 25 entries, involved three winter genotypes (Checo, Windham, Specter) and twenty-two spring genotypes (Table 1), in three replications, planted in autumn during the 2014 and 2015.
Also in 2015, in eight trials, in one replicate, were tested, for the first time, 176 F3 lines, selected from winter/winter or winter/spring crosses pea genotypes, for yield, winter hardiness, plant height and earliness. The area of harvest plot was four m².
The level of resistance to winter hardiness was estimated in the field, early in the spring, in a scale 1 to 9, where score 1 is very resistance and 9 very susceptible. Plant height was measure in cm, total length of plant from the ground till the top to the end of flowering time. The earliness was appreciated like number of days from 1st January till the end of flowering time and yield as kg/ha.
The statistic analyses of data have been evaluated by ANOVA, correlations and linear regressions between study traits.

RESULTS AND DISCUSSIONS

The yield performances and the winter hardiness of winter and spring pea genotypes planted in autumn in two years are presented in the Table 1. It is notice that the all three winter varieties (Checo, Windham, Specter) out

yielded significantly, in average, in the both years, the spring pea genotypes. The yield level of the winter varieties has been almost double against the spring control variety Nicoleta. Of course, the differences between the winter form and the spring form can be higher in the years with a severe winter. In the tested years, 2014 and 2015, as can see the score data, the differences in winter hardiness between the winter and spring forms in both years was not too high, because winters were milder than normal. However, differences in yield, between those three winter varieties and the spring control Nicoleta, range from 1161 till 1876 kg/ha.

Table 1.Yield results of and winter hardiness of several winter and spring pea genotypes sown in autumn.

Genotype	Yield kg/ha (2014)	Yield kg/ha (2015)	Yield mean			Winter hardiness
			kg/ha	Dif. kg/ha	%	1-9
Checo (W)	3300	2800	**3050**	1646	217	1
Windham (W)	1830	3300	**2565**	1161	183	1
Specter (W)	3031	3530	**3280**	1876	234	2
Aurora (S)	850	1455	1153	-251	82	5
Dorica (S)	910	880	895	-509	64	5
Marina (S)	1113	726	920	-484	66	5
Nicoleta (S)	1180	1627	1404	0	100	4
F01-1304 (S)	1380	760	1070	-334	76	5
F09-641 (S)	1903	938	1421	17	101	5
F04-87 (S)	990	1316	1153	-251	82	4
F01-73 (S)	710	1540	1125	-279	80	5
F98-492 (S)	930	1346	1138	-266	81	4
F97-1422 (S)	970	1241	1106	-298	79	4
F99-701 (S)	1060	850	955	-449	68	5
F09-641 (S)	1703	405	1054	-350	75	6
Mona (S)	1200	270	735	-669	52	5
Vedea (S)	870	400	635	-769	45	6
Rodil (S)	560	170	365	-1039	26	6
Eiffel (S)	970	450	710	-694	51	7
Zekon (S)	893	190	542	-862	39	8
F04-148 (S)	750	570	660	-744	47	5
Turbo (S)	1230	610	920	-484	66	5
F00-78 (S)	670	370	520	-884	37	6
F11-1189 (S)	1130	720	925	-479	66	6
LSD 5%	631	672	651	-	46	-

The preliminary data presented in this paper of the 176 F3 lines selected from the winter/winter and winter/spring crosses, demonstrated that it is possible to obtain genotypes which recombined good enough level of winter hardiness with high yield, earliness and different level of plant height. The yield distribution of 176 F3 lines (Figure 1) suggested the selection of genotypes with significant high yield than winter genitor used in the crosses (Specter, Checo and Windham).
The correlation between yield and winter hardiness had (Figure 2) shown a very strong negative relationship among those traits (r=-0.54***). However, the distribution of the lines along regression line, demonstrated the possibility to select the new lines with the same level of winter hardiness like winter parents but with high level of yield than these.

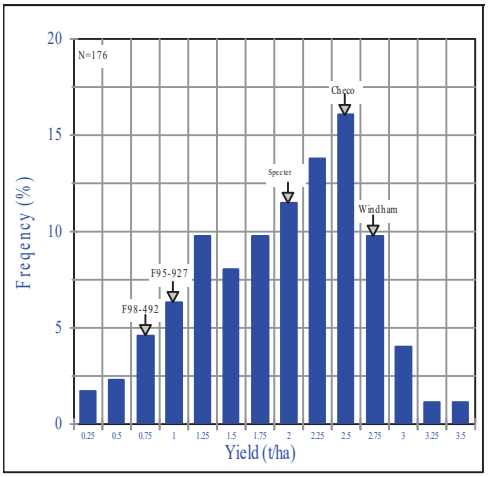

Figure 1. Distribution of 176 F3 winter pea lines after their yield (t/ha)

The yield distribution of 176 F3 lines (Figure 1) suggested the selection of genotypes with significant high yield than winter genitor used in the crosses (Specter, Checo and Windham).
The correlation between yield and winter hardiness had (Figure 2) shown a very strong negative relationship among those traits (r=-0.54***). However, the distribution of the lines along regression line, demonstrated the possibility to select the new lines with the same level of winter hardiness like winter parents but with high level of yield than these.

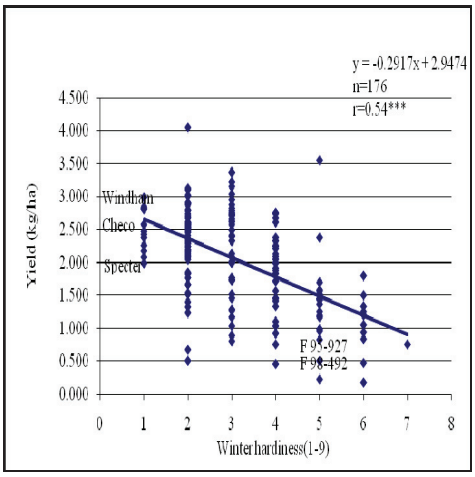

Figure 2. Correlation between winter hardiness and yield data of 176 F3

The data obtained till now form the study of relationship between winter hardiness and plant height indicated the possibility of recombination of both traits of interest (plant height and winter hardiness) (Figure 3) suggesting that, in functions of the end use the production, for forage need to be a tall variety, for high biomass production or mid tall variety for grain type.

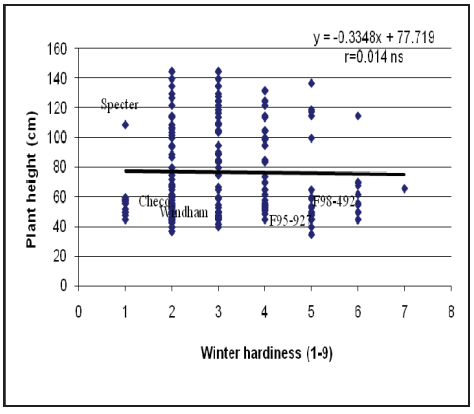

Figure 3. Relationship between winter hardiness and plant height data of 176 F3

No relationship has been found between winter hardiness and earliness data of those 176 lines analyzed (Figure 4).

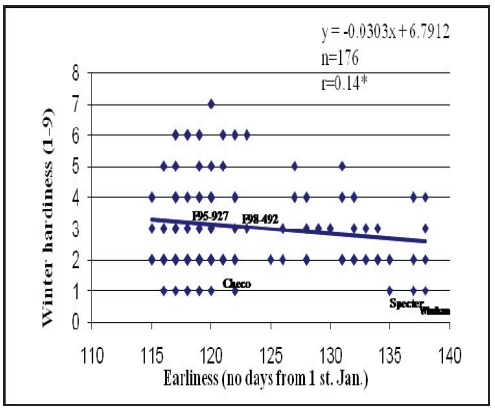

Figure 4. Relationship between winter hardiness and earliness of F3 data of 176 F3

However, high enough correlation between winter hardiness of F4 generation lines and F3 generation lines was observed, demonstrating that trait it is too high inherit and make it easy to be improved.

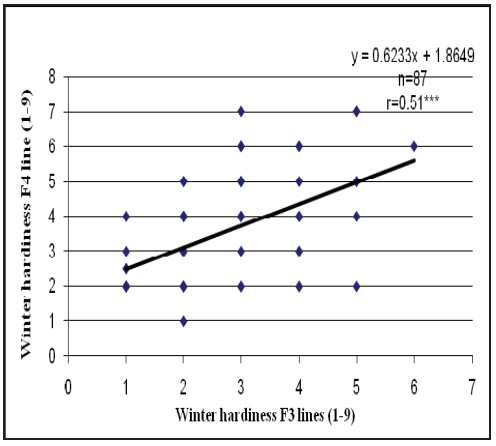

Figure 5. Relationship between winter hardiness F4 and of F3 data of 176 F3

CONCLUSIONS

The conclusion of this study is that will be possible to realize the genetic progress in breeding in winter peas, to select the new varieties with good winter hardiness and being with high yield, different earliness or plant height.

The yield data had shown high yield performances of winter pea's type than spring

type sown in autumn, even in the mild winter condition.

The breeding program just started, from a short time, and it is needed to improve the genetic bases of the germplasm use for all traits, but mainly for winter hardiness.

REFERENCES

Crîngaşu (Bărbieru) A., 2015. Perspectives in winter peas breeding program. Horticulture, LIX: 203-207.

Ceyhan Ercan, 2006. Genetic Analysis of Cold Hardiness in Peas (*Pisum sativum* L.). Journal of Plant Sciences, 1: 138-143.

Lejeune-Henaut I., Bourion V., Eteve G., Cunot E., Delhaye K. and Desmyter C., 1999. Floral initiation in field-grown forage peas is delayed to a greater extent by short photoperiods, than in other types of European varieties. Euphytica 109: 201-211.

Lejeune-Henaut I., Hanocq E., Bethencourt L., Fontaine V., Delbreil B., Morin J., Petit A., Devaux R., Boilleau M., Stempniak JJ., Thomas M., Laine AL., Foucher F., Baranger A., Burstin J., Rameau C., Giauffret C., 2008. The flowering locus Hr colocalizes with a major QTL affecting winter frost tolerance in *Pisum sativum* L. Theor Appl Genet 116:1105–1116.

Murray G.A. and Swensen J.B., 1991. Winter hardiness in pea. Pisum Genet., 23:4-13.

Stoddard FL, Balko C., Erskine W., Khan HR, Link W, Sarker A ., 2006. Screening techniques and sources of resistance to abiotic stresses in cool season food legumes. Euphytica 147:167-186.

Shafiq S, Mather D., Ahmad M., Paull J., 2012. Variation in tolerance to radiant frost at reproductive stages in field pea germplasme. Euphytica 186:831–845.

Weller JL., Liew LC.,Hecht VFG, Rajandran V., Laurie RE., Ridge S.,Wenden B., Blassiau C., Dalmais M., Rameau C., Bendahmane A., Macknight RC., Lejeune-Henaut I., 2012. A conserved molecular basis for photoperiod adaptation in two temperate legumes. Proc Natl Acad Sci USA 109:21158-21163.

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GENETIC CONTROL OF GRAIN SIZE AND WEIGHT IN WHEAT– WHERE ARE WE NOW?

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Abstract

Wheat is one of the world's most important food sources, alongside with rice and maize, directly providing about 50% of human food calories. By 2020, it is estimated that the global demand for wheat will increase by a further 40%, due to the increasing world population. Therefore, higher yield is one of the most important goals in wheat breeding.
Larger grains not only directly relate to grain yield but also have favorable effects on seedling vigour and early growth, thereby promoting and stabilizing yielding ability. Large grain size has been an important trait and it is usually measured in plant breeding practice by one thousand grain weight (TGW), mainly determined by grain width (GW), grain length (GL) and grain thickness (GT), but also by grain shape and density.
Milling and baking quality is also influenced by grain size and shape. Geometrical models indicated that changes in grain shape and size could result in increases in flour yield of up to 5%.
Test weight (volumetric weight), a trait largely used in commercial transactions with wheat, also depends on grain size and shape - larger grains, deviating from spherical shape having lower test weight.
Grain size in wheat is a complex character and any information on its genetic control is useful for increasing breeding efficiency. Identifying molecular markers linked to quantitative trait loci (QTLs) controlling seed size would facilitate selection in early generations and may contribute to improved yield and end-use quality in wheat by accumulating such loci into elite backgrounds.
Grain size in wheat is a quantitative trait controlled by quantitative trait loci (QTL), and numerous QTLs for grain size have been reported. Previous research showed important QTLs on all chromosomes, but most significant QTLs were identified on chromosomes 1B, 1D, 2A, 2B, 2D, 3A, 3B, 3D, 4B, 4D, 5A, 5B, 5D, 6A, 6B, 6D, 7A, 7B, 7D. Grain size is negatively correlated with grain number, mainly due to competition for available assimilates.
Alleviating this correlation should be facilitated by a more detailed knowledge of grain size genetic control.
In this mini-review we will focus on the latest information about the QTLs and genes involved in genetic control of wheat grain size and weight, and the best molecular markers associated with these traits.

Key words*: wheat, grain size, weight, TGW, QTL.*

BACKGROUND

Worldwide food production must be raised by 70% from present level to sustain the estimated population of 9.1 billion by 2050 (Patil, 2013).
Wheat is one of the most important crops worldwide, alongside with rice and maize directly providing about 50% of human food calories (Lin et al., 2015).
It is estimated that the wheat demand will increase by a further 40% before 2020, as a result of world population increase (Rajaram, 2005; Dixon et al., 2009). Since wheat is one of the most important cereals along with rice and maize, annual increase of 1.6%-2% in grain yield is required in the coming years in order to fulfil the global demand (Patil, 2013; Faris, 2014).
To achieve higher yields in wheat, breeding programs focused on obtaining cultivars with best agronomic traits (disease resistance, protein content, grain size and weight, drought tolerance etc.). This achievement can be made through genetic improvements and enhanced plant biology understanding. Grain yield in wheat is determined by the grain number per spike, spike number per plant and grain weight. Among these, the most reliable trait is grain weight, measured as the 1000-grain weight (TGW). Grain weight has a strong positive

correlation with grain size and shape (Gegas et al., 2010). Also, grain shape is characterized by a combination of grain length, grain width, grain length-to-width ratio, and thickness. The complexity of genetic control for grain size and weigh set in motion many studies that lead to the discovery of important QTLs, on almost all chromosomes. Dissection of these complex traits into individual components using molecular maps is useful to obtain information about genetic control and relationship between the components (Wu et al., 2012; Patil et al., 2012).

Marker association (especially haplotype association analysis) accelerates the process of mapping and detection of important genomic regions and favoured alleles or haplotypes for breeding (Hou et al., 2014).

High-density genetic linkage maps are necessary for precisely mapping QTLs controlling grain shape and size in wheat (Qiu-Honget al., 2015).Advances in field of genetics made possible for the geneticists to identify genes and quantitative trait loci (QTLs) involved in controlling important agronomic traits. Wheat yield, or yield components, is controlled by numerous genes with additive and epistatic effects that are highly interactive with the environment (Zheng et al., 2014).

In this article, we briefly review the current status about the genes/QTLs involved in grain size and weight for yield improvement.

GENETIC ANALYSIS OF WHEAT GRAIN SIZE AND WEIGHT

The domestication of wheat, 10000 years ago, was instrumental in the transition of human behaviour from hunter-gatherer to farmers. One of most important modifications that occurred in wheat and other cereal crops was the increase in size of seeds (Faris, 2014).

Grain size (and weight) is a major component of wheat yield. Larger grains not only directly relate to grain yield but also have favourable effects on seeding vigour and early growth, thereby promoting and stabilizing yielding ability.

Large grain size has been an important trait and it is usually represented in plant breeding practice by one thousand grain weight (TGW), mainly determined by grain width (GW), grain length (GL) and grain thickness (GT) (Zhenqi et al., 2010).

TGW is a complex trait and any information on its genetic control is useful for increasing breeding efficiency (Giura and Saulescu, 1996). TGW, a trait largely used in commercial transactions with wheat, also depends on grain shape, seed number (negative compensation effect between seed size and seed number), spikes per plant, environment conditions, stress tolerance and other factors which make the improvement of this trait a challenge for the geneticists (Zanke et al., 2015; Xuejiao et al. 2015; Weiyu et al., 2015; Quan et al., 2015).

Milling and baking quality is also influenced by grain size and shape. Geometrical models indicated that changes in grain shape and size could result in increases in flour yield of up to 5% (Röder et al., 2008; Rasheed et al., 2014).

Wang et al. (2015) studied the TaGS5 genes (physically mapped on 3AS and 3DS). Analysis of association of *TaGS5-A1* alleles with agronomic traits indicated that cultivars with *TaGS5-A1b* possessed wider grain width and higher TKW (45.8g), as well as significantly lower plant height, spike length, and internode length below spike than those of cultivars with *TaGS5-A1a*(44.9g; $P < 0.05$) over 3 years.Expression analysis of the *TaGS5-A1* gene indicated that *TaGS5-A1b*allele possessed significantly higher expression level than *TaGS5-A1a* allele in differently developmental seeds.

Another study, focused on the *TaGS5* genes, was carried out by Ma et al. (2015).

In this study, *TaGS5* homoeologues in wheat were isolated and mapped on chromosomes 3A, 3B and 3D (designated as TaGS5-3A, -3B and -3D).

TaGS5-3A was classified as a type II serine carboxypeptidase, whose functions are closely related to cell division. Two alleles of *TaGS53A*, *TaGS5-3A-T* and *TaGS5-3A-G* were identified in wheat accessions, and a functional marker was developed to discriminate them. Association analysis revealed that *TaGS5-3A-T* was significantly correlated with larger grain size and higher thousand grain weight.

The results of Ma et al. (2015) suggests that *TaGS5-3A* is a positive regulator of grain size

and its favoured allele *TaGS5-3A-T*exhibits a larger potential application in wheat high-yield breeding. More recently, Kumar et al., 2016, published their analysis and were showed that grain length and width are genetically independent and the most significant QTL was identified on chromosome 4B, and could be an ortholog of major rice grain size and shape gene *GS3* or *qGL3*. Also, they found major and stable locus on the homeologous region of *TaGASR7* (7A) gene. This gene (*TaGASR7*), which is an ortholog of *OsGASR7*, a gibberellin- regulated gene that controls grain length in rice. Dong et al., (2014) identified *TaGASR7-A1*, in wheat, based on the sequence similarity with rice, and this gene was mapped on the telomeric end of 7AL with strongest effect on grain length. Nevertheless, the study by Kumar et al. (2016) suggested that GASR7 region may play an important role in the genetic control of seed development and grain shape and size in wheat.

Grain filling between anthesis and maturity is the main mechanism that determines the final grain weight. At this stage there are three physiological processes occurring simultaneously: dry matter accumulation, water accumulation and subsequent desiccation, and grain morphological expansion.

Grain filling can be divided into two components: rate and duration. The rate and duration of grain filling both contribute to final grain weight.

Dry matter accumulation is a process of deposition of starch (~60–70% of the mature grain weight), proteins (8–15%), and other nutrients (e.g. minerals, vitamins and fibres) (Quanet al., 2015).

Considering that starch accounts for about 70% of the grain endosperm, Hou et al. (2014), focused on the conversion of sucrose to starch. Sucrose synthase catalysis is the first step in the conversion of sucrose to starch, that is, the conversion of sucrose to fructose and UDP-glucose by the wheat sucrose synthase genes *TaSus1* and *TaSus2* (located on chromosomes 7A/7B/7D and 2A/2B/2D, respectively). Five favoured haplotypes were identified at TaSus2-2A (*Hap-A)*, TaSus2-2B (*TaSus2-2B-Hap-H)*, TaSus1-7A (*Hap-1* and *Hap-2*), and TaSus1-7B (*Hap-T*) (Hou et al., 2014).

During grain filing stage, drought, heat and other abiotic stresses greatly affect growth and productivity of wheat. Grain filling in wheat depends on two major sources of carbon: current photosynthate in leaves and non-leaf organs; and carbohydrates stored in the stem and leaf sheath from stem elongation to the early phase of grain filling.

Conserved water-soluble carbohydrates (WSC; composed mainly of fructans, sucrose, glucose, and fructose, with the main reserve as fructans at the late stages of WSC accumulation) at early grain filling play an important role in partial compensation of reduced carbon supply (Zhang et al., 2014; Li et al., 2015).

WSC accumulation and utilization depend on growing conditions and genotypes. Among three segments of the main stem (peduncle, penultimate internode and the remainder segments), the remainder segments are the major storage sites and the major source for WSC mobilization during the grain filling period. In general, WSC accumulate until 10–20 days after anthesis, and the reserved WSC can reach more than 40% of total stem dry weight in wheat (Zhang et al., 2014).

Under diverse drought stress conditions, WSC in lower internodes showed significant positive correlations with TGW, especially at the flowering stage under well-watered conditions (WW) and at grain filling under drought stress (DS).

Mobilization of WSC during grain filling can potentially contribute about 10-20% of the final grain weight under non-stress conditions, and up to 70% or more of grain dry matter under drought stress in wheat (Zhang et al., 2014; Li et al., 2015).

Drought tolerant wheat cultivars have a high capability of WSC accumulation, coupled with higher mobilization efficiency, stronger sink activity and longer duration of grain filling.

Zhang et al. (2014) found correlation between TGW and four types of WSC, viz. (1) total WSC at the mid-grain filling stage (14 days after flowering) produced by leaves and non-leaf organs; (2) WSC contributed by current leaf assimilation during the mid-grain filling; (3) WSC in non-leaf organs at the mid-grain filling, excluding the current leaf assimilation; and (4) WSC used for respiration and

remobilization during the mid-grain filling (Remo).

Variation in stem WSC among wheat genotypes is an important genetic factor involving grain weight and yield under drought stress conditions. WSC content is a complex quantitative trait controlled by polygenes, and the small effects of many independent QTL limit their direct use for marker-assisted selection in breeding programs.

QTL associated with stem WSC have been reported in perennial ryegrass, rice, maize, barley, and wheat. In wheat, QTL for WSC were mapped on chromosomes 1A, 2D, 4A, 4B, 5D, 6B, 7B and 7D (Zhang et al., 2014).

Favourable alleles for WSC of Total, Leaf, Non-leaf and Remo highlighted by Bing Zhang et al. in 2014: Xcfd17-2D (Remo, WW) had the same favourable WSC alleles (Xcfd17-2D_{223}) in peduncle, lower internode and the whole stem estimates; Xgwm181-3B_{131} and $_{161}$ (Leaf, DS), Xgwm610-4A_{167} (Leaf, WW),Xgwm513-4B_{144} (Leaf, DS), Xgwm165.1-4D_{199} (Non-leaf, WW), Xwmc517-7B_{188} (Non-leaf, WW) had positive effects both in lower internode and the whole stem. Higher WSC were associated with Xgwm169-6A_{203} (Remo, WW) and Xgwm537-7B_{205} (Leaf, DS) in both the peduncle and lower internode. Xbarc125-3D_{147} (Total) contributed to higher WSC in lower internodes, not only under well-watered conditions, but also under drought stress.

WSC alleles that exhibited significantly positive contributions to TGW on an individual basis identified by Zhang et al., in 2014 were Xcfd17-2D_{223}, Xcfd53-2D_{263}, Xgwm181-3B_{140} and $_{161}$, Xgwm389-3B_{116}, Xbarc125-3D_{147}, Xgwm358-5D_{162} and Xgwm537 7B_{205}.

In Li et al. (2015) study, five of the 16 favorable WSC alleles, individually contributed to significantly higher TKW, respectivelyXbarc181–1B_{187}, Xgwm148–2B_{165}, Xgwm261–2D_{203}, Xgwm149–4B_{153} and Xgwm358–5D_{162}.

WSC can make a positive contribution to TKW under different environment conditions. Pyramiding target favourable alleles is not only effective for obtaining genotypes with higher stem water-soluble carbohydrates, but also is effective for enhancing TKW under drought conditions.

High stem WSC has already been suggested as a criterion for wheat breeding under drought stress. With marker-assisted selection, accumulation of favourable alleles for WSC should play an important role in future wheat breeding programs (Li et al., 2015).

Final grain weight (height and volume) is also influenced by the water accumulation during grain filling. Water is essential to transport photo assimilates and other nutrients into developing grains. It also provides a suitable environment for metabolic processes, and directly takes part in the synthesis of storage products (Xieet al., 2015).

Grain morphology changes along with dry matter and water accumulation. Immediately after fertilization, grain length, width, height (thickness), and thus volume increase rapidly. The first dimension to reach its maximum value is grain length (~15 d after anthesis), followed by grain width, height, and volume (~28 d) (Lizana et al., 2010; Hasan et al., 2011), corresponding to the period of endosperm cell enlargement.

A positive relationship between carpel size at anthesis and final grain weight was found, consistent with earlier reports in wheat (Calderini et al., 1999; Hasan et al., 2011), barley (*Hordeum vulgare* L.; Scott et al., 1983), and sorghum (*Sorghum bicolor* (L.) Moench; Yang et al., 2009). Larger carpels accelerated the initial and rapid grain filling rates (mainly the former), advanced the onset of grain filling, and slightly extended grain filling duration, resulting in higher grain weight. Moreover, larger carpels increased maximum grain water content, grain water absorption and loss rates, and grain dimensions.

The carpel size mediates final grain weight mainly through its effects on the initial phase of grain filling.

The significant QTLs identified by Xieet al. (2015) in their study, were scattered on 18 chromosomes, individually explaining 6-39% of the phenotypic variation. Taken together, QTL coincidences among final grain weight, carpel size, grain dry matter and water accumulation, and final grain dimensions were found on 16 chromosomes, with the increasing alleles usually conferred by the same parents, indicating pleiotropy or the tight linkages of functionally related genes. A large number of

coincident QTLs were observed on chromosomes 2A (36 QTLs for 12 traits), 3B (37 QTLs for 13 traits), 4A (39 QTLs for 14 traits), 5A (16 QTLs for 13 traits), 5DL (20 QTLs for 12 traits), and 7B (49 QTLs for 12 traits), which would offer the opportunity for improvement of multiple grain filling traits simultaneously (Xieet al., 2015).

In another study, Yue et al. (2014), focused on the enzymes involved in fructan synthesis in higher plants, emphasizing 6-SFT as a key enzyme in fructan biosynthesis.

Using the genomic sequence of 6-SFT, 6-SFT-A1 locus, was mapped on chromosome 4A, and revealed that SNP in 6-SFT-A1 gene was associated with wheat seedling drought resistance.

Based on sequence differences in 6-SFT among genomes, A genome-specific primer pair was designed for chromosome location. PCR results showed that the 6-SFT-A2 gene was also located on chromosome 4A. Furthermore, three 6-SFT-A2 haplotypes, designated HapI, HapII, and HapIII, were identified based on the 13 SNP/InDel sites.

Yue et al.(2014) developed two cleaved amplified polymorphic sequence (CAPS) markers to distinguish the three haplotypes. The cleaved PCR products were easily distinguished on agarose gels.

The mean TGW of HapIII was higher than that of both HapI and HapII. HapIII haplotype possessed a significantly positive effect on TGW and, therefore, should be a beneficial allele for improving grain yield (Yue et al., 2014).

Jaiswal et al. (2015) analysed sequence polymorphism in the promoter region of*TaGW2-6A* (negative regulator of grain-width and grain-weight) and found two novel SNPs (one SNP present in CGCG motif) in the promoter region. Among the five haplotypes identified in this study, Hap5 (G_A_G_A) had significantly higher TGW than other haplotypes except Hap2, which did not show any significant difference from Hap5. A user-friendly CAPS marker for the causal SNP was also developed for exploitation of the variation in *TaGW2-6A* gene for improvement in TGW and other associated agronomic traits through marker-assisted selection (MAS) in wheat.

Qin et al. (2014), analysed haplotypes of *TaGW2-6B* and their effects on TKW and interaction with haplotypes at *TaGW2-6A*.Haplotype association analysis indicated that *TaGW2-6B* has a stronger influence than *TaGW2-6A* on TKW, and *Hap-6B-1* was a favoured haplotype increasing grain width and weight that had undergone strong positive selection in global wheat breeding.

Furthermore, haplotype interaction analysis between *TaGW2-6A* and *TaGW2-6B* showed additive effects between the favoured haplotypes. *Hap-6A-A/Hap-6B-1* was the best combination to increase TKW. Based on the -593 A/G polymorphism a CAPS marker was developed and association analysis indicated that *Hap-6A-A* increased TKW by more than 3.1g.

Recent studies reported that transcript abundance of *TaGW2-6A* is negatively associated with the grain width, but the transcript levels of *TaGW-2B* and *TaGW-2D* were positively associated with the grain width in the same bread wheat accessions, suggesting that triplicate homoeologues of *TaGW2* might have different functions in grain development, and that there is a balance among three genes finally determining the grain size in bread wheat.(Jaiswal et al., 2015).

Lu et al. (2015) studied one of the key phytohormones synthesized in the root, cytokinin (CTK) that regulates many important plant processes by controlling cell division and tissue differentiation.

Cytokinin is one key hormone in controlling grain size and weight by regulating endosperm cell numbers of crops and also can enhance grain weight by regulating grain filling patterns of crops. Seed numbers per plant and seed weight are improved by silencing the *TaCKX1* gene in wheat.

At present, wheat CKX genes have been isolated: *TaCKX1* and *TaCKX4* (Chang et al., 2015) on chromosome 3A, *TaCKX2* on 7A or 7B, and *TaCKX2.1*, *TaCKX2.2*, *TaCKX3*, *TaCKX5*, and *TaCKX6* on 3DS.

The results of Lu et al.,2015 study indicated that the allelic variation of*TaCKX6a02* had significant correlation with grain size, grain weight, and grain filing rate (GFR) in the recombinant inbreed line (RIL) population,

explaining 17.1~38.2% of phenotype variations in different environments.

A specific marker, TKX3D, was designed and can be used in marker-assisted selection (MAS) for grain size, weight, and GFR in common wheat.

Chang et al. (2015) associated the CKX genes with flag leaf chlorophyll content after anthesis, as well as grain weight. In high plant leaves, chlorophyll, including chlorophyll a and b, is the main photosynthetic pigment in chloroplasts, and its amount directly affects plant photosynthetic efficiency. Increased chlorophyll content in crop-species leaves increases in both biomass production and grain yield. As chlorophyll is the main pigment in photosynthesis, its abundance and stability in the leaf significantly affects grain filling and crops yield.

Results of Chang et al. (2015) showed that the variation of *TaCKX4*significantly associate with chlorophyll content and grain weight in the RIL population. Through mapping analysis, *TaCKX4,* was closely linked to Xwmc169 on chromosome 3AL. *TaCKX4* co-segregated with a major QTL for both grain weight and chlorophyll content of flag leaf at 5~15 days after anthesis. This QTL explains 8.9~22.3% phenotypic variations of the two traits across four cropping seasons.

From the three identified genotypes, genotype-A corresponded to higher wheat chlorophyll content and grain yield. Furthermore, the study indicated that copy number variation in *TaCKX4* (not the allelic variation) could significantly influence wheat chlorophyll content and grain weight. The locus linked to *TaCKX4* showed good stability and reliability in varied environments and genetic backgrounds, making this useful for improving the effectiveness of MAS for chlorophyll level and grain weigh in wheat breeding.

Cell wall invertase (CWI) is a critical enzyme for sink tissue development and carbon partition and has a high association with grain weight.

Ma et al. (2012) characterized the CWI genes and designed a pair of complementary dominant markers (CWI21 and CWI22), based on the two allelic variation of at the *TaCWI-A1* locus (*TaCWI-A1a* and *TaCWI-A1b*; chromosome 2A). The lines with *TaCWI-A1a* allele had significant higher TKW (45g) compared to the lines with *TaCWI-A1b* allele (42.6g). QTL analysis indicated that *TaCWI-A1*could explain 4.8% of phenotypic variance for grain over 2 years.

Another study on CWI was carried out by Jiang et al. 2015. This study, based on isolated *TaCWI* genes from chromosomes 4A, 5B and 5D, found two SNPs that were detected in the promoter region of *TaCWI-4A*, and four SNPs and two Indels were present in the *TaCWI-5D* gene. Discrimination of *TaCWI-4A* and *TaCWI-5D* haplotypes was accomplished by CAPS markers, viz.,*caps4A* and *caps5D*. The results indicated that *Hap-5D-C* at *TaCWI-5D* was significantly associated with higher TKW in 348 Chinese modern cultivars grown in multiple environments. In rainfed production regions, *Hap-4A-C* was favoured because it brought more seeds, but in well irrigated conditions, *Hap-4A-T* was favoured in modern breeding because of higher TKW.

The *TGW6* gene encodes a novel indole-3-acetic acid-glucose hydrolase and plays a significant role in improving TGW and yield in rice.

Hanif et al. (2016) isolated and characterized*TGW6*orthologs in bread wheat and developed functional markers to validate the *TaTGW6* gene association with TKW and yield. The analysis of ortholog*TaTGW6-A1* revealed two haplotypes, *TaTGW6-A1a* and *TaTGW6-A1b*. Haplotype *TaTGW6-A1a* identified by the dCAPS conferred higher grain weight and yield (higher TKW).

The QTL for yield at the *TaTGW6-A1* locus explained 17.4% of the phenotypic variance in average yield over four environments.

ADDITIONAL GENES WITH EFFECT ON GRAIN SIZE AND WEIGHT

Zheng et al. (2014) studied a member of the transcript elongation factor gene family, *TaTEF.TaTEF-7A* was located on chromosome 7A and was flanked by markers Xwmc83 and XP3156.3. Subcellular localization revealed that TaTEF-7A protein was localized in the nucleus. This gene was expressed in all organs, but the highest expression occurred in young spikes and developing seeds.

Overexpression of *TaTEF-7A* in Arabidopsis thaliana produced pleiotropic effects on vegetative and reproductive development that enhanced grain length, silique number, and silique length.

Haplotype–trait association analysis of the Chinese wheat mini core collection revealed that *TaTEF-7A*was significantly associated with grain number per spike. Phenotyping of near-isogenic lines (NILs) confirmed that TaTEF-7A increases potential grain yield and yield-related traits.

The presence of favoured haplotype, *Hap-7A-3*, showed a positive correlation with yield in a global set of breeding lines. These results suggest that *TaTEF-7A* is a functional regulatory factor for grain number per spike and provide a basis for marker-assisted selection.

Another interesting gene family with positive effect on TKW encoding for plant stress association proteins (SAPs). Thus, *TaSAP1*, a member of the stress association protein (SAP) gene family from wheat is involved in response to several abiotic stresses, including drought, salt and cold. Chang et al., 2013, found that *TaSAP1-A1* locus, located on chromosome 7A, was significantly associated with TGW, number of grains per spike, spike length, peduncle length and total number of spikelets per spike in multiple environments. Also, this team identified three markers T7AM5, T7AM2606 and T7AM39 located in the promotor region with six haplotypes.

CONCLUSIONS

According to the most recent data, the grain size and weight are under complex genetic control and at the same time influenced by the environment. At present, there are identified genes/QTLs significantly associated with grain shape, size and weight. Moreover, several genes/QTLs are involved in response to several abiotic stresses and associated with grain traits and yield. The presence of molecular markers, associated with grain traits, constitute a premise for the improvement of grain potential, by pyramiding the best alleles with additive effect, using MAS.

REFERENCES

Calderini D. F., Abeledo L. G., Savin R., Slafer G. A., 1999.Effect of temperature and carpel size during pre-anthesis on potential grain weight in wheat. Journal of Agricultural Science 132, 453–459.

Chang C., Lu J., Zhang H. P., Ma C. X., Sun G., 2015. Copy Number Variation of Cytokinin Oxidase Gene Tackx4 Associated with Grain Weight and Chlorophyll Content of Flag Leaf in Common Wheat. PLoS ONE 10(12): e0145970. doi:10.1371/journal.pone.0145970.

Chang J., Zhang J., Mao X., Li A., Jia J., Jing R., 2013. Polymorphism of TaSAP1-A1 and its association with agronomic traits in wheat. Planta 237:1495-1508.

Cheng X., Chai L., Chen Z., Xu L., Zhai H., Zhao A., Peng H., Yao Y., You M., Sun Q., Ni Z., 2015. Identification and characterization of a high grain weight mutant induced by gamma radiation in wheat (*Triticum aestivum* L.).BMC Genetics 16:127.

Dixon .J, Braun H.J., Kosina P., Crouch J., 2009.Wheat facts and futures. Mexico D.F.: CIMMYT

Gegas V.C., Nazari A., Griffiths S., Simmonds J., Fish L., Orford S., Sayers L., Doonan J. H.,Snapea J. W., 2010. A Genetic Framework for Grain Size and Shape Variation in Wheat. The Plant Cell, Vol. 22:1046–1056.

Giura A., Saulescu N. N., 1996. Chromosomal location of genes controlling grain size in a large grained selection of wheat (*Triticum aestivum* L.).Euphytica 89: 77-80.

Hanif M., Gao F., Liu J., Wen W., Zhang Y., Rasheed A., Xia X., He Z., Cao S., 2016. TaTGW6-A1, an ortholog of rice TGW6, is associated with grain weight and yield in bread wheat. Molecular Breeding36:1.

Hasan A. K., Herrera J., Lizana C., Calderini D. F., 2011. Carpel weight, grain length and stabilized grain water content are physiological drivers of grain weight determination of wheat. Field Crops Research 123, 241–247.

Hou J., Jiang Q., Hao C., Wang Y., Zhang H., Zhang X., 2014.Global Selection on Sucrose Synthase Haplotypes during a Century of Wheat Breeding. Plant Physiology, Vol. 164, pp. 1918–1929.

Jaiswal V., Gahlaut V., Mathur S., Agarwal P., Khandelwal M. K., Khurana J. P., Tyagi A. K., Balyan H. S., Gupta P. K., 2015. Identification of Novel SNP in Promoter Sequence of TaGW2-6A Associated with Grain Weight and Other Agronomic Traits in Wheat (*Triticum aestivum* L.).PLoS ONE 10(6): e0129400. doi:10.1371/journal.pone.0129400.

Jiang Y., Jiang Q., Hao C., Hou J., Wang L., Zhang H., Zhang S., Chen X., Zhang X., 2015. A yield‐associated gene TaCWI, in wheat: its function, selection and evolution in global breeding revealed by haplotype analysis. TheorAppl Genet. 128(1):131-43.

Justin D. Faris, 2014. Wheat Domestication: Key to Agricultural Revolutions Past and Future. In Genomics of Plant Genetic Resources, Vol. 1,

Chapter 18, pp. 439-464, Springer Science+Business Media Dordrecht.

Kumar A., Mantovani E. E., Seetan R., Soltani A., Echeverry-Solarte M., Jain S., Simsek S., Doehlert D., Alamri M. S., Elias E. M., Kianian S. F., Mergoum M., 2016. Dissection of genetic factors underlying wheat kernel shape and size in an Elite x Non adapted cross using hight density SNP linkage map. The Plant Genome, Vol. 9, No. 1.

Li W., Zhang B., Li R., Chang X., Jing R., 2015. Favorable Alleles for Stem Water-Soluble Carbohydrates Identified by Association Analysis Contribute to Grain Weight under Drought Stress Conditions in Wheat. PLoS ONE 10(3): e0119438.doi:10.1371/journal.pone.0119438.

Lizana X. C., Riegel R., Gomez L. D., Herrera J., Isla A., McQueen-Mason S. J., Calderini D. F., 2010. Expansins expression is associated with grain size dynamics in wheat (*Triticum aestivum* L.). Journal of Experimental Botany 61, 1147–1157.

Lu J., Chang C., Zhang H. P., Wang S. X., Sun G., Xiao S. H., Sun G., Xiao S., Ma C. X., 2015. Identification of a Novel Allele of TaCKX6a02 Associated with Grain Size, Filling Rate and Weight of Common Wheat. PLoS ONE 10(12): e0144765.doi:10.1371/journal.pone.0144765.

Ma D. Y., Yan J., He Z. H., Wu L., Xia X. C., 2012. Characterization of a cell wall invertase gene TaCwi-A1 on common wheat chromosome 2A and development of functional markers.Mol Breeding 29:43–52.

Ma L., Li T., Hao C., Wang Y., Chen X., Zhang X, 2015. TaGS5-3A, a grain size gene selected during wheat improvement for larger kernel and yield. Plant Biotechnology Journal, pp. 1–12.

Patil R. M., Tamhankar S. A., Oak M. D., Raut A. L., Honrao B. K., Rao V. S., Misra S. C., 2013. Mapping of QTL for agronomic traits and kernel characters in durum wheat (*Triticum durum* Desf.), Euphytica, Volume 190, Issue 1, pp 117-129.

Qin L., Hao C., Hou J., Wang Y., Li T., Wang L., Ma Z., Zhang X., 2014. Homologous haplotypes, expression, genetic effects and geographic distribution of the wheatyield gene TaGW2. Plant Biology 14:107.

Rajaram S., 2005. Role of conventional plant breeding and biotechnology in future wheat production. Turk J Agric For 29:105–111.

Rasheed A., Xia X., Ogbonnaya F., Mahmood T., Zhang Z., Mujeeb-Kazi A., He Z., 2014.Genome-wide association for grain morphology in synthetic hexaploid wheats using digital imaging analysis. BMC Plant Biology, 14:128.

Röder M. S., Huang X. Q., Börner A, 2008. Fine mapping of the region on wheat chromosome 7D controlling grain weight.FunctIntegr Genomics. 8(1):79-86.

Scott W. R., Appleyard M., Fellowes G., Kirby E. J. M., 1983. Effect of genotype and position in the ear on carpel and grain growth and mature grain weight of spring barley. Journal of Agricultural Science 100, 383–391.

Su Z., Hao C., Wang L., Dong Y., Zhang X., 2010. Identification and development of a functional marker of TaGW2 associated with grain weight in bread wheat (*Triticum aestivum* L.). TheorAppl Genet 122:211–223.

Wang S., Zhang X., Chen F., Cui D., 2015. A Single-Nucleotide Polymorphism of TaGS5 Gene Revealed its Association with Kernel Weight in Chinese Bread Wheat. Front. Plant Sci. 6:1166.doi: 10.3389/fpls.2015.01166.

Wu Q. H., Chen Y. X., Zhou S. H., Fu L., Chen J. J., Xiao Y., Zhang D., Ouyang S. H., Zhao X. J., Cui Y., Zhang D. Y., Liang Y., Wang Z. Z., Xie J. Z., Qin J. X., Wang G. X., Li D. L., Huang Y., Yu M. H., Lu P., Wang L. L., Wang L., Wang H., Dang C., Li J., Zhang Y., Peng H. R., Yuan C. G., You M. S., Sun Q. X., Wang J. R., Wang L. X., Luo M. C., Han J., Liu Z. Y., 2015. High-Density Genetic Linkage Map Construction and QTL Mapping of Grain Shape and Size in the Wheat Population Yanda1817 × Beinong6.PLoS ONE 10(2): e0118144.doi:10.1371/journal.pone.0118144.

Wu X., Chang X., Jing R., 2012. Genetic Insight into Yield-Associated Traits of Wheat Grown in Multiple Rain-Fed Environments.PLoS ONE 7(2): e31249.doi:10.1371/journal.pone.0031249.

Xie Q., Mayes S. and Sparkes D. L., 2015. Carpel size, grain filling, and morphology determine individual grain weight in wheat. Journal of Experimental Botanydoi:10.1093/jxb/erv378.

Yang Z., van Oosterom E. J., Jordan D. R., Hammer G. L., 2009. Preanthesis ovary development determines genotypic differences in potential kernel weight in sorghum. Journal of Experimental Botany 60, 1399–1408.

Yue A., Li a., Mao X., Chang X., Li R., Jing R., 2015. Identification and development of a functional marker from 6-SFT-A2 associated with grain weight in wheat. Mol Breeding 35 (Issue 2):63.

Zanke C. D., Ling J., Plieske J., Kollers S., Ebmeyer E., Korzun V., Argillier O., Stiewe G., Hinze M., Neumann F., Eichhorn A., Polley A., Jaenecke C., Ganal M.W., Röder M. S., 2015. Analysis of main effect QTL for thousand grain weight in European winter wheat (*Triticum aestivum* L.) by genome-wide association mapping. Front. Plant Sci. 6:644.

Zhang B., Li W., Chang X., Li R., Jing R., 2014. Effects of Favorable Alleles for Water-Soluble Carbohydrates at Grain Filling on Grain Weight under Drought and Heat Stresses in Wheat.PLoS ONE 9(7): e102917.doi:10.1371/journal.pone.0102917.

Zheng J., Liu H., Wang Y., Wang L., Chang X., Jing R., Hao C., Zhang X., 2014. TEF-7A, a transcript elongation factor gene, influences yield-related traits in bread wheat (*Triticum aestivum* L.). Journal of Experimental Botany, Vol. 65, No. 18, pp. 5351–5365.

Zheng J., Liu H., Wang Y., Wang L., Chang X., Jing R., Hao C., Zhang X., 2014. TEF-7A, a transcript elongation factor gene, influences yield-related traits in bread wheat (*Triticum aestivum* L.), Journal of Experimental Botany, Vol. 65, No. 18, pp. 5351–5365.

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THE INFLUENCE OF SEVERAL ABIOTIC FACTORS ON *FUSARIUM* SPP. BIOLOGY

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Abstract

Wheat (Triticum aestivum L.), one of the most widely grown winter cereal crop in Romania, is grown on approximately 2 million ha. Fusarium species affect yield and grain quality because of mycotoxins production. Fusarium spp. is one the most frequently pathogenic species of wheat and understanding its biology provides information regarding the optimal timing to implement specific control measures in order to stop the infection process of the disease.
The occurrence and development of toxigenic fungi affects stored products causing quality depreciation, products aggregation and also toxins and allergens production. Micromycetes development on stored cereal seeds is favoured by temperature, light and atmospheric moisture present in storage units, and their fluctuations in time.
The aim of our research was to determine the influence of some biological parameters (temperature, pH, light and culture media) on the vegetative fungal growth and development, under controlled conditions. The biological material consisted in one strain of Fusarium spp., isolated from caryopses of wheat, from samples taken from a storage unit in Paulesti, Prahova County. It was established that the fungus develops in a large scale of pH, forming specific colonies between low-acid and high alkaline values (pH 4-10). Optimal temperature values were between 20 and 28 Cº, with a 6º C minimum and no growth above 36 Cº. Very good sporulation and vegetative growth was obtained under continuous light conditions.

Key words: *wheat, biologic parameters, Fusarium spp.*

INTRODUCTION

Wheat (*Triticum aestivum*) is one of the most important crops cultivated in Romania due to its favorable growth conditions.
Wheat (*Triticum* spp.) (Donner et al., 2000) is a cereal grain, originally from the Levant region of the Near East and Ethiopian Highlands but now cultivated worldwide. In 2010, world production of wheat was 651 million tons, making it the third most-produced cereal after maize (844 million tons) and rice (672 million tons). However, pathogens that contaminate wheat may survive for extended periods (Berghofer et al., 2003; Cabanas et al., 2008; Gashgari et al., 2010). Wheat was also found to be contaminated in variable amounts by potentially toxigenic fungi including *Aspergillus, Alternaria* and *Fusarium* (Halt, 1998; Tournas and Katsoudas, 2008). These fungi are present in soil and plant material, cause the decay of stored grain and food (Herrman, 2002).
Fungal growth, especially *Aspergillus flavus* and *Fusarium* spp. in wheat, facilitated by hot and humid conditions, poses a major risk through production of mycotoxins (Radoi et al, 2011). Infection of grains in the field by fungi could result in the production of mycotoxins during cultivation, harvesting, storage, transport and processing.
In order to maintain high quality wheat for both short- and long-term storage, grain seeds must be protected from weather, growth of microorganisms, and pests (Cristea et al., 2008, Mali et al., 2015, Berca et al., 2015, Berca and Cristea, 2015).
The most important species of fungi and mycotoxins that could contaminate maize grains are *Aspergillus flavus* and aflatoxins, *Fusarium verticillioides*, *F. proliferatum* and fumonisins and *F. graminearum* and *F. tricinctum* and trichothecenes and zearalenone (Ittu et al., 2010).
Despite decades of intense research, the moulds infection is still a major challenge for scientists

(Munkvold, 2003). Micromycetes' development on stored grains is conditioned by temperature and atmospheric humidity present in stored areas and by its fluctuations in time (Cristea et al., 2004, Mardare et al., 2015). Toxigenic moulds are present, due to various climatic factors, in different stages of food and feed production, including crop growth, harvesting, transport, storage and handling (Beyer et al., 2006).The most common genera of fungi identified in stored maize grains are *Aspergillus, Penicillium* and *Fusarium*. (Cristea, 2005, Pana et al., 2014, Mardare et al., 2014).
Our research was focused on establishing the influence of several biologic parameters on the fungi's growth and development.

MATERIALS AND METHODS

Studies on the influence of abiotic factors, such as temperature, pH value, light and culture medium on the growth and sporulation of *Fusarium* spp. strain, were performed in laboratory conditions. The biological material consisted in wheat caryopses from Glosa cultivar, taken from a storage unit in Paulesti, Prahova County. The grains were placed in a wet room, where the mycelium formation was observed after 3 days. The *Fusarium* spp. strain was obtained by classic isolation technique using Ulster method (Hulea et al., 1969, Raicu et al., 1978) and successive sub-culturing in Petri dishes of 10mm diameter with potato-dextrose-agar growth medium, incubated at 24ºC (Radu et al., 2011) .
The temperature influence on the growth and sporulation of *Fusarium* spp. was monitored between 2ºC and 40ºC, in order to establish the minimum, optimal and maximum growth value. Mycelia disks of 5 mm diameter were inoculated in Petri dishes with PDA medium, incubated in thermostats, at temperatures between 2ºC and 40ºC, and colonies were observed and measured at a 3 days interval for a period of 15 days.
The influence of pH values on *Fusarium* spp. biology was determined using PDA culture medium with modified pH values using sodium hydroxide or hydrochloric acid solutions. The fungus was inoculated on medium with pH values from 3 to 11. Colonies diameters were measured at a 3 days interval, observing also the sporulation process, during 12 days.
It was also observed the fungus reaction to light by incubating mycelia disks on PDA medium at continuous light, continuous darkness, light/dark alternating for 8h/16h and 12h/12h.
In order to establish which nutrient substrate is most favorable for the growth and sporulation of *Fusarium* spp. were chosen different media: potato-dextrose-agar, malt-agar (semi-synthetic medium), Czapex Dox agar (synthetic medium) and natural media like wheat seeds, rice seeds, barley seed (Constantinescu, 1974).

RESULTS AND DISCUSSIONS

The temperature is a key factor in the development of infections and for the pathogen occurrence and growth. As shown in table 1, the *Fusarium* spp. strain has a minimum growth point at 4ºC, with weak vegetation mass, without sporulation. Between 12ºC and 18ºC, it can be observed an increased vegetation mass, with good sporulation of the fungus. The optimal temperature for growth and sporulation of this isolate is situated between 20ºC and 28ºC, when the fungus presented colonies of 80 mm diameter and abundant sporulation, after 15 days of observation (Figures 1 and 2). After 30ºC the fungus declines in development, sporulation is weaker. The maximum growth temperature is 36ºC, the colony diameter barely reached 35 mm, and sporulation is absent. After 38ºC, the fungal growth is completely inhibited (Figure 3).

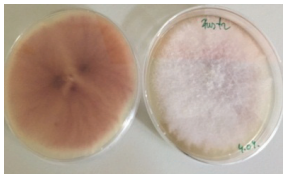

Figure 1 *Fusarium* spp. on PDA medium

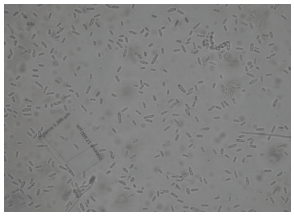

Figure 2 *Fusarium* spp. micro conidia

Table 1. The influence of temperature upon *Fusarium* spp. colony growth and development

T°C/days	3 days	6 days	9 days	12 days	15 days	Observations after 15 days	
	Colony diameter in mm						
2^0	0	0	0	0	0	Vm 0	0
4^0	0	1	4	5	5	Vm ±	0
8^0	0	5	9	15	17	Vm+	Sp+
12^0	2	8	16	23	27	Vm+	Sp+
14^0	3	18	26	38	44	Vm++	Sp+
16^0	8	20	30	34	39	Vm++	Sp++
18^0	13	24	40	47	57	Vm++	Sp+ +
20^0	13	26	38	57	80	Vm+++	Sp+++
22^o	22	34	45	68	80	Vm+++	Sp+++
24^0	23	36	46	70	80	Vm+++	Sp+++
26^0	24	45	70	73	80	Vm+++	Sp+++
28^0	23	50	64	73	80	Vm+++	Sp+++
30^0	11	48	58	68	70	Vm+++	Sp+++
32^0	8	21	28	32	35	Vm++	Sp++
36^0	2	4	6	7	7	Vm+	Sp+
38^0	0	0	0	0	0	0	0
40^0	0	0	0	0	0	0	0

Legend: Vm± = very poor vegetative mass, Vm+ = poor vegetative mass, Vm++ = good vegetative mass, Vm+++ = very good vegetative mass, Sp+ = poor sporulation, Sp++ = good sporulation, Sp+++ = abundant sporulation, Sp± = very poor sporulation, 0 = fungus did not grow/sporulate.

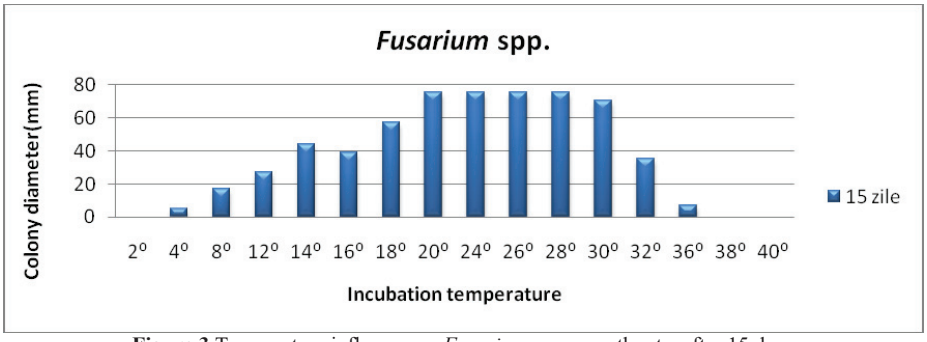

Figure 3. Temperature influence on *Fusarium spp.* growth rate, after 15 days

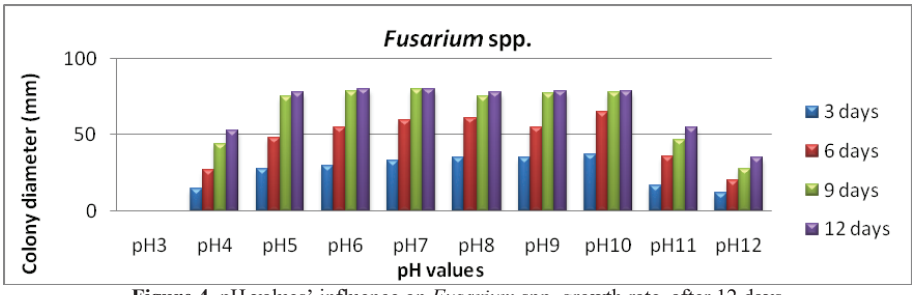

Figure 4. pH values' influence on *Fusarium* spp. growth rate, after 12 days

Regarding the influence of pH value on the development of *Fusarium* spp. fungus, after 12 days on evaluating the results of experiments it was observed that there is a wide range of pH

values substrates development, from strong acid up to high alkaline (Figures 4 and 5).

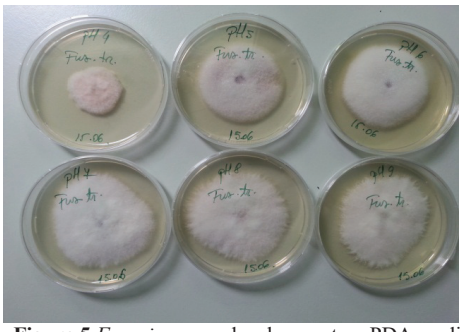

Figure 5.*Fusarium* spp. development on PDA medium with different pH values (4-9)

At pH value 4, the colonies had a good vegetative mass and conidia were observed. Optimal pH values are between 5 and 9, with very good vegetative mass and sporulation. On high alkaline culture medium, the fungus grew less vegetative, but sporulation was good.

In terms of the light influence towards the development of *Fusarium* spp., as it can be observed in table 3, the fungal colonies have developed very well in light exposure. On permanent light or alternatively light/darkness exposure, the vegetative mass of colonies was rich, velvety mycelium and sporulation was abundant. When kept into darkness during the whole observation period, the fungus formed colonies with very little vegetative mass, and conidia were rare on mycelium surface.

Table 2. Light exposure influence on fungus growth

Lightness	***Colony development***
24 hours lightness	Rich vegetative mass, velvety mycelium, abundant sporulation
Alternative lightness/darkness	Rich vegetative mass, velvety mycelium, white-pink color, abundant sporulation
Light/Darkness alternance (12h/12h)	Rich vegetative mass, felt appearance mycelium, white-pink color, rich sporulation
Light/Darkness alternance (8h/16h)	Good vegetative mass, weak sporulation
Continuous darkness	Good vegetative mass, weak sporulation

Regarding the influence of the culture substrate on the pathogen's growth the data presented in table 4, show that nutritive medium has an impact on *Fusarium* spp. growth and sporulation. The fungus grew and developed preferentially on natural mediums (wheat, rice, barley), but also had a very good vegetative mass and sporulation on semi-synthetic medium Potato-dextrose-agar, the colony diameter having reached maxim value of 80 mm. On malt-agar medium and Czapek synthetic medium the vegetation and sporulation were good.

The observations regarding the culture mediums' influence highlights that the pathogen shows plasticity on the natural growth medium.

Table 3. The influence of different culture media on *Fusarium* spp. development

Culture medium		***Observations of vegetative mass and sporulation***
Natural substrates	Barley seeds	Very good vegetative mass; good sporulation
	Wheat seeds	Very good vegetative mass; abundant sporulation
	Rice seeds	Weak vegetative mass; good sporulation
Semi-synthetic media	PDA	Very good vegetative mass; abundant sporulation
	Malt 2%	Good vegetative mass; good sporulation
Synthetic medium	Czapek Dox	Good vegetative mass; good sporulation

CONCLUSIONS

The optimal growth and development temperature for *Fusarium* spp. isolate is between 20ºC and 28ºC, with a minimum value of 4ºC. After 38ºC, the fungus does not grow.

Continuous light exposer, followed by the variant with light/darkness (12h/12h) enhanced the best growth and development of *Fusarium* spp. strain.

The pH reaction substrate was optimal for values between 5.0 and 9.0.

The most favourable culture substrate was Potato-dextrose-agar (semi-synthetic medium), with very good mycelia development and abundant sporulation, followed by the natural substrates wheat and barley. A good development of *Fusarium* spp. was determined also by Czapex Dox and Malt 2% media.

Studies regarding fungus biological parameters play a decisional role in forecasting and warning on disease induced by fungus *Fusarium* spp.

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REFERENCES

Berca L., Cimponeriu G.D., Cristea S. (2015). Distribution of *Alternaria* sp. on *Brassica napus* seeds from growing fields affected by alternaria black spot in Calarasi County. Journal of Biotechnology 208, S5-S120, pg 115.

Berca L., Cristea S. (2015): Research on micoflora present on rapeseed (*Brassica napus*) in the south region of Romania. Romanian Biotechnological Letters, Vol.20, No.5, pg. 10809-10813.

Berghofer, L.K., A.D. Hocking, D. Miskelly and E. Jansson, (2003). Microbiology of wheat and flour milling in Australia. Int. J. Food Microbiol., 85: 137-149.

Cabanas, R., M.R. Bragulat, M.L. Abarca, G. Castella and F.J. Cabanes, (2008). Occurrence of *Penicillium verrucosum* in retail wheat flours from the Spanish market. Food Microbiol., 25: 642-647.

Constantinescu O., (1974), Metode si tehnici in micologie. Ed. Ceres, Bucuresti.

Cristea (Manole) M.S, Cristea S., Zala C. (2015): Research on micoflora present in the caryopses of wheat (*Tritcum aestivum*) in the S-E of Romania, in terms of 2014. Romanian Biotechnological Letters, vol.20, No 1, 10182-10189.

Cristea S., Oprea M., Cristea M.C., Braileanu B. (2004). Cercetari privind parametrii biologici ai ciupercii *Fusarium graminearum* isolate de pe semintele de porumb. Lucrari stiintifice seria A, Vol. XLVII, Agronomie, pg. 291-294.

Cristea S., Georgescu M., Patrascu N., Groza O., Ion L. (2008). Research regarding the pathology and anathomy of the seed – the extension of the wheat kernel. Lucrari stiintifice, seria A, vol. LI, USAMV, Bucuresti.

Cristea S., (2005). Fitopatologie, vol. 2, Ed. Cris Book Universal, Bucuresti.

Donner, D.A., B. Belderok and J. Mesdag, 2000. Bread-Making Quality of Wheat: A Century of Breeding in Europe. Springer, New York, ISBN-13: 9780792363835, Pages: 416.

Gheorghies C., Cristea S. (2001): Fitopatologie, vol.1, Ed. Ceres, Bucuresti.

Gashgari, R.M., Y.M. Shebany and Y.A. Gherbawy, 2010. Molecular characterization of mycobiota and aflatoxin contamination of retail wheat flours from Jeddah markets. Foodborne Pathogens Dis., 7: 1047-1054.

Halt, M., 1998. Moulds and mycotoxins in herb tea and medicinal plants. Eur. J. Epidemiol., 14: 269-274.

Herrman, T., 2002. Mycotosins in feed grains and ingredients. MF-2061, Department of Grain Science and Industry, Kansas State University Agricultural Experiment Station and Cooperative Extension Service.

Hulea Ana, (1969). Ghid pentru laboratoarele de micologie şi bacteriologie. Ed. Agro-silvica, Bucuresti.

Matei R.F., Israel F., Cristea S., Smeu I., Radu A. (2011). Quantitative study of Deoxynivalenol and Ochratoxin accumulation in synthetic media. Romaniam Biotechnological Letters, Vol.16, No.1, pg.33-39.

Mali S., Cristea S., Toader M., Zala C., Berca L., (2015). Barley seed micoflora and their influence on quality inicators. Lucrari stiintifice, vol. 58(2), Seria Agronomie, USAMV Iasi.

Mardare E.S., Cristea S., Gadea M., Tamba-Berehoiu R., (2015). The influence of some abiotic factors on the development of *Alternaria* spp. Pathogen. Romanian Biotechnological Letters, vol. 20(5).

Mardare E.S., Cristea S., Zala C., 2014. Researches on the micoflora of sunflower's achenes from hybrids cultivated in Fetesti area, Ialomita County. Scientific Papers, Agronomy, Vol.57 no.2, USAMV Iasi.

Munkvold G.P., 2003. Cultural and genetic approaches to managing mycotoxins in maize. Annual Review of Phytopatholoy, Vol.41, 99-116.

Pana M., Cristea S., Cernat S., Negrila E. (2014). The mycoflora on barley - the varieties extension certificated at ARDS – Teleorman. Lucrari stiintifice 57(2), seria Agronomy, pg. 217-220.

Radu E., Cristea S., Zala C. (2011). Reserch on the biological features of *Alternaria brassicae* pathogen isolated on rape. Scientific papers, USAMV Bucharest, Series A, Vol. LIV, pg. 350-355.

Raicu C., Baciu D., (1978). Patologa semintei. Ed. Ceres, Bucuresti.

Tournas, Y.H. and E.J. Katsoudas, 2008. Microbiological quality of various medicinal herbal teas and coffee substitutes. Microbiol. Insights, 1: 47-55.

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LEATHER HYDOLYSATE EVALUATED AS BIOACTIVE POTATO FERTILIZER

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Abstract
Leather industry discharges enormous amount of chrome containing leather solid wasted which creates a major disposal problem. Tanned leather solid waste is a complex of hard-to-degrade proteins and chromium. The biotechnological sector allows us to use the waste materials as bacterial substrate for enzyme production. The present work covers potential application in the potato bio-growth as fertilizer. The hydrolysate results from bacterial conversion of leather components. Bacteria was isolated from the composting of leather and incubated into a minimal media for 120 hours at 35°C. In the optimization process maximum proteinase production was 1.223 U/ml. The results obtained suggested that leather debris containing amino-acids and proteins andcan be applied as organic nitrogen soil input.

Key words*: bacterial isolation, leather degradation, proteinase,fertilizer for potato growth.*

INTRODUCTION

Proteolytic enzymes are involved in breakage of the long chain molecules of proteins into shorter fragments – peptides and eventually into their components, amino-acids (Anson, 1938).
Low commercial value protein waste of animal origin such as skins and the manufacturing processes of leather produce annual tones of worthless material. Through the biotechnological methods this waste materials can be used for obtaining hydrolytic enzymes, which can be applied as agricultural fertilizer.
Nitrogen management is perhaps the most important aspect of successful potato (*Solanum tuberosum* L.) production.
The high cost of chemical fertilizer along with the related ecological and health hazards necessitate finding out an alternative nutrient sources to sustain the crop yield without any adverse effect on soil and environment. The aim of this work was the isolation of bacterial strains able to produce proteases involved in leather degradation and the use of the concentrated fermentation liquids as plant fertilizer.

MATERIALS AND METHODS

The strains used in experiments were isolated from composting of chromium tanned leather (leather debris were included in soil samples and incubated at room temperature for 3 months), according to classical microbiological methods.
The leather debris was kindly provided by the National Research and Development Institute for Textiles and Leather Bucharest.
The bacteria were screened for their ability to degrade proteins on selective medium (Habib et al., 2012).
The selected bacteria were grown in minimal media (MM) (g/L - 1.0g $NaCl_2$, 0.05 g $CaCl_2$, 0.7 g KH_2PO_4, 0.9 g $MgSO_4$, 2.38 g K_2HPO_4, 3,0 g sucrose, with 0.6 g leather as nitrogen source, pH 7,2) (Israel et al., 2012).
After inoculation, the bacterial fermentation was performed at 35°C for 120 hours, with stirring (135 rpm). The turbidity (OD600 nm) was measured at 24 h, 36 h, 48 h, 72 h, 96 h and 120 h of incubation and monitored for growth and protease activity.

Biomass separation and concentration of enzymes

At the end of fermentation process the cultivation medium was separated by centrifugation at 9000 rpm, at 4°C, for 20 minutes. The supernatant (fermentation liquid) was collected and concentrated ten times at 60°C, using the rotary evaporator.

The proteinase activity

Proteolytic activity was measured spectrophotometrically at 578 nm, following the method of Anson (1938). The reaction mix contained 0.5 mL enzymatic solution and 1 mL casein 1% in phosphate buffer 0.2M (pH 7), incubated at 37°C for 10 min. Enzymatic reaction was stopped with 2 mL oftrichloroaceticacid 5%. The reaction mix was kept 30 min at room's temperature and then it was filtrated. For every 0.5mL filtrate was added 0.5mL HCl 0.2N, 2mL NaOH 0.5N and 0.6 mL Folin-Ciocâlteu 1:2. After 30 min at room's temperature the extinction was measured.

One unit of proteases activity is defined as the amount of enzyme that releases 1µmol tyrosine per minute, under analysis condition.

Testing leather hydrolysate as bioactive agricultural fertilizer

Liquid fermentation media obtained after biomass separation, named leather hydrolysate, was used as plant biofertilizer.

Different concentrations of leather hydrolysed (15%; 20%; 35%) were added in dilution of 1:10 mL in 100 mL plastic pots with 40 g of soil, and 4 week sold potato plants grown from the meristem were planted. Two potato varieties were used in experiments: *Solanum tuberosum* var. *Christian)* and *S. Tuberosum* var. *Roclas.* All the pots were watered regularly. After 6 days of sowing all plants were uprooted and washed. The growth parameters (plants length and number of leaves) were observed.

RESULTS AND DISCUSSIONS

Three bacterial strains designated DA7, DA10 and DA13 were isolated from the compost with chromium tanned leather. Their growth determined after the cultivation in minimal medium for 120h is presented in table 1.

Table 1. Growth efficiency (OD600nm) of the selected bacterial strains in minimal medium for 24-120 h

Samples	24 h	48 h	72 h	96 h	120h	Average
DA 7	0.239	0.381	0.487	0.699	0.917	0.544
DA 10	0.389	0.592	0.721	0.938	1.019	0.731
DA 13	0.244	0.390	0.515	0.725	0.997	0.574

The proteolytic activity of the selected strains was determined. The maximum enzymatic activity, 1.223 U/mL was obtained with the strains DA10, after 120h of cultivation in minimal medium with chromium tanned leather as nitrogen source.

The fermentation broth was concentrated 10 times and evaluated as biofertilizeron the growth of *Solanum tuberosum* L. plants. It was observed that the added leather hydrolysates exerted a beneficial effect on the plant growth. Plant and root length increased in the treated variants over the untreated ones. Similar effect has been reported by different authors (Kim et al., 2005; Bose et al., 2013;Vasileva-Tonkova et al., 2009) when feather hydrolysate was used as nitrogen supplement in soil.

The strain DA13 was less efficient, event at 35% concentration, the results being correlated with the enzymatic activity determined previously (Figure 1).

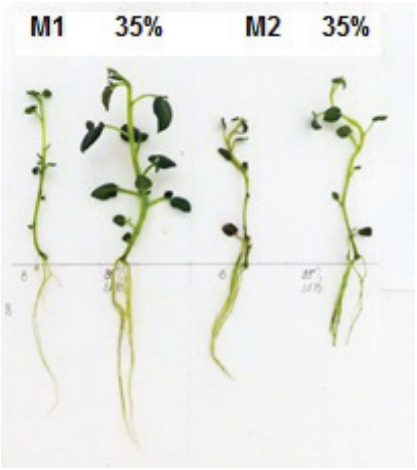

Figure 1. The efficiency of DA13 fermentation broth treatment at 35%.
M1 – Christian variety; M2 – Roclas variety

The best results were obtained with the concentrated broth from DA10 strain, for both potato varieties (Figure 2).

Among the concentrations used in treatments, the most efficient variant was 35% (Figure 3).

Differences between potato varieties were also observed: the highest plants were observed at Roclas variety, but the most vigorous were the plants from Christian variety.

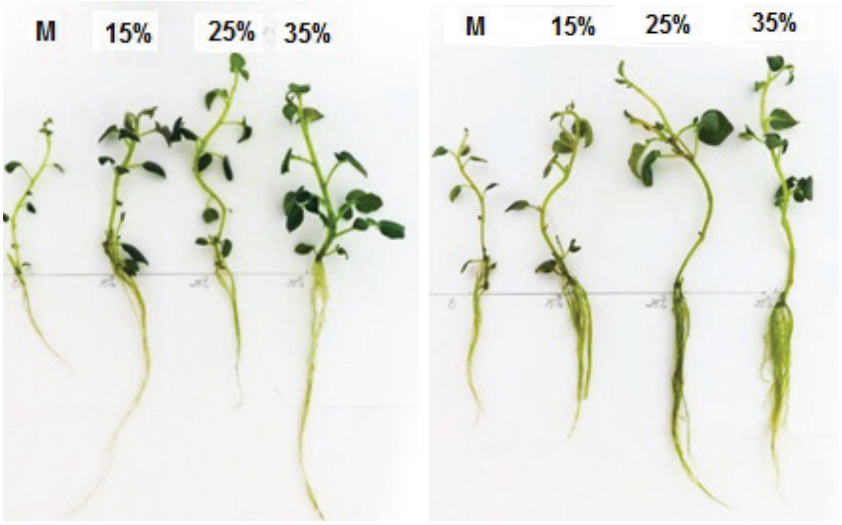

Figure 2. The effect of the application of the DA10 fermentation broth in different concentrations (15%, 25%, and 35%) on potato plants from Christian (left) and Roclas (right) varieties.

Figure 3. The efficiency of DA10 fermentation broth treatment at 35%. M1 – Christian variety; M2 – Roclas variety

CONCLUSIONS

Three new proteolytic bacterial strains were selected from compost (soil with leather debris composted during 3 months). Among them, the strain designated DA10 exhibited both the highest rate of growth and the best proteolytic activity in minimal medium with chromium tanned leather as nitrogen source.

The fermentation broths obtained with all the bacterial strains were used as biofertilizer for potato plants. Differences between bacterial treatments and potato varieties were observed: the best strain was DA10 in concentration of 35%, and the most vigorous were the plants from *Solanum tuberosum L*. Christian variety.
The results obtained demonstrate that the fermentation broths could contain hydrolytic enzymes able to liberate amino-acids that stimulate the plant growth.

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REFERENCES

Anson, M.L., 1938, The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin, J. Gen. Physiol. 22, 79-89

Carey, EE. W. H. Allaway, O.E. Olson. 1977. Control of Chromium Concentraion in Food Plants. 2. Chemistry of Chromium in soils and its availability to Plants. J Ag. Food Chem. Vol 25(2) p. 305-309.

Ciavetta, C. and P. Sequi. 1989. Evaluation of chromium release during the decomposition of leather meal fertilizers applied to the soil. FertReearch 19: 7-11.

El-Safey, E. M, 1994 - "Production of microbial a-amylases under solid-state incubation conditions in the open air", Sci. A thesis, Bot. and Microbiol. Dept., FacSci, Al-Azhar Univ., Cairo, Egypt.

Habib S.M.A., Fakhruddin A.N.M., Begum S., Ahmed M.M., 2012, Isolation and screening of thermostable extracellular alkaline protease producing bacteria from tannery effluents. J. Sci. Res. 4: 515-522

Israel-Roming, F., Gherghina, E, Luta, G., Balan, D., Popa,A., Stefan,R., Cornea, C.P., Studies on biodegradation of tanned leather, Scientific Bulletin, Series F, Biotechnologies, vol. XVI, p.119-123

Korkmaz H., Hür H., Dinçer S., 2004, Characterization of alkaline keratinase of *B. licheni formis* strain HK1 from poultry waste, Annals of Microbiology 54 (2), 201-211.

Lupescu I., Groposila-Constantinescu D., Jurcoane S.,Diguta C., Cozea A., Tcacenco L.,2007, Production of lipases by strain of the non-conventional yeast *Yarrowialipolytica* and isolation of crude enzyme, Roumanian Biotechnology letter, Vol 12, No3, pp 3261-3268.

Moore S., Stein W.H., 1948, Photometric ninhydrin method for use in the chromatography of amino acids, J. Biol. Chem. 176: 367-388.

Tapai (Stoica) Mihaela, 2009, Biosinteza unor amilaze utilizate în hidroliza unor materii prime agricole, Teza de Doctorat, p.65-66, Bucuresti.

Zarnea G., Mencinicopschi Gh, Brăgărea S. T., 1980, „Bioingineria preparatelor enzimatice microbiene", EdituraTehnicăBucureşti.

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INCREASING THE ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND ASSIMILATORY PIGMENTS CONTENT BY OPTIMIZING THE *IN VITRO* GROWTH CONDITIONS OF *LYCIUM BARBARUM* PLANT

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Abstract
According to information published in various specialized articles and those taken from traditional beliefs, goji fruit is considered to be an important antioxidant, antidiabetic, and a natural source with excellent effects on the cardiovascular system and in decreasing the level of cholesterol in the human body. The present study was conducted to develop a method of optimizing the content of antioxidants. Therefore, we initiated the in vitro culture of goji plants from meristematic apexes of plants harvested from 30-day-old germinated seeds generated from the substrate septic consisting of peat mixed with perlite. The culture medium used was Murashige & Skoog supplemented with 0.5 mg / l IBA (indolyl butyric acid) and 0.5 mg / l BA (benzyladenine). Plants containers were exposed to light of different colors fluorescent tubes from Osram company and the following lengths wave: white; blue- 473 nm; green- 533 nm; yellow 580 nm; red-680 nm. Chamber growth temperature was $23^0C \pm 2^0C$, between light and $20^0C \pm 2^0C$, during hours of darkness, photoperiod of 16 hours light and 8 hours darkness. At the age of 60 days these vitro seedlings have undergone physiological and biochemical analyzes. Red light, green and yellow stimulates growth length of vitro seedlings and blue light produce small and stocky plants and even growth slowing. Blue light has increased the total content of assimilating pigments and content of antioxidants (phenols, flavonoids) were increased by the yellow and white light. The antioxidant capacity was also higher in vitroplants grown in blue light and lowest in red light.

Key words*: antioxidant capacity, fluorescent tubes, Lycium barbarum L., phenols.*

INTRODUCTION

Since ancient times plants have been acknowledged to be the source of medicines for human healthcare. Antioxidant property is one of the most valuable phytomedicinal values in plant to be used as natural remedies. Through various scientific findings, it has been proven that the consumption of antioxidants is useful in the prevention and treatment of a number of disorders related to oxidative damages. In food industry, the synthetic antioxidants have been frequently used to be incorporated in the food products as a measure to control lipid oxidation reaction. For instance, butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate and tertiary butyl hydroquinone are among the most widely used synthetic antioxidants. Nevertheless, the usage of those synthetic products has raised some questions pertaining to their possible risks of toxicity.

With regard to antioxidant properties, *Lycium barbarum* is one of the herbs that have been recognized to be the source of this phytomedicine. It belongs to the family of *Solanaceae* and found wild around the hills in the Ningxia region of China, and in remote areas of central China near inner Mongolia. Therefore, this has initiated the urge for the searching and discovery of antioxidant substances from natural sources likewise in medicinal plants. *L. barbarum* polysaccharide (LBP) is reported to have efficient immunomodulatory properties and inhibiting tumour growth. It has been recognized to be an

exotic super fruit and very often included in foods especially in preparing Chinese tonic soups. Another interesting finding reported by Li and his contributors (2007) had proven that the polysaccharide of *L. barbarum* is effective in counter-acting oxidative stress damage. In addition to this, Feng and his contributors (2001) indicated that *L. barbarum* is also effective in providing protection for retina from oxidant injury in diabetic subjects.

As a signal and energy source, light is one of the most important environment factors for plant growth and development. Compared with light intensity and photoperiod, light quality shows much more complex effects on plant morphology and physiology. Specific spectrum stimulates different morphological and physiological responses. Red light (R) and blue light (B) absorbed by photosynthetic pigments are more effective than other wave lengths (Pfündel and Baake, 1990). It is well known that red light influences stem elongation, root to shoot ratio, chlorophyll content, photosynthetic apparatus (Appelgren, 1991; Aksenovaetal., 1994; Sæba et al., 1995). Blue light causes physiological responses via phototropins, including phototropism hypocotyl elongation, leaf expansion, stomatal opening, leaf anatomy, enzyme synthesis, chloroplast movements, and genes expression (Christie, 2007; Inoue et al., 2008; Wang et al., 2009). Red light is the primary light source affecting biomass production and elongation through the phytochrome photoreceptor (Sager and McFarlane, 1997). Blue light also affects photomorphogenic responses (e.g., regulation of leaf flattening and compact appearance) through phototropins and cryptochromes acting in an independent and/or synergistic manner with the phytochromes (de Carbonnel et al., 2010; Kozuka et al., 2013).

There is no information available regarding the relationship between the light absorbed by *L. barbarum* plants and the mechanisms underlying the physiology and secondary metabolism under the influence of different light spectra. Secondary metabolites are formed in order for the plant to overcome potential stressful conditions. In plant tissues such as stems and leaves, the secondary metabolite synthesis can change due to environmental, physiological, biochemical and genetic factors (Wink, 2010; Zhao et al., 2005) with light being one of the most influential factor (Kopsell et al., 2004; Kopsell and Sams, 2013). One of the main groups of secondary metabolites is the phenolic group. This group is among the most ubiquitous groups of secondary metabolites in the plant kingdom and represents an example of metabolic plasticity enabling plants to adapt to biotic and abiotic environmental changes (Wink, 2010). Their concentration depends on season and varies at different stages of growth and development (Seigler, 1998; Wink, 2010). Phenolics are pigments exhibiting radical scavenging activity, as well as protective activity against fungi, bacteria, viruses and insects (Lattanzio et al., 2006; Seigler, 1998). Physiological changes are triggered by exposure to varying wave lengths (Samuolien˙e et al., 2013). However, as goji is not locally found in România, thus the acquisition of extracts can only be made through *in vitro* seed germination whereby the seeds were obtained from the imported dried goji berries. Thus, the *in vitro* germination of the species was carried out accordingly beforehand to produce *in vitro* seedlings to serve as the source of plant materials.

The objective of the present study was to determine the efects of diferent wavelengths of fluorescent tubes by investigating the antioxidant capacity, content of assimilating pigments and content of antioxidants (phenols, flavonoids). The results of this study would be used to give guidance on light colour sources design for goji cultivation in a controlled environment.

MATERIALS AND METHODS

1.1. Plant material, growth conditions and light treatments

The culture substrate used in the in vitro cultures experiments consisted of basal medium (MB) Murashige - Skoog (1962) (MS) agar medium, which consisted of macronutrients, Fe EDTA and trace elements, mineral mix according to the original recipe, but with increased addition of vitamins: pyridoxine HCl, nicotinic acid and thiamine HCl (1 ml / l each of the original recipe to where indicated 0.5 mg / l), to which was added m-inositol 100 mg / l, sucrose 30 g / l and agar - agar 10 g / l; this

basic medium (MB) were added as growth regulators 0.5 mg / l IBA (indolyl butyric acid) and 0.5 mg / l BA (benzyladenine). Before autoclaving of the culture medium, the pH value was adjusted to 5.5 with HCl or NaOH, depending on the basicity or acidity of the final medium. For autoclaving, 15 ml of the medium were placed into the clear glass culture containers that were temperature resistant, 8 cm height and 4 cm diameter. After portioning the culture medium, the culture containers were filled with aluminium foil. Sterilization of the containers and culture media was performed by autoclaving at 121 ° C for 21 minutes.

The plant material used for the initiation of vitro cultures was the meristematic apexes of goji plants with a length of about 1 cm and 2-3 leaf primordia, harvested from seedlings regenerated from zygotic embryos that have sprouted from 30 days old seeds that were germinated on a septic substrate consisting of peat mixed with perlite. After cooling of the culture medium, was performed the inoculation of explants on culture medium and then the inoculated containers were transferred into growth chamber that were placed on racks exposed to a temperature ranged from 23°C ± 2°C, in the light regime, 20°C ± 2°C, during darkness and a photoperiod of 16 h light / 24h. Fluorescent tubes emitting coloured light (Osram company) were used, length 590 mm, Ø 26 mm, 120 lux light intensity and the colour varies depending on wavelength: 473 nm blue; green- 533 nm; yellow 580 nm; red-680 nm; white 380-760 nm (used as a control in our experiment).

1.2. Quantification of phenols, flavonoids, assimilating pigments content, antioxidant capacity and fluorescence

Chlorophyll fluorescence was evaluated by measuring ϕPSII on 5 leaves per treatment. ϕPSII was measured at normal light regime.

Assimilatory pigments were measured with a non-destructive portable chlorophyll content meter (CCM 200 Plus, Opti-Sciences Ltd.) that measures optical absorbance, the readings being expressed as CCI units.

Extracts were prepared by macerating 5 g of ground fresh plant in 95 ml of distilled water or 30% w/v ethanol for 24 hours. Total phenolic content was assessed using the Folin Ciocalteu reagent method, by spectrophotometric readings at 760 nm of the colour of incubated extracts (Makri, 2008).

Total flavonoid content was determined according to the method described by Makri (2008), by evaluating the absorbance at 510 nm of extracts reacted with 5% $NaNO_2$ and 10% $AlCl_3$.

In this study, the method of DPPH assay was used and the comparative antioxidant assessments were conducted mainly to compare the antioxidant capacity of this species by exposing the vitro plantlets to light coloured fluorescent tubes of different wavelengths. Free radical scavenging activity was determined in the DPPH method. In such assay, DPPH is best corresponds to a model radical which will be reduced by antioxidant properties derived from the extracts. DPPH is a relatively stable free radical and can be reduced by electron-rich radical scavengers from medicinal plant extracts. Free radical scavenging activity was determined in the DPPH method (Herald, 2012), measuring the decolouration of DPPH solution reacted with extracts at 515 nm for 3 hours.

1.3. Statistical analysis

The statistical analyses conducted were represented by analyses of variance among treatments and the Tukey test at $p<0.05$, the results being expressed as means and standard errors.

RESULTS AND DISCUSSIONS

The research made by us on the goji in vitro plants, cultivated on Murashige-Skoog medium culture exposed 60 days in light colored fluorescent tubes have allowed revealing that the lighting regime favored synthesizing antioxidants. As can be seen in figure 3 and 4 yellow light treatment determines the highest amount of polyphenols, followed by the quantity produced in vitro plants grown under white light where flavonoid synthesis is the biggest. Comparing the results of the extract obtained in the control variant of vitroseedlings illuminated with fluorescent white tubes with those obtained from the vitroseedlings illuminated with fluorecent colored tubes we found that in terms of content of assimilating pigments, vitroseedlings grew in blue light

contain the highest amount of assimilating pigments and remains the same in red and yellow light and decreases in green light (Figure 1).

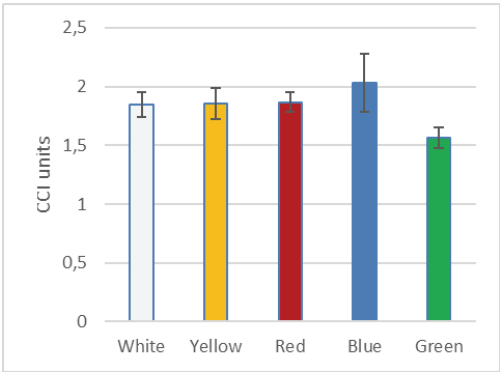

Figure 1. Assimilatory pigments in goji plants (*-significant differences from control plants at $p < 0.05$).

A high productivity with red light treatment is known (Darko et al., 2014), and is described in other species such as *Lycopersicum esculentum* (Suyanto et al., 2012). The amount of carotenoids is related to green and blue light treatments (0.4 to 0.48 mg / g). These pigments absorbing especially at these wavelengths (Govindjee et al., 1960).

From a physiological standpoint, the values of the chlorophyll fluorescence PSII and Fv/Fmϕ were not significantly altered suggesting a minimal impact on plant physiological condition as a result of the use of colored light sources. The difference in content of chlorophyll expressed through light absorption by leaves wasn't statistically significant in vitroseedlings illuminated to all variants of light (Figure 5).

In this study, the antioxidative proprieties of *L.barbarum* were measured spectrophotometrically by DPPH assay. DPPH (diphenyl picrylhydrazyl) assay is the most widlely reported method for screening of antioxidant activity of many plant drugs. The basis of DPPH assay activity relies on the concept of delocalisation of spare electron over the molecule of DPPH. Upon addition of substance with hydrogen donor property, DPPH will undergo reduction process and the colour of solution changes from deep violet to yellow. These disappearance of DPPH radical chromogens reflects the presence of antioxidant in the tested extracts (Molyneux, 2004). The colour changes after reduction can be quantified by its decrease of absorbance at wave length 517 nm. The quantified reduction of absorbance reflected the reduction capability of DPPH radical by antioxidative agents, namely the tested extracts.

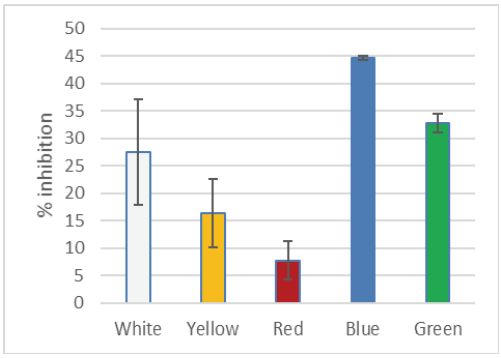

Figure 2. Free radical scavenging activity in goji plants (*significant differences from control plants at $p < 0.05$).

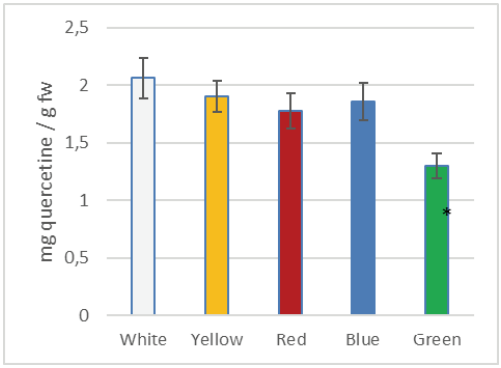

Figure 3. Total flavonoid content in goji plants (*significant differences from control plants at $p < 0.05$).

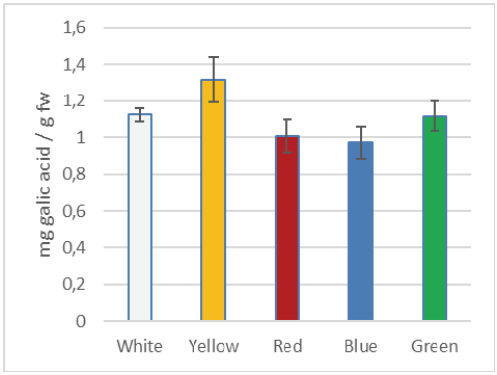

Figure 4. Total phenolic content in goji plants (*significant differences from control plants at $p < 0.05$).

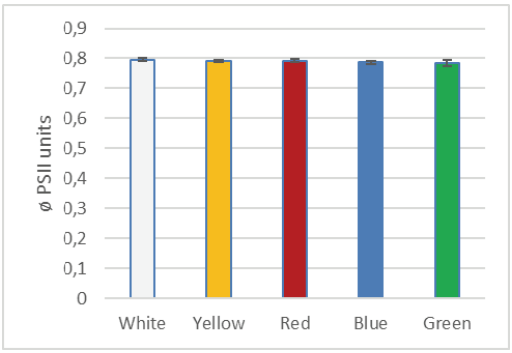

Figure 5. Chlorophyll fluorescence in goji plants (*significant differences from control plants at $p < 0.05$)

DPPH assay method is preferred due to its simplicity, convenience and time-saving properties. The concept involved in this method is particularly focusing on the ability of the tested extract to scavenge a stable DPPH free radical. The DPPH assay method is preferred due to its convenience which can evaluate the activities in a relatively short duration of time (Molyneux, 2004).

The extract which functions as antioxidant reacted with DPPH, chemically named after 1-diphenyl-2-picrylhydrazyl thus resulted in the formation of 1-diphenyl-2-picrylhydrazine. This conversion is visible by the change of colour from deep violet to pale yellow or almost colourless.

Osman and his collaborators have shown in a study done in 2012 that the leaves and stems of the two months old *L. barbarum* were the most optimum extract with the greatest antioxidant activities. After assessing the antioxidant properties of *L. barbarum* by DPPH assay, we have found that almost all variants of vitroseedlings possessing considerable activity of antioxidant with the two months old leaf and stem. Nevertheless, among all the extracts assessed, the most optimum extract which possesses optimum antioxidant activity was seen to be in the leaf and stems *L. barbarum* illuminated with blue light which was found to be higher (44.6%) than that of vitroplants illuminated with others colours of light (Figure 2), especialy with red light (7.73%).

After 60 days of vitro culture, if comparing vitro seedlings in terms of growth, we can see that the red light, green and yellow stimulates increase in length of vitro seedlings and blue light produce small and stocky and even slowing growth of plants (Figure 6).

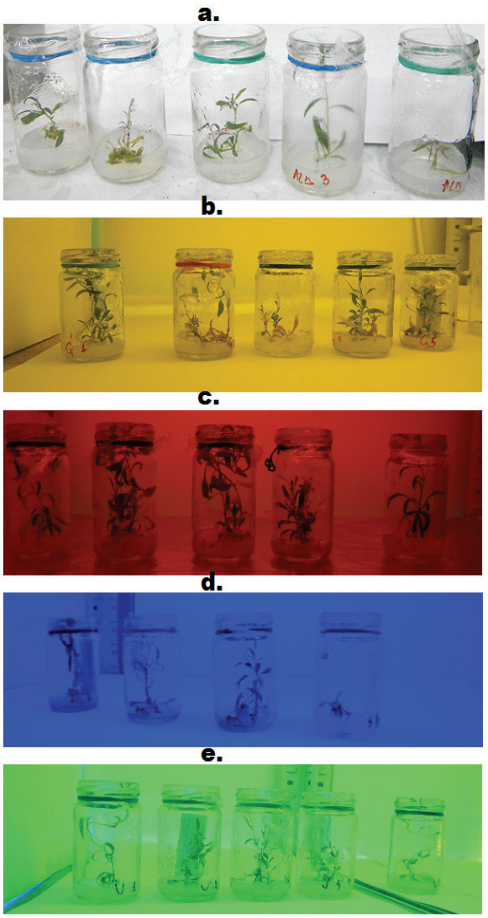

Figure 6. Aspects of *Lycium barbarum* vitro seedlings in the 60th day after initiation of vitro cultures exposed to fluorescent tubes of different colors: a- white; b-yellow; c-red; d- blue; e- green.

CONCLUSIONS

The results from the present study clearly suggested that *Lycium barbarum* plants may serve as an excellent source of antioxidants and bearing health promoting factors not only by the consumption of the berries, but also by optimally consuming leafs and stems of the plants.

These findings are also beneficial in providing a useful benchmark in determining the optimum colour of light for this species at its best, most promising antioxidative effects.

The results of this study would be used to give guidance on light colour sources design for goji cultivation in a controlled environment.
In addition, it may serve as a basis for even more extensive researches to be done on this species with the focus of interest directed towards its phytomedicinal values, hence would be incorporated into health-promoting supplementary foods and pharmaceutical preparations.

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REFERENCES

Aksenova, N. P., Konstantinova, T. N., Sergeeva, L. I., Machá˘cková, I., and Golyanovskaya, S. A., 1994. Morphogenesis of potato plants in vitro. I. Effect of light quality and hormones. J. Plant Growth Regul. 13, 143–146. doi: 10.1007/BF00196378.

Appelgren, M., 1991. Effects of light quality on stem elongation of Pelargonium invitro.Sci.Hortic.45,345–351.doi:10.1016/0304-4238(91)90081-9.

Christie, J. M., 2007. Phototropin blue-light receptors. Annu. Rev. Plant Biol. 58, 21–45.doi:10.1146/annurev.arplant.58.032806.103951

Darko, E., Heydarizadeh, P., Schoefs B., Sabzalian M.R., 2014. Photosynthesis under artificial light: the shift in primary and secondary metabolism. Phil. Trans. R.Sc. B. 369: 2013243.

de Carbonnel, M., Davis, P., Roelfsema, M.R.G., Inoue, S.-i., Schepens, I., Lariguet, P.,Geisler, M., Shimazaki, K.-i., Hangarter, R., Fankhauser, C., 2010. The Arabidopsis PHYTOCHROME KINASE SUBSTRATE2 protein is a phototropin signaling element that regulates leaf flattening and leaf positioning. PlantPhysiol. 152, 1391–1405.

Feng HJ, Jie YC, Yun PL, Gui ZL., 2001. The Effect of *Lycium barbarum* (Wolfberry) on Antioxidant Activity in the Retina of Diabetic Rats. Available from:http://www.sunlifegoji.com/images/stories/files/wolfberry_eye.pdf .

Govindjee, R., Rabinowitch, E., Thomas M.B., 1960. Inhibition of fotosynthesis in certain algae by extreme red light.Biophys J.1, 91-7.

Herald T.J., Gadgil P., Tilley M., 2012. High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. Journal of the Science of Food and Agriculture. 92(11).2326-31.

Inoue,S.I.,Kinoshita,T.,Matsumoto,M.,Nakayama,K.I.,Doi,M.,andShimazaki, K. I., 2008. Blue light-induced autophosphorylation of phototropin is a primary step for signaling. Proc. Natl. Acad. Sci. U.S.A. 105, 5626–5631. doi: 10.1073/pnas.0709189105.

Kopsell, D.A., Kopsell, D.E., Lefsrud, M.G., Curran-Celentano, J., Dukach, L.E., 2004.Variation inlutein, _-carotene, and chlorophyll concentrations among Brassicaoleracea cultigens and seasons. HortScience 39, 361–364.

Kopsell, D.A., Sams, C.E., 2013. Increases in shoot tissue pigments, glucosinolates,and mineral elements in sprouting broccoli after exposure to short-durationblue light from light emitting diodes. J. Am. Soc. Hortic. Sci. 138, 31–37.

Kozuka, T., Suetsugu, N., Wada, M., Nagatani, A., 2013. Antagonistic regulation ofleaf flattening by phytochrome and phototropin in Arabidopsis thaliana. PlantCell Physiol. 54, 69–79.

Lattanzio, V., Lattanzio, V.M.T., Cardinali, A., 2006. Role of phenolics in theresistance mechanisms of plants against fungal pathogens and insects. In:Imperato, F. (Ed.), Phytochemistry: Advances in Research, Research Signpost.Trivandrum, India, pp. 23–67.

Li XM, Mab YL, Liu XJ., 2007. Effect of the *Lycium barbarum* polysaccharides on age-related oxidative stress in aged mice. J Ethnopharmacol. 2007;111:504–511.

Makri, O., Kintzios S., 2008. Ocimum sp. (Basil): Botany, cultivation, pharmaceutical properties and biotechnology, Journal of Herbs, Spices & Medicinal Plants, 13(3): 123-150.

Molyneux, P., 2004. The use of free radical diphenylpicryl-hydrayl (DPPH) for estimating antioxidant activity. Songklanarin J. Sci. Technol. 2004;26:211-219.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tabaco tissue culture. Physiol. Plant, 15: 473-497.

Osman, N.I., Awal A., Sidik, N.J. and Abdullah, S., 2012. Antioxidant activities of in vitro seedlings of Lycium barbarum (goji) by diphenyl picrylhydrazyl (DPPH) assay, Research international journal of Pharmacy and Pharmaceutical Sciences , issn- 0975-1491, vol 4, suppl 4.

Pfündel, E., and Baake, E., 1990. A quantitative description of fluorescence excitation spectra in intact bean leaves greened under intermittent light. Photosynth.Res.26,19–28.doi:10.1007/BF00048973.

Sæba, A., Krekling, T., and Appelgren, M.,1995. Light quality affects photosynthesis and leaf anatomy of birch plantlets in vitro. Plant Cell Tissue OrganCult.41,177–185.doi:10.1007/BF00051588.

Sager, J.C., McFarlane, J.C., 1997. Radiation. In: Langhans, R.W., Tibbits, T.W. (Eds.),Plant Growth Chamber Handbook. North Central Region Research Publication,Iowa State University Press, pp. 1–29.

Samuolien˙e, G., Brazaityt˙e, A., Sirtautas, R., Vir˘sil˙e, A., Sakalauskait˙e, J.,Sakalauskien˙e, S., 2013. LED

illumination affects bioactive compounds inromaine baby leaf lettuce. J. Sci. Food Agric. 93, 3286–3291.
Seigler, D., 1998. Plant Secondary Metabolism. Kluwer Academic publishers, Dordrecht, p. 759.
Suyanto, H., Rupiasih N. N., Dewi, H., .2012. Influence of light wavelengths on growth of tomato. Jurnal Bumi Lestari (Journal of Environment), Volume 12 No. 2.
Wang,H.,Gu,M.,Cui,J.,Shi,K.,Zhou,Y.,andYu,J.(2009).Effects of light quality on CO2 assimilation, chlorophyll-fluorescence quenching, expression of Calvin cycle genes and carbohydrate accumulation in Cucumis sativus. J. Photochem. Photobiol. B Biol.96,30 37.doi:10.1016/j.jphotobiol. 2009.03.010.
Wink, M., 2010. Functions and Biotechnology of Plant Secondary Metabolites.Annual Plant Reviews, 3. Wiley-Blackwell, Oxford, p.410.
Zhao, J., Davis, L.C., Verpoorte, R., 2005. Elicitor signal transduction leading toproduction of plant secondary metabolites. Biotechnol. Adv. 23, 283–333.

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PRELIMINARY RESULTS REGARDING THE TESTING OF TREATMENTS WITH LIGHT-EMITTING DIODE (LED) ON THE SEED GERMINATION OF *ARTEMISIA DRACUNCULUS* L.

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Abstract

Regardless of the food diet (raw, vegan, vegetarian, carnivore), consumption of sprouted grains of different plants, especially vegetables, is encouraged, due to their therapeutic, regenerative and alimentary benefits. Seeds and sprouts have in their composition 20 - 30 times higher amounts of enzymes, minerals and nutrients than mature plants. Artemisia dracunculus L. *(tarragon) is a medicinal and aromatic perennial herb which belongs to the Asteraceae family. It was chosen for the experiment due to its benefits, the essential oil of tarragon displaying antibacterial, antioxidant, antihyperglicemic activity. The plant is also known for enhancing digestion and having a pleasant spicy aroma.*

Studies show that light-emitting diode (LED), light exposure improves the quality of the growth, metabolism and accumulation of bioactive substances. As a result, the effect of different LED light colors on Artemisia dracunculus L. *(tarragon), seed germination was studied. The Artemisia dracunculus* L. *(tarragon), seed were exposed to white, red, blue or green LED light, over a photoperiod of 16 hours, for seven days. The red LED exposure determined a higher degree of germination and longer hypocotil height. The blue LED exposure determined a better development of cotyledons.*

The experimental results obtained contribute with useful information in order to establish a method of easily growing fresh sprouts of Artemisia dracunculus L. *(tarragon), for therapeutic and culinary use, under exposure to a low-carbon, power saving and inexpensive lightning means.*

Key words: *Artemisia dracunculus* L., *LED, seed germination.*

INTRODUCTION

Artemisia dracunculus L. is a perennial herb belonging to the *Asteraceae* family, native to Europe, southern Russia in the area of the Caspian Sea, China and western and central North America. Stems are ascending and richly branched. The plant displays lanceolate, alternate, entire leaves on a plant 45-75 centimeters high (Hemphill and Hemphill, 1984). Under certain conditions, the plant bears small, greenish-white, sterile flowers in terminal panicles. Under typical north temperate zone conditions these are seldom seen. The root is thin, branched and deep up to 30 - 40 cm. Germinative capacity lasts 2 - 3 years. *Artemisia dracunculus* L. is extremely popular as a culinary herb due to its licorice or anise flavour, which adds a special, refreshing taste to sauces and main dishes (Fox, 1970). The leaves and young stems are used for flavoring various dishes and pickles. In the food industry it is used for seasoning of canned meat and vegetables in different marinades, cucumbers and pickles in vinegar (Hassanzadeh et al., 2016). Aerial parts of the plant have traditional therapeutic use in human medicine. The active principles are eupeptic, ensuring normal digestion (Kalantari et al., 2013). Empirical medicine assigns diuretic properties to this plant. The plant is recommended in treating liver and kidney diseases, ascites, appetite and digestion stimulation, in rheumatism, headaches, toothaches (Ribnicky et al., 2004).

French tarragon does not produce viable seed, while Russian tarragon does (Fox, 1970). Consequently, purchased tarragon seed will, of necessity, be of the Russian variety, which is a taller (to 150 centimeters), coarser, weedy plant. The result of this development of a non-seeding plant is that French tarragon must be

reproduced vegetative, without seeds (Hartmann and Kester, 1983). Vegetative propagation differs from seed propagation in that vegetative plant parts, such as stems, roots, bulbs, and leaves are used, rather than seed (Adriance and Brison, 1955). Traditionally, this has been done by dividing existing plants. The process is inexpensive, rapid, simple, and does not require specialized techniques like grafting or budding (Hartmann and Kester, 1983). Due to the necessity of asexual reproduction, French tarragon remains a premium-priced herb plant. The strong and aromatic smell of some species of *Artemisia* genus is due mainly to high concentrations of volatile terpenes, constituents of their essential oils, especially in leaves and flowers. The chemical composition of essential oils from the *Artemisia* genus has been extensively studied in several species from around the world. (Balza and Towers, 1984; Vienne et al., 1989; Venskutonis et al., 1996). Many studies have shown that *Artemisia* species display significant intraspecific variations in the terpene constituents of their essential oils. In some cases, the variation in the volatile components of these plants may occur during plant ontogeny or growth at different altitudes. The quality and yield of essential oils from *Artemisia* species is influenced by the harvesting season, fertilizer and pH of soils, the choice and stage of drying conditions, the geographic location, chemotype or subspecies, choice of plant part or genotype, or extraction method (Abad et al., 2012).

Variants of *Artemisia dracunculus* L. possess anti-diabetic compounds (Eisenman at al., 2014). There are studies that prove the antyhyperglicemian effect of *Artemisia dracunculus* L. The botanical extract of *Artemisia dracunculus* L. improves insulin action (Obanda et al., 2014). In experiments carried on mice, *Artemisia dracunculus* L. polyphenols complexed to soy protein displayed enhanced bioavailability and hypoglycemic activity on the subjects (Ribnicky et al., 2004; Ribnicky et al., 2006).

There are many studies that present the phytochemical benefits of growing plants under LED exposure. For example, in a study on the antioxidant activity of pea (*Pisum sativum* L.) seedlings, the contribution of red light to significant β-carotene expression and antioxidant activity for nutrition and health benefits and blue light to seedling weight and chlorophyll induction of radiated pea (*Pisum sativum* L.), seedlings are emphasized (Wu et al., 2007). Studies on algae show that the biomass and fatty acid production was improved under LED light stress (Choi et al., 2015; Zhao et al., 2013). LED lights were found to increase bioactive substances at low energy costs in culturing fruiting bodies of *Cordyceps militaris* (Yi et al., 2014). LED treatment's enhancing effects were also studied on basil, *Ocimum basilicum* (Bantisa et al., 2016), leaf lettuce, *Lactuca sativa*, (Chang and Chang, 2014, Chen et al., 2014).

LED treatment was also used, along with magnetic field and laser light field, modulated at audio frequencies, on colonies of *Alternaria alternata*, for the purpose of increasing the inactivation and inhibition percentage over these fungi (Niculiță et al., 2008; Dănăilă-Guidea et al., 2008; Ristici et al., 2008). Light irradiation with laser diodes was used also in experiments on vegetables, such as tomato seeds, *Solanum lycopersicum* L., (Niculiţă et al., 2006) or on seedlings of some annual ornamental species (Dănăilă-Guidea et al., 2011). Results show that the germination rate increases for irradiated seeds. In our experimental researches, we chose to use light-emitting diode (LED), exposure to determine seed germination of *Artemisia dracunculus* L. (tarragon).

MATERIALS AND METHODS

For the experiment there were used seeds of *Artemisia dracunculus* L. (tarragon), obtained by conventional horticultural methods. The seeds were in their dormant phase and procured from commercial producer.

Microbial contamination is a major issue for the *in vitro* culture, which causes most of the germplasm losses. Aseptizing the surface of the seeds of *Artemisia dracunculus* L. (tarragon) had the following stages (Badea and Săndulescu, 2001; Cachiță – Cosma et al., 2004):

- immersion of the seeds in a solution of ethylic alcohol ($C_6H_{12}O_6$) 70% and keeping it for 1 minutes;
- successive washing of the seeds, three times for 10 minutes, in aseptized distilled water, in order to remove traces of the aseptizing agent

which, if not removed, would be harmful to the development of the inoculums.

After the process of aseptisation, the seeds of *Artemisia dracunculus* L. (tarragon), were placed in sterile, covered, transparent plastic recipients, on sterile gauze, procured from pharmacy. A number of 10 seeds of *Artemisia dracunculus* L. (tarragon), was placed in each recipient.

The necessary nutrients for the seed germination and plant development were provided by a solution of *Vitaflora Universal* procured from commerce. *Vitaflora Universal* contains the following nutrients: N 7.000%; P_2O_5 3.000%; P 1.300%; K_2O 5.000%; K 4.200%; B 0.014%; Cu 0.007%; Fe 0.020%; Mn 0.010%; Mo 0.001%; Zn 0.007%; Mg 0.010%.

For the first day of inoculation, a dilution of 1/10 of *Vitaflora Universal* solution was used. For the rest of the days of the experiment, a dilution of 1/20 was used. The nutritive solution was diluted in aseptized distilled water. Five milliliters of solution were poured over the seeds every two days.

The LED bulbs were also procured and installed on different lamps, each correspondding to a recipient inoculated with *Artemisia dracunculus* L. seeds. The recipients were placed at 15 cm distance from the LED bulb. The LED bulbs procured had the specifications from Table 1.

Table 1. The LED bulb's specifications

Light colors / Technical specifications	Warm white	Red	Blue	Green
Power (W)	5	5	5	5
Tension (V)	220	220	220	220
Wavelength (nm)	470-640	640	470	525
Color temperature (K)	3000	3000	3000	3000
Light flux (lm)	200	200	200	200
Incandescent bulb equivalent (W)	20	20	20	20
Fascicle angle (°)	120	120	120	120
Dimensions (mm)	80*40	80*45	80*45	80*45
Life span (h)	15000	15000	15000	15000
Screw base	E27	E27	E27	E27

The seeds of *Artemisia dracunculus* L. (tarragon), were incubated at 23°C ± 2°C, with a photoperiod of 16 h and a temperature of 20°C ± 2°C during the dark period of 8 h.

The biomass of *Artemisia dracunculus* L. was dried in oven for 16 h at 105°C (Windham et al., 1987).

The experiments were repeated 2 times. Each experimental variant consisted in three repetitions. The measurements were done for each individual inoculate.

For statistical procedures, average value and standard deviation were determined for the analyzed parameter.

RESULTS AND DISCUSSIONS

The analysis methods of the studied biological material consisted of quantitative elements. As a result, the monitoring methods in order to carry out the testing of treatments with light-emitting diode (LED), on the seed germination of *Artemisia dracunculus* L. involved both, the morphometric measurements of the number of germinated seeds, hypocotyls and cotyledons, and the weight measurements of the fresh weight and dry weight sprouts. The white LED treatment was used as control experimental variant.

The percentage of germinated seeds under red LED treatment had a medium value of 90% ± 5%, higher than the control experimental variant, treated with white LED, which had a medium percentage of 80% ± 5% germinated seeds. The blue LED treatment led to a medium percentage of 50% ± 5% germinated seeds, while the green LED treatment led to a medium percentage of 40% ± 5% germinated seeds of *Artemisia dracunculus* L. (Figure 1).

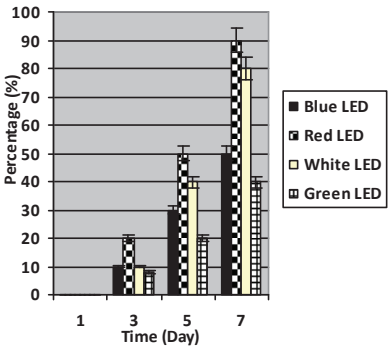

Figure 1. Medium values of the percentage of germinated seeds of *Artemisia dracunculus* L. under the influence of light-emitting diode (LED) treatments

The hypocotyl length of the *Artemisia dracunculus* L. seedlings under blue LED treatment had a medium value of 6 ± 0.34 cm,

higher than the red LED treatment variant, which had a medium percentage of 5 ± 0.34 cm. The white LED and green LED treatment led to a medium hypocotyl lenght of 4 ± 0.34 cm (Figure 2).

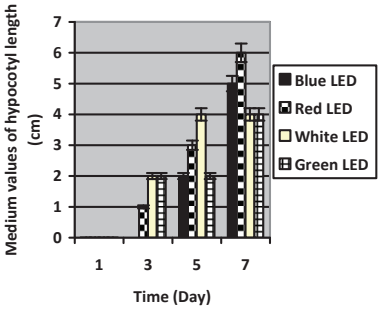

Figure 2. Medium values of the hypocotyl length at *Artemisia dracunculus* L., obtained under the influence of light-emitting diode (LED) treatments

The cotyledon length of the *Artemisia dracunculus* L. seedlings under blue LED treatment had a medium value of 0.6 ± 0.03 cm, higher than the red, green and white LED treatment variants, which had a medium percentage of 0.5 ± 0.03 cm (Figure 3).

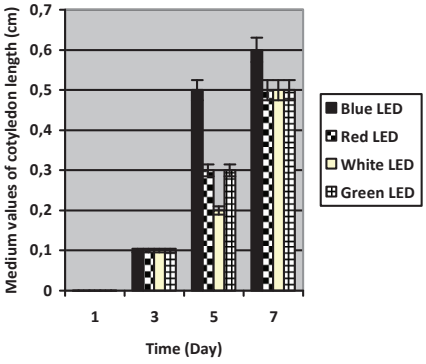

Figure 3. Medium values of the cotyledon length at *Artemisia dracunculus* L., obtained under the influence of light-emitting diode (LED) treatments

Traditional light technologies like high-pressure sodium, metal halide or fluorescent lights produce distinct light spectrum and wavelengths that are effective, but not necessarily optimized for plant growth. LEDs, on the other hand, have the flexibility to deliver specific wavelength combinations and lighting strategies that may yield faster and more favorable results for plant growers and researchers (Choi et. al, 2015, Yi et. al, 2014). LEDs retain their spectral profiles when dimmed, providing consistency in spectrum as researchers adjust intensities throughout the light cycle. Unlike fluorescent bulbs, LED output remains vastly more stable as chamber temperature decreases, providing consistency in spectrum for a wide range of temperatures (Chen, 2014). The experimental results obtained, by the testing of treatments with light-emitting diode (LED) on the seed germination of *Artemisia dracunculus* L., are in agreement with the previous studies, which state that LED treatments induce greater biomass production (Figure 4).

Figure 4. Sprouted of *Artemisia dracunculus* L. seeds on sterile gauze

The influence of the LED treatments over the *Artemisia dracunculus* L. biomass quantity was determined through the quantitative weighing of the fresh vegetal material (fresh weight) and of the dry vegetal material (dry weight). The red LED treatment determined a higher medium quantity of biomass than the blue, green and white LED treatments, represented by a fresh medium weight of 0.0490 ± 0.0020 g and a dry medium weight of 0.0028 ± 0.0002 g (Figure 5).

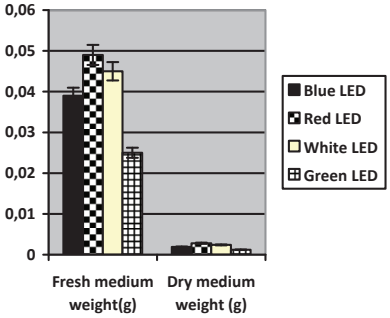

Figure 5. Fresh weight and dry weight of the *Artemisia dracunculus* L. sprouts, obtained under light-emitting diode (LED) treatment

The red LED treatment generated the best results in seed germination (90% of germinated seeds in the last day of the testing), and in hypocotyl length (the longest seedlings had a hypocotyl of 6 cm in the last day of the testing). The blue LED treatment exposure determined a better cotyledon development, cotyledons reaching 0.5 cm in length in the last days of the testing.

The green LED treatment determined poor results in both seed germination and sprouts development.

In our study, we chose to use light-emitting diode (LED), exposure to determine seed germination, due to numerous advantages (higher percentage of germinated seeds, a faster growth, longer hypocotyl size), which were proved during the experiment.

CONCLUSIONS

The monitoring of experimental research of preliminary results regarding the testing of treatments with light-emitting diode (LED), on the seed germination of *Artemisia dracunculus* L., allowed us to obtain, experimental results that indicate the conclusions:

- red LED treatment generated the best experimental results (90% percentage of germinated seeds). The red LED exposure determined a higher degree of germination and longer hypocotil height. The blue LED exposure determined a better development of cotyledons.
- the variety of *Artemisia dracunculus* L. (tarragon) displays great potential for *in vitro* studies, for therapeutical and culinary development;
- the percentage of germinated seeds of *Artemisia dracunculus* L. was higher in the case of the experimental variant which used red LED treatment;

Therefore, we studied a method of growing sprouted fresh tarragon (*Artemisia dracunculus* L.), all over the year, and obtained results for seed germination under LED exposure. The experimental results obtained contribute with useful information in order to establish a method of easily growing fresh sprouts of *Artemisia dracunculus* L. (tarragon), for therapeutic and culinary use, under exposure to a low-carbon, power saving and inexpensive lightning means.

The preliminary experimental results contribute to the completion of the necessary *in vitro* conditions for the obtaining of fresh vegetal biomass of *Artemisia dracunculus* L., both as germinated seeds and as sprouts, which can be destined to the therapeutical or alimentary consume.

REFERENCES

Abad M. J., Bedoya L. M., Apaza L., Bermejo P., 2012. The *Artemisia* L. Genus: A Review of Bioactive Essential Oils. Molecules, 17(3):2542-2566.

Adriance G. W., Brison F. R., 1955. Propagation of Horticultural Plants. 2nd edition. McGraw-Hill Book Company, Inc., NY, New York.

Badea E. M., Săndulescu D., 2001. Biotehnologii Vegetale. Ed. Fundaţia Biotech, Bucureşti.

Balza F., Towers G. H. N., 1984. Dihydroflavonols of *Artemisia dracunculus*. Phytochemistry, 23(10):2333-2337.

Bantis F., Ouzounis T., Radoglou K., 2016. Artificial LED lighting enhances growth characteristics and total phenolic content of *Ocimum basilicum*, but variably affects transplant success. Scientia Horticulturae, 198:277-283.

Cachiţă – Cosma D., Deliu C., Rakosy – Tican L., Ardelean A., 2004. Tratat de biotehnologie vegetală, Vol. I. Ed. Dacia, Cluj-Napoca.

Chang C.-L., Chang K.-P., 2014. The growth response of leaf lettuce at different stages to multiple wavelength-band light-emitting diode lighting. Scientia Horticulturae, 179:78-84.

Chen X.-L., Guo W.-Z., Xue X.-Z., Wang L.-C., Qiao X.-J., 2014. Growth and quality responses of 'Green Oak Leaf' lettuce as affected by monochromic or mixed radiation provided by fluorescent lamp (FL) and light-emitting diode (LED). Scientia Horticulturae, 172:168-175.

Choi Y.-K., Kumaran R. S., Jeon H. J., Song H.-J., Yang Y.-H., Lee S. H., Song K.-G., Kim K. J., Singh V., Kim H. J., 2015. LED light stress induced biomass and fatty acid production in microalgal biosystem, *Acutodesmus obliquus*. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 145:245-253.

Dănăilă-Guidea S., Ristici E., Livadariu O., Niculiţă P., Popa M., Ristici M., Ioniţă F., Patroi A., 2008. Effects induced by simultaneous magnetic and modulated laser fields on fungi cultures *in vitro*. Volume of communications for International Scientific Conference „Physics in Agricultural Research", POLONIA, Lublin,7-12.

Dănăilă Guidea S.M., Niculiţă P., Ristici E., Popa M., M. Ristici, Burnichi F., Drăghici M., Geicu M., 2011. The influence of modulated red laser light on seedlings of some annual ornamental species (*Dianthus caryophyllus* and *Petunia hybrida*). Roumanian Biotechnological Letters, vol.16 (6) Supplement 1, p 34-38.

Eisenman S. W., Poulev A., Struwe L., Raskin I., Ribnicky D. M., 2011. Qualitative variation of anti-

diabetic compounds in different tarragon (*Artemisia dracunculus* L.) cytotypes. Fitoterapia, 82(7):1062-1074.

Fox H. M., 1970. Gardening with herbs for flavor and fragrance. Dover Publications, Inc., NY, New York City, 289-290, 298, 302.

Hartmann H. T., Kester D. E., 1983. Plant Propagation: Principles and Practices, Fourth edition. Prentice-Hall, Inc., Englewood Cliffs, N. J.

Hassanzadeh M. K., Najaran Z. T., Nasery M., Emami S. A., 2016. Chapter 92 - Tarragon (*Artemisia dracunculus* L.) Oils. In: Preedy V. R. (Ed.), Essential Oils in Food Preservation, Flavor and Safety, Academic Press, Elsevier, 813-817.

Hemphill J., Hemphill R., 1984. Herbs, their cultivation and usage. Poole, Dorset: Blandford Press; New York, N. Y.

Kalantari H, Galehdari H., Zaree Z., Gesztelyi R., Varga B., Haines D., Bombicz M., Tosaki A., Juhasz B., 2013. Toxicological and mutagenic analysis of *Artemisia dracunculus* (tarragon) extract. Food and Chemical Toxicology, 51:26-32.

Niculiță P., Dănăilă-Guidea S., Livadariu O., Popa M., Ristici M., Ristici E., 2006. Influence of laser diode red beams on germination rate of tomato seeds, art. no. 67851M. Proceedings of the Society of Photo-Optical Instrumentation Engineers, Bellingham, USA, ROMOPTO, Eighth Conference on Optics, Proceedings Paper, Bellingham, USA, Bellingham.

Niculiță P., Dănăilă-Guidea S., Livadariu O., Ristici E., Popa M., Ristici M., Patroi A., Negoita M., 2008. Testing the effect induced by the simultaneous treatment with magnetic field and laser light field, modulated at audio frequencies, on colonies of *Alternaria alternata*. Romanian Biotechnological Letters, 13(2): 3643-1650.

Obanda D. N., Ribnicky D. M., Raskin I., Cefalu W. T., 2014. Bioactives of *Artemisia dracunculus* L. enhance insulin sensitivity by modulation of ceramide metabolism in rat skeletal muscle cells. Nutrition, 30(7-8):S59-S66.

Ribnicky D. M., Poulev A., O'Neal J., Wnorowski G., Malek D. E., Jäger R., Raskin I., 2004. Toxicological evaluation of the ethanolic extract of *Artemisia dracunculus* L. for use as a dietary supplement and in functional foods. Food and Chemical Toxicology, 42(4):585-59.

Ribnicky D.M., Poulev A., Watford M., Cefalu W.T, Raskin I., 2006. Antihyperglycemic activity of Tarralin™, an ethanolic extract of *Artemisia dracunculus* L.. Phytomedicine, 13(8):550-557.

Ristici E., Dănăilă-Guidea S., Livadariu O., Ristici M., Niculiță P., Popa M., Pătroi A., Kappel W., 2008. Modulated laser light and magnetic fields simultaneously applied on *Alternaria alternata* fungi cultures, Book of Abstracts of 9th IBWAP 2008 Constanta, ("9th International Balkan Workshop on Applied Physics"), România, Constanța, 143.

Venskutonis R., Dapkevicius A., Gramshaw J.W., Baranauskienë M., 1996. Composition of volatile constituents in Tarragon (*Artemisia dracunculus* L.) at different vegetative periods. In: Venskutonis R, Dapkevicius A., Gramshaw J. W., Baranauskienë M. (Eds.), Flavour Science, 46-51.

Vienne M., Braemer R., Paris M., Couderc H., 1989. Chemotaxonomic study of two cultivars of *Artemisia dracunculus* L.: ("French" and "Russian" Tarragon). Biochemical Systematics and Ecology, 17(5):373-374.

Windham W. R. , Robertson J. A., Leffler R. G., 1987. A comparison of methods for moisture determination of forages for near infrared reflectance spectroscopy calibration and validation. Crop Science, 27(4):777-783.

Wu M.-C., Hou C.-Y., Jiang C.-M., Wang Y.-T., Wang C.-Y., Chen H.-H., Chang H.-M., 2007. A novel approach of LED light radiation improves the antioxidant activity of pea seedlings. Food Chemistry, 101(4):1753-1758.

Yi Z.-L., Huang W.-F., Ren Y., Onac E., Zhou G.-F., Peng S., Wang X.-J., Li H.-H., 2014. LED lights increase bioactive substances at low energy costs in culturing fruiting bodies of *Cordyceps militaris*. Scientia Horticulturae, 175:139-143.

Zhao Y., Wang J., Zhang H., Yan, C., Zhang Y., 2013. Effects of various LED light wavelengths and intensities on microalgae-based simultaneous biogas upgrading and digestate nutrient reduction process. Bioresource Technology, 136:461-468.

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AMOUNT OF DNA EXTRACTED FROM DIFFERENT TISSUES OF APPLE TREES IN SPRINGTIME CAN BE USED TO DESCRIBE THE LEVEL OF INFECTION

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Abstract

Phytoplasma spread in apple trees in a systemic manner or in certain tissues. The unpredictable spreading complicates the definition of a single detection method and requires the use of alternative ones. In this paper are compared the results obtained in three plantations (Turan, Korce and Bitincke), regarding the amount of DNA extract from apples with results obtained from the use of the DAPI staining method for the detection of the infection from the leaves of the same trees. Sampling pool was 10 out of 100 trees which material from roots, stalks and wood were preserved in dark and cold conditions for 24 hours prior to DNA extraction. DNA was isolated from materials mentioned above and leaves were also analyzed by DAPI staining. The data about the amount of extracted DNA from each sample were used to compare the level of infection from different tissues categories and were related to the intensity of staining with DAPI. Results have indicated that the tissues with the highest presence of Phytoplasma spp. *in springtime, based on the amount of detected DNA are stalks; and the collection of Korce has the higher level of infection from all analysed collections. At this collection all sampled categories of tissue are infected, meaning that there is a systemic spreading of the disease, compared to the other collections where infection is located only in certain tissues.*

Key words: *DAPI staining, detection, infection, phytoplasmatic DNA, systemic spreading.*

INTRODUCTION

Apple is one of the species of fruit trees cultivated in the region of Korca. The region has about 3 thousand hectares planted with apples and an annual production potential of 13 thousand tons, which constitutes about 62% of the productive potential of the whole country (ProMali, 2014). The great economic importance of apple for the entire southeast region and beyond rises the importance of their preservation from bacterial, viral or phytoplasma diseases. Phytoplasmatic borne infections are caused by phytoplasmas, a category of organisms similar to bacteria, which lack the cell wall (Seemuller, 1990). They are intracellular parasites that grow and multiply in phloematic tissues of the plant. The spread of these organisms from one plant to another is done in two main ways; by grafting (Vindimian *et al*., 2002; Bliefernicht and Krczal, 1995; Baric *et al*., 2008; Ciccotti *et al*., 2008) or by insect vectors (Seemuller, 1990). In the salives glands of the insects phytoplasmas from the infected plant can multiply and are simultaneously spread when their rostrum is inserted in the phloem of the other plants. In winter time, these microorganisms live in the roots of apple trees; while in spring begin to invade other parts such as trunks, stems, and leaves (Schaper and Seemuller, 1982). Exhibiting symptoms of infested trees are various but the most typical is the emergence of parallel branches like a broom. The damage caused by this category of pathogens is considered important and according to data from European countries it can reduce the yield from 10-80%. The infection can cause reduction of the fruit dimensions, weight and quality of the fruit as well as the decrease of the rate of growth of the tree (Myrta A., 2012). To avoid this situation is important to keep a constant control of sanitary conditions in the plantation. This is achieved through various methods; however difficulties arise because phytoplasmas cannot be cultured in vitro like bacteria. One preliminary detection technique is DAPI staining but the accuracy of the method depends on the number of infected phytoplasmas.

Meanwhile, molecular techniques based on PCR amplification of pathogenic genome

fragments provides more accurate and specific results (Ahrens and Seemuller, 1992; Baric, S. *et al*., 2008). The aim of this work is to use the data obtained from the DNA extraction of apples to understand the scale of phytoplasmatic infection, before the implementation of PCR molecular method.

MATERIALS AND METHODS

Plant material: Plant material was collected from three collections located in Korca district, named Bitincke, Korce and Turan, from late winter to early spring 2015. According to sampling procedure described by Rekab *et al*., 2010 were selected 10 from 100 trees. Various categories of plant tissues as roots, trunk and stems were sampled and used to extract phytoplasmic DNA; leaves from the same trees were analyzed by DAPI staining.

In total 150 samples were collected for identification by fluorescent microscopy and 450 samples to extract DNA (Table 1). The collected samples were transported to the laboratory in specific conditions; in darkness and constant temperature.

DNA Extraction: was based on Kirkpatrick *et al.,* 1987. Phloem from the roots, trunk and stems was set apart from the rest of the plant material using a scalpel. 0.5 grams of the phloemes from each category were grinded in a mortar in the presence of grinding buffer. After double grinding, the obtained homogenate passed two centrifugation cycles after which was incubated with CTAB buffer (Doyle, J. J., and Doyle, J. L. 1990) at 60°C for 30 min. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1). After centrifugation, the aqueous layer was precipitated with a two-third volume of -20°C isopropanol and was centrifuged at 15.000 g with a microcentrifuge. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in 100 µl of water. DNA content was digested with 50µg/ml of RNase A at 37°C for 30 min followed by two extractions with chloroform/isoamyl alcohol, ethanol precipitation, and washing of the pellet with ethanol (Ahrens, U., and Semuller, E. 1992).

Measuring the quantity and quality of DNA: Measurements of absorbance were conducted in spectrophotometer, at wavelengths of 260 and 280 nm, and concentration was calculated according to Sambrook *et al*., 1989.

DAPI staining: samples were transported to the laboratory in cold boxes in 4°C and analyzed within 5 hours from the time of collection. The method used was modified from Romero J, 2001. At first we did longitudinal sections from the fixed samples, and then they were placed from sterile distilled water to 5% glutaraldehyde solution. Fixed samples were transferred to fresh 0.1 M phosphate buffer pH 6.9. The last stage included the Thermo Fisher Scientific DAPI staining 1×working solution, which makes phytoplasmas visible. Prepared preparations were observed with fluorescent microscope with 10x, 40x and 100x magnifications.

Figure 1. Symptomatic plants in Turan plantation.

Table 1. The number of samples collected for DNA analysis

Plantations	Sample categories		
	Root	*Trunk*	*Stalks*
Bitincke	50	50	50
Korce	50	50	50
Turan	50	50	50

RESULTS AND DISCUSSIONS

Two main methods were used to verify the presence of phytoplasma infection in apple collections of Korca region in South - Eastern Albania, the quantitative analysis of DNA extracted from roots, trunk and stems and DAPI staining of the leaves. Table 2 describes

the results of DNA extraction only for one plantation (Korce). It is processed in the same way for 2 other plantations.

Table 2. The quantity and quality of DNA extracted from different categories of plant material, for Korca plantation.

Nr	Category	Quality OD 260 / OD 280	Quantity (ng/µl)	Status
1	**Stalks**	**2**	**130**	Contaminated
	Trunk	1.2	100	Healthy
	Root	1.3	90	Healthy
2	**Stalks**	1.2	90	Healthy
	Trunk	1.2	50	Healthy
	Root	**1.5**	**100**	Contaminated
3	**Stalks**	**1.5**	**100**	Contaminated
	Trunk	1.2	70	Healthy
	Root	1.3	90	Healthy
4	**Stalks**	**1.8**	**100**	Contaminated
	Trunk	1.3	80	Healthy
	Root	1.4	90	Healthy
5	**Stalks**	1.2	90	Healthy
	Trunk	1.2	50	Healthy
	Root	**1.5**	**100**	Contaminated
6	**Stalks**	1.2	60	Healthy
	Trunk	**2**	**100**	Contaminated
	Root	**1.8**	**160**	Contaminated
7	**Stalks**	**1.7**	**170**	Contaminated
	Trunk	1.4	90	Healthy
	Root	1.3	40	Healthy
8	**Stalks**	1.4	90	Healthy
	Trunk	**1.7**	**220**	Contaminated
	Root	1.3	40	Healthy
9	**Stalks**	**1.62**	**130**	Contaminated
	Trunk	**1.5**	**100**	Contaminated
	Root	1.3	90	Healthy
10	**Stalks**	1.2	80	Healthy
	Trunk	**2**	**120**	Contaminated
	Root	**1.5**	**100**	Contaminated

Extraction of DNA was made under the protocol of enriching the sample with phytoplasmas, which aims to isolate the DNA of phytoplasmas and eliminate the maximum amount of plant DNA (this does not exclude the possibility that a small amount of the extracted DNA comes from the plant).

Table 3 describes the results on the presence of DNA at different categories of tissues from the three collections (Table 3).
At Bitincka plantation DNA was extracted from 60% of the stem samples but was not detected in root and trunk; at Turan collection 50% of the stem samples contained DNA. The situation is different in Korce plantation where DNA was detected in all three categories of samples. Considering that extraction conditions and protocol were the same for all the sampled material, we conclude that the amount of DNA could be used to determine the level of infection at various tissues. Our data have showed that the tissues with the highest presence of phytoplasmas in springtime are stalks, and the collection of Korce has the highest level of infection from all analysed collections. At this collection all sampled categories of tissue are infected, meaning that there is a systemic spreading of the disease, compared to the other collections where infection is located in certain tissues only.

Table 3. Detection of DNA collected from three collections, expressed as the percentage of positive and negative results for different categories of tissues

Plantations	**Sample categories**					
	Root		*Trunk*		*Stalks*	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Bitincke	0	100	0	100	40	60
Korce	40	60	40	60	50	50
Turan	0	100	0	100	50	50

To evaluate the credibility of these conclusions was used further analysis of samples, through DAPI staining and PCR molecular method. The results of these two techniques supported these conclusions drawn from the evaluation of the quantity and quality of DNA. (Table 4).
Pictures taken by fluorescent microscope showed that Korce plantation has the highest degree of phytoplasmatic infection, because all the samples taken were positive and the fluorescence intensity was higher compared with the two other plantations (Figure 2).

Table 4. Detection of the phytoplasmatic infection by leaves DAPI staining.

Plantations	**Positive results**
Korce	100%
Turan	80%
Bitincke	70%

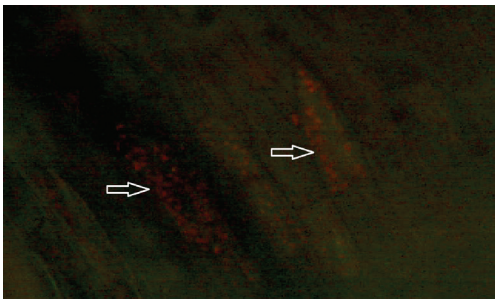

Figure 2. Fluorescent phytoplasma-like bodies in the phloem of leaf main nervure tissue.

CONCLUSIONS

The quantity and quality of DNA extracted from different tissues can be used as a preliminary indication on the presence of phytoplasmas infection at apple trees.

The quantitative analysis of DNA isolated from Turan, Korce and Bitincke plantations have showed that the tissues with the highest degree of infection were the stalks from the collection of Korce where all sampled tissue categories were infected, suggesting a systemic spread of the disease, compared to other collections where infection was located only in certain tissues.

Fluorescent microscopy also showed that Korce's collection had the highest degree of phytoplasmatic infection according to DAPI staining.

Results from both methods are in accordance and further investigation of the presence of phytoplasmas will continue based on amplification of the specific gene fragments.

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REFERENCES

Ahrens, U., and Seemuller, E. 1992. Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82:828-832.

Baric, S., Kerschbamer, C., Vigl, J., and Dalla Via, J. 2008. Translocation of apple proliferation phytoplasma via natural root grafts - a case study. European Journal of Plant Pathology 121: 207-211.

Bliefernicht, K., and Krczal, G. 1995. Epidemiological studies on apple proliferation disease in southern Germany. Acta Horticulturae 386: 444-447.

Ciccotti, A.M., Bianchedi, P.L., Bragagna, P., Deromedi, M., Filippi, M., Forno, F., and Mattedi, L. 2008. Natural and experimental transmission of *Candidatus* Phytoplasma mali by root bridges. Acta Horticulturae 781: 459-464.

Doyle, J.J., and Doyle, J.B. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Kirkpatrick, B.C., Stenger, D.C., Morris, T.J., and Purcell, A. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science 238: 197-200.

Myrta A., 2012.-Bazat e virusologjisë bimore, 141-155

(ProMali., 2014). Korca apple, new regional brand for identifying and promoting apple of Korca. www. promalisnv.org.

Rekab, D., Pirajno, G., Cettul, E., De Salvador, F.R., Firrao, G. 2010. On the apple proliferation symptom display and the canopy colonization pattern of "*Candidatus* Phytoplasma mali" in apple trees. European Journal of Plant Pathology 127: 7-12.

Romero J (2001) XI Curso Internacional Teorico-Practico de Deteccion e Identificacion de Virus, Viroides y Fitoplasmas. Instituto Nacional de Investigacion y Tecnologia Agraria Alimentaria (INIA). Madrid, p 2-9.

Schaper, U., and Seemuller, E. 1982. Condition of the phloem and the persistence of mycoplasmalike organisms associated with apple proliferation and pear decline. Phytopathology 72: 736-742.

Seemuller, E. 1990. Apple proliferation. In: A.L. Jones and H.S. Aldwinckle (eds.) Compendium of Apple and Pear Diseases. American Phytopathological Society. St. Paul, Minnesota. Pp. 67-68.

Vindimian, M.E., Ciccotti, A., Filippi, M., Springhetti, M., and Deromedi, M. 2002. Spread of apple proliferation by root bridges (Abstr.). Petria 12: 375.

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COMPARISON OF THREE DETECTION METHODS OF PHYTOPLASMA AT APPLE TREES PROVES THE ADVANTAGE OF AMPLIFICATION OF SPECIFIC 16SrADN

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Abstract

During 2015, samples from 30 apple trees growing in three plantations of Korca were tested for phytoplasmic infections. The last have been difficult to detect due to their low concentration especially in woody hosts and their erratic distribution in the sieve tubes of the infected plants. After the evaluation of the sanitary status of three apple collections in Korca district based on classical symptomatology and DAPI staining method, in order to give a final assessment, one 16SrADN sequence of phytoplasma genome was amplified from DNA extracted from roots, stems and trunks. Quality of DNA is of key importance in molecular diagnostics, since it can affect the final result. It depends on sampling material and which plant tissue is examined. Results were used to identify the infected tissues. Results obtained by classical, cytological and molecular methods, were compared to understand the sanitary status of collections and to compare the effectiveness of each of them.

Key words: *apple trees, amplicons, molecular methods, phytoplasma genome, sanitary status.*

INTRODUCTION

Phytoplasmas are non-cultivable plantpathogens organisms of the *Mollicutes* class. Apple is the main host of "*Candidatus Phytoplasma mali"*, the agent of a severe disease named Apple Proliferation (Seemuller *et al*., 1994).Classification of phytoplasmas is based on molecular analysis of 16S ribosomal gene (16SrDNA). Phytoplasmas belonging to different ribosomal groups and subgroups have been described till this time (Jarausch *et al*., 2000). Diagnosis of this disease in nature is difficult,that was the reason we followed three methods to identify phytoplasmas.
Field survey and symptomatology was the first method.A widely used method for identifying phytoplasmas was also DAPI staining (4', 6-diamidino-2-phenylindole), which stain phytoplasmas due to the ability to be connected with DNA regions rich with A and T.Phytoplasmas are made visible by fluorescence microscopy. This is a relatively sensitive method but its effectiveness depends on the number of phytoplasmas (Kartte, S., and Seemuller, E. 1991).
Nowadays, phytoplasmas detection is basedprimarily on molecular methods as PCR, which is considered as a sensitive, reliable and specific (Ahrens, U., and Seemuller, E. 1994; Googwin *et al*., 1994; Saillard *et al*., 1994).

The aim of this work is to evaluate the sanitary condition of the three plantations of apples in Korce (Albania), through the comparison of phenotypic characteristics, fluorescent staining byDAPIand molecular analysis by PCR.

MATERIALS AND METHODS

Plant material: Plant material was sampled from three plantations in the district of Korca (Korce plantation, Turan, Bitincke).In each of the plantations for every 100 trees, 10 were sampled as follows: 5 samples were taken for each of the three categories of materials (roots, trunk, stem).
A total of 450 samples were collected from 30 trees of apple cultivars *Golden delicious*,

Starking and *Rennete.* After samples were collected in plastic bags on which were written the number of the tree, the category of material and date of the collection, were transported to the laboratory in specific conditions, in boxes on constant temperature 4 °C.

Field observation was conducted randomly according to predefined schemes (schemes X, Y, Z).

DNA extraction: DNA extraction was made based on the sample enrichment protocol for phytoplasmas, using the MLO buffer described by Kirkpatrick *et al*., (1987) with some minor modifications. The usage of MLO aimed to enrich the sample with phytoplasmas making possible the extraction of their DNA and eliminating as far as possible the DNA of the plant.

The second buffer that was used was CTAB by (Doyle and Doyle., 1990).DNA was extracted from three different categories of tissues: roots, trunk, stalks.

The selection of primers and the amplification process: one primer pair was used to amplify the ribosomal sequence, according to Schneider and Semuller, 1993 (Table 1).PCR mixture had a volume of 40 µl containing 100 to 200 ng of template DNA, 0,5 µM of each primer, 10 µM the four dNTPs, 0,2 units of Gold Star polymerase, and 1x buffer. It was cycled 35 times at the following conditions: 30 s of denaturation at 95°C, 75 s of annealing at 55°C and 90 s of extension at 72°C, (Sakai *et al*., 1988).

Gel Electrophoresis: 1.2% agarose gel in TAE was used to analyze products multiplied by PCR.

Table 1. Oligonucleotide primers used for phytoplasma detection.

Primer	Target	Primer sequence (5'-3')
fCPD	Fragment PD67	CCATAGCGAATGTTTAAAAC
rCPD	Fragment PD67	CAGTGCGAAAATTGGTTAAT

RESULTS AND DISCUSSIONS

Phytoplasma detection using ribosomal primers: Primer pair fCPD/rCPD amplified the target DNA in all samples from infected apple trees (Figure 1).

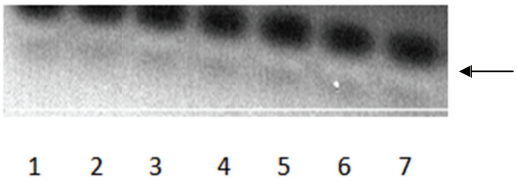

Figure 1. Examples of the results obtained with ribosomal primer pairs fCPD/rCPD, in amplifying template DNA extracted from apple trees.((From left to right: 1, 2. Leaves from Korca plantation; 3. Root from Korca plantation; 4, 5. Trunk from Korca plantation; 6, 7. Stalk from Korca plantation.

PCR results match with the results of DAPI staining of leaf material from the same trees (Figure 2).The intensity of the staining proves the presence of infection at sampling material, which resulted positive from the amplification of the ribosomal fragment of phytoplasmas as well.

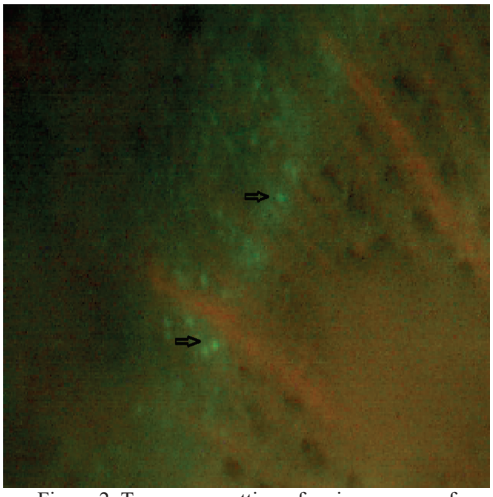

Figure 2. Transverse cutting of main nervures of symptomatic apple leaves, DAPI staining.

Symptomatic rating of apple plants conducted in the field during the spring-summer 2015,

complies with both techniques used for detection of phytoplasmas.
The marked trees displayed a wide range of symptoms, the most common being parallel branches, chlorotic leaves and leaves with a smaller size than normal (Figure 3). In addition, in three plantations were observed dense presences of insects, which are known to serve as vectors of phytoplasmas.

Results taken from the three methods were used to prepare a table (Table 2), which was used to compare the efficiency of each of them on the detection of the infection at apple trees.

Figure 3. Symptomatic tree in the collection of Turan.

Table no 2 describes the detection rate of phytoplasmas based on three different methods.

Table 2. Phytoplasmic infection rate at three collections of Korca region as detected by symptomatology, DAPI staining and specific PCR.

Plantations	Symptomatic Evaluation	DAPI	PCR
Korce	+	100%	100%
Turan	+	80%	100%
Bitincke	+	70%	100%

The usage of PCR molecular technique, resulted effective for three analyzed plantations, as 100% of the samples resulted infected with apple proliferation. It offers several advantages including specificity and relative simplicity. Polymerase chain reaction is more sensitive than microscopic methods and it is used for the detection of low-titer phytoplasma infections in plants (Ahrens, U., and Seemuller, E. 1994; Googwin *et al*., 1994; Saillard *et al*., 1994).DAPI staining method is faster and costs less than PCR molecular technique.

CONCLUSIONS

The comparison of field survey data with those obtained by cytological and molecular methods showed that that PCR based method is more effective. 100 % of the analysed plants resulted infected by PCR, while DAPI staining method displayed different potential of detection at different collections.

However, for the proper evaluation of sanitary conditions of the apple plantations is important to combine field observation, DAPI staining and PCR.

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REFERENCES

Ahrens, U., and Seemuller, E. 1994. Detection of mycoplasmalike organisms in declining oaks by polymerase chain reaction. Eur. J. Pathol. 24:55-63.

Doyle, J.J., and Doyle, J.B. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Googwin, P. H., Xue, B. G., Kuske, C. R., and Sears, M. K. 1994. Amplification of plasmid DNA to detect plant pathogenic mycoplasmalike organisms. Ann. Appl. Biol. 124:27-36.

Jarausch, W., Saillard, C., Helliot, B., Garnier, M., and Dosba, F. 2000. Genetic variability of apple proliferation phytoplasmas as determined by PCR-RFLP and sequencing of a non-ribosomal fragment. Molecular and Cellular Probes 14: 17-24.

Kartte, S., and Seemuller, E. 1991. Susceptibility of grafted Malus taxa and hybrids to apple proliferation disease. J. Phytopath. 131: 137-148.

Kirkpatrick, B.C., Stenger, D.C., Morris, T.J., and Purcell, A. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science 238: 197-200.

Saillard, C., Barthe, C., Moreno, P., and Bove, J. M. 1994. Detection of *Spiroplasma citri* in plants by immunocapture PCR. IOM Let. 3:531-532.

Sakai, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. 1988. Primer directed enzymatic amplification of DNA with thermostable polymerase. Science 239:487-491.

Schneider, B., Cousin, M. T., Klinkong, S., and Seemuller, E. 1993. Taxonomic relatedness and phylogenetic positions of phytoplasmas associated with diseases of faba bean, sunnhemp, sesame, soybean, and eggplant. Z. Pflanzenkrankh. Planzenschutz. In press.

Seemuller, E., Schneider, B., Maurer, R., Ahrens, U., Daire, X., Kison, H., Lorenz, K.-H., Firrao, G., Avinent, L., Sears, B.B., and Stackebrandt, E. 1994. Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. International Journal of Systematic Bacteriology44(3): 440-446.

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GETTING PLANTS *NICOTIANA TABACUM* THAT SIMULTANEOUSLY EXPRESS HETEROLOGOUS GENE OF TWO ACYL-LIPID DESATURASES CYANOBACTERIUM *DES*C AND *DES*A

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Abstract
An important role in plant resistance to low temperature plays a composition of membrane lipids. With the increase of unsaturated fatty acids (FA) in the membranes of cells decreases the transition temperature of the gel phase in liquid crystal phase. Desaturases are enzymes that contribute to the formation of double bonds in the crystal and thus turn the FA with a saturated in unsaturated. The paper used the plant Nicotiana tabacum, expressing the gene of Δ9 acyl-lipid desaturase (desC) cyanobacterium Synechococcus vulcanus. These plants were transformed with a vector-based pBISN with selective nptII gene, under the control of the 35S cauliflower mosaic virus, that carrying the targeted gene desA: licBM3 by Agrobacterium-mediated transformation. Spend regeneration on Murashige-Skooge medium with the addition of BAP, NAA, cefotaxime, kanamycin. Finally we obtained transgenic plants carrying the two genes heterologous desaturases cyanobacterium.

Key words: *acyl-lipid desaturases, fatty acid, transgenic plants*.

INTRODUCTION

The study of plant resistance to abiotic stress is very important nowadays. Structure of membrane lipids has a significant impact on the adaptation of plants to the action of the stressor. Adaptations of organisms to low temperatures and frost temperature dependent phase transition from gel phase in crystalline phase (Los, D.A. et al., 2013).

Due to the shift range of FA saturated in unsaturated membrane fluidity is increasing and decreasing the temperature of transition from liquid state to phase crystallization. Desaturases are enzymes that contribute to the formation of double bonds between carbon atoms and thus transforming saturated fatty acids into unsaturated.

There are several types desaturases: acyl-lipid, acyl-CoA desaturase, acyl-APB. The classification is due to the substrate specificity of these enzymes. Acyl-CoA using FA desaturases joining coenzyme A, acyl-APB-desaturases - FA with bound acyl-portable protein acyl-lipid desaturases used as the substrate crystal, which are composed of lipids (Maali R., et al. 2007). In plants there are two kinds of enzyme, acyl-lipid and acyl-APB (Los, D.A. and Murata, N., 1998). This paper used the acyl-lipid desaturases cyanobacterium *Synechococcus vulcanus* and *Synechocystis sp.* PCC 6803.

Desaturases cyanobacterium characterized by the fact that the desaturation of their participation is in strict sequence, initially formed double connections in the provisions of Δ9, Δ12 then, and then goes to the provisions desaturation Δ6 and ω3 (Maali-Amiri R., et al 2007). That is, first the reaction involving mono FA and then diene. The main goal of this work is to create a tobacco plant that will simultaneously express two genes heterologous acyl-lipid Δ9 desaturase cyanobacterium Δ12 and that should provide further changes in the membranes and reduced sensitivity transformants to cold.

MATERIALS AND METHODS

The paper used gene *des*C (Δ9) cyanobacterium *Synechococcus vulcanus* and gene *des*A (Δ12) *Synechocystis sp*.PCC 6803. Genetic transformation performed by plants *Nicotiana*

tabacum (*cv. Wisconsin*), expressing the gene of Δ9 acyl-lipid desaturase (Gerasymenko I.M., et al., 2010).
The substrate of the enzyme is in the chloroplast so the 5'-end section of the gene *des*C sequence was attached encoding the transit peptide of the small subunit RTP plant *Arabidopsis thaliana* (gene *ats*1A, NCBI, XI36II) for providing targeting. These genes are in the same reading frame of the reporter gene protein licBM3. The gene RTP::desC::licBM3 was cloned under the control of the constitutive 35S promoter in the binary vector of selective gene *bar*. Hybrid gene *des*A was previously cloned into the expression vector based pBISN with selective gene *nptII,* under control of the 35S cauliflower mosaic virus. This gene is located in one of the reading frame reporter protein lichenase *lic*BM3 thermostable bacterium *Clostridium thermocellum.* (Abdeev R.M., et al., 2009). As used control plant *Nicotiana tabacum* wild type and transform *Nicotiana tabacum*, expressing the gene *gfp* :: *lic*BM3 (Gerasymenko I.M., et al., 2015).

Transformation and receiving plants
The method of cultivation of leaf discs with suspension *A.tumefaciens* conducted genetic transformation of tobacco plant *Nicotiana tabacum cv. Wisconsin* (Draper J., et al., 1991). For transformation of plant material genetic constructs used method of "leaf disks." Night *A.tumefaciens* culture built up in LB liquid medium with the addition carbenicillinum-dinatrlcum (50 mg / l) and rifampicin (50 mg / l) at 100-150 rpm and 26ºS in the dark.
The suspension of bacteria was centrifugated at 5000g for 5 minutes. Residue was stirred in a liquid nutrient medium MS, acetocyringone added at a concentration of 100 mM and this suspension was kept in the dark at 25ºS for 1.5 hours in the dark to induce *vir*-region *Agrobacterium*. As a source of great leaf discs used, normally shaped leaves of plants aged 1-1.5 months:1.5. Leaf blade cut into explants (1-1.5sm² square) and placed into the bacterial suspension. The suspension was cultured for one hour in an incubator at 25ºS in the dark. Then take explants, liberated them from drops suspension was transferred to MS medium and cultivated of *Agrobacterium* for two days at 25ºS (before the appearance *Agrobacterium*).
After culturing explants were transferred to agar nutrient medium Murashige-Skooge (MS) with the addition of phytohormones BAP - 1 mg / l and NAA – 0.1 0.1 mg / l. To stop the growth of *Agrobacterium* was added 700 mg / l cefotaxime as selective marker and kanamycin 100 mg / l. Within 2-3 weeks observed the regeneration potential of transgenic tobacco plants. Seedlings kept in cultivation in vitro under conditions 1ºS 25+, with 16-hour photoperiod, lighting of 100 mkM photons / (m2s).
In areas with intensive regeneration occurs greening seedling establishment and later. Lines plants that remain after the selection were grown in selective medium prior to PCR analysis for the presence of transgene.

Molecular biological analysis
To confirm availability of lines of transgenic *Nicotiana tabacum* regenerants obtained, analyzed the total plant DNA was extracted by CTAB (Berdichevets, I.N., et al., 2010) PCR using the appropriate primers:
desA 949 b-sense
GTTGACACCAACGGTAACGCC,
desA 949 b-antisense
CCAGTTAAAGGTGCGCTCGTAA,
desC 777 b-sense
CCTCAATTGGGGCTTTGTCTTC,
desC 777 b-antisense
AACTGTACCTTGGCGGCAAGA, licBM3 291 b-sense AATACGCCTTTTGTTGCAGTGTTT,
licBM3 291 b-antisense
GTCCGAAGGTCCTGTATAAGTGAAGA

Using techniques developed multiplexed PCR analysis of plants obtained after transformation of *Nicotiana tabacum* italic for the transferred T-DNA binary vector (gene fragments *des*C and *lic*BM3). After electrophoretic separation on agarose gel fragments are observed that correspond to the length gene fragment *des*C, *des*A, *lic*BM3. With 16 lines that were transformed vector pNPB14 (desA:: licBM3), 14 lines showed the presence of the transgene in the genome copies and contained no *Agrobacterium* contamination.

Thus was obtained lines of transgenic plants in the genome which proved the presence of hybrid genes *des*A :: *lic*BM3 and previously transformed *des*C :: *lic*BM3 :: RTP.

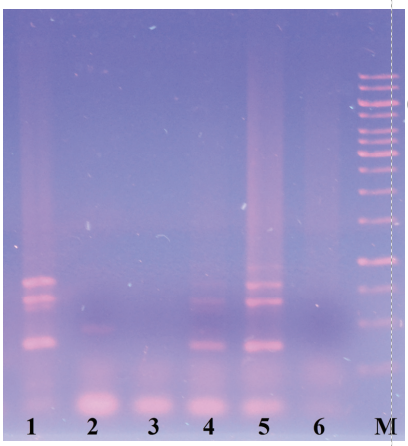

Figure 1. Multiplex PCR analysis of transgenic *N. tabacum* plants for detection of recombinant *lic*BM3, *des*A, and *des*C genes.
1 – *Nicotiana tabacum,* with gene desC :: licBM3 :: RTP;
2 – control *vir*D1;
3 – control H2O;
4 – non transgenic plant;
5 - transgenic plant with genes desC :: licBM3 :: RTP and desA :: licBM3;
6 – non transgenic plant;
M – 100 bp DNA ladder.
*(des*A - 949 bp, *des*C - 777 bp, *lic*BM3 – 642 bp, actin – 351, *vir*D1 – 432 bp.)

Gene expression by the reporter protein is not checked, as used in the plant *Nicotiana tabacum*, which were previously transformed gene *des*C :: *lic*BM3.

RESULTS AND DISCUSSIONS

The method of *Agrobacterium*-mediated transformation of plant lines were obtained *Nicotiana tabacum*, simultaneously expressing two genes acyl-lipid desaturases *des*C cyanobacterium *Synechococcus vulcanus* and *des*A *Synechocystis sp*.PCC 6803.

This was proven by polymerase chain reaction (PCR) which allows the insertion of genes to confirm the presence amplicon *des*A – 949 bp, *des*C - 777 bp, *lic*BM3 - 642 bp (Fig. 1) On track number 1 contains DNA of the plant *Nicotiana tabacum,* with gene *des*C :: *lic*BM3 :: RTP; track 2 control that contains DNA GV3101; track 3 contains a set of primers used to identify hybrid genes with the addition of H2O; track 4 contains DNA of the plant *Nicotiana tabacum*, with gene *des*C :: *lic*BM3 :: RTP held the transformation, but contains one gene desaturase; track 5 contains the DNA of a plant carries 2 genes of desaturases (*des*A and *des*C); track 6 contains DNA of the plant *Nicotiana tabacum* wild type; M – 100 bp DNA marker.

Regenerated plants from which the material seized were grown in vitro on MS selective medium (with the addition of a selective agent kanamycin). After selection all selected clones are transferred to regeneration medium with the same selective agents. After 2-3 months on of selected callus have been arising intensely green cell regeneration, including during further cultivation of seedlings been arising. With 16 lines was confirmed that the insertion of genes in 14 lines. It should be noted that the obtained regenerated not different from control plants phenotype (Fig 2.).

It should be noted that the obtained regenerated not different from control plants phenotype. From previous studies we can conclude that plants that synthesize additional enzymes desaturases thus changing the composition of membrane lipids.

This is done by increasing the proportion of unsaturated fatty acids. In plants expressing the gene of Δ9 acyl-lipid desaturases an increase in the proportion of oleic acid as a substrate of the enzyme is stearic acid. A plant gene expressing Δ12-acyl lipid desaturases observed increase in linoleic acid as a substrate of the enzyme is oleic acid. A very important factor is consistency desaturation FA, which is in strict sequence.

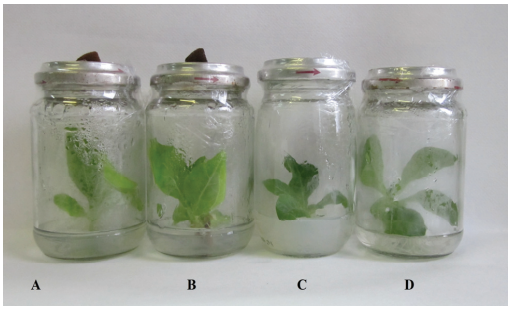

Figure 2. A- control plant *Nicotiana tabacum*,
B – control plant with gene *GFP::licBM3,*
C,D – plants with gene *desA::licBM3* and gene *RTP::desC::licBM3*

First, dual connections are formed between atoms of carbon (C = C) in position Δ9, Δ12 then in position, and then in the provisions of Δ6 and ω3. The formation of an acid depends not only on the level of expression of the corresponding enzyme, but also on the availability of substrate needed for this reaction, so very important to the adjustment of membrane lipids is available substrates in anticipation providing appropriate functioning of enzymes. As for our work, we used tobacco plants expressing the gene targeting *des*C the signal in chloroplasts, the gene for additional *des*A cyanobacterium *Synechocystis* sp.PCC 6803 has been provided with a substrate reaction desaturation (oleic acid). We can assume that in these plants observed shift range of FA rich in unsaturated oleic due to the increase in the share and linolenic acids.

You can also assume that plants that express both genes Δ9 and Δ12-acyl lipid desaturases is more likely providing desaturases other substrate (Δ6 and ω3).

CONCLUSIONS

As a result *Agrobacterium*-mediated transformation was obtained plants *Nicotiana tabacum*, which also carry two genes *des*C and *des*A desaturase cyanobacterium *Synechococcus vulcanus Synechocystis sp*.PCC 6803 respectively. These genes are characterized by different substrate specificity, and Δ9 acyl-lipid substrate provides desaturase work Δ12-acyl lipid desaturase. This leads to an increase in the proportion of unsaturated FA composed of lipids membrane tobacco plants, and thus increase plant resistance to abiotic stresses.

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REFERENCES

1. Abdeev, R.M., Abdeeva, I.A., Bruskin, S.S., Musiychuk, K.A.,Goldenkova-Pavlova, I.V., and Piruzian, E.S., 2009. Bacterial thermostable betaglucanases as a tool for plant functional genomics, Gene. 436:81–89.
2. Berdichevets, I.N., Shimshilashvili, H.R., Gerasymenko, I.M., Sindarovska, Y.R., Sheludko, Y.V., and Goldenkova-Pavlova, I.V., 2010. Multiplex PCR assay for detection of recombinant genes encoding fatty acid desaturases fused with lichenase reporter protein in GM plants, *Anal. Bioanal. Chem.*, 397: 2289–2293.
3. Draper J., Scott R., Armitage P., 1991. Plant Genetic Transformation and Gene Expression. A Laboratory Manual, Moscov, Russia, 194- 232.
4. Gerasimenko, I.M., Golovach, I.S., Kishchenko, E.M.,Sakhno, L.A., Sindarovskaya, Ya.R., Shimshilashvili, Kh.R., Shelud'ko, Yu.V., 2010 and Goldenkova-Pavlova, I.V., Preparation and analysis of transgenic plants carrying genes Δ9 and Δ12 desaturases of cyanobacteria,
Inform. Vestn. Vavilov. Obcsh. Genet. Select.14:127–133.
5. Gerasymenko I.M., Sakhno L.O., Kyrpa T.M., Ostapchuk A.N., Khadjiev T.A., Goldenkova-Pavlova I.V., Sheludko Y.V. 2015. Characterization of *Nicotiana tabacum* plants expressing hybrid genes of cyanobacterial Δ9 or Δ12 acyl–lipid desaturases and thermostable lichenase.
Russian J. Plant Physiol. 62(3):283-291.
6. Los, D.A. and Murata, N., 1998. Structure and expression of fatty acid desaturases, Biochim. Biophys. Acta, 1394:3–15.
7. Los, D.A., Mironov, K.S., and Allakhverdiev, S.I., 2013. Regulatory role of membrane fluidity in gene expression and physiological functions, Photosynth. Res., 116:489–509.
8. Maali-Amiri, R., Goldenkova-Pavlova, I.V., Yur'eva, N.O., Pchelkin, V.P., Tsydendambaev, V.D., Vereshchagin, A.G., Deryabin, A.N., Trunova, T.I., Los', D.A., and Nosov, A.M., 2007. Lipid fatty acid composition of potato plants transformed with the Δ12-desaturase gene from cyanobacterium, Russ. J.Plant Physiol., 54: 600–606.
9. Maali, R., Shimshilashvili, Kh.R., Pchelkin, V.P., Tsydendambaev, V.D., Nosov, A.M., Los, D.A., and Goldenkova-Pavlova, I.V., 2007. Comparative expression in *Escherichia coli* of the native and hybrid genes for acyl-lipid delta(9) desaturase, Genetika, 43:176–182.

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THE INFLUENCE OF GENOTYPE AND SOIL TILLAGE SYSTEM ON FORMING PRODUCTION COMPONENTS FOR SWEET CORN UNDER SOIL-CLIMATE CONDITIONS SPECIFIC TO ROMANIAN PLAIN

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Abstract
Knowing the fact that sweet corn valorization is mainly done, in our country, as fresh product, for boiling purposes and less for manufacturing, productivity elements and the production of corn cobs for ach area unit, constitute, nowadays, priority amelioration tasks, considering that an ever significant part of sweet corn yield begins to be meant, in Romania too, to the manufacture as frozen beans or tins, this fact leading to an economic effectiveness of this crop, only for production levels found over certain limits. Research aimed at testing the behaviour of a sweet corn foreign and local assortment, hybrids which belong to different precocity categories, under the circumstances of practising two soil tilage systems (conventional system and minimum tillage system). After analysing and comparing the experimental results, one has established the superiority of the yields achieved by the minimum tillage system, system which had as result a better preservement of water in the soil, having direct impact on the crop achievement, each of the tested hybrids valorizing very well their genetical endowement concerning the productive capacity.

Key words: *hybrid, conventional system, minimum system, yield.*

INTRODUCTION

Sweet corn could be, for most areas of our country, a plant extremely important both for direct consumption (corn) and as a raw material for manufacturing domestic and especially foreign.

Being a short-day plant, adapted to light the intense, sweet corn in the early stages of growth, has a high resistance to drought, because the root system deeply, but water shortages during training tasseling and grain can adversely affect production(Hallauer A. R., J.B. Miranda, 1988), which is why, the success of this crop requires choosing the most appropriate technological links, so efficiency culture is maximized in terms of input minimum knowing that ecological plasticity recognized this species enables its adaptation with relative ease, the culture conditions completely changed in context of climate change (Haş I., 2004).

The research was conducted during 2014-2015 in pedo-climatic characteristics Draganesti–Vlasca location, jud.Teleorman and include comparative study on sweet corn genotypes of native and foreign origin, hybrids belonging to different groups precocity, for research to identify the most efficient consisting of corn genotypes for pedo-climatic characteristics of the Romanian Plain.

MATERIALS AND METHODS

Experience with sweet corn was one type bifactoral placed in the field after the subdivided parcels method in three repetitions, experimental factors are the following:

Factor A: tillage system with 2 degrees:
- a_1 - classical system;
- a_2 - minimum system.

Factor B: genotype grown by 9 degrees:
- b_1 - *PRIMA*
- b_2 - *SPIRIT F1*

- b_3 - *CANDLE F1*
- b_4 – *DELICIUL VERII*
- b_5 - *SWEET THING F1*
- b_6 - *SHIMMER F1*
- b_7 - *DULCIN*
- b_8 - *CHALLENGER F1*
- b_9 - *JUBILEE F1*

The experimental plot was constituted of three rows for each variant with a length of 5.04 m to 18 plants per row, the distance between lines of 70 cm. There were thus obtained the elementary plots sown area of 10.5 $m^2$and a surface of 7.02 m^2collected.

For the calculation and interpretation of experimental results is used the method of analysis of variance according to the settlement experience in the field.

During the research analyzes and determinations were made on the main elements of productivity as follows:

- number of cobs per plant;
- length of cobs (cm);
- diameter of the cobs (cm) was measured with calipers;
- the weight of the corn cob with husk (g) was obtained by weighing;
- production of cobs with husk (to/ha) was calculated by multiplying the number of ear weight cobs per plant and the number of plants per unit area achieved.

RESULTS AND DISCUSSIONS

Production components and production of cobs achieved per unit area constitute major objectives in choosing the most valuable genotypes of corn, to put your best worth gene pool, knowing that only levels of production which exceeded certain limits are economically efficient.

Table 1.Number of cobs/plant based on genotype and tillage system

GENOTYPE (HYBRID)	COB/PLANT				DIFFERENCE			
	(No.)		(%)		(No.)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	1.42	1.53	87	88	-0.21	-0.21	ooo	ooo
2. SPIRIT F1	1.59	1.73	98	99	-0.04	-0.01	oo	-
3. CANDLE F1	1.62	1.76	99	101	-0.01	0.02	-	-
Average early hybrids	1.54	1.67	94	96	-0.26	-0.20	ooo	ooo
4. DELICLIUL VERII	1.61	1.69	99	97	-0.02	-0.05	-	ooo
5. SWEET THING F1	1.68	1.78	103	102	0.05	0.04	***	**
6. SHIMMER F1	1.72	1.81	106	104	0.09	0.07	***	***
Average extra early hybrids	1.67	1.76	102	101	0.12	0.06	***	***
7. DULCIN	1.48	1.71	91	98	-0.15	-0.03	ooo	a
8. CHALLENGER F1	1.76	1.82	108	105	0.13	0.08	***	***
9. JUBILEE F1	1.79	1.88	110	108	0.16	0.14	***	***
Average mid early hybrid	1.68	1.80	103	103	0.14	0.19	***	***
AVERAGE EXPERIENCE	1.63	1.74	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.03; DL 1% = 0.04;DL 0.1%= 0.05

A decisive role in the production of cobs per unit area playfully number of cobs per plant. Some authors argue that it is preferable to have a single cob plants than two or three because the greater number of cobs per plant would be detrimental to their length and weight.

Also, high density planting the same result negative for this purpose (Jugenheimer, Robert W., 1976).Experimental field sowing of the crop was made at the distance between rows and between plants in the row recommended for maize (Haş I., 2004).

Analyzing the experimental results on the number of trained cobs per plant (Table 1) we observe the superiority of hybrids grown under minimum tillage system, by charging system which yielded a number between 1.53 and 1.88

cobs/plant compared to the classic work for which the number of kernels was between 1.42 and 1.79 cobs/plant.
The lowest number of cobs formed on a hybrid plant was registered early *Prima* and the largest number of kernels was obtained by *Jubilee F1* hybrid, semi early, regardless of tillage system practiced.
From the point of view of this character, best behaved mid early hybrids, followed by extra early hybrids those early hybrids in both tillage systems.
Poor development of the ears, possible deformation of them is due to the significant drop in temperature in June, during the development of maximum ear and formation of husk very tight around the ear may hinder the development of silk and therefore binding flowers (Jugenheimer, Robert W., 1976).
The length of the ears (Table 2) was between 16.6 cm and 19.6 cm in classic tillage system, respectively between 17.3 cm and 22.3 cm for practicing minimum tillage system the biggest ears in the case of hybrids forming mid early, followed by hybrids extra early hybrids and the times.

Table 2. The length of the ears based on genotype and tillage system

GENOTYPE (HYBRID)	LENGTH EAR				DIFFERENCE			
	(cm)		(%)		(cm)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	16.6	17.3	91	88	-1.6	-2.4	ooo	ooo
2. SPIRIT F1	18.2	19.7	100	100	0.0	0.0	-	-
3. CANDLE F1	18.4	19.6	101	99	0.2	-0.1	-	-
Average early hybrids	17.7	18.9	97	96	-1.4	-2.5	ooo	ooo
4. DELICLIUL VERII	17.2	18.2	95	92	-1.0	-1.5	-	-
5. SWEET THING F1	18.5	21.3	102	108	0.3	1.6	-	***
6. SHIMMER F1	18.9	20.7	104	105	0.7	1.0	***	***
Average extra early hybrids	18.2	20.1	100	102	0.0	1.1	-	***
7. DULCIN	17.9	18.2	98	92	-0.3	-1.5	-	ooo
8. CHALLENGER F1	18.8	19.9	103	101	0.6	0.2	***	-
9. JUBILEE F1	19.6	22.3	108	113	1.4	2.6	***	***
Average mid early hybrid	18.8	20.1	103	102	1.7	1.3	***	***
AVERAGE EXPERIENCE	18.2	19.7	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.33;DL 1% = 0.45;DL 0.1%= 0.59

Of sweet corn genotypes tested in the experiment were noted extra early hybrid *Shimmer F1* hybrid with a length of 18.9 cm and ears of hybrid *Jubilee F1* with19.6 cm in conventional tillage system. By practicing minimum tillage system were highlighted in terms of length of ears, extra early hybrid *Sweet Thing F1* with 21.3 cm and *Jubilee F1* hybrid with 22.3 cm, the latter being superior to other hybrids tested, regardless of tillage system practiced.
Regarding cobs diameter (Table 3) revealed that this character values were between 4.33 cm and 4.83 cm by practicing classical tillage system, and 4.38 cm to 5.09 cm in the case of the minimum system work, the use of this system fairly wide variations resulting from the hybrid to another, compared with the system in which it classic variations were quite close.

Table 3. Diameter ears based on genotype and tillage system

GENOTYPE (HYBRID)	DIAMETER EAR				DIFFERENCE			
	(cm)		(%)		(cm)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	4.33	4.38	94	92	-0.27	-0.37	o	oo
2. SPIRIT F1	4.56	4.87	99	103	-0.04	0.12	ooo	***
3. CANDLE F1	4.58	4.76	100	100	-0.02	0.01	o	-
Average early hybrids	4.49	4.67	98	98	-0.33	-0.24	ooo	ooo
4. DELICLIUL VERII	4.53	4.56	98	96	-0.07	-0.19	ooo	ooo
5. SWEET THING F1	4.69	4.79	102	101	0.09	0.04	***	***
6. SHIMMER F1	4.67	4.77	102	100	0.07	0.02	***	*
Average extra early hybrids	4.63	4.70	101	99	0.09	-0.13	***	ooo
7. DULCIN	4.45	4.48	97	94	-0.15	-0.27	ooo	o
8. CHALLENGER F1	4.78	5.06	104	107	0.18	0.31	***	***
9. JUBILEE F1	4.83	5.09	105	107	0.23	0.34	***	***
Average mid early hybrid	4.69	4.88	102	103	0.26	0.38	***	***
AVERAGE EXPERIENCE	4.60	4.75	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.022;DL 1% = 0.029; DL 0.1% = 0.038

By cultivating sweet corn in the classic tillage, cobs weight was, on average, 282.2 g hybrids early, 294.1 g and 296.1 g extra early hybrids in the mid early while ago practicing minimum tillage system is a significant difference between groups in weight cobs such precocity: 298.7 g hybrids early in the 305.7 g –309.1 g hybrids extra early and mid early, the results practicing this system tillage is superior to the conventional system (Table 4).

Table 4. Cobs weight based on genotype and tillage system

GENOTYPE (HYBRID)	WEIGHT COBS				DIFFERENCE			
	(g)		(%)		(g)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	256.3	284.9	88	94	-34.5	-19.6	ooo	ooo
2. SPIRIT F1	294.3	305.9	101	100	3.5	1.4	***	***
3. CANDLE F1	295.6	305.2	102	100	4.8	0.7	***	***
Average early hybrids	282.1	298.7	97	98	-26.2	-17.5	ooo	ooo
4. DELICLIUL VERII	288.5	304.1	99	100	-2. 3	-0.4	ooo	oo
5. SWEET THING F1	296.2	306.8	102	101	5.4	2. 3	***	***
6. SHIMMER F1	297.6	306.2	102	101	6.8	1.7	***	***
Average extra early hybrids	294.1	305.7	101	100	9.9	3.6	***	***
7. DULCIN	283.3	302.9	97	99	-7.5	-1.6	ooo	ooo
8. CHALLENGER F1	301.4	311.2	104	102	10.6	6.7	***	***
9. JUBILEE F1	303.6	313.2	104	103	12.8	8.7	***	***
Average mid early hybrid	296.1	309.1	102	102	15.9	13.8	***	***
AVERAGE EXPERIENCE	290.8	304.5	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.33;DL 1% = 0.45;DL 0.1% = 0.59

Of the nine genotypes analyzed were noted *Challenger F1* mid early hybrids and *Jubilee F1*, cobs weight exceeding 300 g in both tillage systems.

Opposite the first hybrid that formed early cobs with an average weight of 256.3 g in conventional tillage system, respectively 284.9 minimum tillage system.

The other eight tested hybrids practicing minimum tillage system had a significant influence on weight cobs they exceeding 300 g, regardless of hybrid analyzed.

In terms of our country where sweet corn is consumed primarily as boiled corn, cobs with husk production is the main type of production economic interest because the sale is made in the form cobs cob wrapped in corn husks.

Weight cobs with kernels number per plant are the two basic components of production per unit area cobs.

Hybrid's productions made in the study ranged from 21.83 to 32.61 t/ha under classical system tillage and 26.15 to 35.33 t/ha when used minimum tillage system values minimum being recorded early first hybrid in both tillage systems, differences in production between the two tillage systems in all genotypes tested is about 4 t/ha for minimum tillage system (Table 5).

The highest production capacity showed an early hybrid *Jubilee F1* hybrid that achieved a yield of 32.61 t/ha in conventional tillage system, respectively 35.66 t/ha by practicing minimum system.

Table 5.Cobs production based on genotype and tillage system

GENOTYPE (HYBRID)	PRODUCTION				DIFFERENCE OF PRODUCTION			
	(To/ha)		(%)		(To/ha)		(%)	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	21.83	26.15	77	82	-6.69	-5.76	ooo	ooo
2. SPIRIT F1	28.07	31.75	98	99	-0.45	-0.16	ooo	-
3. CANDLE F1	28.73	32.22	101	101	0.21	0.31	*	**
Average early hybrids	26.21	30.0	92	94	-6.93	-5.61	ooo	ooo
4. DELICLIUL VERII	27.86	30.83	98	97	-0.66	-1.08	ooo	ooo
5. SWEET THING F1	29.86	32.77	105	103	1.34	0.86	***	***
6. SHIMMER F1	30.71	33.25	108	104	2.19	1.34	***	***
Average extra early hybrids	29.48	32.28	103	101	2.87	1.12	***	***
7. DULCIN	25.15	31.07	88	97	-3.37	-0.84	ooo	ooo
8. CHALLENGER F1	31.83	33.98	112	106	3.31	2.07	***	***
9. JUBILEE F1	32.61	35.33	114	111	4.09	3.42	***	***
Average mid early hybrid	29.86	33.46	105	105	4.03	4.65	***	***
AVERAGE EXPERIENCE	28.52	31.91	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.19;DL 1% = 0.25;DL0.1% =0.33

CONCLUSIONS

The number of ears trained on the plant was higher in the case of hybrids grown in the system minimum tillage, the lowest number of ears trained on a plant being registered hybrid early *Prima* and the largest number of cobs were obtained by early hybrid *Jubilee F1* regardless of tillage system practiced.

Biggest ears length was obtained for mid early hybrids, hybrids extra early and follow the times, pointing out extra early *Shimmer F1* hybrid with a length of 18.9 cm and *Jubilee F1* hybrid with 19.6 cm conventional tillage.

By practicing minimum tillage system stood out in terms of length of ears, *Jubilee F1* hybrid, which is superior to others hybrids tested in both tillage systems.

Use of minimum tillage has the effect of variations from a fairly wide hybrid another in terms of the diameter of the ears compared to the system in which it classical variations were quite close.

In terms of the number of rows of kernels on the cob formats best behaved extra early hybrids, regardless of tillage system practiced.

The number of grains formed all had substantial variations in the case of extra early and mid early hybrids in both tillage systems, early hybrids being the weakest in terms of this character.

In terms of the weight of ears stood mid early hybrids *Challenger F1* and *Jubilee F1*, cobs weight exceeding 300 g in both tillage systems, while early hybrid *Prima* kernels which consists of an average weight of 256.3 g in conventional tillage system, respectively 284.9 minimum tillage system.

The smaller productions cobs were obtained hybrid early first in both systems tillage, the highest productive capacity of showing early hybrid *Jubilee F1*, hybrid which were obtained yields of over 32 t/ha in both tillage systems, differences in production between the two tillage systems in all genotypes tested is about 4 t/ha for minimum tillage system.

Therefore, we conclude that, for specific climatic conditions Romanian Plain is recommended to cultivate hybrids of corn in the category extra early and mid early, under minimum tillage system.

REFERENCES

Hallauer A. R., J.B. Miranda, 1988, Quantitative Genetics în Maize Breeding, Iowa State Univ. Press, Ames Iowa, 224-238.

Haș I., 2004, Particularități tehnologice pentru cultura porumbului în Transilvania, Editura Boema Turda, 21–45.

Jugenheimer, Robert W., 1976, Corn improvement seed production and uses, John Wiley & Sons Inc, 278-345.

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THE INFLUENCE OF TECHNOLOGICAL LINKS UPON SWEET CORN YIELD QUALITY UNDER SOIL-CLIMATE CONDITIONS OF THE ROMANIAN PLAIN

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Abstract

Within the framework of the present climate changes, it is ever recommended to grow varieties characterized by a large ecological plasticity, varieties which can successfully pass over the extreme droughty periods which may affect both the yield and its nutritive quality. Thus, the sweet corn, variety resistant to hydrical stress conditions, remarked itself by a higher calory content and a superior nutritive value in comparison with the regular corn, representing both an important source of microelements: magnezium (37-48 mg/100 g grains), potassium (270 mg/100 g grains, phosphorous (69 mg/100 g grains), natrium (16 mg/100 g grains) a source of B complex vitamins, C and E vitamins. Knowing the fact that the nutritive value represents one of the main requests in cobs trade the influence of technological links upon sweet corn yield quality, in conditions of crop irrigation lack, has been tested within a bi-factorial trial. Recorded experimental results emphasized the inferiority of the hybrids grown in the classical soil tillage system in comparison with the minimum tillage soil system, thus, both the grown genotype and the soil tillage variant significantly influence the level of production and the grains quality.
Thus, it is requested to find an alternative for animal proteins, namely vegetal proteins, which even incomplete, being cholesterol free, may constitute alternatives for a healthy food.

Key words: *genotype, production, quality, technology, nutritive value.*

INTRODUCTION

The sugar content is the major factor that confers sweetness beans, sweet corn, sweet taste or causing their super sweet (Hallauer A. R., J.B. Miranda, 1988).
The proteins accumulated in corn grains are essential for the human body to produce new cells and repair of damaged for various reasons. In addition, protein plays important role in growth and development. Although only proteins of animal origin contain all nine essential amino acids, and plant proteins can combine incomplete in such a way as to obtain the same effect.
Moreover, proteins in plants are healthier than animals because do not increase cholesterol levels in the blood.
The research was conducted during 2014-2015 in pedo-climatic characteristics village Draganesti - Vlaşca, Teleorman County and focused determinations relating to the content in sugars, starch, total nitrogen and protein from beans from many genotypes of sweet corn domestic and foreign origin, hybrids with different vegetation periods, for research purposes consisting in identifying the most valuable genotypes nutritional aspect that can be grown in pedo-climatic characteristics of the Romanian Plain.

MATERIALS AND METHODS

The research was conducted in a bifactorial experience arranged by the subdivided parcels method in four replications, experimental factors under study are the following:
Factor A: tillage system with 2 degrees:
- a_1- classical system;
- a_2- minimum system.
Factor B: genotype grown by 9 degrees:
- b_1 - *PRIMA*
- b_2 - *SPIRIT F1*
- b_3 - *CANDLE F1*
- b_4 – *DELICIUL VERII*
- b_5 - *SWEET THING F1*
- b_6 - *SHIMMER F1*
- b_7 - *DULCIN*
- b_8 - *CHALLENGER F1*
- b_9 - *JUBILEE F1*

By combining the two experimental factors resulting 18 experimental variants, the results achieved are calculated according to the method of variance analysis, according to the settlement experience in the field.
Characters grain quality pursued during the research were laid following:
- sugar content - determined colorimetric;
- total nitrogen content - determined by the Kjeldahl method mineralization, being and the calculation of crude protein;
- starch content - determined by the method Schrool.

RESULTS AND DISCUSSIONS

The chemical analyzes sweetness grain corn produced in the experimental field to focus on the content of mono- and di-sugars, particularly glucose and fructose, found in the highest amount in grains and the concentration of which affects decisively sweet taste.
After determining the sugar content (Table 1), it was found that this indicator ranged between 2.5 – 3.7 g when used classical system tillage and 3.1 – 4.4 g when corn was grown in minimum tillage system, the latter system leading to an intense accumulation of sugars in the berries, regardless of genotype analyzed.
The higher the sugar content was obtained for mid early hybrids, hybrids from which it was recorded an average content of 3.4 g sugars in the classic tillage, and 4.1 g sugars in the minimum tillage system.
The lowest sugar content was obtained with early hybrid *Prima*, regardless of tillage system practice: 2.5 g in the classic tillage system and 3.1 g in the minimum tillage system.
Most valuable hybrids in terms of sugar content were extra early hybrid *Sweet Thing F1* and early hybrid *Jubilee F1*,hybrids amount of sugars in grains was 3.6 – 3.7 g under the conventional tillage system and 4.4 g when using minimum tillage system.
The lowest sugar content was obtained with early hybrid *Prima*, regardless of tillage system practice: 2.5 g in the classic tillage system and 3.1 g in the minimum tillage system.
Most valuable hybrids in terms of sugar content were extra early hybrid *Sweet Thing F1* and early hybrid *Jubilee F1*,hybrids amount of sugars in grains was 3.6 – 3.7 g under the conventional tillage system and 4.4 g when using minimum tillage system.
Unlike ordinary corn, high starch content which is a positive character because it is the most important raw material for industrial starch, corn starch mellitus indicates a high level of harvesting late or inadequate preservation (Jugenheimer, Robert W., 1976).
Analyzing the experimental results regarding the starch content of the grain (Table 2) show that the favorable impact of practicing minimum tillage system on this indicator, decreasing significance; starch content compared to the values registered in terms of the classic tillage system, indifferent analyzed the genotype of corn this fall having a direct impact on improving food value of grain.
The lowest content of starch was registered in early hybrid *Spirit F1* in the classic tillage system, and extra early *Sweet Thing F1* in the minimum tillage system, these hybrids are superior in terms of culinary other genotypes of corn tested in experience.
On the opposite side stood the extra early hybrid *Deliciul verii* and the mid early hybrid *Dulcin,* hybrids that registered the highest starch content, namely 18.54 – 18.55 g in classic tillage system, these hybrids registering the high starch content and conditions of use minimum tillage system, the values of this indicator was 17.89 g or 17.93 g.

Table 1.Sugar content by genotype and tillage system

GENOTYPE (HYBRID)	SUGAR CONTENT				DIFFERENCE			
	(g/100g)		(%)		(g/100g)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	2.5	3.1	78	81	-0.7	-0.7	ooo	ooo
2. SPIRIT F1	3.4	3.7	106	97	0.2	-0.1	-	-
3. CANDLE F1	3.2	3.9	100	103	0.0	0.1	-	-
Average early hybrids	3.0	3.6	95	94	-0.5	-0.7	ooo	ooo
4. DELICLIUL VERII	2.9	3.3	91	87	-0.3	-0.5	o	ooo
5. SWEET THING F1	3.6	4.4	113	116	0.4	0.6	**	***
6. SHIMMER F1	3.4	3.8	106	100	0.2	0.0	-	-
Average extra early hybrids	3.3	3.8	103	101	0.3	0.1	*	-
7. DULCIN	3.1	3.8	97	100	-0.1	0.0	-	-
8. CHALLENGER F1	3.5	4.1	109	108	0.3	0.3	*	*
9. JUBILEE F1	3.7	4.4	116	116	0.5	0.6	***	***
Average mid early hybrid	3.4	4.1	107	108	0.7	0.9	***	***
AVERAGE EXPERIENCE	3.2	3.8	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.26;DL 1% = 0.34;DL 0.1% = 0.45

Table 2.The starch content by genotype and tillage system

GENOTYPE (HYBRID)	STARCH CONTENT				DIFFERENCE			
	(g/100g)		(%)		(g/100g)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	18.38	17.76	101	102	0.15	0.27	-	**
2. SPIRIT F1	18.03	17.24	99	99	-0.20	-0.25	o	oo
3. CANDLE F1	18.12	17.33	99	99	-0.11	-0.16	-	o
Average early hybrids	18.17	17.44	100	100	-0.16	-0.14	o	-
4. DELICLIUL VERII	18.54	17.89	102	102	0.31	0.40	***	***
5. SWEET THING F1	18.06	17.23	99	99	-0.17	-0.26	o	oo
6. SHIMMER F1	18.16	17.38	100	99	-0.07	-0.11	-	-
Average extra early hybrids	18.25	17.50	100	100	0.11	0.03	-	-
7. DULCIN	18.55	17.93	102	103	0.32	0.44	***	***
8. CHALLENGER F1	18.17	17.27	100	99	-0.06	-0.22	-	oo
9. JUBILEE F1	18.14	17.36	100	99	-0.09	-0.13	-	-
Average mid early hybrid	18.28	17.52	100	100	0.17	0.09	*	-
AVERAGE EXPERIENCE	18.23	17.49	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.16;DL 1% = 0.21;DL 0.1% = 0.28

Observe also increase the starch content in direct proportion to the increase of the growing season hybrids tested, the highest content of starch is registered mid early hybrids, followed by extra early hybrid and the early hybrids, in both systems tillage.

Total nitrogen in corn mellitus derives largely from zein and glutein proteins that were identified cysteine, alanine, asparagine, glicoolul, proline, serine along with essential amino acids methionine, leucine, isoleucine, histidine, phenylalanine, lysine and tryptophan.Other compounds isolated from corn, which is present in the structure of the heterocyclic nitrogen, vitamins: niacin, pantothenic acid, thiamine, riboflavin, folic acid and pyridoxines (Sprague G.F., S.A. Eberhart, 1977).

By practicing a conventional tillage, total nitrogen content in grains of corn ranged from 268 - 312 mg, the highest content was recorded in the case of mid early hybrids, followed by those extra early and the early hybrids (Table 3).

When using minimum tillage system, there is a significant increase in the total nitrogen content in the grain, regardless of genotype analyzed in comparison with the classic tillage system, this index values ranging between 319 and 363 mg.

Of the nine genotypes behaved myself tested the early hybrid *Jubilee F1* in both tillage systems, with a total nitrogen content of 312 mg in the classic tillage system and 363 mg at minimum tillage system.

By analyzing the protein content of the grains we find that this indicator is higher in the practice of minimum tillage system compared to conventional tillage systems (Table 4).

Thus, if the conventional system where the protein content was between 2.58 and 2.97 g, in the minimum tillage system the accumulated protein in beans was between 3.26 and 3.76 g.

First they stood in hierarchical order, mid early hybrids, extra early hybrids and the early hybrids in the minimum tillage system while, if conventional system, hierarchy, in terms of protein content, was : extra early hybrids, the early hybrids and mid early hybrids.

The lowest protein content was recorded at early hybrid *Prima* (2.58 g in the classic tillage system and 3.26 g in the minimum tillage system), the maximum being obtained at mid early hybrid *Challenger F1* in both tillage systems, respectively 2.97 g in the classic tillage system and 3.76 in the minimum tillage system.

Table 3.Total nitrogen content by genotype and tillage system

GENOTYPE (HYBRID)	NITROGEN CONTENT				DIFFERENCE			
	(mg/100g)		(%)		(mg/100g)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	268	319	90	92	-29	-26.0	ooo	ooo
2. SPIRIT F1	303	346	102	100	6.0	1.0	***	-
3. CANDLE F1	309	349	104	101	12.0	4.0	***	***
Average early hybrids	293	338	99	98	-11.0	-21.0	ooo	ooo
4. DELICLIUL VERII	276	329	93	95	-21.0	-16.0	ooo	ooo
5. SWEET THING F1	309	358	104	104	12.0	13.0	***	***
6. SHIMMER F1	302	353	102	102	5.0	8.0	***	***
Average extra early hybrids	296	347	100	101	-4.0	5.0	ooo	***
7. DULCIN	288	333	97	97	-9.0	-12.0	ooo	ooo
8. CHALLENGER F1	307	356	103	103	10.0	11.0	***	***
9. JUBILEE F1	312	363	105	105	15.0	18.0	***	***
Average mid early hybrid	302	351	102	102	16.0	17.0	***	***
AVERAGE EXPERIENCE	297	345	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 1.78;DL 1% = 2.39;DL 0.1% = 3.13

Table 4. Crude protein content by genotype and tillage system

GENOTYPE (HYBRID)	CRUDE PROTEINE CONTENT				DIFFERENCE			
	(g/100g)		(%)		(g/100g)		SIGNIFICATION	
	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage	classic tillage	minimal tillage
1. PRIMA	2.58	3.26	93	92	-0.19	-0.29	ooo	ooo
2. SPIRIT F1	2.77	3.62	100	102	-0.01	0.07	-	***
3. CANDLE F1	2.83	3.69	102	104	0.05	0.14	***	***
Average early hybrids	2.72	3.52	98	99	-0.15	-0.08	ooo	ooo
4. DELICLIUL VERII	2.87	3.32	103	94	0.09	-0.23	***	ooo
5. SWEET THING F1	2.81	3.65	101	103	0.03	0.10	***	***
6. SHIMMER F1	2.85	3.63	103	102	0.07	0.08	***	***
Average extra early hybrids	2.84	3.53	102	99	0.19	-0.05	***	ooo
7. DULCIN	2.48	3.36	89	95	-0.30	-0.19	ooo	ooo
8. CHALLENGER F1	2.97	3.76	107	106	0.19	0.21	***	***
9. JUBILEE F1	2.88	3.69	104	104	0.10	0.14	***	***
Average mid early hybrid	2.77	3.60	100	101	-0.01	0.16	-	***
AVERAGE EXPERIENCE	2.78	3.55	100	100	Mt.	Mt.	Mt.	Mt.

DL 5% = 0.020; DL 1% = 0.026; 0.1% DL = 0.035

CONCLUSIONS

The sugar content of the grains was higher by practicing minimum tillage system, the highest level of sugars being recorded for mid early hybrids in both tillage systems.

The starch content of grains increased in direct proportion to the increase of the growing season hybrids tested, the highest starch content was recorded at mid early hybrids, followed by extra early and early hybrids in both tillage systems.

By practicing minimum tillage system the total nitrogen content increases significantly in the grain, regardless of genotype analyzed compared to the classic tillage system.

The protein content of beans find that this indicator is higher in the practice of minimum tillage system compared to the conventional tillage system, the first hovering followed by the mid early hybrids, extra early and early hybrids.

REFERENCES

Hallauer A. R., J.B. Miranda, 1988, Quantitative Genetics în Maize Breeding, Iowa State Univ. Press, Ames Iowa, 224-238.

Jugenheimer, Robert W., 1976, Corn improvement seed production and uses, John Wiley & Sons Inc, 278-34.

Sprague G.F., S.A. Eberhart, 1977, Corn breeding.pp. Corn and Corn Improvement, American Society of agronomy, Madison, WI, p. 305-36.

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GENETIC ANALYSIS OF QTLs CONTROLLING CARPEL NUMBER IN CITRUS

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Abstract

Purpose of this research is to determine the markers controlling carpel number in mandarin. Progenies obtained by hybridization between the Clementine mandarins (C. clementina Hort. ex Tan.) and Orlando tangelos (C. reticulata Blanco x C. Paradise Macf.) have been used in this research. Morphological and molecular marker data were analyzed in SAS software by using GLM and REGRESSION module. Population includes variation in respect to carpel number, which transgressive segregation was observed and distribution was positively skewed. Based onvariance analysis made by using GLM option in SAS software, ten markers were associated with carpel number All loci explained 100% of the variation for carpel number. OPW19.25, a RAPD marker explained 43% of total variation and OPM20.23 explained 22% of total variation. These results revealed that two loci had major effect in respect to carpel number and other loci had a minor effect. This research revealed significant clues about genetic mechanism of carpel number in mandarin fruit. These markers should be further investigated for applicability and conversion to more specific markers such as SCAR and CAP. This was the first report of the genetic mechanism and molecular markers associated with carpel number in citrus.

Key words*: regression analysis, inheritance, QTL.*

INTRODUCTION

Citrus is the fruit group with higher production worldwide, approximately 120 million tons annually (FAO, 2012). It exists in diploid forms generally, but occasionally exists in triploid and tetraploid forms (2n = 2x = 18). Improving citrus types in respect to important fruit characteristics requires new techniques due to high level of heterozygosity and apomixis. Mutation breeding and somatic hybridization are mostly used in current improvement programs, but these methods rarely contributed to improve the fruit character (DAVIES and ALBRIGO, 1994). Molecular genetic technology can provide new methods which will remove/reduce the obstacles mentioned above.
To date, genetic mapping studies have been focused only on some rootstock features which allows farming under several stress conditions: apomixes (GARCIA et al., 2000), salt resistance (TOZLU et al., 1999) and tristeza virus resistance (ROOSE, 2000). Many characters are controlled by quantitative trait loci (QTL) and genetic maps were usually based on quantitative traits (CHEN et al. 2007). Reports of mapping efforts on fruit characters are scarce (GULSEN etal., 2011). Inheritance of commerciality important fruit characters of citrus fruits is unknown. Data about the characteristics of other fruits, for example easy peeling, flesh color, puffing, granulation, pipiness, and aroma and carpel membrane thickness have not been reported. The purpose of this study was to investigate the association between molecular markers and carpel number in a segregating population derived from a cross between mandarin and tangelo.

MATERIALS AND METHODS

164 progenies derived from the hybridization between Clementine mandarin (*C. clementina* Hort. ex Tan.) and Orlando tangelos (*C. reticulata* Blanco x *C. paradise* Macf.) available in mandarin collection of Alata Horticultural Research Station as described by GULSENet al. (2010). Data file including fruit characteristics and molecular DNA markers were analyzed by using GLM and REGRESSION module in SAS software. Variance analysis has been implemented by GLM module first in respect to all DNA markers. All markers detected to be significant at 5% alpha level were subjected to advanced regression analysis by using REGRESSION module.

RESULTS AND DISCUSSIONS

The population used in this study indicated transgressive segregation between 9 and 14 carpels where parents Clementine and Orlando had 10 and 11 carpels, respectively. Distribution of hybrids to carpel number was as follows: 17 individuals with 9 carpels, 20 with 10 carpels, 18 with 11 carpels, 6 hybrids with 12 carpels and one with 13 and 14 carpels (Figure 1). Positive skewness were observed among the hybrids and segregation was transgressive meaning progenies indicated more or lower values for the trait. Distribution is bell-shaped also indicating quantitative control.

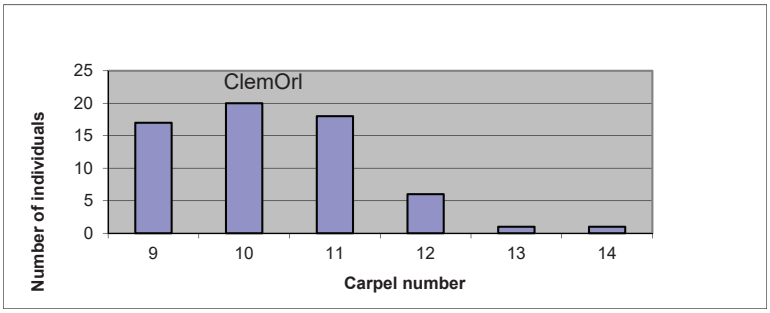

Figure 1. Distribution of F1 hybrids based on carpel number.
Abbreviations: Clementine, Cle; Orlando tangelo, Orl

In GLM analysis in SAS software detected 10 molecular markers that contributed 1% or more to carpel number in the population studied (Table 1). The first 5 loci in Table 1 explained 80% of the total variation, which was very significant. The markers explaining 5% or more of variation: OPW19.250 explained 43%, OPM20.230 22% and ISSRHVH $(CA)_7$ 5%. These 10 markers were subjected to regression analysis. The regression model explained 90% (R^2) of the total variation for carpel number and intercept value calculated was 0.69102. This was consistent with our observation from the Figure 1.

Table 1. SAS Abstracts obtained with the slice number analyze and SAS software 602 markers and 63 F1 hybrid slice number data have been shown with the explained variation ratios under partial R^2

Marker	R^2	F-value	Pr> F
OPW19.250	0.4321	15.98	0.0007
OPM20.230	0.2233	12.96	0.0018
ISSR HVH(CA)7	0.0532	3.47	0.0782
SRAPEM14ME7a	0.0459	4.42	0.0516
SSRAG14.150	0.0417	3.34	0.0851
SRAPEM2ME7b	0.0378	2.68	0.1188
SRAPEM10ME5a	0.0325	4.84	0.0465
SRAPEM7ME12a	0.0279	3.25	0.0928
SRAPEM16ME1a	0.0184	1.87	0.1919
SSRGA01.200	0.0153	2.55	0.1361

Hybrids derived from Clementine mandarin and Orlando tangelo were used in this research. F1 hybrids were characterized for carpel number by simply visually counting them. Molecular marker data were available from GULSEN et al. (2010). They have been used in SAS software in order to determine molecular markers associated with the carpel number. It was concluded that carpel number was controlled by quantitative loci (several genes). By using these molecular markers regression model explained more than 90% of the total variation. This was the first report related to genetic mechanism of carpel number in citrus. Thus this provided valuable insight into possible genetic mechanism of a fruit trait in

addition to fruit acidity previously reported by Fang et al. (1997). Developing genetic maps and molecular markers can provide important tools for citrus breeding programs. As we emphasized, in citrus which have long juvenility period and apomixes, the tools allowing early selection could play an important role in speeding improvement programs.

There is lack of studies of morphological characters which are quantitatively controlled in citrus. FANG et al. (1997) revealed that the population showed binary distribution but it is not significant in inter-class difference in the research which was carried out in order to find related markers for citrus acid level. It could be understood that the genes which has fewer effects played role besides the major gens. OPW19.250 marker determined in this study explained 43% of total variation alone. The second important locus, OPM20.230, explained 22% alone. Other 8 loci explained low level of variations. It could be said that loci in respect to carpel number have major effects and other loci have minor effects. This situation resembles the situation in acid accumulation. The obtained results could be used in order to increase our understanding of the genetic mechanism of important traits and speed up the breeding programs. Genetic studies about carpel number in citrus have not been reported yet. As mentioned above carpel number varied between 9 and 14. Carpel number of mandarins should not neither so few nor so many. When compared with orange, mandarins have fewer carpels in their fruits.

CONCLUSIONS

In this study the statistical analyses revealed significant findings on possible genetic mechanism of carpel number in citrus. First it has been showed that the segregation for the carpel number was transgressive in citrus, in which the progenies exceed their parents. Secondly, we detected two very significant loci that explained 44 and 22% of the total variation. Carpel number varied between 9 and 14 while parents had 10 and 11 carpels in their fruits. The population indicated positive skewness toward higher carpel number. The regression analysis indicated that 10 markers were placed in the regression model and explained more than 90% of the total variation for carpel number in citrus. This information may provide an important base for further research on this trait or other similar traits. Applicability of these markers for early selection of progenies of citrus with long juvenility should be further investigated. In addition, conversion of these markers to more locus specific markers such as SCAR and CAP) is necessary.

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REFERENCES

Chen C., Bowman K.D., Choi Y.A., 2007. EST-SSR genetic maps for Citrus sinensis and Poncirus trifoliata, Tree Genetics and Genomes 4: 1–10.

Davies F.S., Albrigo L.G., 1994. Citrus.CABI, Wallingford, UK.

Fang D.Q., Federici C.T., Roose M.L., 1997. Development of molecular markers linked to a gene controlling fruit acidity in *Citrus,* Genome 40: 841-9.

FAO, 2012, www.fao.org.

Garcia R., Asins M.J., Carbonell E.A., 2000.QTL analysis of yield and seed number in *Citrus,* Theoritical Applied Genetics 101: 487-493.

Gulsen O., Uzun A., Canan I., Seday U., Canihos E., 2010. A new citrus linkage map based on SRAP, SSR, ISSR, POGP, RGA and RAPD markers, Euphytica 173: 265-277.

Gulsen O., Uzun A., Seday U., Kafa G., 2011.QTL analysis and regression model for estimating fruit setting in young Citrus trees based on molecular markers. Scientia Horticulturae 130:418-424.

Roose M.L., Feng D., Cheng F.S., Tayyar R.I, Federici C.T., Kupper R.S., 2000.Mapping the Citrus genome. Proc. I. Int. Soc. Hort. Sciences, R. Goren and E. E. Goldschmidt, Eds., ActaHorticulturae, Leuven, Belgium.Pp: 25–32.

TozluI., Guy C.L., Moore G.A., 1999.QTL analysis of morphological traits in an intergeneric BC1 progeny of Citrus and Poncirus under saline and non-saline environments.Genome 42:1020-29.

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PHYTOHORMONE–LIKE PRODUCING *BACILLUS* INCREASE TOMATO SEEDLINGS QUALITY

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Abstract

Plant growth promoting activity is one of the attributes when searching for beneficial strains of bacteria. Our study aimed to present the potential of Bacillus sp. 83.2s and B. subtilis Bce2 to increase plant growth and vigour. The study is based on phytohormone - like evaluation in the selected Bacillus strains using classic biochemical analysis. Likewise, the plant beneficial effect was also evaluated by growth promotion studies on tomato seedlings, were biometric parameters and chlorophyll content index (CCI) were analyzed. The data have been processed into the following indicators: indole-3-acetic acid in bacterial cultures, and emergence, seedling height and vigour, shoot and root dry weight and CCI in tomatoes seedlings. Both bacterial strains produce high amounts of IAA phytohormones, from 10.4 - 13.8 µg/ml, in normal growth medium, up to 16.3 - 16.6 µg/ml, when 5 mM of tryptophan is added in the medium as auxin precursor. Moreover, B. subtilis Bce2 seed treatment, increased emergence index and seedlings vigour compared to the untreated control and exceeded the commercial growth regulator (Vimpel 77%) in terms of emergence percent, seedlings dry weight, and chlorophyll content index.

Key words*: Bacillus, tomato seedlings.*

INTRODUCTION

Tomato is a main crop in the greenhouse production system, cultivated with good results also in open fields. The main technology for growing tomatoes in our country is from seedlings. Regarding these aspects the quality of planting material is an important issue in tomatoes production. Seedlings quality is even more important in organic production. For healthy and vigorous planting material there are several studies recommending beneficial microorganisms inoculation.

Plant beneficial microorganisms are natural environmental microbes that could be exploited in terms of organic agriculture and biodiversity maintenance. The *Bacillus* genus includes a large number of plant growth-promoting rhizobacteria (PGPR). *Bacillus* bio-based products are highly appreciated in organic farming, not only for their plant protection and growth promotion abilities, but also for their endospore forming ability that gives stable, long lasting, easy to formulate and preserve inoculums.

Among the complex mechanisms involved in plant growth promotion, bacteria can produce phytohormone-like compounds and lytic enzymes, and can increase mineral uptake, nutrients availability and reconvert agricultural wastes (Goswami et al., 2016).

The aim of the preset study is to analyze plant growth promoting ability and phytohormone production in two biocontrol strains of *Bacillus* spp. Plant beneficial activity was evaluated in tomato seedlings maintained in growth chamber conditions.

MATERIALS AND METHODS

Bacterial inoculum

Two bacterial strains were previously selected for their plant beneficial activity (Sicuia et al., 2015; Dinu et al., 2015). Bacterial strains used in this study were Romanian isolates *Bacillus* sp.83.2s and *B. subtilis* Bce2. These bacteria were routinely grown on Luria Bertani medium at 28°C. For bacterial cell suspension, 48h old broth cultures were centrifuged at 3750rpm, for 15minutes, at 10ºC and the pellet was resuspended in phosphate buffered saline (PBS) up to 10^8cfu/ml. The concentration was estimated spectro-photometric at 600nm

wavelength when the optical density reached the absorbance of 1.

IAA synthesis ability

The auxin synthesis in bacterial cultures was evaluated in Luria Bertani broth (LB) and LB supplemented with tryptophan in concentration of 2.5M, and 5M, respectively. Cultures were inoculated with 10% bacterial suspension of 10^8cfu/ml. After 24h of cultivation at 28°C, and 150rpm stirring, the supernatant was harvested by centrifugation and analyzed for auxin quantification. Two ml of each supernatant was treated with 3 drops of o-phosphoric acid, and 4ml Salkowski reagent ($FeCl_3$-$HClO_4$). The homogenate was incubated for 25 min at room temperature. Subsequently IAA was spectrophotometric quantified at 530nm. The results were correlated with a standard curve containing 10 ÷ 100mg IAA/ml.

***In situ* evaluation of plant-growth-promoting activity on tomatoes seedlings**

The study was performed on tomato *Lycopersicon esculentum* Heinz 2274 cultivar. Seeds were surface disinfected, with 70% ethanol, by 3 minutes immersion and subsequently, with sodium hypochlorite 4% by 15 min. immersion, than rinsed ten times with sterile distilled water. For plant growth promotion study, seeds were treated with bacterial cell suspension 10^8cfu/ml PBS, supplemented with 2‰ carboxy-methyl cellulose to ensure the adhesion of the inoculum to the seeds tegument. As positive control a commercial plant growth regulator, Vimpel 77%, was applied as seed treatment and as soil fertigation 20ml/plant, two weeks after seed germination, both treatments in 0.05% concentration. For the negative control, untreated, seeds were immersed in sterile distilled water.

Alveolar trays with peat mixture were seeded with one seed/cell, having 7cm diameter and 8cm depth. Thirty seeds were sown for each experimental variant.

Plants were maintained for five weeks in SANYO MLR-351H growth chamber under a 16 h daylight period. Light intensity, around trays, was approximately 14000 lx. The air temperature was set at 25°C during the day and 16°C during the night. Relative humidity was constant at 70%, and the soil moisture was maintained with tap water.

Plant growth and vigour parameters

The emergence rate (EP) was evaluated one week after sowing.

$$EP\% = \frac{\text{Emerged seedlings}}{\text{Sown seeds}} \times 100$$

Emergence index (EI) was calculated by adapting the formula mentioned by Geetha et al. (2014) for the germination index:

$$EI = \frac{EP\% \text{ in treatment}}{EP\% \text{ in control}}$$

Four weeks after plantlets emerged shoots were measured in order to determine their length. At the end of the experiment, plants were gently removed from the substrate and roots were washed with tap water.

Fresh and dry weight of the shoot and root were taken. The dry weight was determined after drying the plant material at 105°C for at least 3h. To determine the seedling vigour index (SVI) we used two calculation methods, by adapting the formulas presented by Adebisi et al. (2010) and Alirezaie Noghondar and Azizi (2013) for SVI – 1 and SVI – 11, respectively.

$$SVI-1 = \frac{\text{Seedling lenght} \times EP\%}{100}$$

$$SVI-11 = \frac{\text{Seedling dry weight} \times EP\%}{100}$$

Chlorophyll content index

Relative chlorophyll content was estimated with a Chlorophyll Content Meter (CCM-200plus, Opti-Sciences) in intact leaf samples, using a non-destructive method. The Chlorophyll Content Index (CCI) values are determined by the absorbance ratio at two wave lengths. One wavelength falls within the chlorophyll absorbance range while the other serves to compensate for mechanical differences such as tissue thickness (www.apogeeinstruments.com).

$$CCI = \frac{\text{Transmittance\% at 931nm}}{\text{Transmittance\% at 653nm}}$$

The CCI values are proportional with the chlorophyll content in the sample.

RESULTS AND DISCUSSIONS

IAA synthesis in *Bacillus* cultures

Bacteria cultures of *Bacillus* sp.83.2s and *B.subtilis* Bce2 were analyzed for IAA quantification after 24hours of growth in simply LB broth and LB supplemented with tryptophanas auxin precursor. In this respect the optical densities determined at 530 nm were related to the IAA calibration curve with known auxin content.

IAA quantification showed that *Bacillus* sp. 83.2s strain is a better auxin producer in LB broth than *B.subtilis* Bce2 strain. However, when the growth medium was supplemented with tryptophan, Bce2 revealed to produce more IAA than the other strain tested (Table 1).

Table 1. IAA amount in tested *Bacillus* cultures

Experimental variants	IAA (µg/ml)		
	LB broth	LB & 2.5 mM tryptophan	LB & 5 mM tryptophan
Bacillus sp. 83.2s	13.8	15.6	16.3
B. subtilis Bce2	10.4	16.1	16.6

As the results shown, the two analyzed strains are producing higher amounts of auxin(Figure 1)compared to other plant beneficial strains of *Bacillus* sp. mentioned in the literature. Acuña et al. (2011) mentioned that *Bacillus* sp. MQH-19 strain produced only 3 to 6 µg IAA/ml. In similar growth conditions, *Bacillus* sp. Q3 strain produced 3.76 to 10.62 µg IAA/ml (Starovic et al., 2013). However, in yeast malt dextrose broth with or without tryptophan, the amount of IAA would significantly increase 5 to 8 fold (Mohite, 2013).

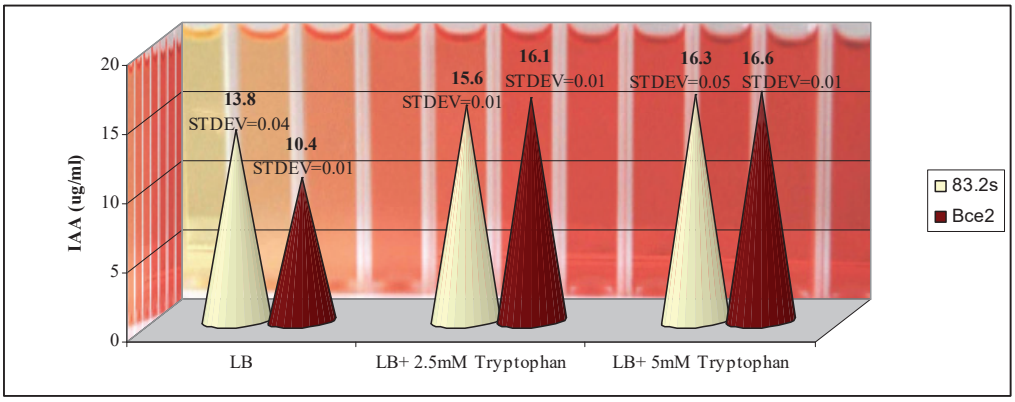

Figure 1. Comparative IAA production in *Bacillus* sp.83.2s and *B. subtilis* Bce2 cultures (after 24h of incubation)

Plant growth promotion

Bacterial seed treatment promoted the tomatoes seedling growth (Table 2). All seeds germinated and seedlings emerged when Bce2 treatment was applied. The other bacterial treatment, 83.2s, provided the same emergence rate (90%) as in the positive control, treated with Vimpel. While in the untreated control the emergence percentage was 83.3%.

Table 2. Tomato seedlings growth promotion with bio-based treatments

Specifications	**EP%**	EI	**Seedling length (cm)**	**Shoot dry weight (g)**	**Root dry weight (g)**	**Seedling vigour DW/SH**	SVI-1	SVI-11
Bacillus sp. 83.2s	90%	1.08	8.7	2.38	0.28	0.31	7.83	2.39
Bacillus. subtilis Bce2	**100%**	**1.2**	9.3	**2.77**	**0.31**	**0.33**	9.30	**3.08**
Vimpel 77% Positive control	90%	1.08	**11.0**	2.23	0.25	0.23	**9.90**	2.32
Untreated control	83.3%	-	8.6	1.92	0.20	0.25	7.16	1.77

The Vimpel treatment induced seedling elongation in a higher rate than the other treatments (Figure 2). However, excessive stem elongation is considered a drawback (Figure 3). Comparing seedlings height (SH) with their dry weight (DW), it can be noticed that Bce2 treatment enhanced seedlings growth promotion with a better ratio (as DW/SH).
Regarding seedling weight the best results were registered in the bacterial treatment with Bce2 strain (3.08g), followed by 83.2s (2.66g) and Vimpel (2.48g). Both bacteria treatments increased plantlets biomass (as DW), compared to the untreated control, as well as if comparing with the positive control. Bce2 increased seedlings DW with 45.3%, 83.2 with 25.5% and Vimpel with 17% compared to the untreated control that developed 2.12 g of DW. Also compared to the positive control (Vimpel) the two bacterial treatments, Bce2 and 83.2s, led to an increased DW content, with 24.2% and 7.3%, respectively (Figure 2).

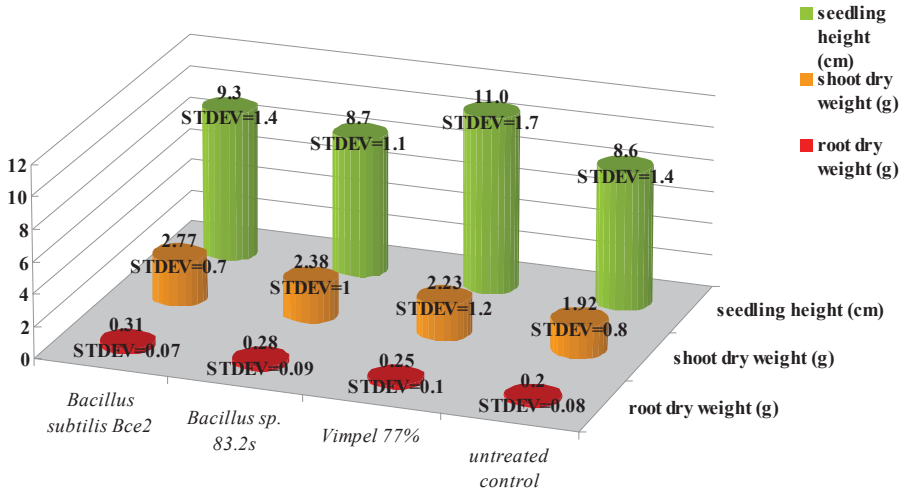

Figure 2. The influence of *Bacillus* treatments on vegetative growth of tomato seedlings

Figure 3. Tomato seedlings with bio-based treatments

The seedling vigour index (SVI), as calculated with Adebisi et al. (2010) and Alirezaie Noghondar and Azizi (2013) formulas, reveal *B.subtilis* Bce2 and Vimpel as competitive treatments for growth promoters.

Relative chlorophyll content

The highest CCI values were registered in the Bce2 bacterial treatment followed by 83.2s treatment (Table 3).

Table 3. Relative chlorophyll content in tomato leafs during seedling growth

Specifications	Weeks after emerging							
	1st		2nd		3rd		4th	
	CCI	STDEV	**CCI**	STDEV	**CCI**	STDEV	**CCI**	STDEV
Bacillus sp. 83.2s	**13.2**	4.26	**25.13**	6.38	**28.69**	5.79	**34.76**	5.5
B. subtilis Bce2	**14.44**	3.86	**26.18**	6.08	**28.89**	5.26	**37.12**	3.99
Vimpel 77% Positive control	**11.51**	2.74	**23.3**	4.68	**24.91**	4.02	**27**	4.78
Untreated control	**11.32**	1.47	**21.15**	5.18	**26.71**	4.28	**33.54**	6.7

A positive correlation was observed between seedlings vigour (as DW/SH) and their relative chlorophyll content. This aspect is more evident in the experimental variant treated with Vimpel, especially after the fertigation, when the chlorophyll content was significantly slowed. The lowest CCI values, registered after the second treatment with Vimpel, could be due to a growth stimulation that leaded to an increased seedling elongation without promoting seedling vigour (correlation of Tables 2 and 3).

CONCLUSIONS

The two strains proposed in the present study, *Bacillus* sp. 83.2s and *B. subtilis* Bce2, revealed plant growth promotion activity *in vitro* and *in vivo* in seedlings trials. Both strains produced IAA phytohormone. In normal culture conditions a higher amount of IAA was produced by 83.2s strain (13.8µg/ml). However, when auxin precursor was added into the growth medium Bce2 strains showed a higher capacity of tryptophan conversion that lead to an IAA production of 16.6 µg/ml.

Bacillus subtilis Bce2, applied as seed treatment, increased the germination percent and seedlings vigour compared to the untreated control and exceeded the commercial growth regulator (Vimpel 77%) in terms of germination, shoot and root dry weight, and chlorophyll content index.

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REFERENCES

Acuña J.J., Jorquera M.A., Martínez O.A., Menezes-Blackburn D., Fernández M.T., Marschner P., Greiner R., Mora M.L., 2011. Indole acetic acid and phytase activity produced by rhizosphere bacilli as affected by pH and metals. Journal of Soil Science and Plant Nutrition, 11 (3): 1-12.

Adebisi M.A., Okelola F.S., Alake C.O., Ayo-Vaughan M.A., Ajala M.O., 2010. Interrelationship between seed vigour traits and field performance in new rice for africa (Nerica) genotypes (*Oryza sativa* L.). Journal of Agricultural Science and Environment, 10(2):15-24.

Alirezaie Noghondar M., Azizi M., 2013. Seed harvesting time afects seedling emergence, vigour and growth: Case study of *Rumex turcomanicus* Czerep. (*Polygonaceae*). Notulae Scientia Biologicae, 5(2):244-248.

Dinu S., Sicuia O.A., Constantinescu F., 2015. The influence of several abiotic factors on some bacterial strains of phytosanitary use. Romanian Journal of Plant Protection, 8:1-6.

Geetha K., Chaitanya K., Bhadraiah B., 2014. Isolation and characterization of PGPR isolates from rhizosphere soils of Green gram in Warangal district of Telangana. Int J Pharm Bio Sci, 5(4): (B) 153 – 163.

Goswami D., Thakker J.N., Dhandhukia P.C., 2016. Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. Cogent Food & Agriculture, 2(1):1127500.

Mohite B., 2013. Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. Journal of Soil Science and Plant Nutrition, 13(3): 638-649.

Sicuia O. A., Grosu I., Constantinescu F., Voaideş C., Cornea C. P. 2015. Enzymatic and genetic variability in *Bacillus* spp. strains with plant beneficial qualities. AgroLife Scientific Journal, 4(2): 124-131.

Starovic M., Josic D., Pavlovic S., Drazic S., Postic D., Popovic T., Stojanovic S., 2013. The effect of IAA producing *Bacillus* sp. Q3 strain on marshmallow seed germination. Bulgarian Journal of Agricultural Science, 19 (3): 572-577.

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INFLUENCE OF DIETHANOLAMINE SALT OF 4-NITROBENZOIC ACID IN CALLUS CULTURE AT *MOMORDICA CHARANTIA* L.

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Abstract

Medicinal plants and their products are an important solution to improve the treatment of people in the whole world. Momordica charantia L. *is a well-known species for its biological activity (antioxidant and antimicrobial activity) and contains a complex of beneficial compounds such as: vitamins, minerals and antioxidants that can be used for treating a wide range of illnesses, especially diabetes.*
Plant tissue culture is an important and facile method for the somatic variability induction and tissue lines selection inorder to obtain valuable secondary metabolites. The use of the substances that control growth and synthesis capacity of tissues allow us to produce under aseptic conditions significant quantities of plant metabolites. The controlled conditions give to the tissue culture a suitable microenvironment for the successful growth and biosynthesis. Phytohormons and other substances with the same effect can determine in tissue culture the increase of biosynthetic capacity which can lead to obtaining and selection of proliferative tissue lines producing secondary metabolites.
The present work aims to study the influence on M. charantia L. *tissue culture of a new biological active compound, diethanolamine salt of 4-nitrobenzoic acid (4-NO_2BA DEA), synthesized by the Institute of Chemistry Timisoara of Romanian Academy. In this research we used 6 hormonal balances in which we associated 4-NO_2BA DEA with cytokinin BAP and also the new compound alone on the MS culture medium, for the selection of tissue lines with high growth capacity.We note that increasing the amount of 4-NO_2BA DEA in the MS culture medium could have beneficial effects on tissue culture at M. charantia.*

Key words*: auxins, callus, Momordica charantia* L.

INTRODUCTION

Momordica charantia L. is a medicinal plant commonly known as bitter melon, balsam pear, bitter cucumber, or bitter gourd, karela (India), fukwa (China), and ampalaya (Philippines). The plant can grow on different types of soils. The flower comes into blossom about one month after planting.
Bitter gourd has a beneficial effect in the treatment of cancer, viral infections (HIV, herpes, Epstein Barr, hepatitis, influenza and measles), bacterial infections (*Staphylococcus*, *Streptococcus* and *Salmonella*), bitter digestive aid (dyspepsia and sluggish digestion), but is well known for the hypoglycemic effect (Budrat and Shotipruk, 2008; Kim et al., 2003), this plant being known as the insulin plant. The main constituents of *Momordica charantia* L. that are responsible with the antidiabetic effects are: triterpene (charantin), protein, steroid, alkaloid, inorganic, lipid and phenolic compounds (Grover Yadav, 2004). The callus culture under the hormone influence allows the selection of tissue lines with high growth capacity, which can be used in the secondary metabolites production (Simina et al., 2014).
Benzoic acids and their derivatives are important compounds involved in various physiological processes in plants. Furthermore, they regulate seed germination (Ng et al., 2003; Crisan et al., 2014;Crisan et al., 2009;Crisan et al., 2007), have been functionally associated with disease resistance and stress tolerance in plants (Dempsey et al., 1999). Ethanolamine salts of different substituted benzoic acids are new compounds synthesized by the Institute of Chemistry Timisoara of Romanian Academy, some of them revealing auxin-likeplant growth regulatory activity on *Arabidopsis thaliana* and *Cucumissativus* L. (Crisan et al., 2014). In contrast to corresponding benzoic acids, the

alkanolamine salts are water soluble, significantly influencing the plant growth activity. This research focuses on testing of new compound 4-NO_2BA DEA in association or not withcytokinin BAP in order to study the growth capacity of *Momordica charantia* L. tissue culture.

MATERIALS AND METHODS

Momordica charantia L. callus was cultivated on MS medium (Murashige and Skoog, 1962) supplemented with 6 variants of hormonal balances, representing combinations of auxins, cytokinins in which we associated auxin 4-NO_2BA DEA with cytokinin BAP and also the new tested compound alone on the MS culture medium, for the selection of tissue lines with high growth capacity.

The new compound, 4-NO_2BA DEA, was obtained by controlled method, *via* proton exchange reaction, from 1:1 molar amounts of 4-nitrobenzoic acid and diethanolamine, in acetone solvent.The different variants of hormonal balances used in our experiment are showed in Table 1.

Table 1. Phytohormonal variants

Hormonal Balance	Phytohormons (mg/l)	
	*4-NO_2BA DEA	**BAP
BH1	1.5	0.0
BH2	1.5	1.0
BH3	1.0	0.0
BH4	1.0	1.0
BH5	0.2	0.0
BH6	0.2	1.0

*4-NO_2BA DEA = 4-nitrobenzoic acid
**BAP = 6-benzylaminopurine

RESULTS AND DISCUSSIONS

Based on the results presented in Table 2, it is showed that the effect of the hormonal balances in the callus culture of *Momordica charantia* L. has a significant influence on the growth of callus at *Momordica charantia* L., instead the combined effect of the hormonal balance and the duration of culture did not show a significant effect on the growth of callus at *Momordica charantia* L.

Table 2. Variance analysis regarding the effect of hormonal balances and *in vitro* culture duration on the growth of *Momordica charantia* L. callus

Source of variation	SP	GL	S^2	Test F
Total variation	14854.5	95		
Hormonal balance	4072.5	5	814.5	5.67**
Culture duration	223.5	3	74.5	0.52
Balance x Duration	224.5	15	15.0	0.10
Error	10334.0	72	143.5	

Regarding the effect of *in vitro* cultivation period on callus growth at *Momordica charantia* L. (Table 2) we can see that in the first 14 days of culture, the growth of *Momordica charantia* L. callus culture recorded an amplitude variation of 1.4%, with values between 114.8 % after seven days and 116.2% after 14 days of culture. After 21 days of culture,a decrease in the amplitude variation to 3.1% with the average of 113.1% is observed. The decrease in the amplitude measurements continue until reaching 0.8%, with an average value of 112.3% at the end of the determinations.

Table 3.The effect of *in vitro* culture duration on the growth of *Momordica charantia* L. callus

Culture duration (days)	Callus growth (%) compared to baseline		Relative values (%)	Difference/ Significance
14 - 7	116.2	114.8	101.22	1.4
21 - 7	113.1	114.8	98.52	-1.7
28 - 7	112.3	114.8	97.82	-2.5
21 - 14	113.1	116.2	97.33	-3.1
28 - 14	112.3	116.2	96.64	-3.9
28 - 21	112.3	113.1	99.29	-0.8

$DL_{5\%}$=6.9 $DL_{1\%}$=9.1 $DL_{0.1\%}$=11.9

Based on the results obtained (Table 4), we can say that the duration of *in vitro* culture does not have an influence on callus growth of *M. charantia* L. throughout the period of our determinations. It also notes that in the first 7 days of culture, the effect of hormonal balance on callus growth does not occur. But after 14 days of culture, significant differences between hormonal balances BH1 (4-NO_2BA DEA 1.5mg/l), BH3 (4-NO_2BA DEA 1.0 mg/l) and

BH4 (4-NO_2BA DEA 1.0mg/landBAP 1.0mg/l)can be observed. After 21 days of culture we can note that there is a differentiating effect of hormonal variations, recording significant differences between BH1 (4-NO_2BA DEA 1.5mg/l), BH2 (4-NO_2BA DEA 1.5mg/l and BAP 1.0mg/l) and BH3 (4-NO_2BA DEA 1.0 mg/l), which last until the end of measurements (28 days).

Table 4.The effect of hormonal balance and *in vitro* culture duration on the growth of *Momordica charantia* L. callus

Hormonal balance	Culture duration (days)					
	7	**14**	**21**	**28**	$\bar{x} \pm s_{\bar{x}}$	$S_{\%}$
BH1	x118.0a	x119.0ab	x115.0ab	x114.5ab	116.6±1.3	4.41
BH2	x122.5a	x128.0a	x127.0a	x125.0a	125.6±4.2	13.28
BH3	x115.0a	x116.0ab	x110.0b	x108.0b	112.2±1.8	6.44
BH4	x109.5a	x109.5b	x106.5b	x108.0b	108.4±2.5	9.17
BH5	x108.5a	x107.5b	x103.0b	x103.0b	105.5±1.4	5.38
BH6	x115.5a	x117.5ab	x117.0ab	x115.5ab	116.4±3.8	13.05
$\bar{x} \pm s_{\bar{x}}$	114.8±2.1	116.2±2.6	113.1±2.8	112.3±2.7	114.1±1.3	
$S_{\%}$	9.19	11.08	12.12	11.64	10.96	

$DL_{5\%}$=16.9 $DL_{1\%}$=22.4 $DL_{0,1\%}$=29.1

Under the aspect of callus growth variation (Figure 2) in case of using the hormonal balance 4-NO_2BA DEA 1.5mg/l and BAP 1.0 mg/l it is noted a proportional increase during the duration of the callus culture until 19 days, when it reaches a maximum gain of 28 % compared to the original value. Subsequently in the last nine days of culture a decreasing trend is observed, so in the end a 25% growth increase is achieved.When using the hormonal balance 4-NO_2BA DEA 1.5 mg/l, there is a regressive evolution of callus growth from the fourth day of culture, when it is recorded the maximum size equivalent to 118.5% compared to the original value and finally ending after 28 days of culture to 114.5% compared to originalvalue.

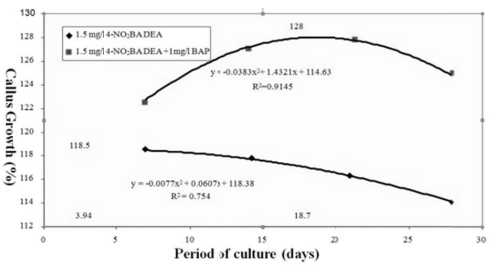

Figure 1. The growth rate of *M. charantia* L. callus under BH1 (4-NO_2BA DEA 1.5 mg /l) orBH2 (4-NO_2BA DEA 1.5 mg /l and BAP 1.0 mg /l)hormonal balances

Decreasing the amount at 1mg/l 4-NO_2BA DEA has led to the regression of callus growth from the fourth day of culture, when it is recorded a maximum size equivalent to 115.6% from baseline and finally ending after 28 days of culture at 107 5% compared to baseline (Figure 2). When 4-NO_2BA DEA (1.0 mg/l) and BAP (1.0 mg/l) is combined, there is again a regressive evolution of callus growth from the fourth day of culture, when it is recorded a maximum size equivalent to 109.6% compared with the original value, reaching 108%. Then a period of stagnation isfollowed which will be recorded until the end of measurements.

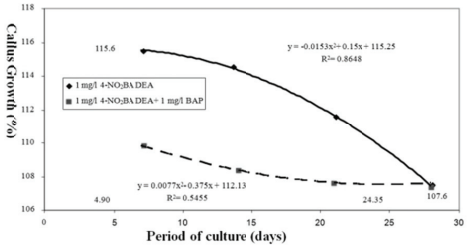

Figure 2. The growth rate of *Momordica charantia* L. callus BH3 (4-NO_2BA DEA 1.0 mg /l) or BH4 (4-NO_2BA DEA 1.0 mg /l and BAP 1.0 mg /l)hormonal balances

The callus growth variation is showed in Figure 3.The use of a concentration like 0.2 mg/l of 4-

NO_2BA DEA leads to a proportional increase in the duration of culture until about 16 days of culture and then it is followed by a period of stagnation for 3 days.After 19 days of culture it is observed a downward trend since the beginning of determinations. The use of the hormonal balance 4-NO_2BA DEA 0.2 mg/l and BAP 1.0 mg/l has led to the regression of callus throughout the period of the measurements leading to a lower value (103.5%) than that obtained in the first determination (109.5%).

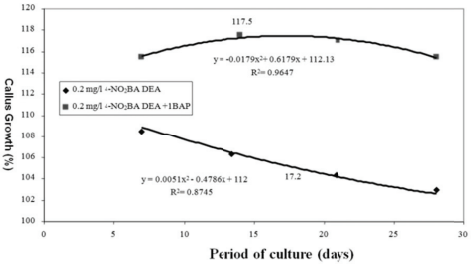

Figure 3. The growth rate of *Momordica charantia* L. callus under BH5 (4-NO_2BA DEA 0.2 mg/l)or BH6 (4-NO_2BA DEA 0.2 mg/l and 1.0 mg/l BAP) hormonal balances

CONCLUSIONS

Based on results carried out during our research we can conclude thatthe use of different concentrations of 4-NO_2BA DEA with or without BAP has a significant effect on the callus growth of *Momordica charantia* L.

Each of the hormonal balance used recorded a percentage increase of callus growth in the first 14 days of culture, followed by a slowing of the callus growth.

This preliminary study shows that the growth capacity of *Momordica charantia* L. tissue culture increases proportionally with the increasing concentrations of 4-NO_2BA DEA used.

REFERENCES

Budrat P., Shotipruk A., 2008. Extraction of Phenolic Compounds from Fruits of Bitter Melon (Momordica charantia) with Subcritical Water Extraction and Antioxidant Activities of These Extracts. Chiang Mai Journal of Science, 35(1):123-130.

Crisan M.E., Bourosh P., Maffei M.E., Forni A., Pieraccini S., Sironi M., Chumakov Y.M., 2014. Synthesis, Crystal Structure and Biological Activity of 2-Hydroxyethyl-ammonium Salt of *p*-Aminobenzoic Acid.Plos One, 9(7):e101892.

Crisan M, Grozav M, Bertea C., 2009. *Arabidopsis thaliana* seed germination and early seedling growth are inhibited by monoethanolamine salts of *para*-halogenated benzoic acids. Journal of Plant Interaction, 4(4)271DOI: 10.1080/17429140903063072.

Crisan M, Grozav M, Kurunczi L, Ilia G, Bertea C., 2007. Inhibitory Effects of some Synthetic Monoethanolamine Salts of *para*-Substituted Benzoic Acids and Corresponding Benzoic Acids on Cucumber Seed Germination. Journal of Plant Interaction, 2(1) 53–61.

Dempsey D.M.A., Shah J., KlessigD.F., 1999.Salicylic acid and disease resistance in plants.Critical reviews in plant sciences, 18:547-575.

Grover J.K., Yadav S.P., 2004. Pharmacological actions and potential uses of Momordica charantia: A Review. Journal of Ethnopharmacology, 93(1):123-132.

Kim D.O., Seung W.J., Lee C.Y.,2003. Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chemistry, 81(3):321-326.

Murashige T., Skoog F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15(3):473-497.

Ng P.L.L., Ferrarese M.L.L., Huber D.A., Ravagnani A.L.S., Ferrarese-Filho O., 2003. Canola (*Brassica napus* L.) seed germination influenced by cinnamic and benzoic acids and derivatives: Effects on peroxidase, Seed Science and Technology, 31(1):39–46.

Simina A., Botau D., Popescu S., 2014. The evaluation of somatic variability in the callus of bitter melon (*Momordica charantia* L.) using molecular methods. Journal of Horticulture, Forestry and Biotechnology,18(3):87-91.

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EXTRACTION AND ANALYTICAL METHODS OF CAPSAICINOIDS - A REVIEW

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Abstract

Bioactive natural products are a main source of new drugs, functional foods and food additives. Chilli pepper is a very important plant used worldwide as a vegetable, a spice and an external medicine. Capsaicin, the pungent principle of Capsicum sp. (69%) is one of the best-known natural compounds. The extraction of the capsacinoids can be made in many ways, with different types of organic solvents, but the yield varies with peppers variety and the conditions of extraction processing. The use of supercritical fluids, especially carbon dioxide, in the extraction of oleoresins, has increased during the last two decades, because it is a simple, inexpensive, fast, effective and solvent-free sample pretreatment technique in comparison with traditional methods (maceration, Soxhlet). Also, the level of capsaicinoids in the Capsicum fruits can be quantified by organoleptic, spectrophotometry, thin-layer chromatography, gas chromatography and high-performance liquid chromatography methods. Of these, high-pressure liquid chromatography (HPLC) is considered the most reliable and rapid method available for the identification and quantification of capsaicinoids.

Key words**:** *organic solvents, supercritical fluid extraction, HPLC, peppers.*

INTRODUCTION

Plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives. The production of valuable secondary metabolites is an attractive alternative to the that of whole plant extract (Namdeo et al., 2007).

GENUS *CAPSICUM*

The most important components in the group of secondary metabolites are derived from the biologically active components of the*Capsicum* species. (Tilahun et al., 2013).

The genus *Capsicum*, which originates from tropical and humid zones of Central and Southern America, belongs to the *Solanaceae* family and includes peppers of important economic value.

More than thirty of *Capsicum* species exist, five of which are domesticated and these are *C. pubescens, Capsicum baccatum, C. annuum, Capsicum chinense, Capsicum frutescens*, the last three species being widely spread and having the most pungent fruits.

Pepper pungency is measured in Scoville Heat Units (SHU). This measurement is the highest dilution of a chile pepper extract at which heat can be detected by a taste panel (Bosland et al., 2007).

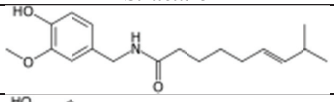
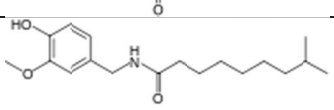
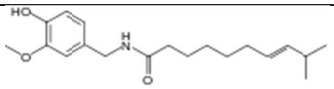
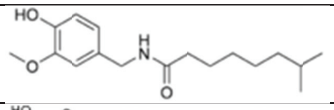
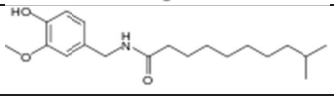
Capsicum is the only genus known to produce capsaicinoids and capsaicin is the major and the most active, pungent compound of chilli peppers (Yamaguchi et al., 2010).

It is represented by 69%, dihydrocapsacin by 22%,nordihydrocapsacin by 7% homocapsaicin and homodihydrocapsaicin takes only 1% in the group of capsaicinoids (Gudeva et al., 2013) (Table 1).

According to most reports, capsaicinoids accumulate in the epidermis of the placenta are stored in vesicles on the surface of this tissue, while seeds occasionally absorb capsaicinoids because of their proximity to the placenta and the content in fruit flesh is very low (Moreno et al., 2012).

Recent studies showed that the concentration of capsaicin varies with taxa and genotype, the geographical origin and the climatic conditions (Cisneros-Pineda et al., 2007). The only *Capsicum* which don't contain capsaicin is the sweet pepper.

Table 1: Chemical structure of different capsaicinoids

Compounds	*Structure*
Capsaicin	
Dihydro-capsaicin	
Homo-capsaicin	
Nordihydro-capsaicin	
Homodihydro -capsaicin	

The structural characteristics of capsaicinoids that determine their spicy properties are associated with the presence of an amide bond connecting a vanillyl ring and an acyl chain.
Capsaicin was first crystallized in 1876 by Tresh, who named it and capsaicin's molecular structure was resolved by Nelson and Dawson in 1919, with the following structural formula:

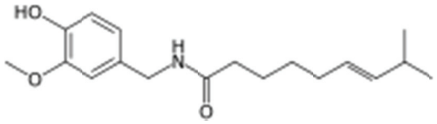


Figure 1: (E)-N-[(4-Hydroxy-3-methoxyphenyl) methyl]-8-methylnon-6-enamide)

The physico-chemical properties of capsaicin are presented in Table 2(Arora et al., 2011).

Table 2: Physico-chemical properties of capsaicin

Properties	*Value*
Molecular weight	305.41 g/ mol
Melting point	62 to 65 °C (144 to 149 °F; 335 to 338 °K)
Boiling point	210 to 220 °C (410 to 428 °F; 483 to 493° K) 0.01 Torr
Flash point	113°C
Stability	Stable. Incompatible with strong oxidizing agents.
Solubility	H_2O- insoluble; alcohols and organic solvents-soluble
UVmax.	227 - 281 nm

CAPSAICINOID EXTRACTION

Capsaicinoid extraction from peppers is typically performed using organic solvents and the extraction efficiencies can vary with peppers, their parts and pre-extraction processing.
Attuquayefio and Buckle (1987) have determined the extraction of capsaicinoids from *Capsicum* fruits and oleoresins using solvents such as acetone, chloroform, methanol, acidified methanol and acetonitrile and they mentioned that acetone resulted in the highest capsaicinoid yields from dehydrated ground *Capsicum*. Later, in another study, Collins et al. (1995) suggested extraction with acetonitrile at reflux, but Barbero et al. (2006) found that acetonitrile is a fairly efficacious solvent, but less than ethanol and methanol.Chinn et al. (2011) studied the effects of solvent type (ethanol, acetone and acetonitrile) on capsaicinoid extraction. Ethanol and acetonitrile were better solvents for capsaicin extraction from fresh samples, while acetone was better for dried pepper parts.
In contrast, Nwokem et al., 2010 chose methanol for obtaining capsaicin, because of high extraction efficiency resulting with reduced amounts of pigments and oils extracted together with capsaicin, as compared to other suitable solvents, like acetone. The highest concentration of capsaicin was 9.177mg/g. Rafajlovska et al., (2011) using methanol, ethanol and n-hexane (ratio 1:20 w/v) for extraction of capsaicinoids from red pungent dried paprika fruits, methanol and ethanol being confirmed to be superior and selected as future extraction solvents.
Haejin et al., (2012) studied the presence of capsaicinoids in samples from different cultivars of hot pepper. Levels of capsaicinoid extraction were observed in the following order: hexane > EtOAC > acetone >MeOH. The maximum amounts of capsaicin and dihydrocapsaicin were extracted in hexane, ranging from 35.1 to 2495 µg/g and 16.8 to 1016 µg/g respectively. So, their study confirmed the use of hexane for extraction of capsaicinoids, using a Soxhlet method.
But, from all organic solvents utilized in extraction process, ethanol was the single non-toxic solvent. So, in a recent study, Xinrong et al. (2014) chose ethanol as solvent for

obtaining red pigment and capsaicinoids from peppers, 90°C, 4 ml/g solvent ratio and 120 minutes extraction time, without remain of toxic solvent.

The microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are new techniques that combine microwave and ultrasound treatments, respectively, with traditional solvent extraction, for extraction of capsaicinoids.

The ultrasound-assisted extraction of capsaicinoids procedure used by Barbero et al., (2008) allowed extraction of the capsaicinoids present in peppers, in a short time (10 minutes), employing methanol as extraction solvent. They concluded that the developed method can be applied for the routine analysis of capsaicinoids in peppers. Barbero et al., (2014) extracted capsaicinoids using methanol, ultrasound power 360 W and 25 ml solvent/0.2 grams, during 15 minutes, at 50°C. The content of capsaicinoids was 1789 μmol/kg fresh weight.

Chuichulcherm et al. (2013) studied capsaicinoids extraction by microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), compared with traditional Soxhlet method, using dried chilies (*Capsicum frutescens* L.). They concluded that the optimum method for extraction of capsaicinoids from *C. frutescens* was UAE method. So, although the capsaicinoids from MAE and UAE were 5.28 and 4.01 mg/g dried chili, respectively, UAE required minimum energy consumption and was evaluated as the most suitable method.

Another extraction techniques involving supercritical fluids have been investigated extensively over the last decades due to the numerous advantages offered in comparison with the conventional techniques of extraction. Apart from having relatively low critical pressure (74 bar), temperature (32°C) and favorable solubility, CO_2 is relatively non-toxic, non-flammable, available in high purity at relatively low cost, and is easily removed from the extract.

The aim of study proposed by Duarte et al., 2004 was to assess supercritical fluid extraction (SFE) of red pepper (*Capsicum frutescens* L.) oleoresins. The influence of pressure and superficial velocity of supercritical CO_2 at 313°K, on the *Capsicum frutescens* oleoresins yield and capsaicinoids content was studied. They found that the supercritical carbon dioxide can be used as solvent to obtain extracts from *Capsicum frutescens* and the highest extraction yields in oleoresins and capsaicinoids were obtained at pressures around 20-22MPa.

More recent, the objective of the study proposed by de Aguiar et al., (2013)was to select a variety of pepper with high concentration of capsaicin and subject it to supercritical fluid extraction (SFE), in order to determine the best conditions of temperature (40°C-60 °C) and pressure (15, 25 and 35 MPa). The conditions that presented numerically higher concentration of capsaicinoids extracted per mass of the sample were 15 MPa and 40 °C, which being considered the most suitable for extraction of capsaicinoids.

Rocha-Uribe et al., (2014) concluded in their study that oleoresin may be extracted from habanero chili powder using supercritical CO_2, providing capsaicinoids and carotenoids free of organic solvents that may be used in food industryfor human consumption.

Santos et al. (2015) extracted capsaicinoids from *Malagueta* pepper (*Capsicum frutescens* L.) using supercritical fluid extraction (SFE) assisted by ultrasound, with carbon dioxide as solvent at 15 MPa and 40°C. The SFE global yield increased up to 77% when ultrasound waves were applied, and the best condition of ultrasound-assisted extraction was 360 W ultrasound power applied for 60 minutes. So, the use of ultrasound represented an efficient manner of producing small scale agitation, enhancing mass transfer on supercritical fluids (SF) extraction processes.

ANALYTICAL METHODS

In the last decade, there has been an increasing demand for new analytical methods that are more reliable and accurate, with short operational time and reduced cost, as well as with minimized use and generation of hazardous substances (Pena-Alvarez et al., 2009).Conventional methods used in determining the level of pungency or capsaicin

concentration are using a panel of tasters (Scoville Organoleptic test method).

High-Performance Liquid Chromatography (HPLC) method is considered the most reliable and accurate method for determining capsaicinoids. Preliminary purification of the extract has been applied before HPLC analysis of capsaicinoids. Thin Layer Chromatography (TLC) and Column Chromatography (CC) methods are also used.Gahungu et al., (2011) extracted capsaicinoids from *Scotch Bonnet* variety using column chromatography on silica gel and then quantitatively evaluated with a reverse phase-high performance liquid chromatography/photodiode array detection (RP-HPLC/PAD). Capsaicin (47.632 mg/g) and dihydrocapsaicin (23.096 mg/g) were the major found capsaicinoids.

Al Othman et al. (2011) chose HPLC method for separation, identification and quantification of capsaicin and dihydrocapsaicin of hot chillies, red chillies, green chillies, green peppers, red peppers and yellow peppers (*Capsicum annum* L). The results showed that hot chillies contained the highest concentration of capsaicin (4249 µg/g) and the highest pungency level (67984.60 SHU), whereas green chilli showed the lowest detected concentration (1 µg/g), green peppers, red peppers and yellow peppers were non pungent.

Later, Chen et al., (2013) analysed by HPLC–UV the presence of capsaicin in the samples of red pepper extracts. The HPLC-UV chromatogram showed that capsaicin content was 9.48 mg/g dry weight.

Also, Barbero et al., (2014) identified in their study the five major capsaicinoids present in peppers using HPLC-MS.Ida Musfiroh et al., (2013) extracted various fruits of *Capsicum* and analyzed the extracts using high performance liquid chromatography. The optimum condition of analysis was attained using a reversed phase system, with a mobile phase of acetonitrile – acetate acid 2% (6:4), a flow rate of 1.0 ml/minute and a detection wavelength of 280 nm using UV detector.

In another study, Zamora et al., (2015) investigated direct spectrophotometric determination of capsaicinoids content in *Chiltepin* pepper as a possible alternative to HPLC analysis. The absorbance of the samples was observed at 215-300 nm and monitored at 280 nm.The results showed that comparative data determined spectrophotometrically and by HPLC on samples ranging from 29.55 to 129mg capsaicinoids/g, so spectrophotometric method can be also, routinely used with a correlation of 0.91 for total capsaicinoid analysis and quality control in pharmaceutical analysis.

UTILISATIONS

Capsaicinoids are important in the food and pharmaceutical industries.

Capsaicin is used in the development of new drugs because it has many beneficial properties, such as antioxidant, antimicrobial, anti-inflammatory and antitumor activities, and contributes to the control of diabetes and pain relief.

It is also used for the anti-convulsive and sedative properties and because stimulate the cardiovascular and respiratory systems. Moreover, capsaicinoids are also utilized as natural inhibitor of pathogenic microorganisms in food industry due to their antimicrobial properties.

CONCLUSIONS

Because of wide spread application of capsaicinoids, techniques have been developed with the objective of reducing extraction time, consumption of the solvent, pollution in analytical laboratories, and sample preparation costs.

Taking into account their benefits, there is a great interest in developing new technologies to obtain concentrated extracts.

So, plants will continue to provide novel products as well as chemical models for new drugs in the coming decades, because the chemistry of the majority of plant species is yet to be characterized.

REFERENCES

Al Othman A. Z., Yacine B.H.A., Mohamed A.H., Ayman G.A., 2011. Determination of capsaicin and dihydrocapsaicin in Capsicum fruit samples using High Performance Liquid Chromatography, Molecules, 16, 8919, 8929, (2011)

Arora R., Gill N.S., Chauhan G., Rana A.C., 2011. An Overview about Versatile Molecule Capsaicin,

International Journal of Pharmaceutical Sciences and Drug Research, 3, (4), 280-286

Attuquayefio V.K., Buckle, K.A., 1987. Rapid sample preparation method for HPLC analysis of capsaicinoids in capsicum fruits and oleoresins, J. Agric. Food Chem., 35, 777-779

Barbero G. F., Liazid A., Palma M., Barroso C.G., 2008. Ultrasound-assisted extraction of capsaicinoids from peppers, Talanta, 75, 1332-1337

Barbero G. F., Ruiz A.G., Liazid A., Palma M., Vera J.C., Barroso C.G., 2014. Evolution of total and individual capsaicinoids in peppers during ripening of the Cayenne pepper plant *(Capsicum annuum* L.), Food Chemistry, 153, 200-206

Barbero G.F., Palma M., Barroso C.G., 2006. Determination of capsaicinoids in pepper by microwave-assisted extraction-high-performance liquid chromatography with fluorescence detection, Anal. Chim. Acta, 578, 227

Bosland P.W., Baral J.B., 2007. Bhut Jolokia'-The World's Hottest Known Chile Pepper is a Putative Naturally Occurring Interspecific Hybrid, Hort Science, 42, (2), 222-224

Chen L., Kang Y.H., 2013. Anti-inflammatory and antioxidant activities of red pepper *(Capsicum annuum* L.) stalk extracts: Comparison of pericarp and placenta extracts, Journal of Functional Foods, 5, 1724-1731

Chuichulcherm S., Prommakort S., Srinophakun P., Thanapimmetha A., 2013. Optimization of capsaicin purification from *Capsicum frutescens* Linn with column chromatography using Taguchi design, Industrial Crops and Products, 44, 473-479

Chinn M.S., Sharma-Shivappa R.R., Cotter J.L., 2011. Solvent extraction and quantification of capsaicinoids from Capsicum chinense, Food and Bioproducts Processing, 89, 340-345

Cisneros-Pineda O., Torres-Tapia L.W., Gutierrez-Pacheco L.C., Contreras-Martin F., Gonzalez-Estrada T., Peraza-Sanchez S.R., 2007. Capsaicinoids quantification in chili peppers cultivated in the state of Yucatan, Mexico, Food Chemistry, 104, 1755-1760

Collins M.D., Wasmund L.M., Bosland P.W., 1995. Improved method for quantifying capsaicinoids in Capsicum using high performance liquid chromatography, HortScience, 30, 137-139

De Aguiar A.C., Luiz P.S., Janclei P. C., Barbero G.F., Godoy H.T., Martínez J., 2013. Supercritical carbon dioxide extraction of Capsicum peppers: Global yield and capsaicinoid content, The Journal of Supercritical Fluids, 81, 210-216

Duarte C., Moldão-Martins M., Gouveia A.F., da Costa S.B., Leitão A.E., Bernardo-Gil M.G., 2004. Supercritical fluid extraction of red pepper *(Capsicum frutescens* L.), J. of Supercritical Fluids, 30, 155-161

Gahungu A., Ruganintwali E., Karangwa E., Zhang X., Mukunzi D., 2011. Volatile Compounds and Capsaicinoid Content of Fresh Hot Peppers (Capsicum Chinense) Scotch Bonnet Variety at Red Stage, Advance Journal of Food Science and Technology, 3, (3), 211-218

González-Zamora A.,Sierra-Campos E.,Pérez-Morales R., Vázquez-Vázquez C., Gallegos-Robles M.A., López-Martínez J.D., García-Hernández J.L., 2015. Measurement of Capsaicinoids in Chiltepin Hot Pepper: A Comparison Study between Spectrophotometric Method and High Performance Liquid Chromatography Analysis, Journal of Chemistry, p. 10.

Gudeva L.J., Mitrev S., Maksimova V., Spasov D., 2013. Content of capsaicin extracted from hot pepper *(Capsicum annuum* ssp. *microcarpum* L.) and its use as an ecopesticide, Hem. Ind., 67, (4), 671-675

Haejin B., Jayaprakasha G.K., Jifon J., Bhimanagouda P.S., 2012. Variation of antioxidant activity and the levels of bioactive compounds in lipophilic and hydrophilic extracts from hot pepper *(Capsicum* spp.) cultivars, Food Chemistry, 134, 1912-1918

Haejin B., Jayaprakasha G.K., Jifon J., Patil B.S., 2012. Extraction efficiency and validation of an HPLC method for flavonoid analysis in peppers, Food Chemistry, 130, 751- 758

Moreno E., Fita A., González-Mas M.C., Rodríguez-Burruezo A., 2012. HS-SPME study of the volatile fraction of Capsicum accessions and hybrids in different parts of the fruit, Scientia Horticulturae, 135, 87: 97

Musfiroh I, Mutakin M., Treesye A., Muchtaridi M., 2013. Capsaicin level of various Capsicum fruits, International Journal of Pharmacy and Pharmaceutical Sciences, 5, (1), 248-251

Namdeo A.G., 2007. Plant Cell Elicitation for Production of Secondary Metabolites: A Review, Pharmacognosy Reviews, 1, (1).

Nwokem C.O., Agbaji E.B., Kagbu J.A., Ekanem E.J., 2010. Determination of Capsaicin Content and Pungency Level of Five Different Peppers Grown in Nigeria, New York Science Journal, 3, (9)

Paduano A., Caporaso N., Santini A., Sacchi R., 2014. Microwave and Ultrasound-Assisted Extraction of Capsaicinoids From Chili Peppers *(Capsicum annuum* L.) in Flavored Olive Oil, Journal of Food Research, 3, 4

Pena-Alvarez A., Ramírez-Mayae E., Alvarado-Suárez L.A., 2009. Analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase microextraction–gas chromatography–mass spectrometry, Journal of Chromatography A, 1216, 2843-2847

Rafajlovska V., Slaveska-Raicki R., Klopcevska J., Srbinovska M., 2011. Extraction of oleoresin from pungent red paprika under different conditions, Mass Transfer in Chemical Engineering Processes, 111, 132

Rocha-Uribe J.A., Novelo-Pérez J.I., Ruiz-Mercado C.A., 2014. Cost estimation for CO2 supercritical extraction systems and manufacturing cost for habanero chili, J. of Supercritical Fluids, 93, 38-41

Santos P., Aguiar A.C., Barbero J.F., Rezende C.A., Martínez J., 2015. Supercritical carbon dioxide extraction of capsaicinoids from malagueta pepper (*Capsicum frutescens* L.) assisted by ultrasound, Ultrasonics Sonochemistry, 22, 78-88

Tilahun S., Paramaguru P., Rajamani K., 2013. Capsaicin and ascorbic acid variability in chilli and paprika cultivars as revealed by HPLC analysis. Journal of Plant Breeding and Genetics, 01, (02), 85-89.

Wang J., Wang Y., Zheng L., Ni S., Fan Z., Yao R., Chen K., 2014. Kinetic Study on Extraction of Red Pepper Seed Oil with Supercritical CO_2, Chinese Journal of Chemical Engineering, 22, 1, 44-50

Wesolowska A., Jadczak D., Grzeszczuk M., 2011. Chemical composition of the pepper fruit extracts of hot cultivars *Capsicum annuum* L., Acta Sci. Pol., Hortorum Cultus, 10, (1), 171-184

Xinrong D., Xia L., Lei D., Fuzhi C., Zuojiao T., Zhonghua L., 2014. Stage extraction of capsaicinoids and red pigments from fresh red pepper (Capsicum) fruits with ethanol as solvent, LWT - Food Science and Technology, 59, 396-402

Yamaguchi M., Yahagi N., Kato H., Takano F., Ohta T., 2010. Capsicum extract and its constituents modulated the production of immunoglobulins in Peyer's patch cells ex vivo, Journal of Functional Foods, 2, 255-262

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ACCELERATED MICROPROPAGATION OF ENDEMIC *FRITILLARIA AUREA* SCHOTT

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Abstract

Fritillaria aurea Schott, an *Iran-Turan element, is a rare alpine geophyte with spotted brown yellow flowers. The plant is endemic to Turkey with dispersion in the South-Eastern Anatolia region. This study reports in vitro culture of Fritillaria aurea previously collected from the provinces of Adiyaman and Malatya in the South-Eastern Anatolia Region and presently cultured at the Ornamental Plants Garden Collection of Faculty of Agriculture, Dicle University, Diyarbakir, Turkey. Bulblets were cultured on MS medium containing different concentrations of TDZ, TDZ andNAA. Callus induction followed by bulb formation was noted on bulblets. The bulblets obtained these were cultured on MS medium containing 40 or80 g/l sucrose to increase bulb diameter. The in vitro regenerated bulblets were rooted on MS medium containing different concentrations of IBA (0.25, 0.50, 0.75 and 1.0 mg/l) supplemented with 30 g/l sucrose. A significant increase in number of bulblets and bulb diameter was noted on F. aurea Schott bulbs.*

Key words: *Bulblets, in vitro, multiplication, sucrose.*

INTRODUCTION

The *Liliaceae* contains approximately 280 genera and 4000 species. There are approximately 430 Liliaceous species in Turkey and there are 49 taxa of *Fritillaria* that grows in wild in Turkey with endemism ratio is 36.53% in *Fritillaria* (Teksen at al., 2011).

The *Fritillaria aurea* Schott is a rare alpine geophyte with spotted brown yellow flowers. The plant is grown at rocky and high places average between 1600-3000 m.

It is an Iran-Turan element and has rich potential for use in alpine ornamental landscaping. The plant is endemic to Turkey and disperses in Mersin, Kayseri, Malatya, Nigde, Sivas and Adiyaman provinces (Bakis et al., 2011).

Although Turkey is signatory to CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora, also known as the Washington Convention), wild populations of the plant are getting reduced with the passage of time largely due to illegal collections for local cut flower industry and traditional medicinal systems, fast urbanisation and very limited measures to conserve local germplasm.

Fritillaria extracts are widely used in traditional Turkish, Chinese, and Indian medicinal systemsto treat cough inflammation and like. *Fritillarias* are widely used to treat cough, lung disorders, lumps beneath the skin and cancers in traditional folk medicines of Turkey.

The rate of natural vegetative multiplication is generally very low in geophytes including *F. aurea* Schott. It takes 5 – 6 years to produce a plant capable of flowering from seed under ideal conditions in the wild. Plant tissue-culture technology is playing an increasingly important role in basic and applied studies, including crop improvement (Brown and Thorpe, 1995). Thus, the techniques like plant tissue culture, could offer an attractive alternative for increasing propagation efficiency especially for commercial production as has been used in many other geophytes and bulbous plants (Bach and Sochacki, 2013).

Fritillaria aurea Schott has huge potential for their use in parks, gardens as indoor and outdoor ornamental plant. Multiplication of plant through plant tissue culture offers many advantages when it is compared to traditional propagation techniques formas propagation that allows accelerated rates of propagation for higher yield of plants in shorter time. Plant tissue also offers a very beneficial pathway for rapid production of clonal elites; where natural production rates of the plants are very slow.

Successful regeneration of plants via organogenesis from vertically sliced bulb halves of this plant has never been reported. This paper reports a protocol for bulblet multiplication leading to acclimatization that also included optimisation of the effects of different concentrations of sucrose and IBA on bulblet growth and root formation. The objectives of the present work are to establish an efficient *in vitro* propagation method for *Fritillaria aurea* Schott as an alternative to the conventional approach using vertically sliced bulbs through *in vitro* manipulation. All stages of organogenesis were identified and complete plantlets were formed and finally successfully acclimatized. Comparison of morphological characteristics between *in vivo* and *in vitro* grown plants was also made.

MATERIALS AND METHODS

Plant materials and experiments

The study made use of *in vitro* regenerated bulblets (Kizil et al., 2013). *Fritillaria aurea* Schott (Figure 1) bulblets were obtained from the previous experiment using MS medium containing0.05mg/l TDZ and 0.10mg/l NAA or 0.10mg/l TDZ and 0.10mg/l NAA under *in vitro* conditions (primary medium).These bulblets were induced on light yellow coloured compact mass calluses and after four months of culture.

The bulblets achieved desired gain in bulblet diameter they were rooted on MS basal medium containing 3×KNO_3 and supplemented with 0.25, 0.50, 0.75 or1.0mg/l IBA, 30.0 g/l sucrose (w/v) and solidified with 6.2 g/l agar (w/v) in Magenta GA^7 vessels for rooting(secondary medium medium).These were cultured on modified MS medium(170 mg/l potassium phosphate, 1900 mg/l potassium nitrate, 100mg/l inositol, 0.5mg/l nicotinic acid, 0.5mg/lpyridoxine, 0.1mg/l thiamine and 2.0mg/l glycine were added to MS basal medium) containing sucrose (40or80 g/l) solidified with 6.2 g/l agarat 24° ± 1°C in sterile Magenta GA^7 vesselsfor28 weeks(tertiary medium).The pH of all cultures medium was adjusted to 5.6 - 5.8 with 0.1 M KOH or 0.1 M HCl before autoclaving at 121ºC, 117.7 kPa for 20 min.

Figure 1. *Fritillaria aurea* Schott growing at its natural habitat

Hardening and acclimatization

The well-developed bulbs were washed thoroughly in running tap water transferred to plastic pots containing sterilised peat moss under greenhouse under controlled conditions of temperature (24º± 1ºC) and light 3000 lux (16/8 h photoperiod) conditions. Potted plantlets were covered with transparent plastic bags to ensure high humidity and watered everyday with water for 15 days to acclimatize them.

All cultures unless otherwise mentioned separately, were grown in Fitotron growth chamber (FitotronSGC 120; Epinal Way, Loughborough, UK) with 16 h of cool white fluorescent light (Philips lamps TLD 36 W/54, Hungary) at a photon flux density of 35 µmol/ m^2/sper day.

All experiments made use of60 explants equally divided into 10 replications. Statistical analysis was performed using IBM SPSS 22 program for windows by comparing means for One Way ANOVA. All values expressed in percentage were arcsine transformed before statistical analysis (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSIONS

Results

The bulblets were induced on callus proliferated by MS medium containing 0.05mg/l TDZ and 0.10mg/l NAA or 0.10mg/l TDZ and 0.10mg/l NAA (primary medium–Figure 2, Figure 3 a).

These bulblets as shown in the materials and methods did not induce roots, therefore there were rooted on MS medium containing 0.25, 0.50, 0.75 or 1.0 mg/l IBA for rooting (secondary medium–Figure3 b). These applications helped to increase rooting percentage, number of roots per bulblet, root length, number of shoots per bulblet, shoot length, bulblet diameter and number of bulblets per explant bulb used as explant variably. Maximum rooting percentage (100%), and number of roots per bulblet(6.35) were note on 0.50 mg/l IBA. Maximum root length (2.73 cm) was noted on 1 mg/l IBA (Table 1).Mean number of roots of on bulblets treated with0.25mg/l IBA were higher compared toother concentrations of IBA. Maximum number of shoots per bulblet (6.44) and number of bulblets per stalk bulbs(11.90) were noted on 0.25 mg/l IBA with significant differences among other concentrations of IBA.

Figure 2.*Fritillaria aurea* Schott plants in *in vitro* bulblets formation on TDZ (Thidiazuron) medium

IBA did not induce meaningful variations in bulblet diameter. The bulblets diameter ranged from 0.58 to 0.64 cm. Maximum shoot length (5.80 cm) and bulblet diameter (0.64 cm) was noted on0.75 mg/l IBA. However, all concentrations of IBA also induced number of bulblets on mother or stalk bulbs used as explant. All concentrations of IBA affected and transformed vegetative bulblet tissues to generative tissues that besides helping in root induction also induced variable number of axillary bulblets on all concentrations of IBA used in the study.

It was thought that it is an important situation for *F. Aurea* Schott induction of productive bulblets under *in vitro* conditions. These IBA induced bulblets were cultured on MS medium containing 40 or80 g/l sucrose to provide increase in their diameter (tertiary medium). MS medium containing 40 g/l sucrose induced higher values for root induction percentage and number of roots per explants, root and shoot length, bulblet diameter and number of bulblets *per* explant compared to the bulblets cultured on 80 g/l sucrose.

Number of roots on modified MS containing 40 g/l application was 1.44, root length as 0.73 cm, shoot length 2.20 cm, bulblet diameter 0.64 cm and number of bulblet as 1.58. Increasing sucrose amount in the medium had affected negatively and investigated characteristics gave low values (Table 2; Figure 3 c, d).

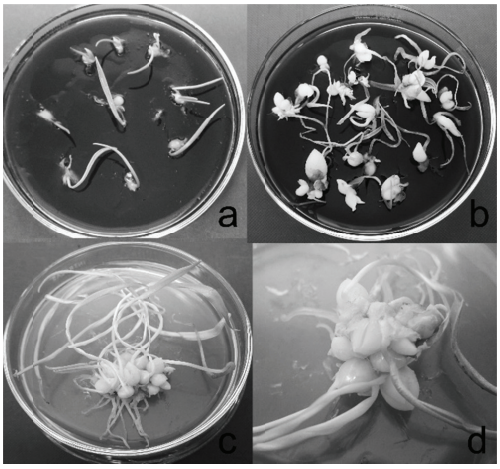

Figure 3. Micropropagation of *Fritillaria aurea* Schott (a) TDZ (Thidiazuron) regenerated bulblets (b) bulblets transferred to IBA (Indole-3-butyric acid) containing medium for rooting (c, d) axiallary bulblet regeneration on stalk bulbs

These bulbs were transferred to pots containing peat and watered daily with 0.5 ml water for a period of 4 weeks until the established and showed profuse growth of green leaves. All of them were physiologically normal and did not show any sign of abnormal growth during culture in the greenhouse; where their pots

were kept under controlled conditions of temperature and humidity.

Table 1. Effects of different IBA (Indole-3-butyric acid) concentrations on some investigated characteristics of *Fritillaria aurea* Schott

IBA (mg/l)	Rooting rate (%)	Number of roots per bulblet	Root length (cm)	Number of shoot per bulblet	Shoot length (cm)	Bulblet diameter (cm)	Number of bulblets per explant
0.25	76.66	4.73	2.60	6.44	1.41 b	0.59	11.90 a
0.50	100.00	6.35	1.95	5.05	5.30 a	0.58	4.65 b
0.75	80.00	5.58	2.65	5.00	5.80 a	0.64	5.45 b
1.00	95.00	4.78	2.73	3.78	4.05 a	0.62	3.55 b

Means within a column followed by the same letter are not significantly different according LSD test at $p \leq 0.05$.

Table 2. Effects of different sucrose concentrations on growth of *Fritillaria aurea* Schott bulbs obtained from different IBA (Indole-3-butyric acid) concentrations

Sucrose (g/l)	Rooting rate (%)	Number of roots per explant	Root length (cm)	Number of shoot per explant	Shoot length (cm)	Bulblet diameter (cm)	Number of bulblets per explant
40	52 a	1.44 a	0.73 a	2.90 a	2.20	0.64 a	1.58 a
80	40 b	0.84 b	0.32 b	1.04 b	1.16	0.49 b	0.84 b

Means within a column followed by the same letter are not significantly different according to LSD test at $p \leq 0.05$.

Discussions

The present study confirmed regeneration of bulblets induced on TDZ and NAA concentrations in agreement with previous studies.TDZ has been shown to induce callus formation in a variety of plant culture systems due to very high rate of cell proliferation. TDZ induced a 30-fold increase in the growth of callus cultures over other plant growth regulators (Capelle et al., 1983) with a relatively high intrinsic activity in agreement with present studies, previous studies also confirm thatTDZ in combination with 2,4-D,IBA (Passey et al., 2003;Yonghua et al., 2005) or NAA (Erisen et al.,2011) were effective to regenerate plantlets from *Fragaria* leaves and *Astragalus* species respectively. However, present studies indicate that the bulblets that were induced on callus proliferated by MS medium containing 0.05mg/l TDZ and 0.10mg/l NAA or 0.10mg/l TDZ and 0.10 mg/l NAA, they failed to increase in diameter. It is assumed that this could be due to the influence of TDZ based a relatively high level of accumulation of minerals or other metabolites in callus tissues that induced stress causing hinderances in the development of tissues in agreement with Murch et al. (1997). Resultantly, to overcome this physiological stress, the induced callus tissues might have modified their metabolic processes that ended up in hinderances to growth of newly regenerated bulblets.

Another explanation could be TDZ induced bulblets failed to elongate due to high carry over effect or partial toxic activity of TDZ in agreement with Huetteman and Preece (1993).The problem was overcome by transfer of bulblets a secondary medium containing IBA lacking TDZ and NAA that created a different balance and improved the growth of bulbs transforming the tissues from vegetative to generative phase. Use of secondary media for shoot proliferation has been reported in many plants like *Malus* (Fasolo et al., 1989), *Pyrus* (Singhaand Bhatia, 1988), *Populus* (Russell and McCown, 1988), and *Rhododendron* (Preece and Imel, 1991). All researchers used primary media to maximize shoot proliferation, and secondary media for their elongation and growth. Some researchers also used alternatively, chilling treatments can be used for shoot elongation. Briggs et al. (1988a, 1988b) also used chilling treatments on TDZ induced *in vitro* grown *Rhododendron* sp. (deciduous azaleas) shoots to 3°C for 2 months; which elongated rapidly once they were shifted to light and warm growing conditions. The results of the present study showed that that IBA acted as recovered to these effects and culture of individual bulblets on it had positive effects on both bulblet regeneration, tissue maturity and production of axillary bulblets.

TDZ induced bulblets changed their response on IBA containing secondary regeneration medium where axillary bulblets and roots were noted that retreated directly from the mother or stalk bulblets produced *in vitro* and were very noticeable after 4 to 5 weeks of culture.

It seemed as if IBA ameliorated the negative impacts of TDZ and all bulblets rooted variably. The supply of IBA increased the root length, root fresh weight, plant height and shoot fresh weight on increased IBA concentrations (0.50 to 2.00 mg/l). Maximum rooting was noted on 0.50 mg/l IBA. Increased TDZ concentrations increased leaf number, plant height and shoot fresh weight. No consistent differences for IBA effects were detected between shoot characters in *in vitro* derived plantlets. Regardless of IBA concentrations, bulblets in 0.50 mg/lIBA produced the best rooting system with vigorous shoot characteristics when they were treated with two concentrations of sucrose. However, 40 g/l sucrose seemed more favourable for development and growth of plants. This affected survival rate of acclimatized plants in as well.

In the present study, for the first time, a protocol for plantlet regeneration on MS medium containing high TDZ concentrations combined with IBA was developed. Regeneration ability of *Fritillaria* species depends on genotype of plant, growing conditions and *in vitro* medium and conditions. Kukulczanka et al. (1989) reported that they had obtained adventives bulblets by using bulb scale and full bulb. The highest regeneration rate was obtained from multiplication of cytokinin and auxins in their study. Contrarily this study showed overcoming negative effects of TDZ and bulblet regeneration using IBA and sucrose. The findings are new and have never been reported for Fritillarias or any bulbous plant species.

Rahimi et al. (2014) reported that plant tissue culture is a technique that has ability of production of a large number of high quality plants. In *Fritillaria imperialis*, the number of regenerated shoots was the highest on MS medium supplemented with 0.5 mg/l TDZ and 30 g/l sucrose; the number of roots was the highest on MS medium supplemented with 0.2 mg/l NAA and 30 g/l sucrose; the *in vitro* bulb diameter is largest. Marija et al. (2011), reported that somatic embryogenesis and whole plant regeneration were achieved from mature zygotic embryos of *Fritillaria meleagris*. They reported also embryogenic callus was derived from mature zygotic embryos after 4 weeks on TDZ containing medium. Somatic embryos at the early stages of development arose from the surface of the embryogenic callus. They had multiplication of somatic embryos, formation of bulblets and shoot development observed on the same medium. The results reported in this study are not in agreement with previous study and have edge over the previous studies. It reports recovery of TDZ regenerated bulblets on IBA and sucrose containing medium. Moreover, the regenerated bulblets were physiologically normal and did not show any abnormality on growth when they were cultured and transferred to pots for growth.

CONCLUSIONS

In conclusion, the present study underlines the importance of primary, secondary and tertiary medium for bulblet regeneration rooting and increasing bulblet diameter of *F. aurea* Schott by organogenesis.

The results also indicate partial cell specific inhibition due to TDZ induced regeneration of under *in vitro* conditions.

The results of the study are novel and could be effectively used during conservation strategies and commercial multiplication studies.

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REFERENCES

Bach, A., Sochacki, D. 2013. Ornamental Geophytes from Basic Science to Sustainable Production; Phsiology and Menagement Systems. Kamenetsky, R and Okubo, H (Eds.), CRC Press, Boca Raton, http//:www.crcpress.com.

Bakis, Y., Babac, M.T., Uslu, E. 2011. “Updates and improvements of Turkish Plants Data Service (TÜBİVES)" In Health Informatics and Bioinformatics (HIBIT), 2011 6th International Symposium on (pp. 136-140). IEEE.

Briggs, B.A., McCulloch, S.M., Edick, L.A. 1988a.Micropropagation of azaleas using thidiazuron. Acta Hort. 226: 205-208.

Briggs, B.A., McCulloch, S.M., Edick, L.A. 1988b.Micropropagation of azaleas using thidiazuron. Acta Hort. 227: 330-333.

Brown, D.C.W., Thorpe, T.A. 1995. Crop improvement through tissue culture. World Journal of Microbiology &Biotechnology, 11: 409-415.

Capelle, S.C., Mok, D.W.S., Kirchner, S.C., Mok, M.C. 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of N6-(Y2- isopentyl) [8-14c] adenosine in callus tissues of *Phaseolulunatus* L. Plant Physiol. 73: 796-802.

Erisen, S., Atalay, E., Yorgancılar, M. 2011. The effect of thidiazuron on the *in vitro* shoot development of endemic *Astragalus cariensis* in Turkey. Turk J Bot. 35: 521-526.

Fasolo, F., Zimmerman, R.H., Fordham, I. 1989. Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. Plant Cell Tiss. Org. Cult. 16: 75-87.

Huetteman, C.A., Preece, J.E. 1993. Thidiazuron-A potent cytokinin for woody plant-tissue culture. Plant Cell Tissue Org. Cult. 33(2): 105-119.

Kizil, S., Sesiz, U., Khawar, K.M., Arslan, N. 2013. Studies on *in vitro* Micropropagation of *Fritillaria aurea* Schott. V. Ornamental Plants Congress, 06-09 May 2013 Yalova, Turkey, Volume II, 602-605.

Kukulczanka, K., Kromer, K., Czastka, B. 1989. Propagation of *Fritillaria meleagris* L. through tissue culture. ActaHortic. 251: 147–153.

Marija, P., Angelina, S., Sladana, J., Milana, T. 2011. Somatic embryogenesis and bulblet regeneration in snakehead fritillary (*Fritillaria meleagris* L.). Afr.J.Biotechnol. 10(72): 16181-16188.

Murch, S.J., KrishnaRaj, S., Saxena, P.K. 1997. Thidiazuron-induced regeneration:A potential stress response. Plant Physiol. 114(3): 177-177.

Passey, A.J., Barrett, K.J., James, D.J. 2003. Adventitious shoot regeneration from seven commercial strawberry cultivars (*Fragaria* x *ananassa* Duch.) using a range of explant types. Plant Cell Replication, 21:397-401.

Preece, J.E.,Imel, M.R.1991. Plant regeneration from leaf explants of Rhododendron "P.J~M. Hybrids'. Scientia Hort. 48 (1-2): 159-I70.

Rahimi, M., Daneshvar, M.H., Heidari, M. 2014. Propagation and bulb formation of *Fritillaria*(*Fritillaria imperialis* L.) Via *in vitro* culture. International Journal of Plant, Animal and Environmental Sciences, 4: 707-710.

Russell, J.A.,McCown, B.H. 1988. Recovery of plants from leaf protoplasts of hybrid-poplar and aspen clones. Plant Cell Rep. 7:59-62.

Singha, S., Bhatia, S.K. 1988. Shoot proliferation of pear cultivars on medium containing thidiazuron and benzylaminopurine. Hort. Sci. 23: 803.

Snedecor, G.W., Cochran, W.G. 1967. Statistical methods. 593 p. The Iowa State University Press, Ames, Iowa, USA.

Teksen, M., Aytac Z. 2011. The revision of the genus *Fritillaria*L. (Liliaceae) in the Mediterranean region (Turkey)., Turk. J. Bot. 35: 447-478.

Yonghua, Q., Shanglong, Z., Asghar, S., Lingxiao, Z., Qiaoping, Q., Kunsong, C., Changjie, X. 2005. Regeneration mechanism of Toyonoka strawberry under different color plastic films. Plant Sci. 168:1409-1424.

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INFLUENCE OF DIFFERENT *IN VITRO* SIMULATORS FOR HYDRIC STRESS FOR GROWTH AND DEVELOPMENT OF POTATO

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Abstract

Necessity of finding genotypes adapted to drought has become urgent due to the effect of this type of stress on potato production. The most important phase, indispensable for improving drought tolerance is to identify genotypes tolerant and sensitive to drought. In this study, to induce in vitro water stress were used polyethylene glycol and sorbitol that was comparable with the basic medium MS, considered control. Determinations were performed 4 weeks after inoculation of mini cuttings belonging to five varieties of plantlets (Ruxandra, Sarmis, Gared, Marvis, Rustic) and the parameters analyzed were next: number of leaves, number of internodes, height of plantlets, root length, weight of fresh plantlet, weight of fresh root. Medium in which was added PEG with different concentrations significantly reduced the average weight of fresh plantlet and root compared with the control medium and medium with sorbitol and significantly reduced the mean number of internodes, the average height of the plantlet, the average root length. This osmotic agent (PEG) can be recommended for in vitro simulation of drought to identify tolerant genotypes to hydric stress.

Key words: *hydric stress, in vitro, osmotic agent, potato.*

INTRODUCTION

Water deficit, extreme temperatures and low atmospheric humidity lead to drought, which is one of the limiting factors affecting crop quality. The amplitude of the effects of drought on potato production depends on phenological calendar, duration and severity of stress. Two critical periods: sprouting and tuberization affect in final tubers production. Potato crop is often considered sensitive to drought and production during successive episodes of drought may be compromised. Necessity to identify genotypes adapted to drought has become urgent because of the effect this type of stress on the growth of potato and production of this crop. Particularly important for production of potato tubers is the precipitation amount during the growing season and the distribution on the vegetative stages. It is estimated that during the growing season are required 250-400 mm precipitations (Bîlteanu, 2001).

The ability of roots to penetrate the soil depends on the power that can exert roots and may be associated with drought tolerance (Tardieu, 1994). If plants cannot take up water from the soil needed to compensate for the lost through perspiration, install wilting phenomenon, a consequence of the drought effects (Ianoşi, 2002).

Drought reduces the growth of roots, at the time of sprouting. Drought installed after plant sprouting inhibits the stolons development, thereby reducing the number of tubers. These processes are irreversible, even though soil moisture subsequently recovers. Capacity of roots to penetrate into the soil depends on the power that roots can exert and may be associated with drought tolerance.

In vitro tissue culture allowed a deeper understanding of the physiology and biochemistry of plants grown under unfavorable environmental (Benderradji L. and colab., 2012).

The most widely used method for the selection of genotypes tolerant to abiotic stress is the *in vitro* selection pressure technique. This is based on the *in vitro* culture of plant cells, tissues or organs on a medium supplemented with selective agents, allowing selecting and regenerating plants with desirable characteristics (Pérez-Clemente and Gómez-Cadenas, 2012).

Polyethylene glycol (PEG), sucrose, mannitol or sorbitol have been used by several workers as osmotic stress agents for *in vitro* selection.
In vitro simulation drought was made to identify varieties with optimum tolerance at drought. Observations showed that was obtained a slowing of regeneration cuttings. Sensitivity to drought was not uniform for varieties analyzed. For *in vitro* selection PEG, sucrose, mannitol and sorbitol were analyzed in several research papers, as agents of osmotic stress. Sorbitol is a sugar alcohol hexahydrate with osmotic effect. Widely water stress in vitro simulation is used polyethylene glycol. Culture media which contain PEG, imitate dry soil, rather than the culture media which have low molecular weight compounds. With the increasing amount of sorbitol and PEG, water absorption becomes difficult for plantlets from nutrient medium and thus is simulate the effect of drought. *In vitro* culture technique minimizes external environmental variations due to nutrient medium defined and controlled conditions and homogeneity of stress applied.

MATERIALS AND METHODS

In Laborator of Vegetal Tissue Culture, of NIRDPSB Brasov (2015) was made a study for identy the adequate agente for induce water stress. Microplantlets from the culture collection were multiplied to each internode and cuttings and were inoculated on Murashige-Skoog medium. Plantlets which were developed were multiplied to obtain nodal cuttings. Cuttings of these plantlets were used as explants, for further multiplication *in vitr*o. As a basic medium was used Murashige-Skoog medium, naphthylacetic acid, sucrose, agar (this was considered control medium). Both PEG and sorbitol were added in the culture medium in 4 concentrations (0.5%; 1.0%; 1.5%; 2.0%). Test tubes with mini cuttings of varieties proposed for *in vitro* water stress test, were placed in the growth chamber by ensuring light and temperature regime required for growth and development of plantlets. After 4 weeks determinations were made for analysis of the following parameters, for a part of plantlets of these varieties: number of leaves and internodes / plantlet and plantlet height, root length, fresh weight of plantlet and root (figures 1, 2, 3, 4, 5). The results were processed by analysis of variance and the significance of differences was determined using the method of multiple comparisons, respectively Duncan test. Experimental differences higher than 5% are considered significant (Săulescu and Săulescu, 1967).
For determine the effect of water stress on developing plantlets the study consisted in an bifactorial experience of two factors (5 x 3), 4 repetitions, including the following factors:
Experimental factor A: variety, with 5 graduations**:**
- a_1- Ruxandra;
- a_2- Sarmis;
- a_3- Gared;
- a_4- Marvis;
- a_5- Rustic.

Experimental factor B – nutrient media used with 3 graduations**:**
- b_1-control medium MS, to which was made no addition of osmotic agent;
- b_2- MS medium, to which was added PEG;
- b_3- MS medium, to which was added sorbitol.

RESULTS AND DISCUSSIONS

Treatments performed with PEG significantly reduced the average weight of plantlet and of root and compared to sorbitol and nutrient medium, considered control (MS) and significantly reduced the average number of internodes, the height of plantlet, root length. From Table 1 it is noted that PEG and sorbitol have no negative influence on the average number of leaves, but PEG reduces the average number of internodes compared to control medium.
For the first element in the study, Duncan test analysis indicates a proximity of values, with no significant differences between the number of leaves formed on MS medium (8.8 leaves) and on medium that contained sorbitol 1% (8.4 leaves), so compared with PEG, sorbitol has a less stressful effect on plantlets, there is a stronger competition in formation of plant leaves depending on the concentration (Table 2). PEG on maximum concentration of 2% had as result hydric stres for leaves formatic, these are in lowest number (7.40).

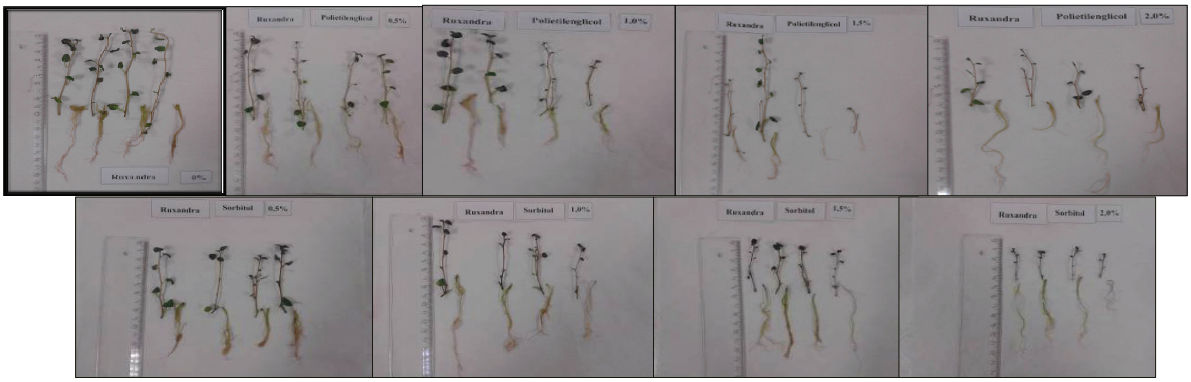

Fig. 1. Effects of water stress simulators (PEG and sorbitol) on growth and development of plantlets and roots for Ruxandra variety

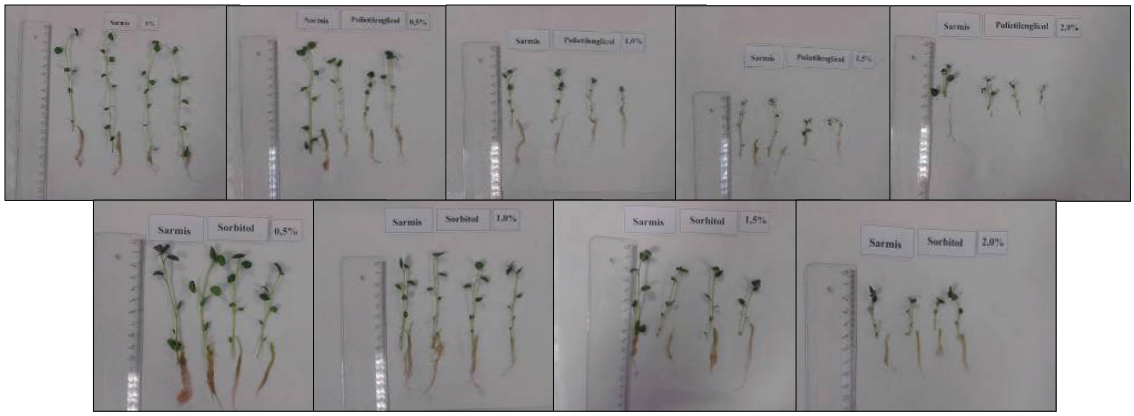

Fig. 2. Effects of water stress simulators (PEG and sorbitol) on growth and development of plantlets and roots for Sarmis variety

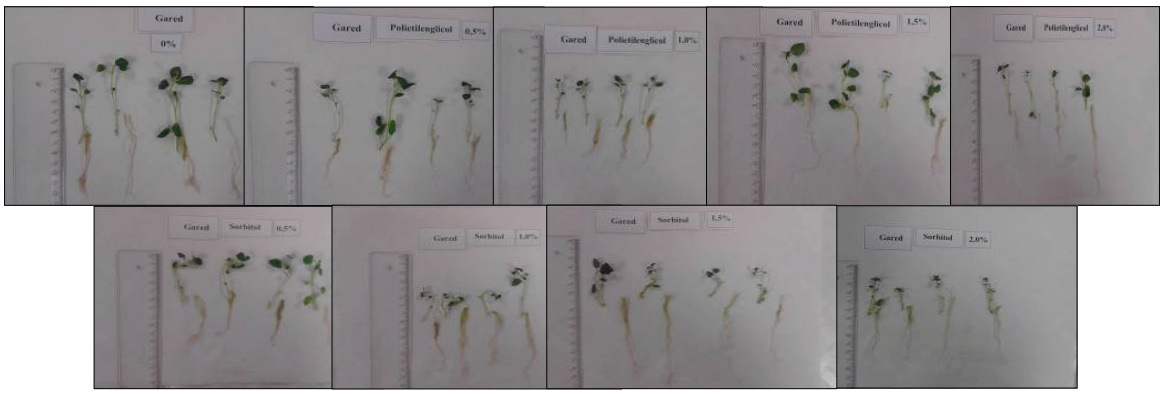

Fig. 3. Effects of water stress simulators (PEG and sorbitol) on growth and development of plantlets and roots for Gared variety

Fig. 4. Effects of water stress simulators (PEG and sorbitol) on growth and development of plantlets and roots for Marvis variety

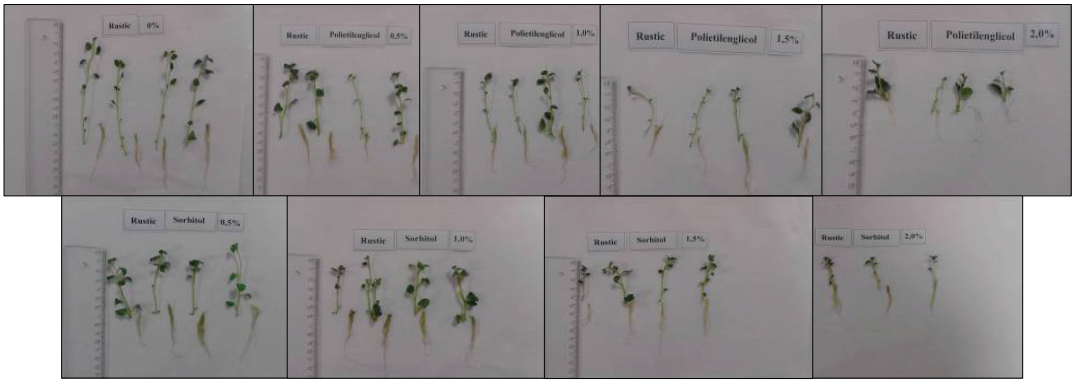

Fig. 5. Effects of water stress simulators (PEG and sorbitol) on growth and development of plantlets and roots for Rustic variety

The average number of internodes / plant indicates the effect of hydric stress attenuation for PEG, on minimum concentration of 0.5% (5.30 internods). Analysis of Duncan test do not record significant differences compared to this value for nutrient medium on which sorbitol was used at concentrations 0.5; 1; 1.5% (BC). Sorbitol 2% is so drastic as 1 and 1.5% PEG (Duncan test D and CD) for inducing *in vitro* drought.

The average height of plantlets (cm) is similarly influenced by the first concentration of two chemicals of water stress accelerators (7.725 and 7.685 respectively) (B), while the last two concentrations studied, indicating a similar effect, but severely on growth *in vitro* plantlets (5.515 cm, with decreasing at 3.645 cm for PEG and respectively 5.29 cm, with decreasing to 3.685 cm by application of sorbitol).

A very decisive indicator of drought simulation in vitro is root length (cm). Concentrations of 1 and 1.5% for sorbitol substance does not describe a relevant effect of water stress (there are no significant differences) with values close the control medium (7.030 cm).

The concentration of 0.5% sorbitol is less conclusive, because should be expected to induce less attenuating stress for increasing root length of microplant, but this concentration results in the formation of a roots with an average length less (6.15 cm), but with a lower average weight close to that obtained using the control medium.

For concentration of 0.5% sorbitol, it would be expected to result in a less pronounced stress on the plant, on the increase in root length, but in fact lead shortening roots, but with a value of average root weight (79,275mg) (B), close to

the value obtained by using of control medium (89,595mg) (A).

PEG-induced very well drought at concentrations of 1.5 and 2%; produces very little roots with weight of 18.610 mg 23.785 mg (Duncan test G, H).

From Table 3 Marvis variety combat drought effect by forming a large number of leaves (with an average of 9.083 leaves). This is followed by Rustic variety (8.222 leaf / plantlet). The average number of internodes grouped in a closely way Marvis, Ruxandra and Rustic varieties (A), followed by Gared and Sarmis varieties (B). For plant height significantly detaches Ruxandra variety which registered a value of 7.269 cm (A), followed by Sarmis variety, which registered 6.892 cm (B). The root has a higher resistance to drought than the plant. It is noted in this sense Ruxandra and Gared varieties, with high and close values (7.111 cm; 7.006 cm) (A), followed by Marvis variety (5.758 cm). On the opposite side the Sarmis variety had a tendency of slow root growth (only 4.6222 cm). For fresh plantlet weight (mg) is distinguished Ruxandra variety (126.142 mg), followed by Rustic variety (120.397 mg).

Gared variety, which in terms of microplants size is located on last place (with an average height of 5.506 cm), also recorded an average low weight of microplant 92.019 mg (situated on the last place). Regarding the average weight of fresh root (mg) detaches Ruxandra variety, with a very high value of 83.9 mg (A), followed by Gared (50.489 mg) and Sarmis variety (48,986). Regarding to this variety from previous analyzes, it appears that shows a tolerance to hydric stress, by producing microplants with a high height (6.892 cm) and with a high number of internodes (4.556). Rustic variety take last place, form roots with an average length small (5.244 cm) and a low weight (34.256 mg) indicating an inability of variety to fight with *in vitro* hydric stress.

Table 1 - Simulators osmotic stress influence compared to the control medium for the elements of growth and development for microplants

The treatment made	Average number of leaves	Average number of internodes	The average height of plantlets (cm)	The average length of root (cm)	The average weight of fresh plantlet (mg)	The average root weight (mg)
Control medium (MS)	8.800 **A**	6.200 **A**	10.70 A	7.025 **A**	207.535 **A**	89.595 **A**
MS+PEG	7.713 **A**	4.663 **B**	5.708 B	5.166 **B**	86.626 **C**	35.750 **C**
MS+sorbitol	8.013 **A**	4.950 **AB**	5.779 **B**	6.711 **AB**	99.928 **B**	60.750 **B**

Media that we are in the same column followed by the same letters are not significant, according to Duncan test

LSD=1.246 LSD=1.416 LSD=1.812 cm LSD=1.252 cm LSD=9.386 mg LSD=4.865 mg

Table 2 - Influence of osmotic stress concentrations simulators on the elements of growth and development of microplants

The treatment made	Hydric stress simulator concentration (%)	Average number of leaves	Average number of internodes	The average height of plantlets (cm)	The average length of root (cm)	The average weight of fresh plantlet (mg)	The average root weight (mg)
Control medium (MS)	-	8.80 **A**	6.20 **A**	10.70 **A**	7.030 **A**	207.535 **A**	89.595 A
MS+PEG	0.5	7.95 **CD**	5.30 **B**	7.725 **B**	6.085 **C**	144.200 **C**	56.235 **C**
	1	7.65 **DE**	4.55 **D**	5.945 **D**	5.025 **D**	83.*285* **F**	44.370 **E**
	1.5	7.85 **CD**	4.90 **CD**	5.515 E	4.825 **D**	71.245 **G**	23.785 **G**
	2	7.40 **E**	3.90 **E**	3.645 **F**	4.730 **D**	47.775 **H**	18.610 **H**
MS+sorbitol	0.5	7.75 **CDE**	5.15 **BC**	7.685 **B**	6.150 **C**	153.085 **B**	79.275 **B**
	1	8.10 **BC**	5.05 **BC**	6.455 **C**	6.835 **A**	118.695 **D**	78.275 **B**
	1.5	8.40 **AB**	5.05 **BC**	5.29 **E**	7.210 **A**	88.445 **E**	54.360 **D**
	2	7.80 **CDE**	4.55 **D**	3.685 **F**	6.650 **B**	39.485 **I**	31.090 **F**

Media that we are in the same column followed by the same letters are not significant, according to Duncan test

LSD=0.4218 LSD=0.3740 LSD=0.3778 cm LSD=0.4015 cm LSD=12.277mg LSD=1.783mg

Table 3 - Behaviour the varieties tested under the influence of treatments made to induce water stress

Variety	Average number of leaves	Average number of internodes	The average height of plantlets (cm)	The average length of root (cm)	The average weight of fresh plantlet (mg)	The average root weight (mg)
Ruxandra	7.528 **CD**	5.194 **A**	7.269 **A**	7.111 **A**	126.142 **A**	83.9 **A**
Sarmis	7.222 **D**	4.556 **B**	6.892 **B**	4.6222 **D**	95. 925 **C**	48.986 **C**
Gared	7.778 **C**	4.667 **B**	5.506 **D**	7.006 **A**	92.019 **D**	50.489 **B**
Marvis	9.083 **A**	5.333 **A**	5.700 **D**	5. 758 **B**	95.378 **C**	46.572 **D**
Rustic	8.222 **B**	5.056 **A**	6.103 **C**	5.244 **C**	120.397 **B**	34.256 **E**

Media that we are in the same column followed by the same letters are not significant, according to Duncan test

LSD=0.3266 LSD=0.3183 LSD=0.3229 cm LSD=0.3638 cm LSD=1.9722 mg LSD=1.458 mg

CONCLUSIONS

Medium with different concentrations in which was additional PEG significantly reduced the weight of fresh plantlets and fresh root as compared with the control medium and sorbitol and also significantly reduced the number of internodes, the height of the plantlet, the root length. The smallest influence of water stress on plantlet height was observed for Ruxandra variety. By using PEG in culture medium, potato plantlets reach 7.83 cm. The roots are the primary sensors of water deficit. Gared and Ruxandra varieties showed a good tolerance to water stress for root length, by applying sorbitol on nutritive medium, plantlet root reaching an average length of 8.13 and 7.22 cm. By addition of PEG, it appears that the same varieties Ruxandra and Gared, shows tolerance to water stress, but PEG being an agent stronger the levels were lower.

REFERENCES

Benderradji L., Brini F., Kellou K., Ykhelf N., Djekoun A., Masmoudi K., Bouzerour. H., 2012. Callus induction, proliferation, and plantlets regeneration of two bread wheat (*Triticum Aestivum* L.) genotypes under saline and heat stress conditions. ISRN Agronomy, Article ID 367851. Bobr. Plant Cell Tissue and Organ Culture; 102 387–395.

Bîlteanu Gheorghe, 2001. Fitotehnie, Volumul 2, Editura Ceres, Bucureşti.

Ianoşi, S. I., 2002. Bazele cultivării cartofului pentru consum, Editura Phoenix, Braşov.

Pérez-Clemente R. M and Gómez-Cadenas A., 2012**.** *In vitro* Tissue Culture, a Tool for the Study and Breeding of Plants Subjected to Abiotic Stress Conditions. In: Annarita Leva and Laura M. R. Rinaldi(Eds.) Agricultural and Biological Sciences ."Recent Advances in Plant *in vitro* Culture", Chapter 5, 91-108.

Săulescu, N.A, Săulescu, N.N., 1967. Cîmpul de experienţă, Întreprinderea poligrafică Sibiu.

Tardieu F., 1994**.** Growth and functioning of roots and of root systems subject to compaction towards a system with multiple signalling. Soil and Tillage Research 30, 217–43.

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EFFECTS OF TREATMENTS WITH ASCORBIC ACID AND GLUTATHIONE ON THE SAUVIGNON BLANC WINE COLOUR DURING BOTTLE AGING

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Abstract

The colour of wine is an important characteristic which contributes to the construction of the quality profile of a wine. This parameter is extremely important especially for white wines, a close connection being present in the consumer's perception between the colour of the wine and its quality level. The appearance of brown shades due to oxidation reactions is equivalent to a decrease in the quality level of the white wine and may indicate even an irreversible degradation of the wine.

The present study evaluates the colour evolution of a Sauvignon Blanc wine during storage in bottles after being treated just before bottling with different combinations and doses of sulphur dioxide (SO_2), ascorbic acid (AA) and reduced glutathione (GSH).The use of various antioxidants aims to reduce and prevent the browning evolution of white wine colour, and many hopes are expressed regarding the newly OIV approved reduced glutathione. However, this preliminary study results indicate that, in certain conditions, these antioxidants may not confer the expected protection and could even damage the final colour of white wine.

Key words*: White wine colour, glutathione, ascorbic acid, browning of white wine, Sauvignon Blanc.*

INTRODUCTION

As the white wine oxidation during aging is an important quality issue in winemaking, finding new methods for protecting the colour, along with the preservation of varietal aromas during the period of storage in bottle, is a priority. Even though a multitude of choices are available at present for improving the white wine stability against oxygen exposure, a complete and easy to apply antioxidant treatment of the white wine, able to ensure long time protection for both aroma and colour, is not yet available. Therefore, several studies have been conducted in the last decades in order to find the best combination and the optimal dose of some antioxidant agents, classical or newly discovered, to be added in wine to prolong its shelf life. One of the most well-known methods used to protect wine from oxidation and to prevent the formation of the secondary characteristics specific to wine aging, is to use agents with special antioxidant properties such as SO_2, ascorbic acid (AA), tannins and last, but not least, reduced glutathione (GSH) (Nikolantonaki et al., 2014). These materials are usually added to wine during the various stages of the production chain, but especially prior to bottling, and act either by reacting with oxygen or by removing or stabilizing the substrates sensitive to oxidation found in the wine, such as polyphenols (Oliveira et al., 2002), aromatic compounds etc.

While many studies already showed the beneficial action of these preservatives on the aromatic characteristics of the wine, their effect on colour conservation was not considered so much of a concern. It is, however, well-known that the colour of the wine is influenced especially by the grape variety, the pH values, the storage temperature, the winemaking protocols and the conditioning treatments applied before the wine bottling (Antoce, 2002).

The above mentioned antioxidant agents, considered essential for the prevention of oxidative processes in wine during aging (Brajkovich et al., 2005; Lavigne Cruège et al., 2003; Ugliano et al., 2011), including the GSH, are able to block quinones forming non-coloured polymers (Singleton et al., 1985; Antoce, 2007).

Previous studies have shown that in combination with small doses of sulphur dioxide, GSH slows down the oxidation rate of aromatic compounds such as volatile thiols, mono-terpenes and esters, and the formation of yellow xanthylium pigments specific for the browning reactions of the white wines (Lavigne and Dubourdieu, 2002; Bouzanquet et al., 2012; Roussis et al., 2007; Sonni et al., 2011). The presence in wine of xanthylium pigments originating from epicatechin determines a major change of its colour as they are two times more coloured than the pigments formed from catechins (Labrouche et al., 2005).

Similarly, although the effect of ascorbic acid as antioxidant agent is generally recognized, its addition in the wines without sufficient sulphur dioxide leads to detrimental reactions of its degradation products and catechins, forming also yellow xanthylium pigments (Barril et al., 2009; Barril et al., 2012).

Despite the existence of numerous studies regarding the role of GSH in protecting the varietal aromas of wines (Papadopoulou and Roussis, 2001, 2008; Roussis et al., 2009), especially regarding volatile thiols, its effect in combination with other antioxidants agents and particularly on the evolution of the white wine colour, has not been equally researched (Kritzinger et al., 2012; Badea and Antoce, 2015). As in July 2015 the OIV included among the allowed oenological practices for must and wine the addition of a maximum 20 mg/l of GSH (Resolutions OENO-TECHNO 10-445 and 10-446/July 2015) the research regarding these treatments has also intensified. This dosage has been approved based on some previous studies. Lavigne-Cruège and Dubourdieu (2002) have proposed a smaller dosage, of only10 mg/L of GSH, for the prevention of the browning phenomenon, while other scientists (Papadopoulou and Roussis, 2001, 2008; Ugliano et al.,2011) proposed the addition of 20 mg/L GSH for protection against wine aging defects.

In this work, the evolution of the colour during 4 months of aging in bottles of a Sauvignon Blanc was studied aiming to test the effect of different combinations and doses of these main wine antioxidants added prior to wine bottling.

MATERIALS AND METHODS

The wine samples have been prepared from the same Sauvignon Blanc base wine with a given level of free SO_2 in which various dosages of GSH, ascorbic acid and combinations of the two were added at bottling time.

The base wine was produced in September 2014 in the Dealu Mare vineyard, Romania, from Sauvignon Blanc grapes harvested from a plantation founded in 2007. The wines were treated and bottled in February 2015, the base wine parameters being included in Table 1 and those of the prepared samples shown in Table 2. The reduced glutathione (GSH) and the ascorbic acid (AA) were provided by Enologica Vason Italy.

Table 1.Physico-chemical parameters of Sauvignon Blanc base wine

Physico-chemical analysis report of the basis wine	
Identification data of the wine	
Cultivar / Product range	SAUVIGNON BLANC
Vintage year	2014
Quality category	CDO
Producer	DOMENIILE DEALU MARE URLATI
Wine physico-chemical parameters	
Free sulfur dioxide (mg/l)	35
Total sulfur dioxide (mg/l)	86
Total acidity (g/l tartaric acid)	6
Volatile acidity (g/l)	0.36
alcoholic concentration (%)	14.5
Sugar (g/l)	1.2
Relative density at 20 °C	0.9912
Nonreducing dry extract (g/l)	19.82

The bottled wine samples were analyzed 4 months after the treatments, by measuring their colour parameters with a computer-controlled double beam spectrophotometer Specord 250 from Analytik Jena AG running the software WinAspect version 2.2.7 and applying the CIELab system.

The computer automatically calculates the trichromatic components (CIE XYZ tristimulus values), *xy* chromaticity coordinates and CIELab colour parameters of the wine samples.Triplicate measurements of the colour parameters for each wine sample were performed by using 1 mm glass cuvettes.

Table 2.Variants of Sauvignon Blanc wines treated with different doses of antioxidants

Wine sample Code	Dosage
SBControl	Control
SBGSH10	10 mg/l Glutathione
SBGSH20	20 mg/l Glutathione
SBGSH30	30 mg/l Glutathione
SBGSH40	50 mg/l Glutathione
SBGSH50	100 mg/l Glutathione
SBAA30	30 mg/l Ascorbic Acid
SBAA40	40 mg/l Ascorbic Acid
SBAA50	50 mg/l Ascorbic Acid
SBAA60	60 mg/l Ascorbic Acid
SBAA70	70 mg/l Ascorbic Acid
SBAA30GSH10	30 mg/l Ascorbic Acid and 10 mg/l Glutathione
SBAA30GSH20	30 mg/l Ascorbic Acid and 20 mg/l Glutathione
SBAA30GSH30	30 mg/l Ascorbic Acid and 30 mg/l Glutathione
SBAA30GSH50	30 mg/l Ascorbic Acid and 50 mg/l Glutathione
SBAA30GSH100	30 mg/l Ascorbic Acid and 100 mg/l Glutathione

The transmittance of wine was recorded every 1 nm over the visible spectrum of 400-700 nm and colour parameters calculated for a D65 standard illuminant and 2° observer angle.

The results have been analysed by using the software Microsoft Excel and the package Chroma Ver. 2.0.

The software automatically calculates the trichromatic components (X, Y, Z) and the basic colour percentages (*x*– the percentage of red and *y*- the percentage of green), as well as the CIELab space system parameters (the colorimetric coordinates L*=clarity, *a**= red/green colour component, *b** =yellow/blue colour component, C* = chroma, H* = hue).

In the CIELab system, an uniform colour space can be very clearly expressed through three dimensions, the Cartesian parameters L*, *a** and *b**. L* represents the luminosity (lightness), *a** defines the place on the red-green axis, while*b** represents the value on the yellow-blue axis.

With the aim of assessing the modifications induced by the treatments, the white wine colour differences were calculated for the CIELab parameters (ΔL^*,Δa^*, Δb^*, $\Delta C_{ab}^*$and ΔH_{ab}^*), as well as the overall colour difference (ΔE_{ab}^*), against the non-treated control sample (SBControl), using the OIV recommended method (OIV- Resolution OENO 1/2006, OIV-MA-AS2-11: R2006).

RESULTS AND DISCUSSIONS

The Sauvignon Blanc variants treated with different doses of antioxidants as presented in the Table 2, were analysed after 4 months from bottling. The values of trichromatic parameters (X, Y, Z) and of the chromaticity coordinates of colour, x-the luminance and y-the chromaticity are presented in the Table 3.

Table 3.Trichromatic parameters and basic colour percentages of Sauvignon Blanc wines after 4 months of bottle aging

	Trichromatic parameters and basic colour percentages				
Wine sample Code	X	Y	Z	x % of red	y % of green
SBControl	88.3994	92.2442	93.5300	0.3224	0.3364
SBGSH10	88.1638	91.6632	92.8239	0.3234	0.3362
SBGSH20	88.0719	91.2673	93.0589	0.3233	0.3350
SBGSH30	86.6174	89.7314	90.9998	0.3240	0.3356
SBGSH50	82.3637	85.4208	87.0727	0.3232	0.3352
SBGSH100	85.8873	88.8166	89.6845	0.3249	0.3359
SBAA30	84.4163	88.5165	89.7646	0.3214	0.3370
SBAA40	91.1027	96.0821	97.4189	0.3201	0.3376
SBAA50	92.0580	97.2069	98.9560	0.3194	0.3373
SBAA60	91.3393	96.4066	97.6347	0.3201	0.3378
SBAA70	92.4338	97.6716	99.3375	0.3194	0.3374
SBAA30GSH10	89.6307	93.8582	95.3269	0.3215	0.3366
SBAA30GSH20	88.8788	93.1425	94.2912	0.3217	0.3372
SBAA30GSH30	89.4165	93.6848	95.8051	0.3206	0.3359
SBAA30GSH50	90.0345	94.5040	95.8355	0.3211	0.3370
SBAA30GSH100	89.1820	94.0326	95.8042	0.3196	0.3370

It can be observed that there are differences between the colour of the samples, even if not very big, as the influence of the base wine characteristics was more important than the treatments themselves.

The parameter X values are high (above 91) for all the samples containing only ascorbic acid, except the case of sample SBAA30, the one containing only 30g/l AA, for which it is only 84.4. All the other samples containing AA and GSH also had high values of X – between 89 and 90, while the samples prepared only with GSH had lower values, especially at higher GSH doses. This means that the main influence was brought about by the treatment with AA. It can also be observed that the control sample SBControl and the wines treated with 10 and 20 mg/l GSH have shown similar colour parameters.

The parameter Y, defining the wine samples luminosity (Table 3) is lower for all the samples treated with GSH alone and for the sample SBAA30 with only 30mg/l AA, which means that they are less transparent than the control

sample or the wines treated with AA or AA-GSH combinations. Regarding the luminosity too, treatments with AA have a more important influence than GSH. Similar conclusions can be drawn with the CIELab method (Table 4), when the values for the clarity L* are taken into account (Figure 1).

Table 4.Chromatic characteristics values of Sauvignon Blanc wines after 4 months of bottle aging

Wine sample Code	L*	*a* *	*b* *	C_{ab} *	H_{ab} *
SBControl	**96.9189**	**1.3322**	**4.1080**	**4.3224**	**1.2599**
SBGSH10	96.6825	1.9211	4.1775	4.5981	1.1398
SBGSH20	96.5199	2.4519	3.7370	4.4698	0.9901
SBGSH30	95.8844	2.4895	4.0573	4.7607	1.0201
SBGSH50	94.0561	2.2711	3.6709	4.3183	1.0160
SBGSH100	95.5037	2.7680	4.3130	5.1254	1.0001
SBAA30	95.2456	0.5411	4.0529	4.0902	1.4380
SBAA40	98.4649	-0.4025	4.1641	4.1836	-1.4744
SBAA50	98.9098	-0.6032	3.9205	3.9667	-1.4181
SBAA60	98.5935	-0.5310	4.2435	4.2766	-1.4464
SBAA70	99.0926	-0.7190	3.9865	4.0508	-1.3924
SBAA30GSH10	97.5748	0.7642	4.0225	4.0945	1.3831
SBAA30GSH20	97.2783	0.6350	4.2308	4.2821	1.4170
SBAA30GSH30	97.5047	0.6749	3.5815	3.6445	1.3844
SBAA30GSH50	97.8347	0.3793	4.1300	4.1474	1.4792
SBAA30GSH100	97.6428	-0.3579	3.8212	3.8380	-1.4770

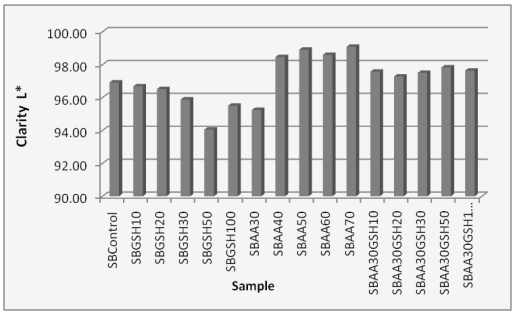

Figure 1. CIELab coordinate L* of Sauvignon Blanc wines after 4 months of bottle aging

As it is already known, when the parameters *a** and *b** are positive, the colour will be in the range of red-orange-yellow and when*a** is negative and *b** is positive, the colour will be in the range of yellow-green. Therefore, as we can observe in Table 4, the wine samples treated only with ascorbic acid (except SBAA30) and the sample with 100 m/l GSH and 30 mg/l AA (SBAA30GSH100) are in the colour range of yellow - green, while the control sample and all the other samples treated with GSH with or without AA, reveal a more or less accentuated tendency towards red-orange-yellow.

From the representation of these values in an*ab* diagram (Figure 2) we can observe that three groups and the control sample are clearly individualized in the space, in direct correlation with the applied treatment: the group treated with different doses of GSH alone, the group treated with a combination of different doses of GSH and 30 mg/l ascorbic acid and the group treated with different doses of AA alone.

While the first two groups containing GSH and the control sample SBControl can be very clearly differentiated in the red-orange-yellow colour space, the group of samples treated only with AA is clearly positioned in the yellow-green space. Thus, it can be concluded that the presence of GSH makes the colour of wine shift toward orange/brown shades.

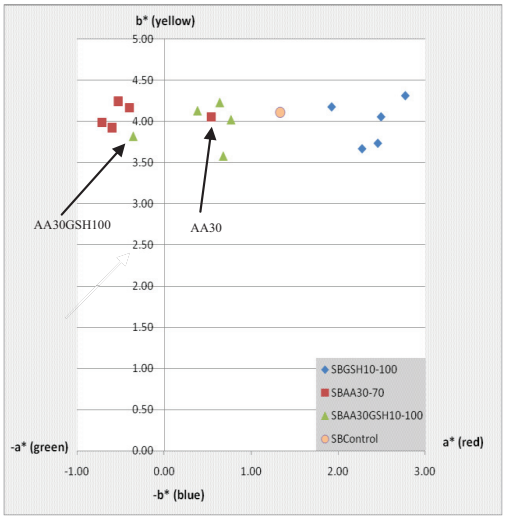

Figure 2.The *ab* diagram describing the position of the colour of Sauvignon Blanc wines treated with ascorbic acid and/or glutathione after 4 months of bottle aging

However, samples treated only with GSH are shifted more toward orange/brown than are the samples treated with the combination of various doses of GSH and 30mg/l ascorbic acid, with the control sample lying in between these groups. This led us to the conclusion that the presence of ascorbic acid in the wine samples has the main positive impact when it comes to the preservation of their yellow-green colour in time.

Another observation is that, although distinct groups are formed in accordance to the type of treatment, in each group an outlier is present: the sample SBAA30 located in the group of samples treated with GSH and AA combinations and sample SBAA30GSH100 located in the group of samples treated only with AA.

The presence of AA30 in the more oxidized GSH-AA group may suggest that the dose of 30 mg/l AA may not be sufficient, only the samples with 40-70 mg/l AA being not oxidized. Conversely, the presence of the sample with 30 mg/l AA and 100 mg/l GSH in the group of not-oxidized samples treated with AA, suggests that a higher dose of GSH, of at least 100 mg/l, is required for protection of colour in Sauvignon Blanc.

After the calculation of the differences in luminosity ΔL_{ab}*, saturation ΔC_{ab}*, position on the red-green Δa* and yellow-blue Δb* space, it was confirmed that the wine samples treated with ascorbic acid had a distinct behavior as compared to the samples treated with GSH or the control sample.

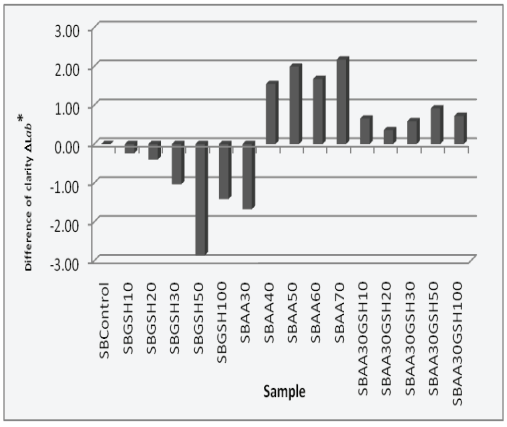

Figure 3.Graphic representation of the difference of luminosity ΔL_{ab}* of Sauvignon Blanc wines after 4 months of bottle aging

As represented in the Figure 3, while the wine samples SBGSH10-100 treated only with GSH and SBAA30 were less transparent than the control SBControl, all other samples containing different doses of AA, with or without GSH, were lighter than the control.

Analysing the variation of the parameter C_{ab}* (chroma), it is easy to observe that the ΔC_{ab}* values are negative for all the samples treated with AA, as such or in combination with GSH, therefore they have a smaller saturation than the control sample SBControl (Figure 4).

The samples treated only with GSH have mainly positive ΔC_{ab}* values, so they are more chromatic (colorful) than the control sample.

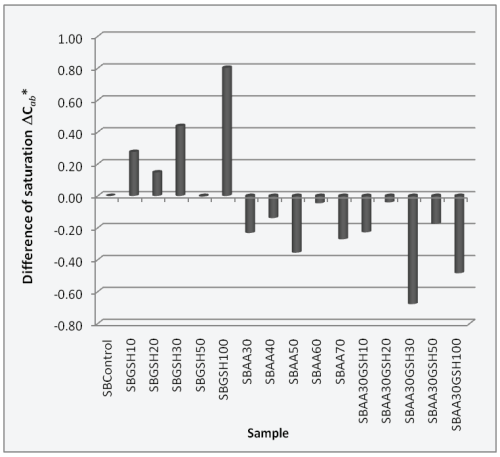

Figure 4. Graphic representation of the difference of saturation ΔC_{ab}* of Sauvignon Blanc wines after 4 months of bottle aging

In order to determine the overall colour difference of the samples against the control, the parameter ΔE_{ab}* was calculated.

For the assessment, it is considered that when the overall colour difference ΔE_{ab}* between two samples is under 0.5 units, the difference is not perceptible, when the difference is situated between 0.5-1 units it is slightly perceptible, while in the case of values between 1.5-3 units the difference is perceptible and above 3 units the colour difference is clearly perceptible.

The values of the colour difference ΔE_{ab}* between the samples treated only with GSH and the control sample are in the range of 0.5-3 units, which means there are perceptible differences between these wines colours and the control sample, the difference mainly increasing with the GSH dose applied (Figure 5).

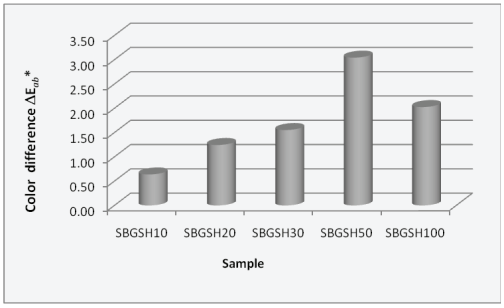

Figure 5. Graphic representation of the overall colour difference ΔE_{ab}* of Sauvignon Blanc wines treated with various doses of GSH against the control sample measured after 4 months of bottle aging

Similarly, the values of the colour difference ΔE_{ab}* between the samples treated only with AA and the control sample (Figure 6)are in the range of 1.5-3 units, all being perceptible by the human eye.

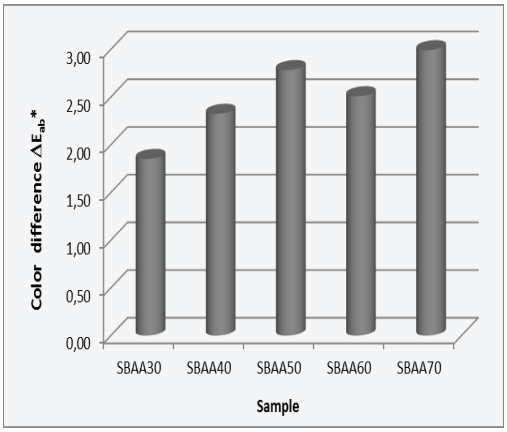

Figure 6. Graphic representation of the colour difference ΔE_{ab}* of Sauvignon Blanc wines treated with various doses of ascorbic acid against the control sample measured after 4 months of bottle aging

In the Figure 7, the differences for samples treated both with GSH and 30 mg/l AA were plotted by taking as control not only the wine without any treatment (SBControl), but also the wine treated with 30 mg/l ascorbic acid (SBAA30Control).

As it can be seen, the colour differences induced by GSH addition are higher, when the effect of those 30 mg/l AA is eliminated (comparison with SBAA30Control), showing that the GSH in the absence of AA produces in time perceptible colour changes.

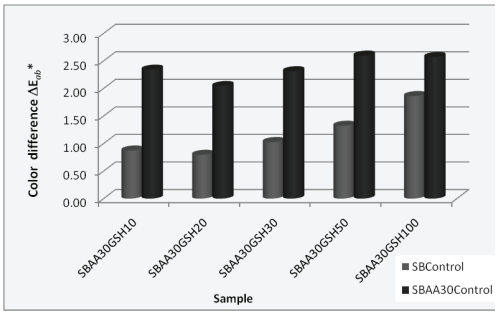

Figure 7. Graphic representation of the colour difference ΔE_{ab}* of Sauvignon Blanc wines treated with 30mg/l ascorbic acid and various doses of GSH against the control samples measured after 4 months of bottle aging

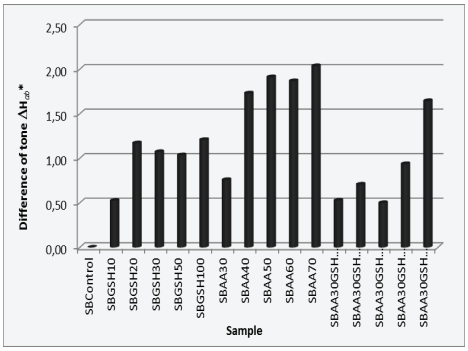

Figure 8. Graphic representation of the difference of hue ΔH_{ab}* between samples of Sauvignon Blanc after 4 months of bottle aging

As for the variation of hue, all the calculated ΔH_{ab}* values are positive (Figure8), therefore, after comparing the wine samples values with the yellow hue value recorded for control sample (SBControl), we can conclude that all the treated wine samples, irrespective of the dose and type of antioxidant added, are greener, which proves they are less oxidised than the untreated base wine.

CONCLUSIONS

The results obtained in this research show that the colour of wine can be clearly influenced by the type and dose of the antioxidants used for its protection.

The addition of ascorbic acid as such in the wine determined obvious changes of the wines colour.

The samples treated with AA are more transparent than the control sample and as

compared to the samples treated only with GSH. All the wine samples treated with ascorbic acid, with or without GSH, are brighter than the untreated wine.

The samples treated only with GSH are darker than the untreated wine.

The wine samples treated only with ascorbic acid (with the exception of SBAA30 and SBAA30GSH100) are located in the *ab* space in the range of yellow-green colour. All the other samples treated with GSH, irrespective if in combination or not with AA, have showed a more or less important tendency to place towards orange/brown tones.

Three groups of treated wines and the control sample are clearly individualized in the colour space, in direct connection with the treatment used: the group treated with increasing doses of GSH, the group treated with the combination of various doses of GSH and the fixed amount of 30 mg/l AA and the group treated with different doses of AA.

The group treated with GSH is placed more toward orange colour than the control sample, while the other two groups, both containing AA with or without GSH are placed more toward green colour.

From green to red, the groups are placed in *ab* space as follows: AA group, AA30-GSH group, untreated wine, GSH group. No major differences were recorded among the samples on the yellow-blue axis, all being in the same range of the yellow space.

Addition of more than 40mg/l of AA proved more efficient in protecting the green component of colour, but using a higher dose of GSH (100 mg/l) in combination with the usually recommended 30mg/l of ascorbic acid (sample SBAA30GSH100) provided the same level of protection of the colour as the wines treated with 40-70 mg/l AA alone.

All the treatments containing AA, irrespective of the dosage or the presence of GSH, determined less oxidised wines compared to the untreated wine.

We can conclude that the usage of ascorbic acid alone or in combination with glutathione ensured a better antioxidant protection to the wines than the treatment with GSH alone. These additions should be made only when a sufficient level of sulphur dioxide is provided.

REFERENCES

Antoce O.A., 2002. Study on the effect of the extraction enzyme quantity on the colour of Merlot must and wines. I. Chromatic parameters evolution during maceration-fermentation process. Scientific Papers. Series B, Horticulture, Volume XLV, ISSN 1222-5312, 196-200.

Antoce O.A., 2007. Oenology. Chemistry and sensory analysis. Universitaria Publishing House, Craiova.

Badea G.A., Antoce O.A., 2015. Glutathione as a possible replacement of sulfur dioxide in winemaking technologies: a review.Scientific Papers. Series B, Horticulture, Volume LIX, ISSN 2285-5653, 123-140.

Barril C., Clark A.C., Prenzler P.D., Karuso P., Scollary G. R., 2009. Formation of pigment precursor (+)-100 methylene-600-hydroxy-2H-furan-500-one-catechinisomers from (+)-catechin and a degradation product of ascorbic acid in a SBdel wine system. J.Agric. Food Chem.,57(20):9539–9546.

Barril C., Clark A.C., Scollary G.R., 2012. Chemistry of ascorbic acid and sulfur dioxide as an antioxidant system relevant to white wine. Analytica Chimica Acta, 732:186–193.

Brajkovich M., Tibbits N., Peron G., Lund C.M., Dykes S.I., Kilmartin P.A., et al., 2005. Effect of screwcap and cork closures on SO2 levels and aromas in a Sauvignon Blanc wine. J. Agric. Food Chem.,53(26):10006–10011.

Bouzanquet Q., Barril C., Clark A.C., Dias D.A., Scollary G.R., 2012. A novel glutathione-hydroxycinnamic acid product generated in oxidative wineconditions. J. Agric. Food Chem., 60(49):12186–12195.

Kritzinger E.C., Bauer F.F., du Toit W.J., 2012. Role of glutathione in winemaking: A review. J. Agric. Food Chem., 61(2):269–277.

Labrouche F., Clark A.C., Prenzler P.D., Scollary G.R., 2005. Isomeric influence on the oxidative coloration of phenolic compounds in a model white wine: Comparison of (+)-catechin and (–)-epicatechin. J. Agric. Food Chem.,53 :9993–9998.

Lavigne-Cruège V., Dubourdieu D., 2002. Role of glutathione on development of aroma defects in dry white wines. In: 13th International Enology Symposium, SBntpellier, 331–347.

Lavigne-Cruège V., Pons A., Chone X., Dubourdieu D., 2003. Rôle du glutathione sur l'évolution aromatique des vins blancs secs. In: 7e Symposium international d'Oenologie, Bordeaux.

Nikolantonaki M., Magiatis P., Waterhouse L.A., 2014. Measuring protection of aromatic wine thiols from oxidation by competitive reactions vs wine preservatives with ortho-quinones. Food Chemistry, 163:61–67.

Oliveira C.M., Silva Ferreira A.C., Guedes de Pinho P., Hogg T.A., 2002. Development of a potentiometric method to measure the resistance to oxidation of white wines and the antioxidant power of their constituents. J. Agric. Food Chem., 50(7):2121–2124.

Papadopoulou D., Roussis I.G., 2001. Inhibition of the decline of linalool and a-terpineol in Muscat wines by glutathione and N-acetyl-cysteine. Italian Journal of Food Science, 13:413–419.

Papadopoulou D., Roussis I.G., 2008. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a SBdel wine medium by glutathione and

N-acetylcysteine. International Journal of Food Science and Technology, 43:1053–1057.

Roussis I.G., Lambropoulos I., Tzimas P., 2007. Protection of volatiles in a wine with low sulfurdioxide by caffeic acid or glutathione. Amer. J. Enol. Vitic., 58(2):274–278.

Roussis I.G., Papadopoulou D., Sakarellos-Daitsiotis M., 2009. Protective effect of thiols on wine aroma volatiles. Open Food Science Journal,3:98–102.

Singleton V.L., Salgues J., Zaya J., Trousdale E., 1985. Caftaric acid disappearance and conversion to products of enzymatic oxidation in grape must and wine. Amer. J. Enol. Vitic., 36:50–56.

Sonni F., Clark A.C., Prenzler P.D., Riponi C., Scollary G.R., 2011. Antioxidant action of glutathione and the ascorbic acid/glutathione pair in a model white wine. J. Agric. Food Chem., 59:3940–3949.

Ugliano M., Kwiatkowski M.J., Vidal S., Capone D., Siebert T., Dieval B., Aagaard O., Waters E.J., 2011. Evolution of 3-mercatohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. J. Agric. Food Chem.,59:2564–2572.

*** Resolution OIV-OENO 446-2015, Treatment of wine with glutathione, approved in OIV General Assembly, in Mainz, Germany, 2015, http://www.oiv.int/oiv/info/enresolution?lang=en accessed in January 2016.

*** Resolution OIV-OENO 445-2015, Treatment of must with glutathione, approved in OIV General Assembly, in Mainz, Germany, 2015, http://www.oiv.int/oiv/info/enresolution?lang=en accessed in January 2016.

*** OIV, 2006, Method OIV-MA-AS2-11, Determination of chromatic characteristics according to CIELab. Compendium of International Analysis of Methods, R2006, http://www.oiv.int/oiv/info/enresolution, accessed in January 2016.

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COLOR CHANGES DURING THE STORAGE IN BOTTLES OF MUSCAT OTTONEL WINE TREATED WITH ASCORBIC ACID AND GLUTATHIONE

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Abstract

The present work aimed to determine the colour evolution after aging in bottle of a white wine from Muscat Ottonel cultivar and the effect of the addition of different antioxidants at bottling time, such as SO_2, reduced glutathione (GSH) and ascorbic acid. Wine colour is an important indicator of its degree of oxidation, therefore the evolution toward a brownish colour is equivalent with some quality loss or even degradation. Considering that colour is also a key parameter that contributes to the perception of the quality profile of a white wine, the study attempted to determine the effect of certain doses and combinations of some anti-oxidants, which are known to ensure the best wine quality preservation during aging of wine in bottles. Our preliminary study shows that, in some cases and conditions, the anti-oxidants used to protect the aroma and the colour of wines end up affecting the colour.

Key words*: Glutathione, ascorbic acid, white wine colour, browning of white wine, oxidation of white wines.*

INTRODUCTION

The protection of white wines from oxidation is a condition for preserving their quality and prolonging their shelf life. The loss of aroma compounds, the browning phenomenon and the precipitation of phenolic substances are characteristics of the white wines oxidation (Kilmartin, 2010).As the wine colour is an important indicator of its degree of oxidation, the occurrence of brownish colour in white wine is equivalent with a decrease in quality, because when such oxidation is apparent, it also means that the aromatic compounds in wine were already affected (Singleton, 1987).

The addition of different conditioning agents, particularly prior to bottling, is the classic method employed to keep the quality of wine, by eliminating or stabilizing the substrates susceptible to oxidation, such as polyphenols, volatile compounds, etc.

Antioxidants such as SO_2, ascorbic acid, reduced glutathione (GSH) have the capacity to reduce and eliminate quinones and are essential for the management of oxidative aging processes of wine (Brajkovich et al., 2005; Lavigne Cruège et al., 2003; Ugliano et al., 2011). It is already known that using SO_2 and ascorbic acid combined in various ratios slows down the oxidation of polyphenols in wine in different proportions (Oliveira et al., 2002). But, as the ascorbic acid is a highly unpredictable molecule, its addition in white wines involves some risk taking. Its degradation products may not be captured by $SO_2$and could react further with any catechins found in wine, determining the occurrence of yellow xanthylium pigments(Barril et al.,2009; Barril et al., 2012)or the appearance of sotolon, which affects the dry white wine aroma (Pons et al.,2010).

The tripeptide glutathione (GSH),a natural antioxidant found in grapes, alsodrew the researchers' attention in the last decade for potential use in wine production or during storage. In July 2015 OIV approved the addition of GSH in must and wine up to a concentration of 20 mg/l(Resolutions OENO-TECHNO 10-445 and 10-446/July 2015).

However, although results of numerous studies on the effect of adding glutathione to wine were recently published, the role of this antioxidant and its complementary action with the most used wine preservatives like sulfur

dioxide and ascorbic acid have to be further examined (Kritzinger et al., 2012; Badea and Antoce, 2015).

The researches have showed that in combination with SO_2, GSH reduces the degradation of aromatic compounds like monoterpenes and esters and slows down the browning reaction of white wines, in particular the formation of yellow pigments of xanthylium (Bouzanquet et al., 2012; Roussis et al.,2007; Sonni et al., 2011).

While a lot of researches have proved that glutathione has an important contribution to the protection of varietal aroma during the aging of wines (Papadopoulou and Roussis, 2001, 2008; Roussis et al., 2009), its effect on the wine colour is not clearly established. Various doses of exogenous GSH that should be added to wine to provide antioxidant protection were proposed by researchers. For muscat-type aroma, the terpineol and linalool protection in wines kept in contact with the air at 20°C for 3 days has been assured by the addition of 20 mg/lGSH (Papadopoulou and Roussis, 2001, 2008).A smaller quantityof only 10 mg/l of GSH has been proposed by Lavigne-Cruège and Dubourdieu (2002) who demonstrated that the formation of aging defects, such as yellow pigments and the loss of the varietal characteristics of wine, can be prevented with this low dosage. However, the effects of glutathione in wines depend on the wine composition and conditions, so that contradictory results have been reported. While it was proved that GSH protects against oxidation, it was reported that it can also contribute to oxidation (Vaimakis and Roussis, 1996) or that glutathione addition may determine the occurrence of higher contents of H_2S during the storage of a wine (Ugliano et al., 2011).

The wine colour is influenced by some factors like the grape variety, the pH values, the storage temperature, the winemaking technologies and the stability treatments applied before the bottling (Antoce, 2002). Numerous studies have demonstrated during last decades a direct correlation between the white wines oxidative stability during aging in bottle, the values of pH, total phenol and total SO_2 contents and the GSH or ascorbic acid dosage added to wines.

In this study we have investigated the color changes after 4 months of bottle storage of a Muscat Ottonel wine treated at bottling time with GSH and/or ascorbic acid in various dosages and conditions.

The tested Muscat Ottonel wines were prepared from a base wine with a given level of sulfur dioxide treated with GSH or/and ascorbic acid in different dosages and combinations, aiming to determine an optimal treatment for this type of wine.

MATERIALS AND METHODS

The studied wine was industrially produced in September 2014 in the Domeniile Dealu Mare Urlati Wine cellar from Prahova County, Romania, from Muscat Ottonel grapes cultivated in their own vineyard. The wine samples have been prepared in February 2015, by bottling the same base wine after treatment with different combinations and dosages of glutathione or/and ascorbic acid, both supplied by Enologica Vason Italy, as represented in the Table 1.

Table 1 - Variants of Muscat Ottonel wines produced in 2014 and treated with different doses of antioxidants

Wine sample Code	Dosage
MOControl	Control
MOGSH10	10 mg/l Glutathione
MOGSH20	20 mg/l Glutathione
MOGSH30	30 mg/l Glutathione
MOGSH40	50 mg/l Glutathione
MOGSH50	100 mg/l Glutathione
MOAA30	30 mg/l Ascorbic Acid
MOAA40	40 mg/l Ascorbic Acid
MOAA50	50 mg/l Ascorbic Acid
MOAA60	60 mg/l Ascorbic Acid
MOAA70	70 mg/l Ascorbic Acid
MOAA30GSH10	30 mg/l Ascorbic Acid and 10 mg/l Glutathione
MOAA30GSH20	30 mg/l Ascorbic Acid and 20 mg/l Glutathione
MOAA30GSH30	30 mg/l Ascorbic Acid and 30 mg/l Glutathione
MOAA30GSH50	30 mg/l Ascorbic Acid and 50 mg/l Glutathione
MOAA30GSH100	30 mg/l Ascorbic Acid and 100 mg/l Glutathione

The physico-chemical parameters of the base wine used are shown in Table 2.

Table 2 - Physico-chemical parameters of Muscat Ottonel base wine

Physico-chemical analysis report of the basis wine	
Identification data of the wine	
Cultivar / Product range	MUSCAT OTTONEL
Vintage year	2014
Quality category	CDO
Producer	DOMENIILE DEALU MARE URLATI
Wine physico-chemical parameters	
Free sulfur dioxide (mg/l)	42
Total sulfur dioxide (mg/l)	80
Total acidity (g/l tartaric acid)	6
Volatile acidity (g/l)	0.33
Alcoholic concentration (%)	13.1
Sugar (g/l)	1.23
Relative density at 20 °C	0.9902
Nonreducing dry extract (g/l)	20.2

The bottled wines were analyzed 4 months after the treatment with the above mentioned doses of antioxidants, by measuring the colour parameters with a computer-controlled double beam spectrophotometer Specord 250 from Analytik Jena AG, running the software WinAspect version 2.2.7, which automatically calculates the trichromatic components (CIE XYZ tristimulus values), the *xy* chromaticity coordinates and the CIELab colour parameters. The obtained results have been analysed by using the software Microsoft Excel as well as the package Chroma Ver. 2.0. For measuring the colour parameters 1 mm glass cuvettes were used, while the standard illuminant was D65and the angle of observer 2°. The transmittance of wine was measured every 1 nm over the visible spectrum between 400-700nm.

The trichromatic components (X, Y, Z) and the basic colour percentages (*x*– the percentage of red and *y*– the percentage of green),as well as their transformation into the CIELab space system parameters (the colorimetric coordinates L*=clarity, *a**= red/green colour component, *b** =yellow/blue colour component, C* = chroma, H* = hue) were automatically calculated by the software. In order to evaluate the changes of colour induced by the treatments, differences of these parameters were calculated against the untreated control (MOControl).The differences of CIELab parameters ΔL*,Δa*, Δb*, ΔC_{ab}* and ΔH_{ab}*, as well as the overall colour difference against the control sample, ΔE_{ab}*, have been calculated in Excel in accordance with the OIV method (OIV- Resolution OENO 1/2006, OIV-MA-AS2-11: R2006).

RESULTS AND DISCUSSIONS

The variants prepared were assessed after 4 months of storage in bottles and the parameters recorded in accordance to the CIEXYZ and CIELab methods. The measured values of trichromatic parameters (X, Y, Z) and of the chromaticity coordinates of colour, luminance *x* and chromaticity *y*are included in Table 3.

We can see that the colour parameters determined are rather close for all the samples, as the base wine imposes its characteristics on all. From these results we can see, however, that the luminosity (parameter Y in Table 3) is lower for all the samples with GSH and the control sample, while the samples with ascorbic acid, irrespective of the dosage, are brighter.

Table 3 - Trichromatic parameters and basic colour percentages of Muscat Ottonel wines determined after 4 months of bottle aging

Wine sample Code	Trichromatic parameters and basic colour percentages				
	X	Y	Z	*x* % of red	*y* % of green
MOControl	**92.7895**	**97.6440**	**101.1128**	**0.3183**	**0.3349**
MOGSH10	91.6838	96.4309	99.3223	0.3190	0.3355
MOGSH20	92.5881	97.4078	100.9868	0.3182	0.3348
MOGSH30	91.5139	96.2079	99.2848	0.3189	0.3352
MOGSH40	91.4817	96.1447	99.3360	0.3188	0.3350
MOGSH50	92.0272	96.7019	100.4661	0.3182	0.3344
MOAA30	92.6896	97.8371	101.0445	0.3179	0.3356
MOAA40	93.7348	99.0493	102.4415	0.3175	0.3355
MOAA50	92.4725	97.6794	100.4128	0.3183	0.3362
MOAA60	94.0322	99.4636	102.5979	0.3176	0.3359
MOAA70	92.6805	97.9860	100.5360	0.3183	0.3365
MOAA30GSH10	92.8468	98.0147	100.9786	0.3181	0.3359
MOAA30GSH20	93.5649	98.8449	102.3002	0.3175	0.3354
MOAA30GSH30	92.2983	97.5105	100.1315	0.3183	0.3363
MOAA30GSH50	93.6449	98.9087	102.4180	0.3175	0.3353
MOAA30GSH100	92.9785	98.2005	101.0512	0.3182	0.3360

The parameter X is smaller for the GSH samples and for the control than in all the other samples, showing that indeed the addition of GSH correlates with some changes in colour.

In the CIELab system, the color can be very precisely defined through the Cartesian parameters L*, *a**, *b**. By definition, if the

parameters *a** and *b** are positive, the colour of the analysed sample will be in the range of red-orange-yellow and if the parameters *a** and *b** have different signs (*a** is negative and *b** is positive)the colour of the analysed sample will be in the range of yellow-green.

Table 4 - Values of chromatic characteristics of Muscat Ottonel wines after 4 months of bottle aging

Wine sample Code	L*	*a**	*b**	C_{ab}*	H_{ab}*
MOControl	**99.0818**	**-0.0377**	**2.8162**	**2.8165**	**-1.5574**
MOGSH10	98.6032	0.0471	3.1526	3.1529	1.5559
MOGSH20	98.9889	0.0037	2.7374	2.7374	1.5694
MOGSH30	98.5148	0.1229	3.0246	3.0271	1.5302
MOGSH40	98.4897	0.1731	2.9480	2.9531	1.5122
MOGSH50	98.7105	0.2009	2.5940	2.6017	1.4935
MOAA30	99.1565	-0.5421	2.9919	3.0416	-1.3907
MOAA40	99.6312	-0.7272	2.9105	3.0000	-1.3260
MOAA50	99.0957	-0.6632	3.2927	3.3588	-1.3720
MOAA60	99.7922	-0.8956	3.0882	3.2154	-1.2885
MOAA70	99.2160	-0.8104	3.4203	3.5150	-1.3381
MOAA30GSH10	99.2272	-0.5625	3.1536	3.2034	-1.3943
MOAA30GSH20	99.5516	-0.6851	2.8637	2.9445	-1.3360
MOAA30GSH30	99.0293	-0.6883	3.3606	3.4304	-1.3688
MOAA30GSH50	99.5765	-0.6505	2.8312	2.9050	-1.3449
MOAA30GSH100	99.3000	-0.6416	3.2321	3.2952	-1.3748

As it can be observed in the Figure 1, the wine samples treated only with glutathione are darker compared to control sample(they have higher L* values), while the rest of samples with ascorbic acid, with or without GSH, resulted lighter than the control sample.

By analyzing the resulted values of the CIELab space parameters, we can observe that all the wines treated with ascorbic acid, with or without GSH, are in the color range of yellow - green, while the samples treated only with GSH showed a slight tendency toward red - orange - yellow.

If we plot these samples on an *ab* diagram (Figure 2) we can easily see that they form three groups, which correlate with the type of treatment: the group treated only with various doses of GSH, the group treated only with various doses of ascorbic acid and the group treated with 30 mg/l ascorbic acid and various doses of GSH.

The first group is clearly differentiated in the color space, while the other two overlap, showing that the main influence on the color is due to the presence of ascorbic acid.

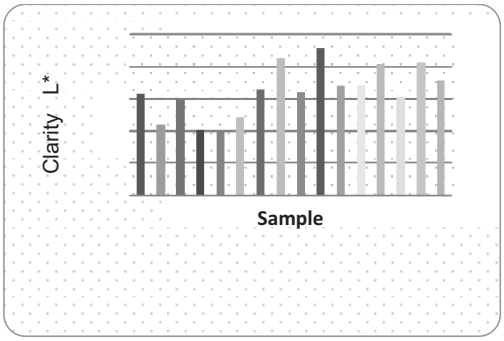

Figure 1.CIELab coordinate L* of Muscat Ottonel wines after 4 months of bottle aging

The samples produced only with GSH acquired a shade of orange, while the rest of samples, with ascorbic acid and with or without GSH did not show the same trend.

If we analyze the differences in brightness ΔL* and saturation ΔC_{ab}*, and the variations of red-green Δa* and yellow-blue Δb* shades, we observe that the wine samples treated with various doses of GSH and/or ascorbic acid, behaved differently from the control sample.

As regarding the variation of the parameter *a** (red-green), most of the Δa* values are negative (Table 5), which means that the samples are greener than the control sample, except for the wines treated only with glutathione, which are redder than the control.

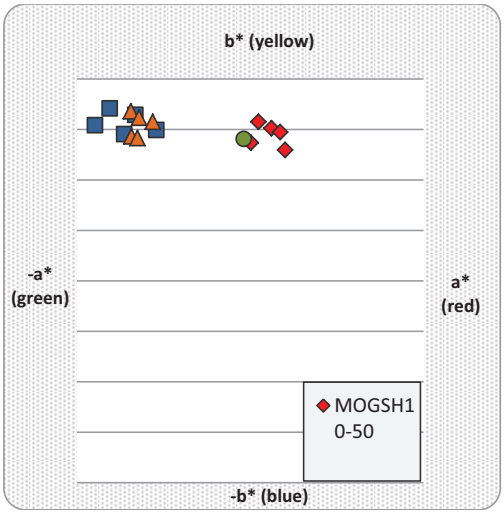

Figure 2. The *ab* diagram describing the position of the colour of Muscat Ottonel wines treated with ascorbic acid and/or GSH determined after 4 months of bottle aging

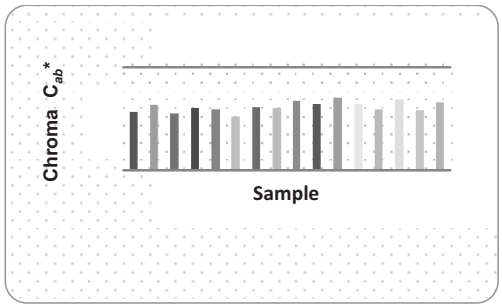

Figure 3. CIELab coordinate C_{ab}* of Muscat Ottonel wines after 4 months of bottle aging

This indicates that the usage of ascorbic acid provided a better antioxidant protection even in combination with glutathione, compared with using only GSH.

The comparison of samples containing ascorbic acid, whether they were treated only with increasing amounts of ascorbic acid or the fixed amount of 30mg/l ascorbic acid and increasing doses of glutathione, with the control sample MOControl showed that the samples treated only with ascorbic acid are predominantly greener than those treated with both antioxidants (Figure 2) and the variations of the parameter *b** (yellow-blue), the Δb* values, are mostly positive, so they were yellower than the control sample. This demonstrates that in the presence of large quantities of ascorbic acid the oxidation process is slowed, while glutathione has antioxidant effect only with the concomitant use of ascorbic acid.

Table 5 - Variations of CIELab coordinates of Muscat Ottonel wines after 4 months of bottle aging

Wine sample Code	ΔL*	Δa*	Δb*	ΔC_{ab}*	ΔE_{ab}*	ΔH_{ab}*
MOControl						
MOGSH10	-0.4786	0.0848	0.3364	0.3364	0.5911	0.0848
MOGSH20	-0.0929	0.0414	-0.0788	-0.0791	0.1287	0.0408
MOGSH30	-0.5670	0.1606	0.2084	0.2106	0.6251	0.1577
MOGSH40	-0.5921	0.2108	0.1318	0.1366	0.6422	0.2077
MOGSH50	-0.3713	0.2386	-0.2222	-0.2148	0.4941	0.2453
MOAA30	0.0747	-0.5044	0.1757	0.2251	0.5393	0.4844
MOAA40	0.5494	-0.6895	0.0943	0.1835	0.8866	0.6713
MOAA50	0.0139	-0.6255	0.4765	0.5423	0.7864	0.5694
MOAA60	0.7104	-0.8579	0.2720	0.3989	1.1466	0.8068
MOAA70	0.1342	-0.7727	0.6041	0.6985	0.9900	0.6885
MOAA30GSH10	0.1454	-0.5248	0.3374	0.3869	0.6406	0.4895
MOAA30GSH20	0.4698	-0.6474	0.0475	0.1280	0.8013	0.6364
MOAA30GSH30	-0.0525	-0.6506	0.5444	0.6139	0.8499	0.5855
MOAA30GSH50	0.4947	-0.6128	0.0150	0.0885	0.7877	0.6066
MOAA30GSH100	0.2182	-0.6039	0.4159	0.4787	0.7650	0.5554

Analysis of the variation of the parameter C_{ab}* (chroma) showed that the ΔC_{ab}* values are mostly positive (except for the samples MOGSH20 and MOGSH50), which means that they have a higher saturation than the control sample (Figure 4), so they have a higher brightness than the control sample.

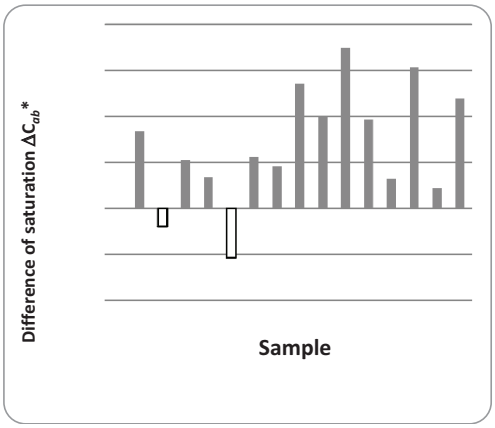

Figure 4. Graphic representation of the difference of saturation ΔC_{ab}* of Muscat Ottonel wines after 4 months of bottle aging

In order to determine whether there are global colour differences between different wine samples, the calculated values of the overall colorimetric difference parameter ΔE_{ab}* (Table 5) have been analysed. In accordance with the use of grey scale to compare two colours, if the difference between those two colours is under 0.5 units, it is considered barely perceptible; if the difference between the two colours is in the range of 0.5-1 units, it is considered slightly perceptible.

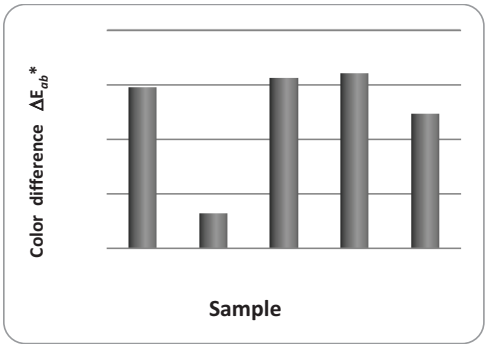

Figure 5. Graphic representation of the overall colour difference ΔE_{ab}* of Muscat Ottonel wines treated with various doses of GSH after 4 months of bottle aging

Overall, the values of the colorimetric difference ΔE_{ab}* between the samples with GSH and the control sample MOControl are situated over 0.5 units, meaning that the difference in colour between the sample wines and the control MOControl is slightly perceptible, except for some samples - namely MOGSH20 and MOGSH50, for which the difference is barely perceptible (Figure 5). The same conclusion can be drawn if we analyse the samples containing only ascorbic acid: the difference in colour between the wines and the control sample MOControl is also slightly perceptible (Figure 6).

There are some slightly perceptible differences between the control sample MOControl and the samples containing increasing doses of ascorbic acid, except for the sample treated with 30 mg/l, which did not show the same behaviour, the difference between this sample and the control sample being barely perceptible.

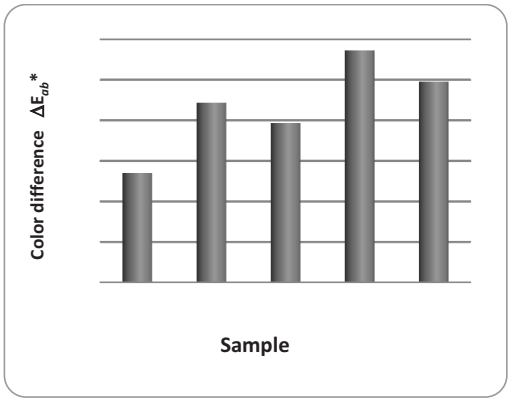

Figure 6. Graphic representation of the colour difference ΔE_{ab}* of Muscat Ottonel wines treated with various doses of ascorbic acid after 4 months of bottle aging

As we can observe in the Figure 7, the differences between samples containing 30 mg/l ascorbic acid combined with ascending doses of glutathione and MOAA30 containing only 30 mg/l ascorbic acid, considered as the control sample MOAA30Control, are barely perceptible, while comparing the same samples with the control sample without ascorbic acid, MOControl the differences are easier to perceive.

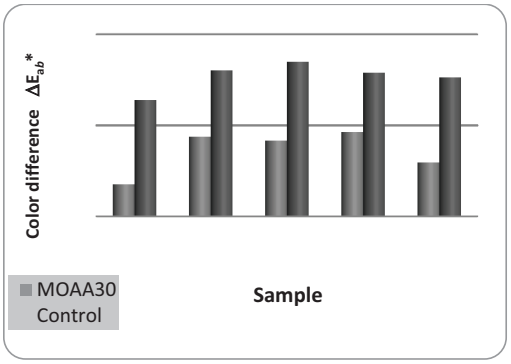

Figure 7. Graphic representation of the colour difference ΔE_{ab}* of Muscat Ottonel wines treated with 30mg/l ascorbic acid and various doses of GSH after 4 months of bottle aging

As regarding the variation of the parameter H_{ab}* (the hue), all the ΔH_{ab}* values are positive, which means that comparing with the control sample MOControl colour (considered yellow as the wine is white) all the wine samples, whatever the treatment applied, were greener, so less oxidised (Figure 8).

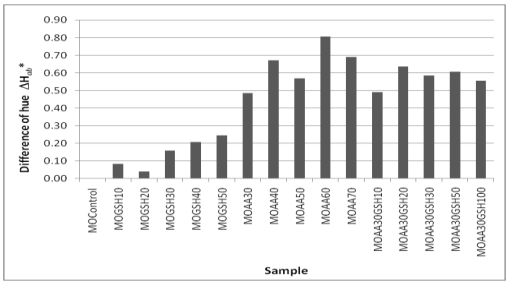

Figure 8. Graphic representation of the difference of hue ΔH_{ab}* between wine samples of Muscat Ottonel after 4 months of bottle aging

With the purpose to summarize the obtained results, we can make the following remarks:

-Regarding the wine samples treated only with GSH, we have noticed that the samples treated with higher doses than 30mg/l GSH showed a pinking effect, developing orange shades.

- Regardingthe overall color differences ΔE_{ab}* of the treated wines and the control sample MOControl, the values were not significantly different, but positive values recorded for the differences of tone ΔH_{ab}* have showed that all the samples treated with glutathione were greener than the control sample, which means that GSH has ensured some antioxidant

protection to the treated wines, even if not very advanced.

-The samples treated only with ascorbic acid in variable quantities evolved differently: all treated samples were greener than the control sample MOControl, which can be interpreted as a slowdown of the polyphenol oxidation, as previous studies demonstrated too. Regarding the variation of the yellow-blue parameter *b**, the most yellow sample, compared to the controlone, was MOAA5, the sample treated with 70mg/l ascorbic acid; thus we can conclude that the use of ascorbic acid had an obvious antioxidant effect.

-Analyzing the determined values of the color components *a** and *b** in the case of wine samples treated with a constant amount of 30mg/l ascorbic acid and varying amounts of glutathione, we found that all samples treated with GSH and ascorbic acid were greener than the control sample MOControl, as the Δa* has negative values, but in the same time the Δb*values are positive, so all the samples were yellower than the control. The overall colorimetric differences ΔE_{ab}* were in all cases barely perceptible (Figure 7), but the difference of hue ΔH_{ab}* had positive values (Figure 8), so the samples were greener than the control, which means that the combination of GSH and ascorbic acid assured a better antioxidant protection to the wines treated in this way, than the use of glutathione alone or in combinations with small quantities of ascorbic acid. This led to the conclusion that low quantities of glutathione, used alone or in combination with small dosage of ascorbic acid have no important effect on the yellow color of the wine, but in higher quantities these antioxidants showed a cumulative effect leading to the intensification of the yellow shades of wine samples. We must keep in mind that although negative values of the difference Δa* between the values of the colour parameter *a**- the red-green component of the color, can mean a slowing down of the polyphenols oxidation, while increasingvalues of the parameter *b**- the yellow-blue component of the colour with the increasing of the ascorbic acid quantity may indicate the formation of yellow xanthylium cation pigments, equivalent with a certain wine oxidation and degradation, as we mentioned previously.

CONCLUSIONS

The measured data revealed that treating wine with varying doses of glutathione alone could not assure an effective conservation of the color characteristics of the wine, which showed a tendency to develop shades of orange specific to browning reactions. In the case of wines treated with varying doses of ascorbic acid, higher doses of ascorbic acid were more appropriate for the conservation of the wine colour, and in the case of variable doses of glutathione together with a fixed dose of 30 mg/l ascorbic acid, the combination of these two antioxidants, (in the presence of small quantities of sulfur dioxide, of course), was found to have a synergistic antioxidant action and to be more effective for the conservation of the wine color characteristics. The glutathione and ascorbic acid in combination with the sulfur dioxide can improve wine stability and prevent the formation of atypical oxidative character during wine aging, but the most appropriate dosage of these antioxidants was not clearly determined until now. It is therefore important to continue the research to determine the minimum/optimal amounts of free sulfur dioxide, as well as the doses of GSH and ascorbic acid necessary to ensure the resistance of wine to oxidation and to extend its shelf life.

REFERENCES

Antoce O.A., 2002. Study on the effect of the extraction enzyme quantity on the colour of Merlot must and wines. I. Chromatic parameters evolution during maceration-fermentation process. Scientific Papers. Series B, Horticulture, Volume XLV, ISSN 1222-5312, 196-200.

Badea G.A., Antoce O.A., 2015. Glutathione as a possible replacement of sulfur dioxide in winemaking technologies: a review.Scientific Papers. Series B, Horticulture, Volume LIX, ISSN 2285-5653, 123-140.

Barril C., Clark A.C., Prenzler P.D., Karuso P., Scollary G. R., 2009. Formation of pigment precursor (+)-100 methylene-600-hydroxy-2H-furan-500-one-catechinisomers from (+)-catechin and a degradation product of ascorbic acid in a model wine system. J.Agric. Food Chem.,57(20):9539–9546.

Barril C., Clark A.C., Scollary G.R., 2012. Chemistry of ascorbic acid and sulfur dioxide as an antioxidant system relevant to white wine. Analytica Chimica Acta, 732:186–193.

Brajkovich M., Tibbits N., Peron G., Lund C.M., Dykes S.I., Kilmartin P.A., et al., 2005. Effect of screwcap and cork closures on SO2 levels and aromas in a Sauvignon Blanc wine. J. Agric. Food Chem.,53(26):10006–10011.

Bouzanquet Q., Barril C., Clark A.C., Dias D.A., Scollary G.R., 2012. A novel glutathione-hydroxycinnamic acid product generated in oxidative wineconditions. J. Agric. Food Chem., 60(49):12186–12195.

Kilmartin P.A., 2010. Understanding and controlling nonenzymatic wine oxidation, Woodhead Publishing Series in Food Science, Technology and Nutrition, 432–458

Kritzinger E.C., Bauer F.F., du Toit W.J., 2012. Role of glutathione in winemaking: A review. J. Agric. Food Chem., 61(2):269–277.

Lavigne-Cruège V., Dubourdieu D., 2002. Role of glutathione on development of aroma defects in dry white wines. In: 13th International Enology Symposium, Montpellier, 331–347.

Lavigne-Cruège V., Pons A., Chone X., Dubourdieu D., 2003. Rôle du glutathione sur l'évolution aromatique des vins blancs secs. In: 7e Symposium international d'Oenologie, Bordeaux.

Oliveira C.M., Silva Ferreira A.C., Guedes de Pinho P., Hogg T.A., 2002. Development of a potentiometric method to measure the resistance to oxidation of white wines and the antioxidant power of their constituents. J. Agric. Food Chem., 50(7):2121–2124.

Papadopoulou D., Roussis I.G., 2001. Inhibition of the decline of linalool and a-terpineol in Muscat wines by glutathione and N-acetyl-cysteine. Italian Journal of Food Science, 13:413–419.

Papadopoulou D., Roussis I.G., 2008. Inhibition of the decrease of volatile esters and terpenes during storage of a white wine and a model wine medium by glutathione and N-acetylcysteine. International Journal of Food Science and Technology, 43:1053–1057.

Pons A., Lavigne V.R., Landais Y., Darriet P., Dubourdieu D., 2010. Identification of a sotolon pathway in dry white wines. J. Agric. Food Chem., 58(12):7273–7279.

Roussis I.G., Lambropoulos I., Tzimas P., 2007. Protection of volatiles in a wine with low sulfurdioxide by caffeic acid or glutathione. Amer. J. Enol. Vitic., 58(2):274–278.

Roussis I.G., Papadopoulou D., Sakarellos-Daitsiotis M., 2009. Protective effect of thiols on wine aroma volatiles. Open Food Science Journal,3:98–102.

Singleton V.L., 1987. Oxygen with phenols and related reactions in musts, wines, and model systems: observations and practical implications. Amer. J. Enol. Vitic.,38:69–77.

Sonni, F., Clark, A. C., Prenzler, P. D., Riponi, C., Scollary, G. R., 2011. Antioxidant action of glutathione and the ascorbic acid/glutathione pair in a model white wine. J. Agric. Food Chem. 59, 3940–3949.

Ugliano M., Kwiatkowski M.J., Vidal S., Capone D., Siebert T., Dieval B., Aagaard O., Waters E.J., 2011. Evolution of 3-mercatohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. J. Agric. Food Chem.,59:2564–2572.

Vaimakis V., Roussis I.G., 1996. Must oxygenation together with glutathione addition in the oxidation of white wine. Food Chemistry, 57:419–422.

http://www.tex.tuiasi.ro/biblioteca/carti/CURSURI/Prof. Dr.IngAugustinMuresan/. Curs 14. Evaluarea culorii.pdf

*** OIV, 2006, Method OIV-MA-AS2-11, Determination of chromatic characteristics according to CIELab. Compendium of International Analysis of Methods, R2006. http://www.oiv.int/oiv/info/enresolution, accessed in January 2016.

*** Resolution OIV-OENO 445-2015, Treatment of wine with glutathione, approved in OIV General Assembly, in Mainz, Germany, 2015, http://www.oiv.int/oiv/info/enresolution?lang=en accessed in January 2016.

*** Resolution OIV-OENO 446-2015, Treatment of must with glutathione, approved in OIV General Assembly, in Mainz, Germany, 2015, http://www.oiv.int/oiv/info/enresolution?lang=en accessed in January 2016

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PHYSICAL CHEMICAL STUDIES REGARDING CIDER STABILITY STORED UNDER AMBIENT CONDITIONS

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Abstract

Multiple cider samples obtained from United Kingdom market were analyzed for their parameters before opening and after 24/48 hour storage on ambient conditions.
We aimed to study the basic parameters such as color, turbidity and sulfites contained in different type of ciders.
The first step to achieve our general aim was to select from the diversity of cider market and to assess the parameters used to conduct this study.
The physical and chemical studies allow us to evaluate not only the diversity of the samples but also the how they behave in different ambient conditions.
This information is essential for the selection of cider consumption and for better understanding of the selected parameters for further beverage studies, especially apple and pear made ones.
Deviant variation from standard was found in more than half from twenty five samples analyzed. The results of previous studies showed that the defective storage conditions of cider might affect not just the bacteria contaminations, but that include also defective physical and chemical composition.
The experiments exposed that weaknesses of the opened product that might become a quality, or more than that, a safety issue through its flawed parameters when these common conditions exist.

Key words**:** *cider, color, sulfites, physical, turbidity.*

INTRODUCTION

The formative taste suggestion is almost regularly reflected through the eyes. But there are various elements to a food article, optical aspect and color are the most distinct provocation. In attributing anticipation to responsive facts, humans are observational creatures.
Set incertitude or clash in the perceptive suggestion we get, we facilitate to depend most on what we observe.
Furthermore we might appraise that the feel of smell and ingest should overcome in conditions of food reflection, it is alerting, that a perceivable deviation could also be commanding. This is due to primal choices of foods and beverages frequent trust on receptions.
Our acuity to detect regular everyday particularities is surely faulty, so appearance may sometimes be an increased trustworthy inception of guidance. Visible briefing of foods gives effectual clues as to other sensitive ranges, emerging wishes about what we are about to consume.
On the other hand what happens is that the sulfites reduce the development of spoilage yeasts and bacteria, however allowing the looked-for fermenting yeasts (like Saccharomyces cer. or others) to grow and to lead the transformation to alcohol.
Fluctuating the color of cider can be caused by some metals. Darkening may give escalation to the reactions between metal (industrial tools or machines) and tannin of cider, helped by atmospheric oxygen. Such changes occur only after unscrewing cider's bottle and contact with air oxygen.
There are fears about the addition of sulfites as a secondary factor beyond measure resulting from the use there of in phases bottling but also because some people are allergic and can be considered a health hazard.
Haziness can be determined by microbial activity, especially in those cases when the fermentation went wrong.
But even after opening the bottles spoilage may occur, especially if the bottles are not stored at proper temperature. Massive infestation and

alteration of ciders can be observed many times with naked eye.
Sometimes, even the product is filtered, the cloudiness can be induced by pectin which is not soluble in alcohol.
An important factor is the origin of cider, the appearance of fresh ciders made from raw apple/pear juice that was not put throughout a filtration process to remove granular elements of pulp or deposit.
The second origin is the pear/apple juice which is already filtered to eliminate bigger elements and is pasteurized to be preserved for a while till will be used in the fermenting process.
Using different analyzers and methods helped to get and collect accurate data regarding cider samples and provided the results of indicators that impacted product physical and chemical changes.
Considering the diversity of the cider on the market, variations of their properties we selected couple of parameters for the study.
The absorption spectrum is influenced by strength: such as the highest values can be found at higher concentrations. Other factors that might influence the absorption are temperature, pH etc.
Chemical stabilizers or inhibitors like sulfites and addition of artificial colors, flavors are frequently inputted in pear/apple juices to prolong their shelf life and make them look have natural properties. Nevertheless these days market demand for fresh and harmless nutrients deprived of artificial additives and presservatives conduct to rise the awareness in consuming of nutrient additives from natural bases.

MATERIALS AND METHODS

Equipment determining total sulfites:
1. Distillation apparatus scrubber
A. 250 ml round bottom flask,
B. Claisen adapter
C. Pasteur pipette with a rubber stopper
D. Graham condenser 300 mm
E. adapter Connection Vacuum adapter
F. 50 ml flask seem G. H. Adapter Reduction
I. adapter connection
J. socket adapter
2. burette 10 ml / 100 ml; 3. vacuum cleaner source/water; 4. 5, 10 and 20 ml volumetric pipette; 5. tube connection to water and vacuum

Photo no.1 - Sulfites determination

Samples must be cold when they are used for analysis to reduce degradation of labile sulfites. In the same time samples should be used in the manner to minimize disclosure to air and heat. After titration the sulfite content, expressed in µg SO2/g food (ppm), as follows:

SO_2 (ppm) = (32.03)(V_{Corr})(N)(1000) / W_{Sample}, Where

32.03 = milliequivalent weight of SO_2
N = Normality of NaOH titrant.
V_{Corr} = volume (mL) of NaOH of normality N required to reach end point, minus the NaOH volume required to titrate the reagent blank.
1000 = factor to convert milliequivalents to microequivalents.
W_{Sample} = Sample weight, in grams.

Formula no.1 - Titration Quantitation (source U.S Department of Agriculture Food Safety and Inspection Service 2016, February)

ALCOLYZER ANALYZING SYSTEM – ALCOHOL DETERMINATION

It is a high precision measurement machine and it was used for the research to get accurate indicators results. It has a pack of common density, concentration tables stored in the software, and new substances are easily introduced as a table or a polynomial. It determinates the sugar content (<0.01 Brix, g/L), alcohol content (<0.01 %v/v, <0.02 Proof); Determination of extract content (Plato, Balling) Quality control of soft drinks (< 0.01 Brix)

Photo no. 2 - Alcolyzer Analyzing System

Sample analysis is performed completely in one measurement cycle that establishes alcohol content, density, sugar content, degree of fermentation, calories.
Measurement of turbidity, color and pH values were delivered in the same time.
Level of measurement accuracy is up to an alcohol content of 12% v /v (but values are revealed up to 30%v /v). It has stoppage due to removal-cleaning between samples and can be adjusted and calibrated really simple (with water and water-alcohol solution). Time for each sample evaluation is four minutes (Anton Paar GmbH, 2016).

EPA, TURBIDIMETER 2100AN 115VAC
Hach 2100 series laboratory turbidimeter is planned to provide the best accuracy and sensitivity in any application.
The 2100AN is prepared with a stable halogen-filled tungsten filament lamp and is ideal for testing higher ranges of turbidity up to 10,000 NTU (Hach Company, 2013).
Additional absorbance, transmittance, and color detection modes make the 2100AN one of the most flexible of any bench to turbidimeter.
It can be used with exchangeable color filters and optional adapters for cell to measure smaller sample volumes. It had built-in printer which provides a data record of calibration and measurement. Turbidity can be interpreted as a measure of the relative clarity of water.

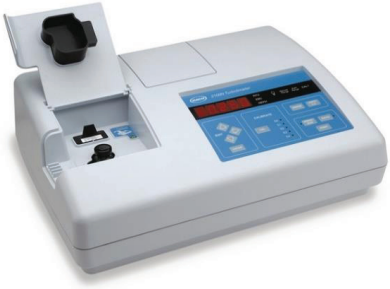


Photo no. 3 – Haze determination

SPECTROPHOTOMETER CECIL
The benefits of using for the research of this spectrophotometer were due8n M optical bandwidth, automatic lamp change, the light reduced for greater precision calibration test automatically at startup.

In order to perform proficiently, thin films must have the appropriate thickness.
Film thickness is often measured, both during and after thin-film coating.
Spectral reflectance measures the amount of light reflected from a thin film over a range of wavelengths, with the incident light at a known angle to the sample surface.
Ellipsometry is similar, except that it measures reflectance at non-normal incidence and at two different polarizations.
Thickness of a few microns can be measured by ultra-violet/visible spectrophotometers, using a specular reflectance accessory.
This method was described by Cori and Wimpfheimer (1999) and may be performed on a sample area as small as 2 mm diameter.

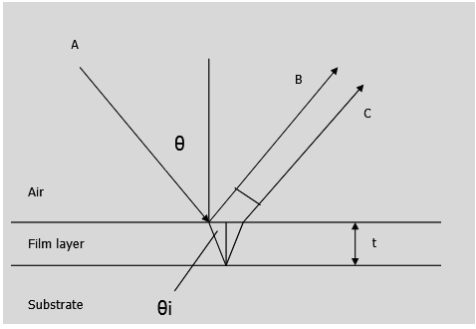


Photo no. 5 – Reflection of light in a thin layer

An incident beam of light, A, hits the surface of the film at an angle θ, from the normal.
Part of the beam is reflected at the front surface as beam B.
Whilst some of the remainder is refracted by the film layer, travels the film, is reflected at the interface of film layer and substrate, and then finally emerges from the film as beam C.
Beams B and C arrive at the detector of the spectrophotometer.
The combined intensity of beams B and C, at any given wavelength, is a function of the phase variance, if any, between the two at that given wavelength.
If the wavelength of the incident beam is continuously varied, as when scanning a spectrum, the result will be a continuous series of maxima at wavelengths, where constructive interference (reinforcement) occurs and minima, at wavelengths where destructive interference (cancellation) occurs.

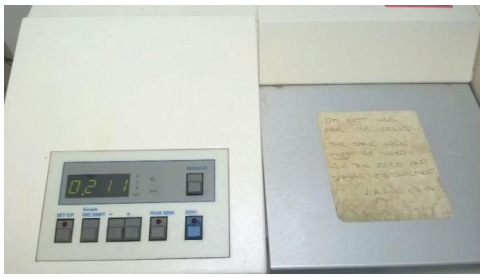


Photo no.4 - CT-310 Chroma Meter Konica

The Chroma Meter CT-310 is a small-sample tri-stimulus colorimeter for measuring the transmittance and color of fluids (Konica Minolta specifications, 2016).
It has three different sample cells (with optical path lengths of 2mm, 10mm, and 20mm) and two sensitivity settings enable measurements of fluids with a wide range of densities.
Other versatile functions include data memory, printout statistical calculations, and data communication. It minimizes the differences in readings between multiple instruments.
Light from the pulsed xenon arc lamp is thoroughly diffused by passing through two diffusion plates and into a mixing chamber, and then enters two optical fiber cables.
Optical fiber cable 1 transmits the light to the enlighten in gend face to illuminate the specimen for measurement; optical fiber cable two transmits light directly to sensors for monitoring the light double-beam feedback system.

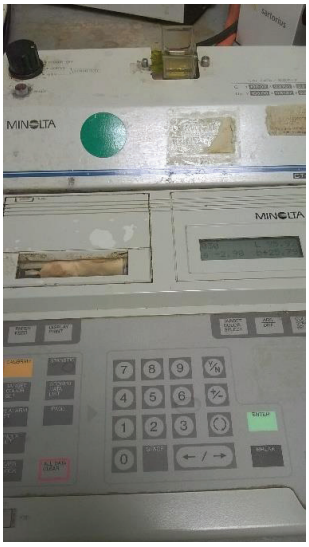


Photo no. 6 – a* and b* determination

METHOD USED

Nowadays there are many methods for the determination of alcoholic beverages, among them the turbidity measurement is the most important because it is a simple and very important indicator in changing the properties of a drink, regardless of technology or final product stage.
Sudden change in turbidity may indicate the source of contamination (biological, chemical, etc.) or can indicate a problem in the manufacturing process.
The aim of this study was to determine differences and to compare the results of different types of cider and how they behave after they are no longer airtight.
A complete study of the composition of the apple juice, fermentation or final product (cider) includes determining data other than those derived exclusively from chemical analysis, buta study of the characteristics of debt, including sensory analysis, all executed by certain rules precisely.
These studies will contribute data to guide students, researchers for future experiments and analyzes in this field.

TURBIDITY

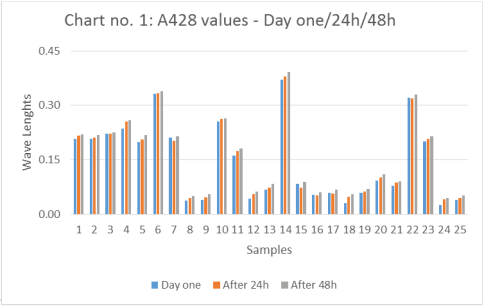
Preparation of sample: cider sample must be degassed prior to testing, otherwise they influence of the bubbles of gas, and will result in higher yields. Decant part of cider in a beaker and mix until all the gas has been released. You can also use ultrasonic waves degassing device. Turbidity measurement parameters used or this study, but general industry and wine, beer, cider are:
EBC - "European Brewery Convention".
NTU - "Nephelometric Turbidity Unit".

Measurement of absorption spectrum- the color Togauge Konicahave used the values and CIEL*bre presenting a* b*(CIELAB) color space is specified by: French international de l'Eclairage Commission, hence the initials.
It defines all the colors perceptible to the human eye and was made to serve as a device independent model to be used as a reference.
The three coordinates of CIELAB represent the brightness of the color (L* = 0 basic black and L* = 100 indicating white diffuse), and its

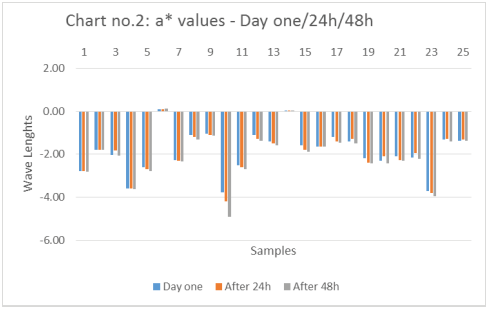
positions between red/magenta and green (a*, negative values indicate green and positive indicated purple) and positions between yellow and blue (b*, negative values indicate blue and positive indicating yellow). For Cecil measuring device was used to measure color related parameters.
Values were expressed in nanometers using wavelength spectrum from 280 to 500 nm, depending on the sample or specification of each product. Before the samples were used for analysis the machine analysis (Cecil) was calibrated according to the wave lengths standard specifications.

RESULTS AND DISCUSSIONS

Experiments in this chapter focused on the differences between the parameters of bottled cider, which occurred after opening and keeping both at ambient room temperature for24 – 48 hours.
They were covered by the sulfur differences and different properties which may affect flavor, aroma, appearance of the product (especially color etc.). The samples used for this study cider were taken from local markets bottled and cans too.
Other further studies will try to reveal pH, acidity, ABV trend for the same types of ciders.
In the chart number one are represented the results of twenty-five assessed values cider samples color spectrum length A428.
These samples were taken in the first working lab for measuring parameters in one day, and for subsequent determinations, respectively 24hours, 48 hours were held at an ambient temperature of 19°Celsius.

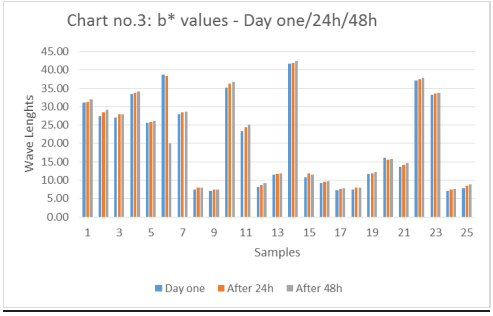


On the day one a twenty samples had values more than 0.04 (on the A428 measurement scale).
A number of thirteen cider samples, representing more than half of total samples tested, had A428 values increased after 24 hour from the opening moment (as the average for total samples was 0.1514).
Go after 48 hour storage a number of twelve sample had a significant increase of the value with an average of 0.256 (A428 wave length).

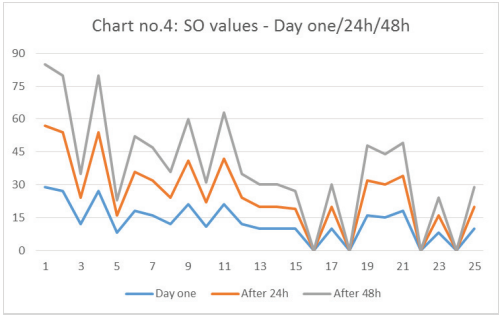


In the second chart are represented the results of the same ciders, for a different parameter (a* values).

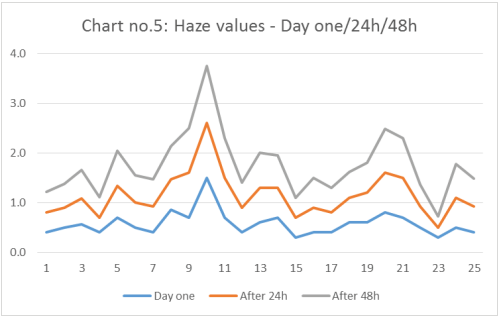
A number of thirteen sample kept the increasing trend from day one to the end (48 hours), with just two positive values, and eleven sample with values between -4.2 and -1.2 after twelve hours of storage.
Six samples (24h measurement) were under day one values with the average values of -1.63333.
After 48 hours just three samples kept the same value like day one result and twenty two samples had increased values with an average of -2.08227.



For the b* measurements almost all the results increased, the highest value was a peak of 11.5 representing a 6.48% growth from day one value. Two sample values have increased from day one to 24/48 hours, but the values were equal from 24 to 48 hours. Between all samples the lowest value is 7.33, representing a dry cider and the highest is 42.06 representing a cloudy cider.



Sulfites values shown on y axis (measured in ppm).
The samples sixteen and twenty-two values were zero since they are canned cider.
In this case is not allowed to use sulfites, which are considered corrosive agents, they might be dangerous through the explosion of the aluminum cans.
There are four results above the value of twenty, due to the addition of sulfites in the fermentation process to help specialy east to end the process, but also to inhibit other microbial forms that may harm the process itself (by altering the taste, smell, appearance, wrong fermentation etc.).
Fourteen samples went from day one to 48 hours of storage values with a small decrease of the amount of sulfites since they were wasted into physical – biological process activities of the cider.
The average of the decrease is 1.428571 (for fourteen samples).
Other ten samples kept a constant value from day one to the end of the experiments.
Turbidity values shown on y axis (measured in Nephelometric Turbidity Unit– NTU).
The haze measurements revealed that a number of seven samples had an average percentage decrease of 22% from initial value of haze from day one to 24 hours and an average 16% from day one to 48 hours, which reveals a small increase of the value from 24 hours to 48 hours measurement based on cider's ongoing biological activity.



CONCLUSIONS

When the temperature is close to refrigeration, the optimum pH is kept, even if the bottle or the can has been opened to about 24hours.
Sulfur dioxide value depends critically on the pH apple juice even is used only in small quantities.
Cider apple/pear juice, when is pressed, it covers a large number of yeasts, molds and some bacteria that are not useful, and only very few of those useful.
Some cider manufacturers in the UK have set a maximum limit of total SO210mg/L, which was challenging to maintain product within these limits.
One reason was the occurrence of smell-urine mouse taste, especially in the mixing phase (for low levels of sulfites).
Although not everybody can spot this taste, there is a risk that those who drink this taste not ably in cans.
For cans the total allowable sulfites that might come into contact with the protection lacquer is around 25 ppm, but not more than this value.
Otherwise the metal of which it is made can may be corroded if the can lacquer cannot protect the result antaction of hydrogen sulphide generated inside.
Some brands of bottled cider in cans are often specially fermented in the total absence of sulfites, exactly what our results showed throughout the experiments.
However, in the study were detected several types of cider, especially cloudy ones (unfiltered type, or traditional recipe), which had sulfites levels over 25 ppm.

This is not necessarily a factor that might increase the risk of corrosion of aluminum cans because some manufacturers choose a double or triple degree of varnishing depending on the action of bottled liquid.
Sulfites levels in the study were determined on the basis of laboratory research and most are normal with standards, with a much lower chance of taste and flavors to the finished product.
However for higher values, this is somewhat normal for products classic unfiltered, cloudy etc. Also, several acts yeast during the fermentation, naturally reducing the content of sulfur dioxide.
Adding the sulfites, after fermentation it is also sometimes used after the fermentation has been completed, products in the shelves, storage and packaging.
This is partly antimicrobial action but also to act as an antioxidant. Or rather, destroy oxidation initiators such as hydrogen peroxideoraldehydes, not leading to unwanted flavors.
In these cases is usually added a fixed quantity of 50 ppm each time (up to the legal limit of 200 ppm for all additions summed together), to achieve a residual result of 30 ppm SO free for the day.
This is due to the fact that the antioxidant properties of sulfur dioxide are not affected by pH.

THE COLOR AND TURBIDITY

The difference is noticeable between a cloudy and a pasteurized cider color. Enzymes change hue apple cider in a dark brown color which is inactivated by heat. For direct apple juice pasteurizing when is obtained, this lead to the color change.
But after pasteurization the process of recovering color, clarity and turbidity makes changes to the product.
The values resulting from the study confirmed that both color values a* and b*, A 428 and verified turbidity cider were closer to the market demands, no significant changes were reported.
We noticed some increased values just between day one and 48 hours storage, due to ambient temperature and oxygen intake that leaded to microbiological and biochemical changes of color turbidity, higher than if the products were kept cold and unopened.
Relationships between color and the cider types were observed. There was an undesirable but not essential relationship between color and cloudiness results as well a helpful but not significant relationship was observed between color and sulfites results. Pear and apple ciders change their color into darken cue because natural enzymes react with oxygen. This might be an indicator because it shows the aroma of the cider in the end.
Further research about cider will try to connect the information gathered until now with experiments regarding pH, acidity. The correlation can be made also with the microbial activity of the cider after pasteurization and how different conditions can affect its own characteristics like ABV, sugar content and many others.
Other researchers observed Significant changes in pH, Brix and viscosity only for cider microfiltered with 0.45 μm (Evonne Lau and col., May 2012). They suggested that same type of particles were present in their cider samples and just the concentration was different for different types of ciders.
Their conclusions were the composition of apples is subject to inconsistency due to season and raw material variations. This might bring unpredictability in the composition and turbidity of pear/apple cider and as a result in the effect of the microfiltration process.
The results of this work indicate that color, turbidity and sulfites content are deeply correlated with the process of various raw materials, additives and with the ciders type. More than that ambient conditions of the unopened bottles accelerated all the biochemical processes.

REFERENCES

Arias, C. R., Burns, J. K., Friedrich, L. M., Goodrich, R. M. and Parish, M. E., 2002, Microbiology
Bamforth C. W., Brewing, 2006, New technologies, Cambridge
Beliciu CM and Moraru CI. 2009. Effect of solvent and temperature on the size distribution of Casein micelles measured by Dynamic Light Scattering. Journal of Dairy Science 92:1829-1839

Beveridge T and Wrolstad RE., 2009, Haze and cloud in apple juices. Critical Reviews in Food Science and Nutrition. 37:1, 75-91
Borza, V., et al., 2007, Journal of Labelled Coumpound and Radiopharmaceuticals 50, (5-6), p. 422
Borza, V., et al., 2007, Journal of Labelled Compound and Radiopharmaceuticals
Chivu, M, 2012, Microbiologia Berii, USAMV Bucureşti, Facultatea de Biotehnologii
Choi and Nielsen, 2005, The effects of thermal and non-thermal processing methods on apple cider quality and consumer acceptability, Journal of Food Quality
Dennis E. Briggs, Chris A. Boulton, Peter A. Brookes and Roger Stevens, 2004, Brewing Science and practice, Cambridge
EURACHEM,1995, Quantifying Uncertainty în Analytical Measurement. Laboratory of the Government Chemist, London. ISBN 0-948926-08-2
F. B. Wright, 2001, Distillation of Alcohol and De-Naturing, 2nd edition
Fontana, M.G, 1986, Corrosion Engineering, 3rd edn, McGraw-Hill, New York
Fritsch J, Moraru CI, 2008, Development and optimization of a carbon dioxide-aided cold microfiltration process for the physical removal of microorganisms and somatic cells from skim milk. J Dairy Sci. 91: 3744-3760
Gustavo V. Barbosa – Cánovas and others,2006, Handbook of Brewing Second Edition
Howell, J. P., Burke, S. D., 1996, The corrosion of aluminum-clad spent fuel în wet basin storage, Proc. Embedded Topical Mtg. on DOE Spent Nuclear Fuel and Fissile Material Management, Reno, American Nuclear Soc., La Grand Park, IL
Jean L. Jacobson, Introduction to Wine Laboratory Practices and Procedures, New York, 2006
Jorgensen A., 1900, Micro-organisms and Fermentation, 3rd ed., London
Neacşu, E., et al., 2007, Aspects related to spent nuclear fuel wet storage management: the water chemistry, IRPA Regional Congress for Central and Eastern Europe, Brasov, Romania
Neacşu, E., et al.,2008, Experience on Safe Wet Storage Spent Nuclear Fuel at VVR-S Research Reactor of IFIN "Horia Hulubei", International Conference of Physical Chemistry, ROMPHYSCHEM 13, Bucharest – ROMANIA,
Ola Norrman, 1999, Home Distillation Handbook, Malmoe, Sweden
Paradh, A.D, Mitchell, W.J., Hill, A.E., 2011, Journal of the Institute of Brewing 117 (4), 498-506
Rowe, L.C., Walker, M.S., 1961, Corrosion, p. 353
Standards Methods for the Examination of Water and Wastewater,1992, 18th Edition, 5120B
Spectroscopy Application Note 1.93, Cecil CE 3055 Specular Reflectance unit, 2016 February
www.en.wikipedia.org/wiki/Turbidity, 2016 February.

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STUDY CONCERNING THE USE OF GERMINATED OAT FLOUR IN OBTAINING DIETARY BREAD

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Abstract

The flour obtained from sprouted grains is successfully used in the composition of functional and dietetic foods. Due to the high content of active ingredients and good bioavailability of their nutritional compounds, the products made from sprouted grain flour have a higher quality in comparison with classic foods. By using white wheat flour and flour from germinated oats in different percentages (5%, 10% and 15%), we obtained a bakery product: bread with sprouted oats, which has been characterized in term of physical-chemical parameters. The addition of germinated oat flour in raw material diminishes gluten content and enhances the ability of flour hydration, which increases the elastic properties of the product and its storage life. Sprouted oat bread is distinguished by a high content of minerals(15% sprouted germinated oat flour added). The amount of carbohydrates is reduced to20% in bread with 15% sprouted germinated oat flour and this product is recommended in the hypoglycemicdiet.

Key words*: bread, sprouted germinated oat flour.*

INTRODUCTION

Production and consumption of functional foods is a strong current trend in healthy eating. These foods have an important biological role in all metabolic processes of the body. These ensure the health, promote the growth and development of the organism and optimize metabolic processes, physiological activity of the organs, the immune system, cognitive performance and the defense against oxidative stress (Alexa 2010; Hurgoiu, 2004).Known as protective food, functional food can be used for a long time without causing adverse side effects, cytoprotective and restorative effects, increasing the body's natural immunity (Zielińska-Dawidziak et al., 2014).

Cereals are the first vegetable matrices used as functional foods. It is known the role of dietary fiber from cereals in ensuring a healthy diet (Finney, 1982; Alexa, 2009). Germination, as process of grain processing, lead to a significant increase in the content of bioactive compounds (vitamins, bioelements, enzymes) of a product, making it the product of functional food (Alexa et al., 2009; Botau et al., 2015). Germinated grain flours have a higher bioavailability of nutritional compounds and permit the production of functional food. Germinated oat is the ideal food for a perfectly healthy diet. Given the large number of nutrients that are found in oat germ, they bring a multitude of benefits to our health, being an important source of protein, unsaturated fats, fiber, vitamins A, B1, B2, B6, D, E and K, iron, phosphorus, magnesium and zinc. Germinated oat are recommended in treating anemia, but also helps to strengthen the immune system, protects the muscles, circulatory system, lungs and improves visual acuity. Being very rich in antioxidants, they protect our cells against free radical damage. Treatment with oat germ is indicated for strengthening immunity being recommended for older people and children. The chemical composition of germinated cereals and their positive effect on organism have been reported in previous studies. (Hidalgo et al., 2013; De Vasconcelos et al., 2013; Marton, 2010; Pandhre et al., 2011).

The research aim was the obtaining a product from white wheat flour with added germinated oat flour in different percentages, which was analyzed in term of physical-chemical parameters. The association of germinated grain in bakery matrix is an innovative solution for obtaining functional foods.

MATERIALS AND METHODS

The characteristics of germinated oat flour

The study of physical-chemical proprieties aimed the determination of content in gluten,

hydration capacity (CH) and acidity of flour samples studied. For the samples of bread, was analyzed ash content, the height to diameter (H / D) and humidity. The gluten content, the ability of moisture, ash content and humidity were expressed as a percentage, acidity was expressed in degrees of acidity, and the ratio of height to diameter (H / D) was obtained by dividing the height of the samples of bread to the diameter of these samples.

Obtaining of germinated oat flour

Germinated oat was obtained in USAMVB laboratory and included: oat washing, seeds germination in Petri dishes under optimal conditions, germs harvesting, drying, grinding and obtaining of dry germ germinated oat flour which is stored until use in obtaining of bread with sprouted oats.

Oats were germinated for 7 days at 20-24^{o}C , after a pre- soaking for 40 hours. Green malt obtained was dried at temperature below 65^{o}C until the moisture below 6% .

Phisical-chemical characterisation of flours

White flour and germinated oat flour were analyzed in term of wet gluten content (G), hydration capacity (CH) and acidity (A) accordingSTAS 90/1988.

Obtaining of bread with germinated oat flour

The bread was made by the direct method for the preparation of the dough.The bread was obtained using white wheat flour and flour from germinated oats in different percentages (5%, 10% and 15%). Dosage of raw materials was done by weighing or measuring according to technological recipes. To obtain bread with 10% germinated oat flour we use 300 g white wheat flour type 480, 180 g Water, 3 g yeast, 30 g oat flour, 4.5 g salt. All materials were homogenized on high speed of mixer for 10 minutes, to obtain the dough. The fermentation was done 40 minutes at 35^{o}C and then the dough was divided and shaped in round forms. The dough was baked 20 minutes at 220 ° C (Moldoveanu, 1992).

Physical-chemical characterisation of bread with germinated oat flour

Regarding sensorial properties it was analysed the product shape, appearance and color of the shell, core sectional appearance, consistency, smell and taste. The main physical-chemical parameters of bread: ration between bread height and diameter (H/D), ash and bread humidity were determined according STAS 91/1983 - "Bread, bakery products and bakery specialties. Methods of Analysis".

Statistical analysis

To determine the significance of differences between flours and breads, data processing was performed by analysis of variance and t-test (Ciulca, 2006). The basic principle of biplot method developed by Gabriel *et al.* 1971 was also used.

RESULTS AND DISCUSSIONS

There were obtained three types of bread containing oat germ meal of 5%, 10% and 15% added to wheat flour, which were characterized in term of physical and chemical parameters. The sensorial properties shown that bread with 5% and 10% oats germ meal was well increased, with well-developed core, uniform porosity, darker compared to the control and pleasant taste. Bread with added 15% oats germ meal was less high, with well-developed core, uniform porosity, but less elastic than the5% and 10% germinated oat flour added. The color was darker than the sample with 10% germinated oat flour added and slightly astringent taste (Table 1).

Table 1. Results on sensorial examination of bread with oat germs flour

Sensorial	Bread with 5% oats germ meal	Bread with 10% oats germ meal	Bread with 15% oats germ meal
Product form	Oval shape, very well brought	Oval shape, well brought	Oval shape, less high
Shell:Skin, color	Color close to the witness, without cracks	Darker compared to the control, without cracks	Darker than the other samples, cracks
Core: Sectional appearance, color, consistency	The corevery well developed,uniform porosity	The core well developed, uniform porosity	The core well developed, uniform, elastic smaller, darker
Smell	Pleasant	Pleasant	Pleasant
Taste	Very pleasant	Pleasant	Slightly astringent taste

The addition of germinated oat flour in wheat flour causes the decrease of the gluten content in bread (Table 2). Gluten content was 26% for bread flour with addition of 5%, 22% for the bread with 10% flour and 20% for the bread with 15% added oat germ meal.

In the study made by MacArtur and D'Appolonia (1984), the values of wet gluten in the samples of flour obtained from wheat varietes Waldron (41.6%), Olaf (36.1%) and Experimental (38.3%) were significantly superior to those established by us. These authors reported a maximum value of the wet gluten content at 41.6% and in our study, there was obtained the value of 26%. This difference was influenced by the type of flour and the genotype used. In our researches was analysed a mixture made from wheat flour and germ germinated oat flour (variety Lovrin 1) and in the study mentioned above was determined wet gluten in the flour varieties of wheat Waldron, Olaf and Experimental without being mixed with flour germinated grain. Therefore, it appears that increasing the quantity of flour from germinated oats determines an significant decrease in wet gluten content in the dough, which is of great practical importance to obtain dietary products.

The white flours hydration capacity (CH) augments with increased addition of germinated oat flour, as showed by the results presented in Table 2.

From the technological point of view this is very important because a higher hydration capacity increases elastic properties of the product and increases the duration of storage. By sprouting, hydrolytic enzymes release chemical compounds that increase the absorption of water and ensure their solubility.

In other studies (Camire and Flint, 1992) was determined hydration capacity of germinated oat flour and cornmeal, settling lower values than those obtained by us. Also, Bhatty (1986) established a capacity of hydration much lower then our results at flours made from oat varieties Scout and Tupper.

The acidity of samples arises once the addition of germinated oat flour, mostly due to higher enzyme activity is flour made from sprouted grains compared to white wheat flour made from the grain endosperm lacking the enzyme equipment In other studies, the maximum degree of acidity found in wheat flour was at the value 3 (Moldoveanu, 1992), higher than the values determined by us.

The ratio between height and diameter (H/D) does not vary in the case of addition of 5% and respectively 10% oat flour. Significant changes in this parameter is recorded in the case of addition 15% germinated oat flour (Table 2).

Values of the first two breads analyzed indicates a ratio H / D balanced while the low value for bread with added 15% germinated oat flour is due to a height lower sample analyzed and a larger diameter, providing an indication that the bread has a lower quality.

Moisture content of bread samples with addition of germinated germinated oat flour does not vary within wide limits. There is a small increase once with augmentation of germinated oat flour percentage added to the sample (Table 2).

Humidity values were within the limits imposed by STAS 878/68 for white bread weighing up to 1 kg (up 43.5%). Moisture core, expressed as a percentage, was between 43 and 43.5% in some samples of white bread (Moldoveanu, 1992), standing out higher values than those obtained in our measurements.

The ash content, that shows the contribution of mineral substances in bread, increases with the addition of germinated oat flour, being at maximum in case of 15% bread with oat flour germinated, enhancing food value product (Table 2).

The values of ash obtained in the study of Maleki et al. (1980) were between 0.41% and 0.43%. Measurements were made at certain flours obtained from several varieties of wheat, such as Eagle, Omaha, Aurora, etc. It appears that these values are significantly lower than those achieved by us, which shows that our product has a high food value due by the use of germinated oat flours.

In Table 2 is presented the statistical analysis of the values obtained regarding flour samples and bread with germinated oat added. Statistical analysis of the values obtained shows that raw material (white flour) with addition of 5% germinated oat flour was significantly superior to other types of gluten analyzed. The hydration capacity (CH) and acidity showed values significantly higher at 15% flour with added sprouted germinated oat flour. Bread with

added 15% germinated barley flour presented values of total ash and moisture significantly superior to other varieties while bread with added 5% and 10% germinated oat flour have identical values, which is significantly superior to other assortment analyzed.

Raw material with addition of 5% germinated barley flour showed significantly superior values towards to the average of gluten, while with the addition of 15% oat flour germ, gluten had significantly lower value.

The capacity hydration and acidity of raw materials with 15% germinated oat showed significantly superior values to the average, while 5% germinated oat showed values significantly below the average. Assortment of bread with 15% added flour from germinated oats had significantly superior values to the average of moisture and ash, while the values significantly lower than the average of those traits were recorded at bread with added 5% flour from germinated oats. The proportion height-diameter (H/D) presented values signifycantly superior to the average at assortments of bread with added 5% and 10% germ germinated oat flour and bread with added 15% germinated oat flour presented value significantly lower than the average.

Table 2. Statistical analysis of flour samples and bread with germinated oat added

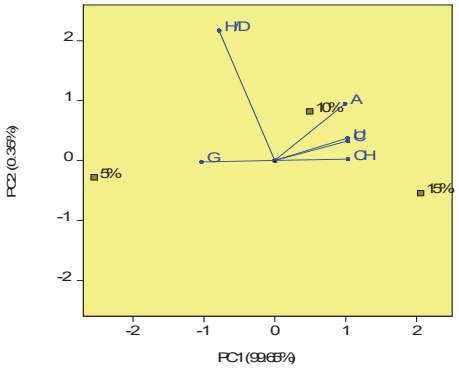
Analyzed sample	Wheat flour with germinated oat flour			Bread		
	Gluten (%)	CH(%)	Acidity(degrees)	Ash(%)	ProportionH/D	Humidity (%)
5% germinated oat	**26a**	60b	1.44b	2.60b	**0.45a**	36.20b
10% germinated oat	22ab	62ab	2.72a	2.90ab	**0.45a**	36.62ab
15% germinated oat	20b	**63a**	**2.80a**	**3.00a**	0.42a	**36.75a**
Average	**22.67±1.76**	**61.67±0.88**	**2.32±0.44**	**2.83±0.12**	**0.44±0.01**	**36.52±0.17**
Dl 5%	4.81	2.40	1.20	0.33	0.04	0.45
Dl 1%	7.54	3.77	1.88	0.51	0.06	0.71
Dl 0,1%	12.85	6.43	3.21	0.88	0.10	1.21

They are considered significant differences between genotypes lettered differently

In the Figure 1 is presented the bread obtained with different percentages of germinated oat flour germs.

Figure 1. Bread with germinated oat flour

The two dimensional biplot representation for physical-chemical proprieties of flour and bread with added germinated oat flour samples (Figure 2) shows that wheat flour with the addition of 15% germinated oat flour associate with highest values for capacity hydration and acidity.



G - gluten, CH – hydration capacity, A – acidity, C – ash, H/D – ratio H/D, U – humidity

Figure 2. Biplot analysis for physical-chemical properties of flour samples and bread with germinated oat added

The highest content of gluten is present in the flour with the addition of 5% germinated oat flour that dropped with increasing amounts of sprout germinated oat flour. Regarding the

bread, the highest values for the amount of ash and moisture is associated with bread with added 15% germinated oat flour. The values of height-to-diameter ratio (H/D) shows the same values for bread with added 5% and 10% germinated oat flour (0.45), which is higher than bread with added 15% of germinated oat flour (0.42).

CONCLUSIONS

The addition of germinated oat flour germ in wheat flour causes the decrease of the gluten content. By addition of 10% oat flour, the hydration capacity increases at 62% and respectivelyat 63% in the case of the addition of 15% oat flour.

The acidity of the samples augments with the addition of germinated oat flour germ flour, this fact is due to higher enzyme activity of germinated oat flour.

The sensorial properties of samples shown that bread with 5% and 10% oats germ meal was well increased, with well-developed core, uniform porosity, darker compared to the control and pleasant taste. Bread with added 15% oats germ meal was less high, with well-developed core, uniform porosity, but less elastic than the 5% and 10% germinated oat flour added.

The contribution of mineral substances in bread arises with the addition of germinated oat flour, being the maximum at the bread with 15% germinated oat flour, which increases the nutritional value of the product.

Two-dimensional diagram for physical-chemical properties of the types of bread tested shows that the wheat flour with the addition of 15% germinated oat flour associate the highest values for hydration capacity and acidity.

The addition of germinated oat flour germ flour in bread recipe increases the nutritional value and dietary potential of bread.

REFERENCES

Alexa E.,2009. Dietetic flouring supplies for children nourishment. Journal of Agroalimentary Processes and Technologies, Timişoara, 13(1): 163-168.

Alexa E., 2010. Alimente făinoase dietetice. Materii prime, caracterizare, tehnologii de obținere. Eurobit Publishing, Timişoara.

Alexa E., Voica V.D., Stoin D., Pop G., Rosu I., Negrea M., 2009. Reserches concerning the physical-chemical and rheological properties of ecological flours, Analele Universitatii din Oradea - Fascicula Chimie XVI, 19-24.

Bhatty R.S., 1986. Physiochemical and Functional (Breadmaking) Proprieties of Hull-less Barley Fractions, Cereal Chemistry Journal, 63(1): 33-35.

Botau D., Pîrvulescu P., Alexa E., Ciulca S., 2015. Obtaining and nutritional characterisation of functional biscuits with cereal germs and *Momordica charantia* extract, Scientific Bulletin. Series F. Biotechnologies, 19: 125-129.

Camire M.E., Flint S.I., 1992. Thermal Processing Effects on Dietary Fiber Composition and Hydration Capacity in Corn Meal, Oat Meal and Potato Peels, Cereal Chemistry Journal, 68(6): 645-647.

Ciulca S., 2006. Metodologii de experimentare în agricultură și biologie, Agroprint Publishing, Timişoara.

De Vasconcelos M.C.B.M., Bennett R., Castro C., Cardoso P., Saavedra M.J., Rosa E.A., 2013.Study of composition, stabilization and processing of wheat germ and maize industrial by-products. Industrial Crops and Products, 42, 292–298.

Finney P.L., 1982. Effect of germination on cereal and legume nutrient changes and food or feed value: a comprehensive review, Recent Advances in Phytochemistry, 17:229-305, Plenum Press New York and London.

Gabriel K.R., 1971. The biplot graphic display of matrices with application to principal component analysis. *Biometrika*, 58(3): 453–467.

Hidalgo A., Brusco M., Plizzari L., Brandolini A., 2013.Polyphenol oxidase, alpha-amylase and beta-amylase activities of Triticummonococcum, Triticumturgidum and Triticumaestivum: A two-year study, Journal of Cereal Science, 58(1): 51–58.

Hurgoiu V., 2004. Alimente funcţionale, Science Publishing House, Cluj-Napoca.

Maleki M., Hoseney R.C., Mattern P.J., 1980. Effects of Loaf Volume, Moisture Content and Protein Quality on the Softness and Staling Rate of Bread, Cereal Chemistry Journal, 57(2):139-140.

Marton, M., Mandoki, Z., Csapo, K.Z. (2010). The role of sprouts in human nutrition. Alimentaria, 3: 81-117.

MacArthur L.A., D'Appolonia B.L., 1984, Gamma Radiation of Wheat. I. Effects on Dough and Baking Proprieties, Cereal Chemistry Journal, 60(6), 456-460.

Moldoveanu G., 1992. Tehnologia panificatiei, Tehnica Publishing, Bucuresti.

Pandhre G.R., Satwase A.N., Syed Imran H., 2011. Studies on drying characteristics and nutritional composition of sprouted wheat and finger millets, International Journal of Current Research, 3(7): 218-221.

Zielińska-Dawidziak M., Piasecka-Kwiatkowska D., Warchalewski J. R., Makowska A., Gawlak M., Nawrot J., 2014. Sprouted wheat grain with ferritin over expression as a potential source of iron for cereal product fortification, European Food Research and Technology, 238(5), 829-835.

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***STAS 91/1983

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SOME PROPERTIES OF CAROB POD AND ITS USE IN DIFFERENT AREAS INCLUDING FOOD TECHNOLOGY

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Abstract

Carob (Ceratonia silliqua) is an evergreen, drought resistant tree. It has a good nutritional value, a long shelf-life (2-3 years) and it is relatively cheap. Due to its high sugar content, carob is naturally sweet. This property makes it usefulin, as an antioxidant in different foods, as a thickener, stabilizer or flavourant in food applications, in ethanol, lactic acid production, in medical applications, in cosmetic emulsionsetc. In food research, new product development of carob could contribute greatly to the promotion of carob as a food source and hence towards its commercial value. Carob's application in the food industry is mainly focused on the extraction of carob bean gum (locust bean gum). The use of the deseeded pod in food is, however, minimal and thus carob's economical market value is low. The current world production of carob extracts is estimated at 315000 tons per year, with Spain being the main producer and exporter (42%) and followed by Turkey with 5% in the Mediterranean and Aegean region. This review is focused mainly onthe properties of carob tree, chemical composition, human and animal nutrition, medical applications, health benefits, polyphenol content, and antioxidant properties of carob pod and the use of carob pod in different areas.

Key words*: carob tree, food technology, antioxidant properties, animal and human nutrition*

INTRODUCTION

Carob pods have been used in many countries as an antioxidant in different foods, as a thickener, stabiliser or flavourant in food applications, in ethanol production,in the production of cosmetics, in animal nutrition, in lactic acid production and in medical applications etc. The use of carob pods in food dates back to ancient times, where the pods are reported that in raw form have been consumed(Brandt, 2002; Haber, 2002; Owen *et al.*, 2003).

The carob pods have got especially polyphenolic compounds, carbohydrates and contain low amounts of insoluble dietary fibers, minerals, lipidsand proteins. Due to its composition carob pods are also used in animal nutrition (Avallone, et al., 1997).

Due to the presence of free sugars, organic acids and amino acids are natural constituents of many fruits and vegetables and play an important role in maintaining quality and determining nutrititive value (Ashoor and Knox, 1982). The nature and the concentration of these constituents in fruits are also of interest because of their important organoleptic properties. Free sugars are one of the most important constituents of fruits and vegetables.

Other using area are in the production of traditional foods such as confectioneries, beverages, in production of bread or pasta in a few countries in the Aegeanregion. It is given below a reviewabout the use of carob pods.

1. Properties of carob tree

Carob is found not only in wild form but also in cultivated forms(Biner et al., 2007).Carobs have been cultivated for 4.000 years. The world's commercial carob is supplied from Portugal and Spain, approximately 100.000 ha of carob trees.The current world production of carob extracts is estimated at 315.000 tonnes per year, although there are no accurate statistics available about annual world production, with Spain being the main producer and exporter (42%) and followed by Turkey with 5% (Biner et al., 2007; Makris and Kefalas, 2004).

The carob tree belongs to a member of legume family, botanically known as *Ceratonia siliqua* L. which is called as Locust bean gum (LBG). The tree is known to be an important component of the vegetation and is economically important. The carob seed consists of three partsi.e., germ, endosperm and husk. In many regions, locally grown vegetables and fruit contribute substantially to

local diet and due to its edible fruits, the plant has been cultivated (Avallone et al.,1997; Dakia et al., 2007; Yousif and Alghzawi, 2000). The fruit is a pod with pulp and seed, the pulp being 90% of its total dry weight (Correia and Martins-Loução, 2004).

Carob trees are resistant against drought; require little maintenance and produce a range of products from the seed and the pod (Fletcher, 1997). It grows very well at between 30° and 45°C but it is also tolerant to the hot and humid coastal areas with hot winds (Zografakis&Dasenakis, 2000, Tous et al., 1996).

The carob tree is used for various purposes suitable for preventing soil erosion and for rural area development in the Mediterranean, in the industry, forestation, prevention, as ornamentals (Turhan et al., 2006; Tous et al., 2009; Gubbuk et al., 2009). "Carob Kernel" or seeds are very important for Locust BeanGum industry (Battle and Tous, 1997b; Karkacier et al., 1995; Gubbuket al., 2010).

2. The chemical composition of Carob Pods

The nonfleshy bean-like fruit of carob tree (Chamberlain 1970; Ayaz et al. 2009), which is called carob pod, is light to dark color and straight or slightly curved in shape. Locust bean gum contains non starch polysaccharides consisting of galactose and mannose in the ratio 1:4 and hence they are known as galactomanan (Parvathy et al., 2005).The fruit and its products, are sold both in large stores and local markets,and they contribute strongly to the diet of people living in the Mediterraneanareas of Europe and also in Turkey (Ayaz et al.2007).

Except for polysaccharides carob pod contains low levels of fat and it is rich in potassium, calcium and polyphenols.

The pod consists mainly of pulp (90%), which is rich in sugars (48–72%), but also may contain a large amount of condensed tannin, which are bitter-tasting chemical compounds that bind proteins. In table 1 is shown the chemical composition of carob pods. It can be seen that the concentration of each component is variable and the composition depends on the variety, climate and growingtechniques (Tous, 1990; Petit et al., 1995; El-Shatnawi, 2000; Morton, 1987) in a wide range.

Table 1: Proximate composition of carob pods (Calixto and Cańellas, 1982; Marakis, 1996; Avalloneet al., 1997; Battle andTous, 1997b; Yousifand Alghzawi, 2000; USDA, 2006)

Chemical constituent	Concentration (g.100 g^{-1})
Moisture	3.6-18
Ash	1-6
Fat	0.2-2.3
Protein	1-7.6
Carbohydrates	48-88.9
Total sugars	32-60
Dietary Fibre	2.6-39.8
Polyphenols	0.5-20

3. Effect of polyphenol content on the antioxidant and medicinal properties of Carob

Phenolics are compounds with an aromatic ring bearing one or more hydroxyl groups. Polyphenols occur in foods of plant origin and because of their antioxidative properties ability to modulate several proteins, polyphenols generally have beneficial effects on human health once consumed (Vinson, 2001; Sakakibaraet al., 2003). However, data on carob's antioxidant properties and the core functionality, with relation to its polyphenolic components, is still limited. Moreover, the profile as well as the nature of polyphenolic components of carob pods are still not fully understood.

Synthetic and natural antioxidants are used successfully to block or delay the oxidation process in meats (Cross et al., 1987).

Due to their safety and toxicity problems of synthetic antioxidants,there is increasing interest in use of natural antioxidants (Li et al. 2011; Ahn, et al., 2002). Moreover, as well as increasing lipid stability,an antioxidant added to a food product may act as an antioxidant in the body, thus reducing the risk of various diseases related to the production of free radicals (Bravo, 1994; Boskou, 1999). Therefore, there is an increasing interest in the natural antioxidants,e.g. polyphenols, present in medicinal and dietary plants,which might help in preventing oxidative damage.

The recent investigations showed that antioxidant properties, responsible for the majority of observed biological effects of carob flour, can to be significantly influenced during

roasting (Sahin, et al., 2009). It was found that certain phenolic compounds can degrade during roasting. Polyphenols exhibit a wide range of biological properties, and the antioxidant activity is the best known. Phenolic antioxidants prevent against oxidative damage of some important biomolecules like DNA, protein, and lipids and leads to degenerative diseases such as cancer, inflammatory, cardiovascular. (Scalbert et al., 2005).

4. Use of Carob Pob in animal nutrition

Main carob bean producer and exporter countries are Spain, Italy, Portugal, Morocco,Greece, Cyprus and Turkey (Roukas, 1994b; Catarino, 1993; Battle and Tous, 1997b; Race et al., 1999; Tunalıoğlu and Ozkaya, 2003). Carob pods and seed seem to be promising as anon-conventional feed resource that can be used for small ruminants feeding. Guessous et al. (1989) reported that increasing lambs fed diets with 200 g/kg carob meal obtained more than 200 g/d and decreased the time needed to reach significant weight. For centuries, due to the high sugar content, carob pods have been used as animal feed (Battle &Tous, 1997a; Würschet al., 1984). When fed to animals in feeding trials, carob pods have been shown to give results similar to those reported for barley. Cattle, horses, goats and sheep have also been reported to feed on the lower leaves and branches of the carob tree (Marakis, 1996).The carob tree is highly recommended for use as feed supplement for animal farming in drought stricken regions (Battle andTous, 1997a).

Carob pulp as a favourable fatty acid composition due to the presence of essential fatty acids, such as linoleic and alpha-linolenic acids (Ayaz et al., 2009)and might represent a natural source of desirable fatty acids in the diets of concentrate-fed animals. In the studies is conducted to evaluate the possibility of feeding carob pulp to livestock have mainly focused on ruminants (Silanikove et al., 2006; Priolo et al., 2000). For humans, carob pods have been used primarily in traditional foods.

5. Use of Carob pod in Food Technology

From the seed of carob, the endosperm is extracted to produce a galacto mannan, which forms locust bean gum, a valuable natural food additive for its strong gel characteristics, which are useful in products such as canned pet food, since they are maintained after heating. The carob pod is used actually as grinded to obtain carob powder, which can be used for human consumption although high tannin content limits this application.

There are controversy statements regard to tannin content of carob pods. According to Battle and Tous(1997a); Würsch et al., (1984); Bravo et al., (1994) carob pod contains a large amount of condensed tannins (16-20%), according to Youssif and Alghzawi, (2000) carob pods contain lower tannin values. Carob leaves have been reported to contain considerably lower values of 0.7% dry matter basis (Silanikove, 2001).

The main use is the production of carob bean gum from the seed endosperm which is used as the food additive (stabilizer and thickener) in food- and pharmaceutical industry. In addition, carob fruits are used in food industry as a source of many products such as gum, sugar and alcohol (Carlson, 1986).

Carob is used in many Arab countries to make a popular drink which is consumed mainly in the month of Ramadan. Carob is also used in preparation of special traditional types of Arabic confectionery. In western countries, carob powder is produced by deseeding of carob pods, yielding of kibbled carob, followed by roasting and milling of the kibbled carob. Carob juice concentrate (CJC) is produced by boiling carob juices without any added ingredients and technological or scientific techniques. Due to its high sugar content, carob was consumed as a food especially in ancient times, as a sweet for children or in emergency situations such as war (Owen et al. 2003).

Throughout the Mediterranean region including Turkey, gently milled carob pods are processed to a cocoa-like flour which issold as a "*carob cocoa*" in big stores and local markets. The milled flour is often added to hot or cold milk for drinking (Morton, 1987).

6. Health benefits

The reason of using carob as a chocolate substitute resides in that carob is an ingredient free from caffeine and the obromine. High glycemic index (GI) and glycemic load (GL) have been proposed to be associated with increased risk of chronic diseases. High GI food intake may elevate postprandial blood

glucose levels, leading to high insulin demand. Some studies have shown that the consumption of low glycemic index food improves blood glucose control, lipid profile and lipo protein concentrations. Some other benefits are known as the prevention of coronary heart diseases, cancer prevention, promotion of anti-allergy effects and vaso-relaxation (Sakakibara et al., 2003).

7. Ethanol/Lactic acid Production

Raw materials containing fermentable sugars (e.g., sugar cane, sugar beet,sweet sorgum and carob), hydrolyzable polysaccharides (wheat, maize,and other starch-containing grains) are used also for bioethanol production. Due to high carbohydrate content, it is possible to use of carob interesting source for bioethanol production.Carob pod has usually been neglected for a long time alternative utilization especially about biotechnological processes and fermentation. In recent years, carob has attracted considerable attention because of high carbohydrate and mineral content (Li et al., 2011). Many high value-added products are produced such as lactic acid,(Turhanet al., 2010) mannitol (Carvalheiro et al., 2011) citric acid (Pramod and Lingappa, 2012) and pullulan (Roukas and Biliaderis, 1995) were produced by using carob via fermentation process.Turhan et al., (2010) performed that ethanol production from carob pod extract by using *Saccharomyces cerevisiae*. The final ethanol concentration, and maximum production rate were found to be 42.6 g/L and 3.37 g/L/h, respectively. Vaheed et al.,(2011) investigated also that ethanol production from carob pod extract. The carob pod is used actually as animal feed or is grinded to obtain carob powder, which can be used for human consumption. The production of ethanol from non sterilized carob pod extracts using *Saccharomyces cerevisiae* could be investigated (Roukas, 1994a, b, c).

CONCLUSIONS

Carob seeds are the largest output of the locust bean gum in food industry. Thus, the industrial target is to get high seeds yield with high nutritional properties. Indeed, carob rich in sugars, polyphenols, fibre and minerals are interesting for health consumer particularly in food industry, medicinal and pharmacological industries. Due to its sweetness and flavor similar to chocolate, the pods milled into flour are used in the Mediterranean region as cocoa substitute for sweets, biscuits, and processed drinks production. And another property it can be concluded that carob pod contains antioxidant substances such as polyphenols, which exhibit a wide range of biological properties, and among these, the antioxidant activity is the best known. Phenolicantioxidants prevent against oxidative damage of some important biomolecules like DNA, protein, and lipids.

Another area for using carob pods is known in production of ethanol and different kind of acids, which is explained below:

1. raw materials containing fermentable sugars (sugar cane, beet and sweet shorgum),
2. polysaccharides that can be hydrolyzed for obtaining fermentable sugars (starch contained in several grains, like maize and wheat) and
3. lignocellulosic biomass

The production of lactic acid using fermentation has several advantages compared to chemical synthesis because of low-cost substrates and low energy consumption. It may be expensive when purified sugars such as glucose and sucrose are used as a feedstock. Therefore, agricultural by products or residues are the cheaper alternatives to refined sugars for lactic acid production (Hofvendahl and Hagerdal, 2000).

The RCSF (a raw carob seed flour) and GERM (grinding of germs) flours are therefore interesting sources of insoluble fibre and compounds with antioxidant activity, lignan in particular. Carob seed flours could be used as an alternative raw material and incorporated as an ingredient in new food formulations. In particular, the antioxidant properties of the carob seed flours make them a potentially interesting ingredient for functional foods.

REFERENCES

Ahn J., Grün I. U., Fernando, L. N., 2002. Antioxidant properties of natural plant extracts containing polyphenolic compounds in cooked ground beef. Journal of Food Science, 67: 1364–1369

Ashoor S.H., Knox J.M., 1982. Determination of organic acids in foods by high-performance liquid chromatography. J. Chromatogr. 299: 288–292

Avallone R., Plessi M., Baraldi M., Monzani A. (1997). Determination of the chemical composition of carob (Ceratonia siliqua): Protein, fat, carbohydrates and tannins. Journal of Food Composition and Analysis, 10: 166–172

Ayaz F. A., Torun H., Glew R. H., Bak Z. D., Chuang L. T., Presley J. M., Andrews R., 2009. Nutrient content of carob pod *(Ceratonia siliqua* L.) flour prepared commercially and domestically. Plant Foods for Human Nutrition, 64: 286–292

Battle I., Tous J., 1997b. *Carob Tree (Ceratonia Siliqua L.*) Promoting the Conservation and Use of Underutilized and Neglected Crops, Vol. 17,Institute of Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetics Resources Institute, Rome.

Battle I., Tous, J., 1997a.Promoting the conservation and use of underutilized and neglected crops (Carob tree, *Ceratonia siliqua L.*). International Plant Genetic Resources Institute (IPGRI), Rome, Italy

Biagi G., Cipollini I., Paulicks B. R., Roth F. X., 2010. Effects of tannins on growth performance and intestinal ecosystem in weaned piglets. Archives of Animal Nutrition, 64: 121–135

Biner B., Gubbuk H., Karhan M., Aksu M., Pekmezci M., 2007. Sugar profiles of the pods of cultivated and wild types of carob bean (*Ceratonia siliqua L.*) in Turkey. Food Chem. 100 (4): 1453–1455

Boskou D., 1999. Non-nutrient antioxidants and stability of frying oils. In D. Boskou & I. Elmadfa (Eds.), Frying of food. Oxidation, nutrient and non-nutrient antioxidants, biologically active compounds and high temperatures (pp. 183–204). Lancaster: Technomic Publishing Co., Inc.

Brandt, L.A. (2002). Carob fibre offers health benefits. Prepared Foods, 171: 51

Bravo, L., Grados, N. and Saura-Calixto, F. 1994. Composition and potential uses of mesquite pods (*Prosopis pallida* L.). Comparison with carob pods (*Ceratonia siliqua* L.). J. Sci. Food Agric. *65*, 303–306

Calixto F. S. Cańellas J., 1982. Components of nutritional interests in carob pods (*Ceratoniasiliqua*). Journal of the Science of Food and Agriculture*,* 33, 1319-1223

Carlson W. A., 1986. The carob: Evaluation of trees, pods and Kernels. Int. Tree Crops J. 3, 281 – 290.

Carvalheiro F., Moniz P., Duarte L.C., Esteves M.P., Gírio FM. 2011. Mannitol productionby lactic acid bacteria grown in supplemented carob syrup. J Ind Microbiol. Biotechnol, 38:221–227

Catarino F., 1993. The carob tree: an exemplary plant. Naturopa 73: 14–15

Chamberlain D.F., 1970. Ceratonia L. In Flora of Turkey and the East Aegean Islands (P.H. Davis, ed.) pp. 7–8, Edinburgh University Press,Edinburgh

Correia P.J.,Martins-Loução M.A., 2004. The use of macronutrients and water in marginal Mediterranean areas: the case of carob-tree. Field Crops Research 91 (1), 1–6.

Cross H. R., Leu R., Miller M. F. 1987. Scope of warmed-over-flavor and its importance to the meat industry. In A. J. St. Angelo & M. E. Bailey (Eds.), Warmed-over-flavor of meat (pp. 1–18). Orlando: Academic Press

Dakia P. A., Bleckerb C., Roberta C., Watheleta B., Paquota M., 2008. Composition and physicochemical extracted from whole seeds by acid properties of locust bean gum or water dehulling pre- treatment Food Hydrocolloids 22: 807–818.

Dakia P. A., Wathelet B., Paquot M., 2007. Isolation and chemical evaluation of carob *(Ceratonia Siliqua L.)*. Food Chemistry, 102: 1368 – 1374.

El-Shatnawi M. J., Mohawesh, Y. 2000. Seasonal chemical composition of saltbuck in semiarid grasslands of Jordan. J. Range Manage. 53: 211 – 214.

Fletcher R., 1997. XIII. Carob agroforestry in Portugal and Spain, The Australian New Crops Newsletter. Available in: http//www.newcrops.uq.edu.au/public1.htm

Gubbuk H., Guven D., Gunes E., 2009. Physical features of some Turkish carob (*Ceratonia siliqua L.*) pods. Bull. UASVM Hortic. 66, 685

Gubbuk H., Kafkas E., Guven D., Gunes E., 2010. Physical and phytochemical profile of wild and domesticated carob (*Ceratonia siliqua L.*) genotypes. Span. J. Agric. Res. 8: 1129–1136

Guessous F., RihaniN., Kabbali A., Johnson W.L., 1989. Improving feding systems for sheep in a Mediterranean rain-fed cereals/livestock areaof Morocco. J. Anim. Sci. 67: 3080–3086

Haber, B. (2002). Carob fibre benefits and applications. Cereal Foods World, 47: 365- 369

Hofvendahl, K., Hagerdal, B.H. (2000). Factors affecting the fermentative lacticacid production from renewable resources. *Enzyme and Microbial Technology*, 26:87–107

Karkacier M., Artik N., Certel M., 1995. The conditions for carob (*Ceratonia siliqua L.*) extraction and the clarification of the extract. Fruit Process. 12: 394–397

Li H.B., Lil D., Zhang Y. R.Y., Gan, F.L., Song, C., Chen F., 2011. "Antioxidant Properties of Chinese Medicinal Plants." In Reactive Oxygen Species and Antioxidants in Higher Plants, edited by S. Dutta Gupta, 331–362. London: Sage

Makris D.P.; Kefalas P.,2004. Carob pods (*Ceratonia siliqua* L.)as a source of polyphenolic antioxidants. Food Technology and Biotechnology 42 (2): 105–108

Marakis S.G., 1996. Carob bean in food and feed: status and future potentials – A critical appraisal. Journal of Food Science and Technology, 33(5): 365-383

Morton J. F. 1987. Carob. In Fruits of warm climates. C F Dowling, Jr. (ed.). Miami, Fl. PP 121 – 124

Owen, R. H., Haubner, R., Hull, W. E., Erben, G., Parvathy K. S., Susheelamma N. S., Tharanathan R. N., Gaonkar A. K., 2005. Asimple non-aqueous method for carboxymethylation of galactomanans. Carbohydrate Polymer, 62: 137–141

Petit M. D., Pinilla, J. M., 1995. Production and purification of a sugar syrup from carob pods. Lebensm. -Wiss. U – Technol. 28: 145-152.

Pramod T., Lingappa K., 2012. Immobilization of Aspergillus niger in Hen Egg White forthe

production of Citric acid using carob pod extract. J Microbiol BiotechnolRes, 2

Priolo A., Waghorn G. C., Lanza M., Biondi L., Pennisi P. 2000. Polyethylene glycol as a means for reducing the impact of condensed tannins in carob pulp: Effects on lamb growth performance and meat quality. Journal of Animal Science, 78: 810–816

Race D., Curtis A., Booth W., 1999. Carob agroforesty industry: an assessment of itspotential for the low-medium rainfall Murray Valley region. Aust. J. Exp. Agric. 39: 325–334

Roukas T., 1994c. Solid-state fermentation of carob pods for ethanol production. Appl. Microbiol. Biotechnol. 41 (3): 296–301.

Roukas T., 1994a. Kinetics of ethanol production from carob pods extract byimmobilized Saccharomyces cerevisae cells. Appl Biochem Biotechnol, 44(1):49–64

Roukas T., 1994b. Ethanol production from nonsterilized carob pod extract by free and immobilized *Saccharomy cescerevisae* cells using fed-batch culture. Biotechnol Bioeng 43(3):189–94

Roukas T., Biliaderis C. G., 1995 Evaluation of carob pod as a substrate for pullulanproduction by Aureobasidium pullulans. Appl Biochem Biotechnol. 55:27–44

Sahin H., Topuz A., Pischetsrieder M., Ozdemir F., 2009. Effect of roasting process on phenolic, antioxidant and browning properties of carob flour. European Food Research and Technology, 230: 155-161

Sakakibara H., Honda Y., Nakagawa S., Ashida H. Kanazawa K. 2003.Simultaneous determination of all polyphenols in vegetables, fruits and teas. Journal of Agricultural and Food Chemistry, 51: 571-581

Scalbert A., Manach C., Morand C., Rémésy C., Jiménez L., 2005. Dietary polyphenols and the prevention of diseases. Critical Reviews in Food Science and Nutrition, 45(4): 287–306.

Sharma B. R., Dhuldhoya N. C., Merchant S. N., 2008. Glimpses of galactomannans. Science Tech Entrepreneur, 3: 1–10

Sikorski Z.E., 2003. Chemical and Functional Properties of Food Components.2nd ed. Pp. 116-117. USA: CRC Press

Silanikove N., Gilboa N., Nitsan, Z., 2001. Effect of polyethylene glycolon rumen volume and retention time of liquid and particulate matter along the digestive tract in goats fed tannin-rich carob leaves. Small Rumin. Res. 40: 95-99

Silanikove N., Landau S., Or D., Kababya D., Bruckental I., Nitsan Z., 2006. Analytical approach and effects of condensed tannins in carob pods *(Ceratonia siliqua*) on feed intake, digestive and metabolic responses of kids. Livestock Science, 99: 29–38

Spiegelhalder, B., Bartsch, H., Haber, B.2003: Isolation and structure elucidation of the major individual polyphenols in carob fibre, Food and Chemical Toxicology, 41: 1727-1738 surface methodology. J Ind Microbiol Biotechnol., 38:101–11

Tous J.,Hermoso J.F., Ninot A., Plana J.,1996.Current situation of carob plant material. In: Proceedings of the III international carob symposium, University of Lisbon, Portugal

Tous, J. (1990). El garrobo, Ed. Muniperna. Madrid, pp. 27-43.

Tous, J., Romero, A., Hermoso, J.F., Ninot, A., Plana, J., Batlle, I., 2009. Agronomic and commercial performance of four Spanish carob cultivars. HortTecnology 19, 465–470.

Tunalıoğlu, R., Özkaya, M.T., 2003. Keciiboynuzu. T.E.A.E.- Bakıs 3 (5): 1–4

Turhan I., Bialka K.L., Demirci A, Karhan M., 2010. Ethanol production from carobextract by using Saccharomyces cerevisiae. Bioresour Technol101:5290–5296

Turhan I., Bialka K.L., Demirci A., Karhan M., 2010. Enhanced lactic acid production fromcarob extract by Lactobacillus casei using Invertase pretreatment. FoodBiotechnol;24:364–74

Turhan I., Tetik N., Aksu M., Karhan M., Certel M., 2006. Liquid-solid extraction of soluble solids and total phenolic compounds of carob bean (*Ceratonia siliqua L.).* J. Food Process. Eng. 29: 498–507.

USDA (United States Department of Agriculture) 2006. Agricultural Research Service, National nutrient database, NDB no. 16055. 7 October 2006

Vaheed H, Shojaosadati SA, Galip H. 2011. Evaluation and optimization of ethanolproduction from carob pod extract by Zymomonas mobilis using response

Vinson J.A. (2001). Phenol antioxidant quantity and quality in foods: Fruits. Journal of Agricultural and Food Chemistry, 49: 5315-5321

Würsch P., Del Vedovo S., Rosset J., Smiley M. 1984. The tannin granules from ripe carob pod. Lebensm.-Wiss. Technol. 17: 351– 354.

Yousif A.K., Alghzawi H.M., 2000. Processing and characterization of carob powder. Food Chemistry, 69(3): 283-287

Zografakis N., Dasenakis D., 2000.Biomass in Mediterranean. Project No. 238: “Studies on the Exploitation of Carob for Bioethanol Production”. Commission of the European Communities, Directorate General for energy and transport. Regional Energy Agency, Region of Crete

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MUSHROOM MYCELIA CULTIVATION ON DIFFERENT AGRICULTURAL WASTE SUBSTRATES

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Abstract

Increasing demand for edible or medicinal mushrooms has led to investigation into the suitability of sawdust and agricultural wastes as substrates for commercial production. Effective use of bio-resources by waste-free processing and production of nutraceuticals or ingredients for functional foods are the main directions in biotechnology. The aim of this work was to evaluate the growth capacity of mushroom mycelia on substrates of sawdust or agricultural wastes mixed with different amendments. Four variants of agricultural substrates (wheat and sorghum each of them mixed with CaSO4 and dolomite amendments) and seven mushroom species (Flammulina velutipes, Laetiporus sulphureus, Ganoderma lucidum, Ganoderma applanatum, Hericium coralloides, Trametes versicolor and Lepista nuda) were used. The mycelial cultures were initially grown in Petri dishes on 2 % malt extract agar or PDA media at 25 ^{0}C in the dark. After one week, the mycelium of each mushroom species was transferred to different sterilized grain, wheat, sorghum or barley straws or sawdust as substrates with various amendments. Various degrees of grain coverage with mycelia depending on substrate and mushroom species were obtained. The results obtained give the possibility of high quality inoculum using cheap renewable resources and the future extension of the research at the mushroom farm for evaluating the effectiveness of this inoculum for fruiting bodies obtaining. Better utilization of these recyclable materials by mushroom cultivation releases important land surfaces and also eliminates the polluting factors from the terrestrial ecosystems. After finishing the culture cycle, the spent substrate represents a valuable reusable resource as constituent material in nutritional mixtures for horticultural cultures, in bioremediation of some degraded soils or contaminated with various pollutants, of waste water, having a positive impact on improving the sorrounding environment.

Key words*: Mushrooms mycelia, agricultural wastes substrates, amendments, growth efficiency.*

INTRODUCTION

Following the agricultural sector activities very high quantities of lignocellulosic wastes result. These are represented by different parts of the cultivated plants (eg. wheat, sorghum, rice) that can be straws, stalks, husks.

Rani et al., 2008 show that the quantity of residual straws rises at 70 million tons per year in India. The European countries are also rich in wheat straws which is the cheapest crop residues in this part of the world (Ćilerdžić et al., 2014; Knežević et al., 2013). A quantity of wheat straw wastes is also available for Europe in some studies and is around 170 million tons (Knežević et al., 2013; Tabka et al, 2006). The problem of removing these wastes is also the subject of other studies around the world. According to some authors the solutions based on burning the sorghum stalks threat the health by respiratory diseases. The storage of the sorghum wastes on the fields can promote the occurence of the pests represented by insects and diseases that can sicken the crops (Rani et al., 2008). The wheat and rice straws are also eliminated by burning, producing in turn problems related to environmental pollution as Yang et al., 2013 show. Solving the problem of lignocellulosic agricultural wastes can be realised by promoting a biotechnological process that involves the use of the Basidiomycetes. Thereby the huge waste quantities coming from the cereal harvesting could constitute a valuable nutrient resource that with the help of a bioconversion technology could be recycled in useful products (Tan and Wahab, 1997).

The use of the Basidiomycetes as a biological conversion technology of the mentioned wastes resides in the mushroom ability to secrete the hydrolysis or oxidising enzymes. The

mushrooms *Pleurotus* and *Lentinus* are two examples of species that own enzymes systems based on endoglucanase, laccase and phenoloxidases (Rani et al., 2008). *Trametes versicolor* is capable to solubilise lignocellulosic materials with the help of an enzymes mixture composed from peroxidases, celulases and glucose oxidases (Vyas et al, 1994). From literature, the *Pleurotus* mushrooms can adapt for growing and fruiting on a large variety of lignocellulosic wastes (Mikiashvili et al, 2006). Taking into account the use of mushrooms as potential straw degradation technology, basidiomycetes mycelium growing technologies on agricultural origin substrates can be investigated and designed. According to some sources the mushroom cultivated on the first place in the world is *Agaricus bisporus*, *Pleurotus ostreatus* being on the second place while on the third place is situated *Lentinula edodes*. Other mushrooms which follow the three most cultivated basidiomycetes worldwide are *Auricula auricula*, *Flammulina velutipes* and *Volvariella volvacea* (Rühl et al, 2008; Carmen Sanchez, 2004; Carmen Sanchez, 2009; Aida et al., 2009; Reis et al., 2011). The *Pleurotus* mushroom, known also as the oyster mushroom can be cultivated with excellent results under semi controlled conditions in a small space by using agricultural waste (Bhatti et al., 2007). Some sources indicate the fact that paddy straws are the best substrate for *Pleurotus* cultivation (Mane et al., 2007). Another important mushroom with increased higher demand namely *G. lucidum* has led to research in what concerns the suitability of sawdust and agricultural wastes as substrates for commercial bag cultivation of the mushroom. The cultivation on solid substrates has been declared as a success (Peksen et al., 2009). A study reveal that fruiting bodies of mushrooms including *G. lucidum* have been produced on solid cultures in a traditional manner using substrates such as grain, sawdust or wood (Peksen et al., 2009).

The aim of this work was to evaluate the growth capacity of mushroom mycelia on substrates of sawdust or agricultural wastes mixed with different amendments.

MATERIALS AND METHODS

Mushrooms culture

Seven mushroom species, such us: *Flammulina velutipes, Laetiporus sulphureus, Ganoderma lucidum, Ganoderma applanatum, Hericium coralloides, Trametes versicolor* and *Lepista nuda* were used for the experiments. The mushroom species were kindly provided from the Culture Collection of Mushrooms of the Faculty of Biotechnology- UASVM, Bucharest.

Growth conditions

Mycelial cultures were initially grown in Petri dishes (90 mm in diameter) on 2% malt extract agar or PDA (potato-dextose-agar) media at 25^0C in the dark. One week after, the mycelium of each mushroom species (agar segments of 10x10 mm) was transferred to different sterilized grain, wheat, sorghum or barley straws or sawdust as substrates with various amendments (Table 1).

Table 1. Variants of agricultural substrates

Variants	Substrates	Amendments
V1	Wheat – 420 g	$CaSO_4$ – 12,6g
V2	Wheat – 420 g	Dolomite – 12,6g
V3	Sorghum – 500 g	$CaSO_4$- 15 g
V4	Sorghum – 500 g	Dolomite – 15 g

After the inoculation the experimental samples were incubated at 25^0C at dark, for 15 days. After this period the growing ability of the fungi species mycelium an the degree of grain coverage with mycelium were evaluated.

RESULTS AND DISCUSSIONS

For the investigation of the growing ability of the fungi on different natural plant substrates, the mycelium of *Flammulina velutipes, Laetiporus sulphureus, Ganoderma lucidum, Ganoderma applanatum, Hericium coralloides, Trametes versicolor* and *Lepista nuda* was used for inoculating four types of experimental variants: V1 (wheat grains with $CaSO_4$) as amendment; V2 (wheat grains with dolomite) as amendment; V3 (sorghum grains with $CaSO_4$) and V4 (sorghum grains with

dolomite). The dolomite is an amendment for soil for long- term, that conains calcium oxide and magnesium, having beneficial effects on the soil. Currently, the dolomite is used with modern soil cutivating technologies. After 3 – 4 weeks from the grains inoculating and incubation in the dark at approximately 24^0C it was found that that depending on the substrate nature, the covering degree of the substrate with mushroom mycelium was different. Thus, in the case V1 (wheat with $CaSO_4$) and V2 (wheat with dolomite) of experimental variants, mycelia of ***Ganoderma lucidum*** invaded the grains at a rate of 75% and 30% respectively (Figure 1). In what concerns the substrate variants V3 (sorghum with $CaSO_4$) and V4 (sorghum with dolomite), the covering degree of the grains with mushroom mycelium was 90% and 100% respectively (Figure 1). Following these experiments it was found that the optimal substrates for mycelium development in the first 4 weeks following the inoculation, where the ones consisting of sorghum grains with different amendments. After 8 weeks of de culture on tested substrates, on variant V1 (wheat with $CaSO_4$) even fruiting bodies developed (although initially the invasion of grains by the mushroom mycelium was more slowly) (Figure 2).

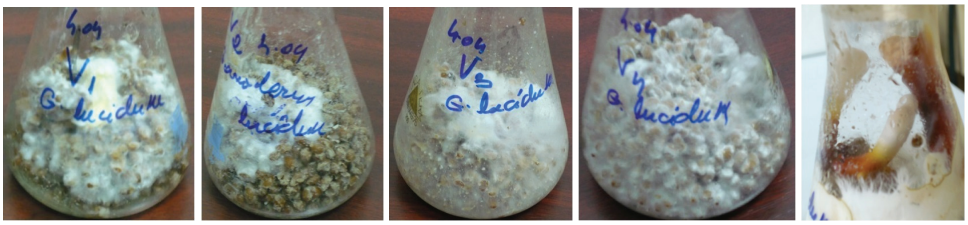

Figure 1. Different degrees of grain coverage with *Ganodema lucidum* mycelium depending on substrate, four weeks after. Figure 2. Fruiting bodies developed after eight weeks of culture on V1

In what concerns the ***Ganoderma applanatum*** species, the mycelium of this mushroom has optimally developed on the substrate represented by wheat with $CaSO_4$. The degree of coverage being 100%. The wheat covered with *G. applanatum* mycelium was used to inoculate a wheat straw substrate. After 8 weeks on this type of substrate, in which the mushroom invaded completely the straws, fruiting bodies primordiums developed (Figure 3).

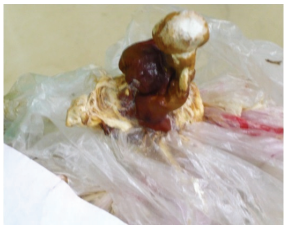

Figure 3. Fruiting bodies of *G. applanatum* developed on sawdust, eight weeks after

Flammulina velutipes is known for being edible and also considering the existing information in the literature (Bao et al., 2009) it has medicinal properties, highlightening immunomodulatory, antitumoral and antioxidant properties. After 6 weeks from *F. velutipes* mycelium grain inoculating it has been found that the covering degree of the substrate was 100% on all the tested variants. On the variants V3 (sorghum with $CaSO_4$) and V4 (sorghum with dolomite) fruiting bodies of this mushroom have appeared (Figure 4). The wheat covered with mycelium was used to inoculate another wheat straws substrate with 3% $CaSO_4$. After 8 weeks on this type of substrate, in which the mushroom invaded completely the straws but without making fruiting bodies.

Figure 4. *Flammulina velutipes* after six weeks on grain substrates mixed with different amendments

In what concerns the ***Laetiporus sulphureus*** species, the mycelium of this mushroom has abundant developed on all the tested substrate variants, except on the substrate variant V4, represented by sorghum with dolomite (Figure 5). The mycelium appearance was dusty and the colour of the mycelium turned from white in the first stage to intense beige to the end of culture period.

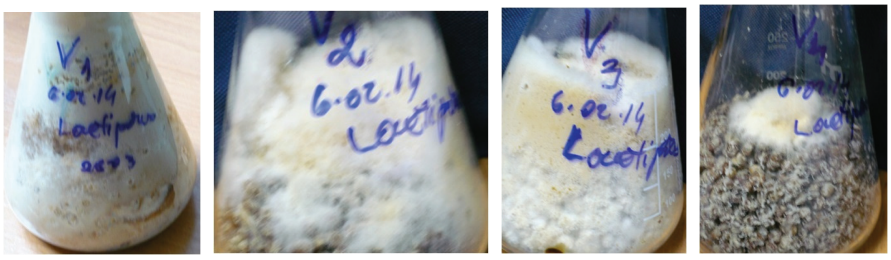

Figure 5. Mycelium of *Laetiporus sulphureus* developed on various substrates

Lepista nuda (*Clitocybe nuda*) is an edible mycorrhizal species, isolated from the ground level, around trees. It distinguishes by the production of a violaceous pigment at the mycelium level (the pigment was initially observed at the carphopores lamellae level). Researches performed on this species revealed antioxidant, antimicrobial properties (Dulger et al., 2002) and immunological effects (Lin et al., 2011). The fungal mycelium has abundant developed only on the variants V1 and V4 (Figure 6).

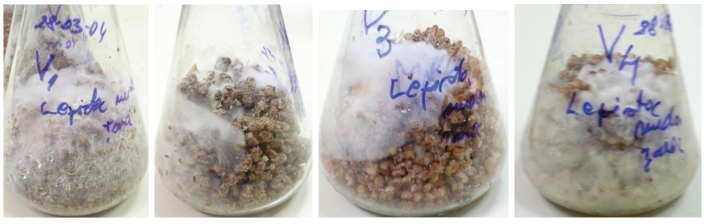

Figure 6. Different degrees of grain coverage with *Lepista nuda* mycelium depending on substrate, four weeks after

Trametes versicolor grows on decaying tree trunks. It has antitumor, hepatoprotective, antiviral, etc (Roupas et al., 2012; De Silva et al., 2013).

On all the tested substrates, the degree of covering was 100 %. On wheat straws substrate after 8 weeks the mushroom mycelium invaded completely the straws developing fruiting bodies primordiums (Figure 7).

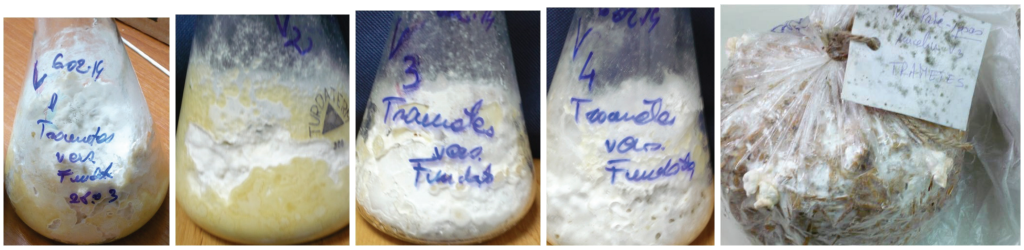

Figure 7. *Trametes versicolor* mycelium developed on wheat and sorghum substrates and fruiting bodies developed from inoculated straw

Hericium coralloides grows on dead hardwood trees. When young, the fungus is soft and edible. *Hericium* has been used as a traditional medicine in China and Japan for many years; but its medicinal uses are still being explored.

Hericium coralloides mycelium developed on all substrate variants investigated. After eight weeks, fruiting bodies were developed on V1 and V3 grain substrate (Figure 8).

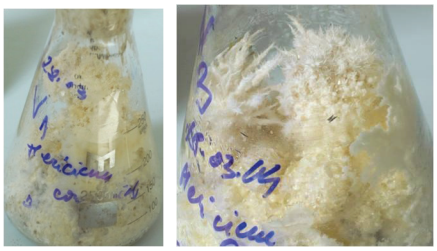

Figure 8. *Hericium coralloides* fruiting bodies developed on V1 and V3 grain substrate

CONCLUSIONS

Several variants with natural plant substrates with different amendments, for optimal substrate evaluation for mycelium growth in order to obtain fungal inoculum were carried out. The mushroom cultivation on different substrates reveals that using agricultural wastes mixed with different amendments, in the most cases, supported a good mycelial growth. Different degrees of grain coverage with mycelium depending on substrate and mushroom species. These results suggests the possibility of high quality fungal inoculum using a series of cheap renewable resources and the future extension of the research at the mushroom farm for evaluating the effectiveness of this inoculum for fruiting bodies obtaining. Better utilization of these recyclable materials by mushroom cultivation releases important land surfaces used for their storage and also eliminates the polluting factors from the terrestrial ecosystems. Furthermore, after finishing the culture cycle, the spent substrate represents a valuable reusable resource as constituent material in nutritional mixtures for horticultural cultures, in bioremediation of some degraded soils or contaminated with various polutants, of waste water, having a positive impact on improving the sorrounding environment.

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REFERENCES

Aida F. M. N. A., Shuhaimi M., Yazid M., Maaruf A. G., 2009. Mushroom as a potential source of prebiotics: a review. Trends in Food Science & Technology, 20(11): 567-575.

Bao H. N., Ushio H., Ohshima T., 2009. Antioxidative activities of mushroom (*Flammulina velutipes*) extract added to big eye tune meat: dose-dependent efficacy and comparison with other biological

antioxidants. Journal of Food Science, 74(2): 162-169.

Bhatti M.I., Jiskany M.M., Wagan H.K., Patan M.A., Magsi M.R., 2007. Growth, development and yield of oyster mushroom, *Pleurotus ostreatus* (Jacq. Ex. Fr.) Kummer as affected by different spawn rates. Pak. J. Bot., 39(7): 2685-2692.

Ćilerdžić J., Vukojević J., Stajić M., Stanojković T., Glamočlija J., 2014. Biological activity of Ganoderma lucidum basidiocarps cultivated on alternative and commercial substrate. Journal of Ethnophamacology, 155(1): 312-319.

De Silva D. D., Rapior S., Fons F., Bahkali A. H., Hyde K. D., 2012. Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. Fungal Diversity, 55(1): 1-35.

Dulger B., Ergul C. C., Gucin F., 2002. Antimicrobial activity of the macrofungus *Lepista nuda*. Fitoterapia, 73(7): 695-697.

Knežević A., Milovanović I., Stajić M., Lončar N., Brčeski I.,Vukojević J., Ćilerdžić J., 2013. Lignin degradation by selected fungal species. Bioresource Technology, 138: 117–123.

Knežević, A., Milovanović, I., Stajić, M., & Vukojević, J., 2013. Potential of Trametes species to degrade lignin. International Biodeterioration & Biodegradation, 85: 52-56.

Lin C. C., Yu Y. L., Shih C. C., Liu K. J., Ou K. L., Hong L. Z., ... & Chu C. L., 2011. A novel adjuvant Ling Zhi-8 enhances the efficacy of DNA cancer vaccine by activating dendritic cells. Cancer Immunology, Immunotherapy, 60(7): 1019-1027.

Mane V.P., Patil S.S., Syed A.A., Baig M.M.V., 2007. Bioconversion of low quality lignocellulosic agricultural waste into edible protein by Pleurotus sajor-caju (Fr.) Singer. Journal of Zhejiang University Science B., 8(10):745-751.

Mikiashvili N., Wasser P. Solomon, Nevo E., Elisashvili V., 2006. Effects of carbon and nitrogen sources on *Pleurotus ostreatus* ligninolytic enzyme activity. World Journal of Microbiology and Biotechnology, 22(9): 999-1002.

Peksen A., Yakupoglu G., 2009. Tea waste as a supplement for the cultivation of *Ganoderma lucidum*. World Journal of Microbiology and Biotechnology, 25(4): 611-618.

Rani P., Kalyani N., Prathiba K., 2008. Evaluation of Lignocellulosic Wastes for Production of Edible Mushrooms. Applied biochemistry and biotechnology, 151(2-3): 151-159.

Reis S.P., Barros L., Martins A., Ferreira C.F.R. I., 2012. Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: An inter-species comparative study. Food and Chemical Toxicology, 50(2):191-197.

Roupas P., Keogh J., Noakes M., Margetts C., Taylor P., 2012. The role of edible mushrooms in health: Evaluation of the evidence. Journal of Functional Foods, 4(4): 687-709.

Rühl M., Fischer C., Kües U., 2008. Ligninolytic enzyme activities alternate with mushroom production during industrial cultivation of Pleurotus ostreatus on wheatstraw-based substrate. Current Trends in Biotechnology and Pharmacy, 2(4): 478-492.

Sánchez Carmen, 2004. Modern aspects of mushroom culture technology. Applied Microbiology and Biotechnology, 64(6): 756-762.

Sánchez Carmen, 2009. Cultivation of *Pleurotus ostreatus* and other edible mushrooms. Applied microbiology and biotechnology, 85(5): 1321-1337.

Stajić M., Vukojević J., Duletić-Laušević S., 2009. Biology of *Pleurotus eryngii* and role in biotechnological processes: a review. Critical Reviews in Biotechnology, 29(1): 55–66.

Tabka M. G., Herpoël-Gimbert I., Monod F., Asther M., Sigoillot J. C., 2006. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. Enzyme and Microbial Technology, 39(4): 897-902.

Tan Y. H., & Wahab M. N., 1997. Extracellular enzyme production during anamorphic growth in the edible mushroom, Pleurotus sajor-caju. World journal of microbiology and biotechnology, 13(6): 613-617.

Vyas B. R. M., Volc J., Šašek V., 1994. Ligninolytic enzymes of selected white rot fungi cultivated on wheat straw. Folia microbiologica, 39(3): 235-240.

Yang W., Guo, F., Wan Z. 2013. Yield and size of oyster mushroom grown on rice/wheat straw basal substrate supplemented with cotton seed hull.Saudi journal of biological sciences, 20(4): 333-338.

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PHYSICAL AND CHEMICAL CHARACTERISTICS OF CROSSED OLIVES AND THEIR CONVENIENCE TO GREEN TABLE OLIVE FERMENTATION BY USING *Lactobacillus plantarum* AS A STARTER CULTURE

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Abstract
Genetic variation was reported as an important factor effects quality of table olive. So that researchers aimed to develop new cultivar which had high table olive characteristics than that's of standard cultivar. This research was aimed to determine characters of raw and processed fruits of 4crossedolive genotypes which had been reported by previous studies as promising cultivar for registration according to agronomic characteristics. Fruits of Manzanilla cultivar which is the most important green table olive cultivar in Spain were used for comparison. Number of olives per kilogram, flesh to seed ratio, water, oil, total and reducing sugar, and phenolic compounds were analyzed. Sensory and salt analyses also were applied to processed olives. For green table olive production; olives were debittered by 2% NaOH and then put in brine which contained 5% salt at pH4,5. At 4th day of keeping the olives in brine, 10^{7}CFU/mL Lactobacillus plantarum were inoculated to the brine for fermentation until pH fall to 3,8.All the olives of genotypes had enough reduced sugar content (>2 %) for fermentative microorganisms and higher olive weight than Manzanilla but only olive of BK013 had higher flesh to seed ratio than Manzanilla. After processing hydroxytyrosol losses were determined in the range of 30,25-88,88 % and processed olives of MT038 had higher hydroxytyrosol content this is precious for nutrition physiology of consumer. Olives of BK013 and GK131had bettertable olive and sensory characteristics so that they have potential for registration as new table olive cultivar.

Key words: *olive crossing, olive genotype, table olive selection.*

INTRODUCTION

Olive industry is seeking new cultivars better suited to modern cultivation techniques and with high quality olive oil and table olive (Bellini et al., 2008). So that generally olive cross breeding studies are aimed to obtain new olive genotypes resistance against diseases and pests, appropriate to machinery harvest, have high olive fruit and oil yield with high quality prosperitiesand less periodicity (León et al., 2008).Genetic variation was reported as one of the important factor effects final quality of table olive (Menz and Vriesekoop, 2010; Ahmed et al., 2007). So that researchers aimed to develop new cultivar which had high table olive charcteristics than that's of standart cultivar (Bellini et al., 2008; Ozdemir et al., 2011).

Classic breeding programs by crossing and selection in the progenies are reported in Turkey (Ozdemir et al., 2011; Arsel and Cirik, 1994), Tunisia (Trigui, 1996), Greece (Pritsa et al., 2003), Israel (Lavee et al.,2003), and Italy (Bellini et al., 2002). A few novel cultivars have been released in olive producer countries in recent years (Lavee et al., 2014; Roca et al., 2011; Ozdemir et al., 2011;Bellini et al., 2008). Olive crossbreeding studies have been carried out since 1990 at Atatürk Central Horticultural Research Institute (Yalova, Turkey). The objective of the study is to obtain new olive cultivars which have and superior table olive characteristics. First stage of the study mainly 10 native and foreign were used as parents and hybridisation studies were realized (Yalçinkaya et al 2002). According to their agronomic characteristics previous studies had indicated that some of these crossed olive genotypes had potential for registration (Ozdemir et al., 2011; Aktepe Tangu et al., 2008). So that this research was aimed to determine the raw and table olive characteristics of these crossed olive genotypes to prepare data and define the suitability to table olive fermentation by using starter culture.

MATERIALS AND METHODS

In this study, 4 olive genotypes were evaluated which were given in Table1. They come from the crosses of foreign Belle d'Espagne (Italian cultivar) and Manzanilla (Spanish cultivar) and Karamürselsu, Tavşanyüreği and Gemlik (Turkish cultivar).

These trees were planted at in 1,5 m x 3 m distance in olive genotype observation orchard of Ataturk Central Horticultural Research Institute in Yalova city of Turkey (40°39'42.1"N 29°17'24.5"E).

These genotypes were chosen on the basis of their high productivity and resistance to diseases and low periodicity.

Table 1. Olive genotypes and their parents

Genotype code	Parents
BK013	Belle d'Espagne X Karamürselsu
MT038	Manzanilla X Tavşanyüreği
GK131	Gemlik X Karamürselsu
GK132	Gemlik X Karamürselsu
Manzanilla	-

Olives were randomly handpicked at 1 maturation index according to Guide for The Determination of The Characteristics of Oil-Olives (International Olive Council, 2011) from this observation orchard.

Method of table olive production

Olives were processed to table olive according to method of Leal Sanchez et al. (2003). Olives were debittered by keeping in 2% NaOH solution until NaOH will reach 2/3 of olive. Then 4 washes performed to remove excess NaOH from olive. Olives put in brine which contained 5% salt at pH4,5 (pH adjusted acetic acid). At 4th day of keeping the olives in brine, 10^7cfu/ml *Lactobacilus plantarum* (ATCC 14917) were inoculated in brine for fermentation. Olives were fermented in brine at 20°C until pH fall to 3,8.

Physical analysis

Number of olives per kilogram and flesh to seed ratio were determined according to official method TS 774. Fruit weight was calculated by weighting the 100 olive fruits. Flesh to seed ratio was calculated by using the ratio of flesh and seed weight of 100 olive fruits.

Water and oil analysis

Water content of olive samples was determined in a conventional oven at 105±2°C (Esti et al., 1998). Before the oil analysis, seed of olives were removed and olives were crushed. After that crushed olives were dried. Oil of the dried olive paste was extracted by soxhlet apparatus for at least 8 hours with petroleum ether extraction at 50°C. Oil content of the olives was calculated at fresh weight (Cemeroglu, 2007).

Reduced sugar analysis

5 g olive paste was weighted and mixed with 5 mlpotassium ferrocyanide (%15) ve 5 ml zincsulfate (%30). This mix was completed to 250 ml with distilled water and filtered through filter paper (40 µm pore diameter). 0.5 ml of the diluted sample, 1.5 ml of distilled water and 1 ml of the dinitrophenol was added into the test tube which was held in 100°C water bath for 6 min and cooled for 3 min with tap water. Absorbance values were determined by spectrophotometer (Shimadzu UV-2900, Japan) at 600 nm wave length within 20 minutes (Ross, 1959).

Total sugar analysis

25 ml ofthe filtrate from prepared sample dor reducing sugar is put intoballoonflask. 5 mlof HCl was added for the inversion and placed on the 70°C water bath. Temperature of sample maintained at 67°C for 5 minutes than temperature was cooled down to 20°C. pH adjusted to 6 by using 5 NNaOH and 0.1N NaOH. After the neutralization it is completed to 50 ml with distilled water. 0,5 mlfrom sample, 1,5 ml pure water and 6 ml dinitrophenol were mixed and heated on 100°C water bath for 6 minutes. Cooled at for 3 minutes under streams. 600 nm wavelength reading was taken in spectrophotometer (Ross, 1959).

Phenolic compound analysis

Hydroxytrosol, oleuropein, luteolin and rutin content of olives analyzed according modified method of Morello et al. (2004). 5 grams of olive flesh homogenized with 50 ml of methanol and macerated in a magnetic stirrer for 2 hours. After that sample was filtered by into the evaporation flask by coarse filter paper and methanol was evaporator at 40°C. Then the

residue was redissolved in 50 ml methanol and filtered into vials through 0,45 µm filters. Terms of HPLC Equipment: injection volume: 20 µl, flow rate: 1,2 ml/min, column temperature: 30°C, detectors: DAD, stoptime: 28 min, mobile phase: 84,6% water –0,4% formic acid – 15% acetonitrile, max pressure: 400 bar, wave length: 240 nm, column features: NC100-5C18-3848 Hichrom.

Salt analysis

Salt content of olives were analysed according to Mohrmethod (titrimetric method). Olive flesh was homogenized and weigthed 10 gin flask. Hot distilled water was added and shaken vigorously for 5-10 minutes. The solution was filtered by filter paper in a 100 ml balloon flask and washed 4-5 times with hot water in to the balloon flask. After completely cooled down, baloon flask was filled to 100 ml with distilled water and 10 ml from this filtrate was added to the flask with 2-3 drops potassium chromate solution.This was titrated with AgNO3 solution until red color was observed in flask (Cemeroglu, 2007).

Sensory analysis

Sensory analysis of table olives were carried out by participation of 18 experienced and trained food and agricultural engineers as panelist. Appearance, color, tissue hardness, ease of seed removing, salt, sourness and eating quality of table olives were analyzed between 0-10 point score by panelist (Panagoet al., 2002).

Statistical analysis

Research plan was performed according to the randomized experimental design (single factor experimental design). Three replicates were tested for each parameter. Analysis of variance was applied with the Duncan multiple comparison test of the means ($p<0,01$) to determine the presence of significant differences among the samples. Statistical analysis was performed by using the JMP v. 5.0 statistical package program (SAS Institute, Cary, N.C., U.S.A.). The physicochemical characteristics of genotypes were used to perform principal component analysis (PCA) with the PNTSYS statistical package program (Applied Biostatistics Inc., New York, USA). Different letters indicate significant difference in same colon of tables.

RESULTS AND DISCUSSIONS

Fruit weigth and flesh to seed ratio are important criterias for table olive cultivar from commercial point of view. Statistically significant differences were observed according to number of olive per kilogram and flesh to seed ratio values of olives. Number of olives per kilogram and flesh to seed ratio of olives were given in Table 2. Number of olive per kilogram and flesh to seed ratio of 48 new table olive genotypes coming from a cross-breeding programme were reported between 103,1- 909,1 and 1,7–10,0 (Medina et al., 2012). Chiquitita was a new registered olive cultivar obtained in a crossbreeding program (Picual x Arbequina) in Córdoba, Spain (Rallo, 1995). Number of olives per kilogram of Chiquitita was reported as 370,37 by Rallo et al. (2008). In this study all genotypes had higher olive weight than Chiquitita and olive weight and flesh to seed ratio values were determined in value range of results of Medina et al. (2012). All olive genotypes had higher fruit weight (lower numer of olives per kilogram) than fruits of Manzanilla. But only olives of BK013 had higher flesh to seed ratio than fruits of Manzanillain this research.

Table 2. Number of olives per kilogram and flesh to seed ratio of olives

Olives	Number of olives per kilogram	Flesh to seed ratio
BK013	127±16,49 e	5,87±0,18a
GK131	159±18,03 c	5,04±0,20 b
GK132	152±13,11 d	5,10±0,17b
MT038	167±16,34 b	5,14±0,21b
Manzanilla	223±14,21 a	4,97±0,23b

Different letters refer statistically significant differences in same colon

Water and oil content was affect sonsory quality and hardness of table olives and sugar content was important for succes of fermentation (Tseng and Montville, 1992, Kalis and Harris 2007). Water, oil, reduced sugar and total sugar content of olives were given in Table 3. Water and oil content of Chiquitita was reported as 19.1% and 60.8 % (Rallo et al., 2008). In this research all olives had higher

water content but only GK131 had higher oil content than Chiquitita were determined. Only olives of GK132 had lower water content then olive of Manzamilla. All the olive of genotypes had higher oil content than olive of Manzanilla. GK131 can also be registered as double purpose olive variety because of their table olive characteristics and high oil content. Similar result has been reported in literature for different olive cultivars (Menz and Vriesekoop, 2010; Nergiz and Engez, 2000).

Sugars are the main soluble components in olive tissues and play an important role, providing energy for metabolic changes (Marsilio et al., 2001). In table olive processing sugars act as carbon source for microorganisms (Tseng and Montville, 1992) for producing secondary metabolites responsible for good characteristics and the distinctive flavor of the commodities (Marsilio et al., 2001).

2% sugar content of olive is reported as enough carbon sources for fermentative microorganisms. If sugar content is not to 2% olive flesh, reduced sugar should be added to brine for successful fermentation (Kailis and Harris, 2007). In this study, all of the olive genotypes had higher reduced sugar than 2% so that there is no need addition of sugar to brine for fermentation. Similar results were found in literature for different olives cultivars (Menz and Vriesekoop, 2010;Kailis and Harris, 2007; Marsilio et al., 2001).

Table 3. Water, oil, reduced sugar and total sugar content of olives (%)

Olives	Dry matter	Oil	Reduced sugar	Total sugar
BK013	66,89±2,16 ab	17,04±0,68 b	3,96±0,18 a	4,18±0,22 a
GK131	65,37±2,81 b	20,73±1,04 a	2,81±0,16 bc	2,97±0,15 c
GK132	63,80±2,16 c	14,87±0,68 e	2,72±0,10 c	3,04±0,13 bc
MT038	68,41±2,28 a	16,02±0,83 c	2,94±0,12 b	3,1±0,15 bc
Manzanilla	67,46±2,33 ab	13,78±0,67 d	2,83±0,12 bc	3,02±0,14 bc

Different letters refer statistically significant differences in same colon

Oleuropein is responsible from bitter taste of oleuropein and its content is reduced during table olive processing (Kailis and Harris, 2007).Phenolic component also highly affect taste of table olives (Pereira et al., 2006). So that quantity of phenolic compounds particularly oleuropein was an important selection criteria for olive of new cultivar candidate. Hydroxytyrosol, luteolin, rutin and oleuropein are main phenolic of olives and their content in olives of genotypes and Manzanilla were given in Table 4. Phenolic compounds especially oleuropein and hydroxytyrosol have important effect on sensory characteristics of table olives (Morelló et al., 2004). Oleuropeinis also responsible from the bitter taste of olives (Pereira et al., 2006). GK132 is remarkable characteristics according to its low oleuropein and high hydroxytyrosol content. Hydroxytyrosol, rutin and oleuropein content of fruits of Intosso, Arabequina Hojiblanca and Duro varieties were reported between 349-1160 mg/kg, 80-500 mg/kg and 63-16500 mg/kg respectively (Gomez Rico et al.,2008; Marsilio et al., 2001; Bianco and Ucella, 2000). There were some differences between literature and our results of phenolic compound analysis. In this research all olives grown under same conditions and cultivation techniques so that genetic factor wasthought as main reason for this difference between analysis results of olives of genotypes.

Table 4. Hydroxytyrosol, luteolin, rutin and oleuropein content of olives (mg/kg)

Olives	Hydroxytyrosol	Luteolin	Rutin	Oleuropein
BK013	1864,10±87,4 c	19,01±2,2 b	84,32±6,5 d	1935,52±112,4 b
GK131	2168,39±103,9 b	13,98±2,1 d	63,20±4,1 e	1314,4± 78,3 d
GK132	2447,62±106,5 a	21,06±1,5 a	102,14±7,2 c	1095,37±82,1 e
MT038	1644,32±98,1 d	15,27±2,0 c	466,32±25,6 a	1397,85±95,8 c
Manzanilla	819,85±42,9 e	14,8±1,9 d	167,387±8,4 b	1987,58±122,1 a

Different letters refer statistically significant differences in same colon

There were statistically significant differences on fruit number per kilogram, flesh to seed ratio, water, oil and sugar contents of raw and table olives. Number of olives per kilogram and flesh to seed ratio of table olives were given in Table 5. Number of olive per kilogram and flesh to seed ratio of NaOH debittered table olives were determined as 174 and 6,39 by Kailis and Harris (2004).

Water, oil and salt content of table olives were shown in Table 6. In this research, processing increased fruit weight of olives because NaOH allowed small amounts of water to penetrate into the olive. Garrido Fernandezet al. (1997) and Romeroet al.(2004) determined the water content of table olives processed by Spanish method (NaOH debittered) between 70,34-73,40 %. Kailis and Harris (2004) reported

water and oil content of table olives which debittered by NaOH between 67-79% and 11-17%. In same report reducing sugar could not be detected in any sample.

Table 5. Number of olives per kilogram and flesh to seed ratio of table olives

Olives	Number of olives per kilogram	Flesh to seed ratio
BK013	123±17,32e	5,69±0,25a
GK131	156±12,61c	4,94±0,26b
GK132	147±15,58d	4,93±0,21b
MT038	161±16,55b	4,97±0,28b
Manzanilla	214±14,58a	4,77±0,26b

Different letters refer statistically significant differences in same colon

Table 6. Water, oiland salt content of table olives(%)

Olives	Water	Oil	Salt
BK 013	64,88±2,56b	17,51±1,22b	2,67±0,17a
GK 131	64,08±2,39bc	20,58±1,16a	2,18±0,18b
GK 132	61,89±2,51c	14,13±0,84d	2,23±0,21b
MT 038	65,90±2,58ab	15,71±0,79c	2,19±0,14b
Manzanilla	64,76±2,36b	13,42±0,86d	2,14±0,09b

Different letters refer statistically significant differences in same colon

In this research water content of table olives determined lesser than literature and but similar to the literature sugar could not be detected in table olives. Reduced and total sugar contents varied among olive cultivars according to processing conditions in processed olives (Kailis and Harris, 2007). In this research all of olives processed by same method so that these differences were caused by genetic difference. Hydroxytyrosol, rutin, luteolin and oleuropein content of table olives were given in Table 7. As a result of debittering, washing and fermentation steps of table olives processing oleuropein were not detected and except MT038 and GK132 luteolin and rutin could not be detected in table olive samples.

Table 7. Hydroxytyrosol, rutin, luteolin and oleuropein content of table olives

Olives	Hydroxytyrosol	Rutin	Luteolin	Oleuropein
BK013	207,24±16,7 d	ND	ND	ND
GK131	583,77±34,0 b	ND	ND	ND
GK132	297,41±15,3 c	ND	3,06±0,2	ND
MT038	638,05±21,5 a	2,20±0,1	ND	ND
Manzanilla	571,88±27,4 b	ND	ND	ND

ND = not detectable, Different letters refer statistically significant differences in same colon

During debittering processing of olives oleuropein was degraded to hydroxytyrosol but hydroxytyrosol was removed by washing steps (Ozdemir et al., 2014; Romero et al., 2004). Also fermentation steps can also reduce the content of hydroxytyrosol (Brenes et al., 1995). Hydroxytyrosol losses were determined in the range of 30,25-88,88 % after the table olive production and lowest (30,25 %) and highest (88,88 %) hydroxytyrosol loss was identified for olive of Manzanilla and BK013 respectively.

Table olives are highly appreciated for both their sensory characteristics and nutritive value (Marsilio et al., 2005). In this study results of sensory analysis of table olives were given in Table 8. General eating quality of the olives is 6,0-8,4 and the average of all of the evaluated sensory criteria was in the 6,1-7,6. As a result of the statistical evaluation of the panelists' points, it is understood that olives of GK131 and GK132 were most popular genotypes. Table olives processed with strain OM13 as adjunct culture, showed better sensory characteristics compared to those processed without starter (Sabatini et al., 2008). Result of sensory evaluation of 174 table olive samples showed that appearance, tissue hardness and salinity had low score, easy of seed removing and color had middle and sourness and bitterness had high scores (Kailis and Harris, 2004). Askolana olive was processed with three different processing method by Marsilioet al. (2005) and they were evaluated with 0-10point sensory test by panalists. Tissue hardness of this table olives was 6,0-7,6.

Table 8. Sensory analysis results of table olives

Evaluated Criteria	BK013	MT038	GK131	GK132	Manzanilla
Appearance	6,0 c	6,3 c	6,7 b	8,5 a	6,7 b
Color	5,6 d	6,4 c	7,6 b	9,0 a	7,3 b
Tissue hardness	7,4 a	6,3 c	7,2 a	7,4 a	6,7 b
Easy of seed removing	6,4 b	6,8 ab	7,2 a	5,8 c	5,6 c
Salt	5,4 c	6,1 b	6,3 b	7,3 a	5,2 c
Sourness	5,0 c	5,3 c	6,0 b	7,1 a	5,0 c
Generaleating quality	6,8 b	6,0 c	8,4 a	8,3 a	6,6 b
The average of thecriteria	6,1	6,2	7,0	7,6	6,2

Different letters refer statistically significant differences in same colon

CONCLUSIONS

In this research raw and processed olives of 4 new table olive genotypes coming from a cross-breeding programme and grown in same condition and processed in same processng methodwere evaluated. Acording to statistical results, genetic diversity of those genotypes were determined as signifcantly effective factoron physical and chemical characteristics of their olives. Fruit weight and flesh to seed ratio were important characters which determine the commercial value of table olives. All of the olives of genotypes had higher fruit weight than olives of Manzanilla and some fruit of other standard cultivar such as Ascolano and Arbequina reported by Kailis and Harris (2007). But only olives of BK013 had higher flesh to seed ratio than that's of Manzanilla. All the olives of genotypes had 2,72-3,96 % reduced sugar content which was enough for fermentative microorganisms. After processing especially debittering and washing steps caused high loss content in oleuropein, hydroxytyrosol, luteolin and rutin.Hydroxytyrosol is a valuable phenolic component in terms of nutrition physiology. After processing, 30,25-88,88 % loss determined in hydroxytyrosol content. Also oleuropein could not detected in olive samples after process. Fruit weight was increased between 1,88-4,04 % because of used NaOH de-bittering step when compared to raw and processed olives. Positive effect of lactic acid bacter starter cultures uses in green olive fermentation and sensory attributes of olive samples were reported when compared to spontonues fermantation (Aponte et al., 2012; Sabatini et al., 2008). In this research all samples were processed by using starter culture and high quality table olives were obtained according to result of chemical and sensory test. GK132 had highest scores except for easy of seed removing in sensory evaluation.GK132 had both highest reduced sugar content before process and sourness value after process. This result maybe related with conversion of sugar into lactic acid by fermentation.GK132 had lowest water and oil content among raw and table olive samples. Table olive characteristics of genotypes varied in a wide range and BK013, GK131and GK132 had good table olive characteristics so that they had potential for registration as new table olive cultivar.Result of this research will be used final selection of breeding program and definition of characteristics of these olives for new cultivar certification by breeding researcher.

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REFERENCES

Ahmed C.B., Rouina B.B.&Boukhris, M. (2007). Effects of water deficit on olive trees cv. Chemlali under field conditions in arid region in Tunisia. Scientia Horticulturae, 113: 267-277.

Aponte M., Blaiotta G., La Croce F., Mazzaglia A., Farina V., Settanni L. & Moschetti, G. (2012). Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation. Food microbiology, 30: 8-16.

Bellini E., Giordani E., Parlati M.V. Pandolfi S., 2002. Olive genetic improvement: thirty years of research.Acta Horticulture, 586:105-108.

Bellini E., Giordani, E., &Rosati, A. (2008).Genetic improvement of olive from clonal selection to cross-breeding programs.Advances in Horticultural Science, 22(2), 73-86.

Bianco A., Ucella N., 2000. Biophenolic components of olives. Food Research International, 33:475- 485.

Brenes M., Rejano L., Garcia P., Sanchez A.H., Garrido A., 1995. Biochemical changes in phenolic compounds during Spanish-style green olive processing. Journal of Agricultural and Food Chemistry, 43(10):2702-2706.

Cemeroglu B., 2007. Food Analysis', Bizim Büro Publication. Ankara, Turkey.

Esti M, Cinquanta L, La Notte E (1998). Phenolic compounds in different olive varieties. Journal of Agricultural Food Chemistry, 46: 32- 35.

Gomez-Rico A., Fregapane G., Salvador, M.D., 2008. Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils. Food Research International, 41:433- 440.

International Olive Council. 2011. Guide for The Determination of the Characteristics of Oil-Olives. COI/OH/Doc. No 1. November 2011, Madrid.

Kailis S.G., Harris D., 2004. Establish protocols and guidelines for table olive processing in Australia. Rural Industries Research and Development Corporation Report, Pub. No: 04/136, Australia, 35-44.

Kailis S. G., Harris D., 2007. Producing table olives. Landlinks Pres., Australia, 76-82.

Lavee S., Avidan B., Ben-Ari G., 2014. Trends in breeding new olive varieties in Israel for quality and economic management. Agricultural Sciences, 5:701-709.

Lavee S., Avidan N., Meni Y., 2003. Askal', a new high-performing oil variety for intensive and super-intensive olive orchards. Olivae, 97:53–59.

Leal Sanchez V.G., Ruiz-Barba J.L., Sanchez A.H., Rejano L., Jimenez-Diaz R., Garrido A., 2003. Fermentation profile and optimization of green olive fermentation using Lactobacillus plantarum LPCO10 as a starter culture. Food Microbiology, 20:421- 430.

Leo´n L., de la Rosa R., 2007. Breeding for early bearing in olive. Hortscience, 42:499-502.

León L., De la Rosa R., Gracia A., Barranco D., Rallo L., 2008. Fatty acid composition of advanced olive selections obtained by crossbreeding. Journal of the Science of Food and Agriculture, 88(11):1921-1926.

Marsilio V., Seghetti L., Iannucci E., Russi F., Lanza B., Felicioni M., 2005. Use of a lactic acid bacteria starter culture during green olive (Olea europaea L cv Ascolana tenera) processing. Journal of the Science of Food and Agriculture, 85:1084- 1090.

Marsilio V., Campestre C., Lanza B., De Angelis M., 2001. Sugar and polyol compositions of some European olive fruit varieties (Olea europaea L.) suitable for table olive purposes, Food Chemistry, 72:485-490.

Medina E., Morales-Sillero A., Ramírez E.M., Rallo P., Brenes M.,Romero C., 2012. New genotypes of table olives: profile of bioactive compounds. International Journal of Food Science & Technology, 47(11):2334-2341.

Menz G., Vriesekoop F., 2010. Physical and chemical changes during the maturation of Gordal Sevillana olives (Olea europaea L., cv. Gordal Sevillana)', Journal of Agriculture and Food Chemistry, 58:4934-4938.

Morello J.R., Romero M.P., Motilva M.J., 2004. Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from Arbequina, Farga, and Morrut cultivars. Journal of Agriculture and Food Chemistry, 52:6002-6009.

Nergiz C., Engez Y., 2000. Compositional variation of olive fruit during ripening. Food Chemistry. 69:55-59.

Ozdemir Y., Guven E., Ozturk A., 2014. Understanding the characteristics of oleuropein for table olive processing. Journal of Food Processing & Technology, 5(5):1-6.

Ozdemir Y., Akcay M.E., Kurultay S., 2011. Melezleme ıslahı ile elde edlen iki amaçlı (sofralık ve yağlık) zeytin çeşit adaylarina genel bir bakış. Bahçe, 40:29-36.

Panagou E.Z., Tassou C.C., Katsaboxakis K.Z., 2002. Microbiological, physicochemical and organoleptic changes in dry salted olives of Thassos variety stored under different modified atmospheres at 4 and 20°C. International Journal of Food Science and Technology, 37:635-641.

Pereira J.A., Pereira A.P., Ferreira I.C., Valentão P., Andrade P.B., Seabra R., Bento A., 2006. Table olives from Portugal: phenolic compounds, antioxidant potential, and antimicrobial activity. Journal of Agricultural and Food Chemistry, 54(22):8425-8431.

Pritsa T.S., Voyiatzis D.G., Voyiatzi C.J., Sotiriou, M.S., 2003. Evaluation of vegetative growth traits and their relation to time to first flowering of olive seedlings. Australian Journal of Agricultural Research, 54:371-376.

Rallo L., 1995. Selection and breeding of olive in Spain. Olivae, 59:46–53

Rallo L., Barranco D., De la Rosa R., León L., 2008. 'Chiquitita'olive. HortScience, 43(2):529-531.

Roca M., León L., De la Rosa, R., 2011. Pigment metabolism of 'Sikitita' olive (Olea europaea L.): a new cultivar obtained by cross-breeding. Journal of Agriculture and Food Chemistry, 59:2049-2055.

Romero C., Brenes M., Yousfi K., García P., García A., Garrido A., 2004. Effect of cultivar and processing

method on the contents of polyphenols in table olives. Journal of Agricultural and Food Chemistry, 52(3):479-484.

Ross, F.A. (1959) 'Dinitrophenol Method for Reducing Sugars'. In Talburt, W. F., Smith, O., (eds.), Patato Processing , 469-470. AVI Publishing Comp., Connecticut.

Sabatini N., Mucciarella M.R. & Marsilio, V. (2008). Volatile compounds in uninoculated and inoculated table olives with Lactobacillus plantarum (Olea europaea L., cv. Moresca and Kalamata). LWT-Food Science and Technology, 41: 2017-2022.

Trigui A., 1996. Improving the quantity and quality of olive production in Tunisia: Unavoidable need and outlook for olive identification and breeding. Olivae, 61:34-40.

Tseng C.P., Montville T.J., 1992. Enzymatic regulation of glucose catabolism by Lactobacillus plantarum in an aerobic chemostat. Biotechnol. Progress, 18:126-131.

Turkish Standards Institute. (1992). TS 774. Turkish Table Olive Standard. Ankara.

Yalçinkaya E., Kaynaş N., Sütçü A.R., Fidan A.E., 2002. Olive cross breeding studies at Yalova–ACHRI. Actahorticulturae, 586:179-182.

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DETECTION OF GENETIC VARIABILITY IN *Pleurotus eryngii* USING TUBULIN-BASED POLYMORPHISM MOLECULAR MARKERS

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Abstract

Pleurotus eryngii is an edible mushroom with important biological functions, such as anticancer, antiviral, immune potentiating, and hypolipidemic activities. Due to its importance from economically, medicinal and ecologically point of view, a special attention was given to this species worldwide. Analyses via molecular markers revealed that Pleurotuseryngii is a species complex comprises at least six varieties (natural isolates and commercial strains). Improvement of culture technologies as well as the increase of biological compounds content involves a better knowledge of the genetic properties of each isolate, candidate for specific applications. For this reason, in this study several strains of Pleurotus eryngii collected from different geographical locations were analyzed in correlation with a wild Pleurotus ostreatus isolate, in order to determine the genetic variability and relationship between species. The genetic diversity of biological material was analyzed using a TBP (tubulin-based polymorphism) molecular marker that relies on the presence of intron-specific DNA polymorphisms of the plant β-tubulin gene family. For the fungal DNA extraction mycelium obtained on plates with PDA was used. Our results revealed that TBP amplification profile of intron 1 showed eight polymorphic bands while TBP amplification profile of intron 2 indicated nine polymorphic bands.The multiple length polymorphism of β-tubulin intron 2 generated amplification profiles which resulted highly specific for each of the analyzed species. However, these results suggested that TBP molecular markers could be used for highlighting the genetic diversity both intra- and inter-specific and the genetic diversity was closely related to the geographical distribution of this fungus.

Key words :*genetic variability, Pleurotus eryngii,* polymorphism, *β*-Tubulin, *TBP molecular markers.*

INTRODUCTION

Pleurotus eryngii, known as king oyster or king trumpet, is an edible mushroom native to regions of Europe, the Middle East, and North Africa but also commercially grown in Japan and the United States. It is the largest species of the oyster mushroom, it has a thick white stem and a small tan cap and is known for its robust, somewhat earthy flavour and more meaty texture. It is a mushroom with generous amounts of nutrients including carbohydrates, proteins, vitamins, minerals and fibres. Various studies revealed that *P. Eryngii* species complex has the most abundant population diversity in the genus *Pleurotus* (De Gioia et al., 2005).Assessment of genetic and phenotypic diversity is necessary to distinguish genotypes of *Pleurotus eryngii* with traits of interest and to identify strains with high yield potential (De Gioia et al., 2005; Zhao et al., 2013).Molecular markers are the tools most widely used to assess genetic diversity (Koebner et al., 2001; Karp et al., 1998). Some of these molecular markers correspond to unknown DNA almost associate within regions of unknown function sequences. Moreover, most of the identified changes do not occur in functionally relevant regions of DNA and thus rarely lead to identification of variations occurring within genes (Bardini et al., 2004). Inter-simple sequence repeats (ISSR) markers have been widely applied for analyses of genetic variance and population structure in many types of organisms (Kauserud and Schumacher, 2003; Wang et al., 2012).

Previous studies have shown that ISSR analyses only showed a relatively low genetic diversity among peanut cultivars despite abundant morphological, physiological, and agronomic variance (Raina et al., 2001). Another molecular marker system termed start codon targeted (SCoT) polymorphism, which is a simple and novel DNA marker system, could detect more polymorphisms compared with several other molecular marker systems (Collard and Mackill, 2009; Xiong et al., 2011). Thon and Royse (1999) developed a set of primers for β-tubulin genes of basidiomycetes and showed that these genes have potential for phylogenetic studies in the Basidiomycotina. Begerow et al. (2004) analyzed 36 fungal β-tubulin sequences to study the evolution of this gene and the phylogeny of basidiomycetes. The multifunctional and essential role of the tubulin proteins is reflected in the conservation of regions within their primary amino acid sequence. The TBP technique is depending on which intron or combination of introns is used as a marker relies on an exon-primed intron-crossing PCR reaction. Therefore, a combinatorial TBP (cTBP) that uses both intron 1 and intron 2 of the β-tubulin genes as the source for genomic polymorphism is expected to produce a greater number of molecular markers associated with each original gene locus. In our studies we tested cTBP method, in order to investigate the genetic variability in several commercial strains of *Pleurotus eryngii* mushrooms collected from different geographical locations and their genetic relationship with an indigenous *Pleurotus ostreatus* isolate. According to our knowledge no studies have been so far performed on TBP method applied to *Pleurotus eryngii* mushrooms.

MATERIALS AND METHODS

Mushrooms culture. Pure cultures of three strains of *P. eryngii: P. eryngii*2600 (Belgium), *P.eryngii* (Romanian producers), *P. eryngii* (German producers) and an indigenous *Pleurotus ostreatus* strain, were obtained by isolating tissue cultures from the fruiting bodies (fig. 1). A small piece of tissue was removed aseptically and transferred into a culture tube containing potato dextrose agar (PDA), and incubated in the dark at 25°C for 7-9 days. These samples were stored at 4°C until further used.

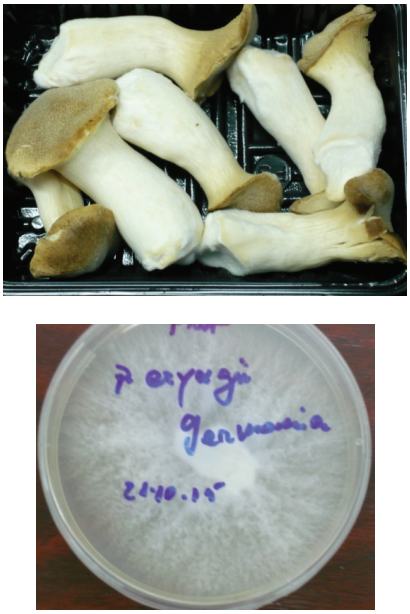

Figure1. Fruiting body and mycelium of *P.eryngii*

DNA isolation

Mycelia for DNA extraction were cultured on PDA Petri dishes with cellophane at 25°C for 5 days. The total DNA was extracted using a Plant Genomic DNA Miniprep Kit (Sigma) according to the protocol specified by the manufacturer. The purity and quality of the genomic DNA were determined through electrophoresis on 0.8 % agarose gel. The DNA solution was stored at -20°C.

TBP PCR Amplification

Intron1 and intron2of the *β*-tubulin gene family were PCR amplified using each 50 ng of template genomic DNA and the following forward and reverse oligonucleotide primers combination:
TBPfex1(5'AACTGGGCBAARGGNCAYTAYAC3');TBPrex1(5'ACCATRCAYTCRTCDGCRTTYTC-3') pair of primers for intron1, TBPfin2(5'GARAAYGCHGAYGARTGYATG3');TBPrin2(5'CRAAVCCBACCATGAARAARTG-3') pair of primers for intron2

(Brevario et al., 2007). PCRs (20 µL) were performed in 1× PCR buffer (10 mMTris.HCl (pH 8.8), 50 mMKCl, and 0.1% v/v Triton X-100), 0.2 mM each dNTP, 2.5 mM MgCl2, 50 ng of template DNA, 1 µM each primer, and 1 U *Taq*polymerase 360 (Promega). Following the initial denaturation step at 94 °C for 3 min, the PCR consisted of 35 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 90 s. The reactions were held at 15 °C after a final extension at 72 °C for 8 min (Bardini& al., 2004). Highlighting products was performed on agarose gel (1.5 %), staining with ethidium bromide and visualization under UV light using UVP BioDocIt system.

Data analysis. Bands generated by TBP-PCR amplification were scored as either present (1) or absent (0) across all accessions to build separate binary data matrices. The dendrogram was constructed with UPGMA cluster analysis using TREECON for windows software (version 1,3b).

RESULTS AND DISCUSSIONS

Molecular analysis results using the markers for intron 1 and intron 2 of β- tubulin gene, previously mentioned, showed polymorphism for both markers. Following TBP - PCR amplification, generated profile of intron1 exhibited 8 polymorphic bands. There were two polymorphic areas: one range between 320-500pb and other between 900 -1500pb (fig. 2)

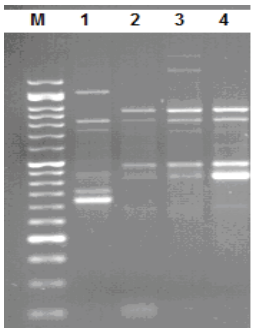

Figure 2. TBP - PCR amplification profile of intron1
M = Molecular marker size (GeneDirex 50 bp);
1= *Pleurotus ostreatus*(wild strain); 2= *P. eryngii* 2600 (Belgium); 3= *P. eryngii* (Romania);4= *P. eryngii* (Germany).

This polymorphism clearly separated *Pleurotus ostreatus* from *P. eryngii* strains. On the other hand, small polymorphic differences were observed between *P.eryngii* strains investigated. These results obtained with TBPfex1/TBPrex1 pair primers for intron1 suggests a polymorphism both at intra- and inter-species level. More information was obtained following TBP - PCR amplification of intron2 with TBPfin2/TBPrin2 pair of primers. Generated profile of intron2 of β- tubulin gene showed nine polymorphic bands (fig.3).

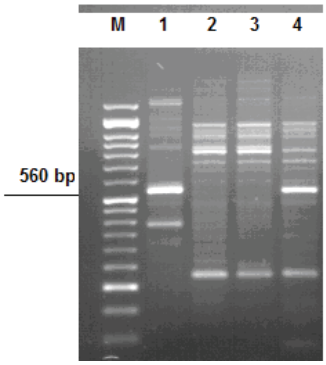

Figure 3. TBP - PCR amplification profile of intron2
M = Molecular marker size (GeneDirex 50 bp);
1= *Pleurotus ostreatus*(wild strain); 2= *P. eryngii* 2600 (Belgium); 3= *P. eryngii* (Romania); 4= *P. eryngii* (Germany)

Regarding the samples of *P. eryngii 2600* (Belgium) and *P. eryngii* originated from Romania we can observe that resulting amplification products showed high uniformity. This suggests that *P. eryngii* from Romania maybe identical with *P. eryngii 2600* from Belgium. But, considering the results obtained with TBPfex1/TBPrex1 pair primers for intron1 that show in *P. eryngii*–Romania sample two non-specific products (in the size of ~1000 - 1500bp) we can concluded there is a small genetic variability between the two *P. eryngii* strains. Contrariwise, in the case of *P. eryngii* originated from Germany we obtained a major amplification product, in the size of ~560 bp, which has similarity with an amplification product with the same size from the *P. ostreatus* isolate (fig. 3).

Clusters analysis

The genetic relationship between the studied *Pleurotus* strains is shown in dendrograms constructed with UPGMA cluster analysis based onpolymorphism for each intron, separately. Thus, UPGMA dendrogram based on polymorphism of intron1 shows two clusters: one includes *P. eryngii* 2600 (Belgium) and *P. eryngii*(Germany) strains, and one includes *P. eryngii* (Romania). The *P. eryngii* group belong to the same branch which shows us a low genetic diversity into this group (fig. 4).

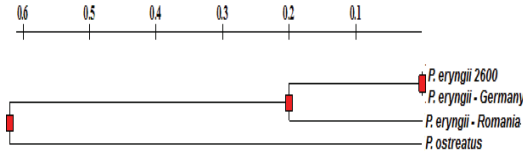

Figure 4. UPGMA dendrogram of *P. Eryngii* strains and *P. Ostreatus* isolate based on polymorphism for intron1.The scale bar means the genetic distance.

UPGMA dendrogram based on polymorphism of intron2 shows also two clusters, one includes *P. eryngii* 2600 (Belgium) and *P. eryngii* (Romania) and the other cluster include *P. eryngii*(Germany) (fig. 5).

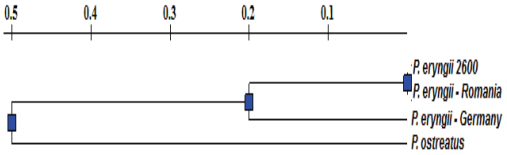

Figure 5. UPGMA dendrogram of *P. Eryngii* strains and *P. Ostreatus* isolate based on polymorphism for intron2.The scale bar means the genetic distance

When the analysis was performed with the amplicons obtained for both introns, clearer results were shown: based onthe molecular polymorphism it could be concluded that the *P.eryngii* commercial strains presented intraspecific differences at beta-tubulin gene level (fig. 6).

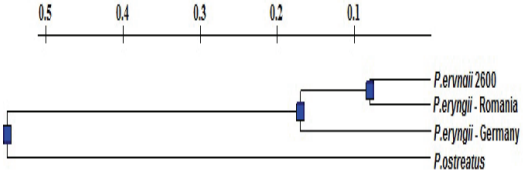

Figure 6. UPGMA dendrogram of *P.eryngii* strains based on the polymorphisms of introns 1 and 2

Moreover, significant interspecific diversity was observed that allow the idea that the primers for intron 1 and 2 of beta-tubulin gene could be used for identification of *Pleurotus* species. However, supplementary studies are necessary for the validation of this idea.Similar polymorphism levels were previously observed in other edible mushrooms, such as *Lentinulae dodes*(99.6%) (Xiao et al., 2010) and *Auricularia polytricha* (99.8%) (Du et al., 2011). Mushroom populations must be divided into groups according to their geographical origins which indicate that the genetic diversity is closely related to the geographical distribution (Zhao et al., 2013).

CONCLUSIONS

Based on the combined data from the cTBP patterns obtained in the PCR amplification with two pair of primers, our result showed a high similarity between *Pleurotus eryngii*2600 (Belgium) and *Pleurotus eryngii*(Romania) and a small genetic diversity between these strains and *Pleurotus eryngii* originated from Germany. This preliminary study regarding determination of genetic variability in *Pleurotus* genus using cTBP method suggests that this technique could be useful for species-specific identification. Whatever, in the future we will increase the number of the mushrooms species / strains that will be analyzed using cTBP technique. According to our knowledge this is the first study performed on *P. Eryngii* using TBP method, but future experiments with an increased number of strains are needed.

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REFERENCES

Bardini, M., Lee, D., Donini, P., Mariani, A., Gianì, S., Toschi, M., Lowe, C., Breviario, D., 2004. Tubulin-based polymorphism (TBP): a new tool, based on functionally relevant sequences, to assess genetic diversity in plant species. Genome, 47: 281.291.

Begerow, D., John, B., Oberwinkler, F., 2004. Evolutionary relationships among B-tubulin gene sequences of basidiomicetous fungi. Mycol. Res., 108 (11): 1257-1263.

Brevario, D., Baird, VMV., Sangoi, S., Hilu, K., Blumetti , P., 2007. High polymorphism and resolution in targeted fingerprinting with combined *b*-tubulin introns. Mol Breeding , 20:249–259

Collard, B.C.Y, Mackill, D.J., 2009. Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant MolBiol Rep 27: 86-93. doi: 10.1007/s11105-008-0060-5.

De Gioia ,T., **Sisto, D**., **Rana, G.L**., **Figliuolo, G**., 2005. Genetic structure of the Pleurotus eryngii species-complex. Mycol. Res., 109 (Pt1): 71-80.

D**u P, Cui BK, Dai YC., 2011.**High genetic diversity in wild culinary medicinal wood ear mushroom, *Auricularia polytricha*(Mont.) Sacc., in tropical China revealed by ISSR analysis. Int J Med Mushrooms 13: 289-297.

Karp, A., Peter, G., Ingram, I., and Ingram, D. 1998. Molecular tools for screening biodiversity. Chapman and Hall, London,U.K.

Koebner, R.M.D., Powell, W., and Donini, P. 2001. Contributions of DNA molecular marker technologies to the genetics and breeding of wheat and barley. Plant Breed. Rev. **21**: 181.220.

Kauserud, H., Schumacher, T., 2003. Genetic structure of Fennoscandian populations of the threatened wood-decay fungus *Fomitopsis rosea* (Basidiomycota). Mycol Res 2: 155-163. PubMed: 12747326.

Raina SN, Rani V, Kojima T, Ogihara Y, Singh KP et al., 2001.RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. Genome 44: 763-772. doi:10.1139/g01-064. PubMed: 11681599.

Thon, M.R., Royse, D.J., 1999. Partial beta-tubulin gene sequences for evolutionary studies in the Basidiomycotina. Mycologia, 91: 468-474.

Wang XM, Yang R, Feng SF, Hou XQ, Zhang YQ et al., 2012. Genetic variation in *Rheum palmatum* and *Rheum tanguticum* (Polygonaceae), two medicinally and endemic species in china using ISSR markers. PLOS ONE 7: e51667. doi:10.1371/journal.pone.0051667. PubMed: 23289054.

Xiao Y, Liu W, Dai YH, Fu C, Bian YB., 2010. Using SSR markers to evaluate the genetic diversity of *Lentinulaedodes*' natural germplasm in China. World J Microbiol Biotechnol 26: 527-536. doi:10.1007/ s11274-009-0202-4.

Xiong FQ, Zhong RC, Han ZQ, Jiang J, He LQ, et al., 2011. Start codon targeted polymorphism for evaluation of functional genetic variation and relationships in cultivated peanut (*Arachis hypogaea* L.) genotypes. MolBiol Rep 38: 3487-3494

Zhao M, Huang C, Chen Q, Wu X, Qu J, et al., 2013. Genetic Variability and Population Structure of the Mushroom *Pleurotus eryngii* var. *tuoliensis*. PLoS ONE 8(12): e83253. doi:10.1371/journal.pone.0083253.

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SCREENING FOR S-LAYER PRODUCTION BY SOME LACTOBACILLI FROM HOME-MADE FERMENTED FOODS

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Abstract

Production of surface-layer proteins has been described for several species of the genus Lactobacillus. They seem to be responsible for a sum of cell wall functions like protection against physico – chemical agents, adhesion, and aggregation among others. In this study, 15 strains of lactobacilli obtained from different fermented vegetables, cereals, and dairy productswere screened for S-layer production. Five strains were able to produce S-layer proteins, with a molecular mass between 40 and 55 KDa, as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis .Four of these strains were selected to test the influence of incubation temperature on the bacterial growth and S-layer production. Although the growth at 42°C was slower than at 37°C, similar amounts of S-layer proteins were produced. The proteins were efficiently extracted with 5M LiCl, especially from the cells grown at 42°C.

Key words*:fermented foods, lactobacilli, S-layer, SDS-PAGE.*

INTRODUCTION

Lactobacilli constitute an important group of lactic acid bacteria (LAB). American Food and Drug Administration classifies them as Generally Recognized as Safe (GRAS) microorganisms. Most species are found in natural habitats rich in carbohydrates, but also in the gastrointestinal tract of human and animal organisms (Slover and Danzige, 2008).
Home-made fermented foods have been shown to be rich sources for manylactic acid bacteria, including lactobacilii, with potential biotechnological application (Wouters et al. 2013, Grosu-Tudor et al. 2014). In Romania, such foods are still produced and consumed at a significant extent. Besides the fermented dairy products, Romanians use fermentation as a common method for cereals, vegetables, and fruits conservation, especially for the cold seasons, when fresh vegetables are hard to find.
Lactobacilli are commonly used as probiotics, therefore, they have to withstand different types of stress like high temperatures during product fabrication or harsh conditions inside gastrointestinal tract (low pH and bile salts, Champagne et al., 2002). This is possible because many *Lactobacillus* strains had developed a mechanism of defense making them suitable to be used as probiotics or in biotechnological industry (Tuohy et al., 2003).
Many *Lactobacillus* species possess at their surfaces an array of single, identical proteins, known as S-layer. This is a bi-dimensional crystalline structure of one, two, three, four or six (glycol-) protein subunits with oblique, tetragonal or hexagonal symmetry (Sleytr, 1997).In the case of lactobacilli, these subunits can be easily disintegrated using denaturing agents such as lithium chloride (LiCl), guanidine hydrochloride (GHCl), and re-assembled due to the non-covalent links (Sara and Sleytr, 2000). The S-layers of *Lactobacillus* species are formed by proteins with a molecular weight of 25 – 71 KDa (Åvall-Jääskeläinen and Palva, 2005), the smallest ones when compared to those from other bacteria, which can reach a molecular weight up to 200 KDa (Sleytr and Messner, 1983).

During the past years, S-layer has been characterized for several species of genus *Lactobacillus* and some features and functions have been assigned to it. These include protection role against physico-chemical factors (Engelhardt and Peters, 1998; Chami et al., 1997), aggregation, and adhesion (Åvall-Jääskeläinen and Palva, 2005) among others.
The aim of the present study was to select S-layer producing strains among some lactobacilli isolated from home-made fermented foods and to study the influence of the incubation temperature on the bacterial growth and S-layer production.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The LAB strains used in this study were isolated from Romanian artisan dairy products (9 strains, namely *Lb. brevis*46.5, *Lb. fermentum*419, *Lb. helveticus*34.9, *Lb. plantarum*6.10, 7.5, 16.1, 26.1, 35.1, 44.2; Zamfir et al. 2006,Grosu-Tudor et al. 2013), fermented vegetables (5 strains, namely *Lb. brevis*403, 530, *Lb. parabrevis*196, *Lb. plantarum*198, 619; Wouters et al. 2013), and fermented cereals (one strain, namely *Lb. oris*P49; Grosu-Tudor et al. 2014). All strains were long-termpreserved at -80°C in MRS medium in the presence of 25% (v/v) glycerol as cryo-protectant. Fresh cultures from the freeze-dried stocks were obtained by transferring twice each strain (2% v/v inoculum) in MRS medium followed by 24 h incubation at 37°C.

Screening for S-layer formation by SDS-PAGE analysis

One milliliter of an overnight culture (OD_{600nm}: 1.5) from each of the 15 LAB strains was centrifuged (10 000 x *g*, 10 min, 4°C) and the deposits were washed with distilled water and centrifuged again. The sediments were resuspended in 50 µl Laemli sample buffer and warmed up at 95°C for 5 minutes. After cooling, the suspensions were centrifuged again applying the same parameters and checked for S-layer proteins production using Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis(SDS-PAGE). The gels were prepared according to Laemmli (1970) method. Therefore, 10% (w/v) polyacrylamide running gel, and 4% (w/v) polyacrylamide stacking gel were used in a BiometraMinigel Twin (Biometra, Germany). Electrophoresis was conducted at a constant intensity of 40 mA in the stacking gel and 60 mA in the migration gel, respectively. Broad range protein molecular weight (MW) marker (10-225 KDa, Promega, USA) was used as reference. Gels were stained with Coomasie Brilliant BlueR250 (Carl Roth GmbH, Germany) to visualize the bands.

Growth and S-layer production by *Lactobacillus* strains

The effect of incubation temperature on the cell growth and S-layer proteins production was studied in *Lb. parabrevis*196, *Lb. brevis*403, *Lb. brevis*530 and *Lb. helveticus*34.9.
Active cultures of the selected strains were grown at two different temperatures (37°C, and 42°C) for 24h. Cell growth was determined by measuring the optical density at 600 nm(OD_{600nm}), pH and cell count (CFU/ml) using solid MRS medium (MRS supplemented with 1.5% (w/v) agar).

Extraction of S-layer proteins

Cells suspensions were obtained by inoculating 100 ml glass bottles containing 50 ml MRS with each strain(2% inoculation rate). The bottles were incubated at 37°C, and 42°C for 24 hours.The obtained bacterial biomass was separated by centrifugation as described before, washed twice with 10 ml of phosphate – buffered saline (PBS), pH = 7.4. The cell pellets were then resuspended in 5 ml of PBS and an equal volume of 5M LiCl was added in order to extract the S-layer proteins. After 2 hours at 37°C and occasionally shaking, the samples were centrifuged and the supernatants with the putative S-layer proteins were transferred to Vivaspin 6 ultrafiltration modules with a 10-kDa MM cut-off (Sartorius Stedim Biotech, Goettingen, Germany).Milli Q water was added over the retentate to a final volume of 5 ml and centrifuged again. Milli Q water was finally added over the retentate to a volume of 3 ml and transferred to a falcon tube. After a final centrifugation, the sediment representing the presumptive S – layer was resuspended in 60 µl Laemmli buffer(Laemmli,

1970). Both the sediment and supernatant were analyzed by SDS – PAGE.

RESULTS AND DISCUSSIONS

Detection of S-layer proteins

A total of 15 lactobacilli were evaluated for their ability to produce S-layer proteins. SDS-PAGE of whole protein extracts from fivestrains, namely *Lb. brevis* 403, 530 and *Lb. parabrevis* 196, isolated from fermented vegetables, and *Lb. helveticus* 34.9 and *Lb. brevis* 46.5 isolated from fermented dairy products, showed intense protein bands with molecular weightsin range of 40 - 55 kDa(figure 1)corresponding, most likely, to S-layer proteins (according to Åvall-Jääskeläinen and Palva, 2005). These protein bands are in the range of the molecular weights described for S-layer proteins from lactobacilli according to these authors. In addition, we observed variations of the molecular weightsbetween the species, but also between the particular strains of the same species (figure 1).

We observed intense formation of presumptive cell surface proteins in*Lb. brevis* 403 and 530, *Lb. parabrevis* 196, and *Lb. helveticus* 34.9. Consequently, these strains were selected for further studies.

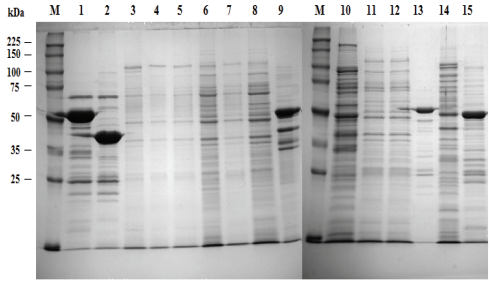

Figure 1. SDS-PAGE profiles of whole cell proteins from 15 LAB strains. Lanes: M: broad range protein molecular weight marker (Promega); 1: *Lb. brevis* 403; 2: *Lb. brevis* 530; 3: *Lb. plantarum* 619; 4: *Lb. plantarum*6.10; 5. *Lb. plantarum*7.5; 6: *Lb. plantarum* 16.1; 7: *Lb. plantarum* 35.1; 8: *Lb. plantarum* 44.2; 9: *Lb. helveticus* 34.9; 10: *Lb. fermentum* 419; 11: *Lb. plantarum* 26.1; 12: *Lb. oris* P49; 13.*Lb. parabrevis* 196; 14: *Lb. plantarum* 198; 15: *Lb. brevis* 46.5.

Growth of the selected strains

After 24 hours of incubation, lactobacilli cells belonging to the four selectedstrains grew well at 37°C reachingcell counts between $4.7x10^8$ and $5.6x10^9$ CFU/ml. The pH values dropped from 6.2 to 3.88 - 4.35 (Table 1). In the case of incubation at 42°C lactobacilli cultures obtained in the experimental conditions displayed a poor development comparing to incubation at 37°C. Cultures belonging to the strains*Lb. helveticus* 34.9 and *Lb. brevis* 530 displayed a mildlyreduced development, with a difference in cell counts of only 1 log CFU/ml comparing to the cell counts obtained at 37°C. These results show good tolerance to higher temperature for the strains *Lb. helveticus* 34.9 and *Lb. brevis* 530. Poor tolerance to higher temperature was marked for *Lb. brevis*403 with a significant decrease of cell counts (3 logs CFU/ml lower comparing to the cell counts obtained at 37°C). The poorest growth was observed for *Lb. parabrevis* 196 with a difference in cell counts of 5 logs CFU/ml comparing to the culture obtained at 37°C. These results indicate important sensibility of thisstrainto temperatures above 37°C.

Table1. Growth parameters of the selected bacterial strains

Temperature	*Strain*	*pH*	*CFU/ml*
37°C	FV 196	4.35	5.6×10^9
	FV 403	4.29	2.9×10^9
	FV 530	4.33	4.7×10^8
	RFF 34.9	3.88	1.7×10^9
42°C	FV 196	5.37	2.2×10^4
	FV 403	4.80	3.1×10^6
	FV 530	4.75	3.6×10^7
	RFF 34.9	4.11	3.3×10^8

Extraction of S-layer proteins

An intense band corresponding to about 40-55KDa, most likely the S-layer proteins, was observed for all tested strains, both grown at 37°C and 42°C (figure 2). Furthermore, after incubation of lactobacilli at 42°C, the bands corresponding to S-layer are similar to those obtained after incubation at 37°C. This points out that these four strains have the capability to produce S-layer both under the optimal cultivation temperature and under temperature stress conditions.

After the 5M LiCl treatment, the putative S-layer proteins were efficiently extracted from most of the strains. With a few exceptions (especially for strains grown at 37°C), in the cells subjected to LiCl extraction (for 2h), the band corresponding to the S-layer proteins was

not detected on SDS-PAGE. The corresponding band was, on the other hand, the major band inthe sediment recovered from the LiCl treatment and ultrafiltration. In some cases, the same bandwas also detected as the major band in the supernatant recovered from the treatment.

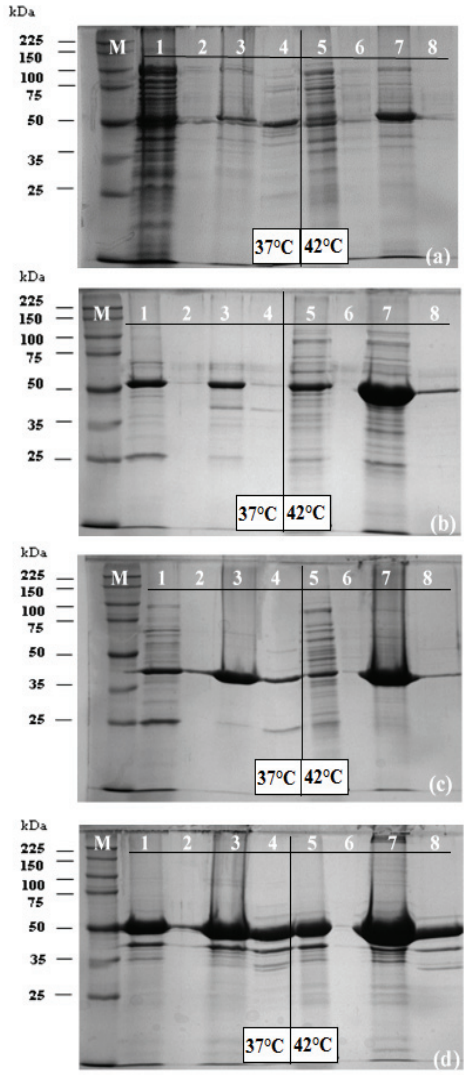

Figure 2. SDS-PAGE analysis of S-layer proteins of (a) *Lb. parabrevis* 196; (b) *Lb. brevis* 403; (c) *Lb. brevis* 530; (d) *Lb. helveticus* 34.9.
Lanes: M: Molecular weight marker; 1 and 5: cells before 5M LiCl treatment; 2 and 6: cells after 5M LiCl treatment (2h); 3 and 7: S-layer proteins extracted with 5M LiCl; 4 and 8: supernatant recovered after 5M LiCl extraction.

Overall, a better extraction was obtained for the strains cultivated at 42°C than 37°C. A possible explanation can be the ratio between the volume of 5M LiCl and the cell density in the suspensions used for extraction (significantly lower for the cultures obtained at 42°C compared with the ones at 37°C), but other processes might be responsible, too. Further studies will be made.

CONCLUSIONS

All four strains selected in our study showed the ability to produce cell surface-associated proteins with molecular weights in the range of 40-55 KDa. The proteins can be efficiently extracted using 5M LiCl. These proteins are produced in similar amounts when cells were grown at 37°C and 42°C and are, most likely, S-layer proteins, offering potential bio(nano)-technological applications to the producing strains.

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REFERENCES

Åvall-Jääskeläinen S., Palva A., 2005. Lactobacillus surface layers and their applications.FEMS Microbiological Reviews. 29: 511-529.

Chami M., Bayan N., Peyret J.L., Gulik-Krzywicky T., Leblon G., Shechter E., 1997. The S-layer protein of Coryne bacterium glutamicum is anchored to the cell wall by its C-terminal hydrophobic domain.Molecular Microbiology 23:483-492.

Champagne C.P., Ross R.P., Saarela M., Hansen K.F., Charalamopoulos D., 2002.Recommendations for the viability assessment of probiotics as concentrated cultures and in food matrices.Int. J.Food Microbiol. 2011: 185-193

Engelhardt H., Peters J., 1998. Structure research on surface layers: A focus on stability, surface layer homology domains, and surface layer-cell wall interactions. J. of Structural Biology. 124: 276-302.

Grosu-Tudor S.S., Stancu M.M., Pelinescu D., Zamfir M., 2014. Characterization of some bacteriocins produced by lactic acid bacteria isolated from fermented

foods. World J. Microbiol. Biotechnol.30: 2459-2469.

Grosu-Tudor S.S., ZamfirM., Van der Meulen R., De Vuyst L., 2013. Isolation of novel homopolysaccharide producing lactic acid bacteria from Romanian raw milk and fermented dairy products. Eur. Food Res. Techno. 237: 609-615.

Laemli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Sara M., Sleytr U.B., 2000. S-layer proteins. J. Bacteriol. 182: 859-868.

Sleytr U.B., Messner P., 1983. Crystalline surface layers on bacteria. Annual Review of Microbiol 37: 311-339.

Sleytr, U.B., 1997. I. Basic and applied s-layer research: an overview. FEMS Microbiol.Reviews. 20: 5-12.

Slover C.M., Danziger L., 2008. Lactobacillus: a review. Clin.Microbiol.Newslett. 30: 23-27.

Tuohy M., Probert H.M., Smejkal C.W., Gibson G.R., 2003. Using probiotics and prebiotics to improve gut health.Drug Discov.Today. 8:692-700.

Wouters D., Grosu-Tudor S.S., Zamfir M., De Vuyst L., 2013. Bacterial community dynamics, lactic acid bacteria species diversity and metabolite kinetics of traditional Romanian vegetable fermentations.J. Sci. Food Agriculture 93: 749-760.

Zamfir M., Vancanneyt M., Makras L., Vaningelgem F., Lefebvre K., Pot B., Swings J., De Vuyst L., 2006.Biodiversity of lactic acid bacteria in Romanian dairy products. Syst. Appl. Microbiol. 29: 487-495.

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EFFECT OF EXTRACTION VARIABLES ON THE OMEGA-3 EICOSAPENTAENOIC ACID (EPA) CONTENT OF (*Nannochloropsis oculata*) MICROALGA OIL

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Abstract

Microalgae are a recognized source of fatty acids and fatty acid-based lipids of potential interest in preparation of functional health products. Unlike terrestrial crops, these photoautotrophic microorganisms can directly produce polyunsaturated fatty acids (PUFA) and, although microalgae are not suitable for direct human consumption, their nutritional value can also be exploited if added to animal feeds. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) belongs to the omega-3 fatty acids group. In recent years, omega-3 fatty acids rich oil has attracted much attention because of its recognized beneficial effect on human health. In this study, response surface methodology was used to investigate the effect of ultrasound-assisted extraction variables including extraction temperature (25-6°C), extraction time (30-90 min.) and solvent: microalga ratio (10:1-30:1 ml:mg) on the omega-3 EPA content of N. oculata microalga. The experimental results showed that the extraction temperature and time were the significant parameters for the EPA rich oil extraction, while the solvent:microalga ratio was insignificant. The optimum oil extraction parameters for the maximum omega-3 EPA content were as follows: extraction temperature, 27.6°C; extraction time, 34.00 min. and solvent:microalga ratio, 21:3 ml:mg. Under the above predicted optimum conditions, the experimental oil yield and omega-3 EPA content were 62.8 % and 16.25%, respectively. The drying of algal biomass by freeze dryer improved the green color intensity and omega-3 EPA content.

Key words*: Omega-3 EPA, oil extraction, optimization, microalga oil, N. oculata.*

INTRODUCTION

The important health benefits are associated with omega-3 polyunsaturated fatty acids (PUFA) particularly with eicosapentaenoic (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*:3) (Gogus & Smith, 2010). Traditionally, main commercial omega-3 fatty acids source is fish. Concerns about the potential danger of contaminants such as mercury, however, often discourage people from eating fish. Another more recently recognized and serious issue is the global decline in wild-harvest fish stocks. Thus, new sources of omega-3 fatty acids must be found in order to reply this growing omega-3 rich oil demand. Microalgae, krill and genetically modified crops are considered as alternative omega-3 fatty acids rich oil sources (Ryckebosch, Bruneel, Termote-Verhalle, Goiris, Muylaert, & Foubert, 2014). Marine microalga *Nannochloropsis oculata* possesses valuable nutrients particularly omega-3 EPA for human health (Lee, Chuang, Su, & Wu, 2013). *N. oculata* contains high amount omega-3 EPA with as much as 5% of its dry biomass (Khozin-Goldberg & Boussiba, 2011). Besides, due to its high omega-3 PUFA levels and its ability to grow easily and rapidly, *N. oculata* is commonly utilized as feed to rear rotifers that are then fed to marine finfish larvae for growth transformation (Chaturvedi, Uppalapati, Alamsjah, & Fujita, 2004). Extraction is one of the fundamental processing steps used for recovering oil from microbial cell for the microalga oil industrialized production. Several methods such as acid heating, autolyze, Soxhlet's, organic solvent, supercritical fluid extraction, mechanical presses, and enzymatic oil extraction have been used to extract the algal oil from the microbial cell and plant materials (Zhou, Zhu, & Ren, 2013). Among these, ultrasound-assisted extraction (UAE) is an alternative to conventional extraction techniques with its inherent advantages (reduction of extraction time, solvent volume,

energy and better extraction efficiency). The enhancement of extraction obtained by using ultrasound is mainly attributed to the effects of acoustic cavitations produced in the solvent by the passage of an ultrasonic wave (Topuz, Gokoglu, Yerlikaya, Ucak, & Gumus, 2015).
In this study, response surface methodology was used to investigate the effect of ultrasound-assisted extraction variables ncluding extraction temperature (25-65°C), extraction time (30-90 min) and solvent: microalga ratio (10:1-30:1 ml:mg) on the omega-3 EPA content of *N. oculata* microalga.

MATERIALS AND METHODS

Materials
The microalga (*N. oculata*, CCMP525) was obtained from NCMA Bigelow lab (East Boothbay, Maine, USA). It was cultivated in 50 lt polyetilen bubble column photobioreactor at a temperature 24±1°C. The culture was enriched with f/2 medium (Guillard, 1975) that is widely used for marine algae, and composed of (mg/l): $NaNO_3$, 75; NaH_2-$PO_4.H_2O$, 5; $Na2SiO_3.9H_2O$, 30; Na_2EDTA, 4.36; $CoCl_2.6H_2O$, 0.01; $CuSO_4.5H_2O$, 0.01; $FeCl_3.6H_2O$, 3.15; $MnCl_2.4H_2O$, 0.18; $Na_2MoO_4.2H_2O$, 0.006; $ZnSO_4.7H_2O$, 0.022; Thiamine HCl, 0.1; Biotin, 0.0005; B_{12}, 0.0005. The ultra-filtrated water with salinity of 25 g/l was used for cultivation. Air was aerated with a flow rate of 250 ml/min for about 20 days continuously. The growth was monitored by counting the cells with the help of Neubauer haemocytometer. The cultured microalgal biomass was concentrated by centrifugation (4000×g, 5 min) and then freeze dried to final water activity of aw: 0.6.

Ultrasound-assisted extraction of omega-3 EPA rich microalgal oil
Hexane was selected as oil extracting solvent according to preliminary trial and study of dos Santos, Moreira, Kunigami, Aranda, and Teixeira (2015). Ultrasound-assisted extraction was performed in an ultrasonic bath. Frequency of ultrasonic bath was fixed at 250 W, 40 kHz and microalga sample was placed into a volumetric flask, made up to volume with the extracting solvent and sonicated for different times at the required temperature. After the extraction, the flask was transferred and cooled to room temperature. The algal oil extract were filtered through Whatman no:1 filter paper. Solvent was evaporated with rotary evaporator at 45ºC and algal oil rich extract was obtained.

Experimental design
In order to obtain the optimal conditions for the extraction of omega-3 EPA rich oil from *N. oculata* biomass and examine the effect of solvent:microalgae ratio, extraction temperature and time on the yield and omega-3 EPA content, a three-variable, three-level Box & Behnken Design (Box & Behnken, 1960) was applied in a response surface methodology (RSM) study by generating second-order polynomial equations (Eq. 1):

$$Y = \beta_0 + \Sigma\beta_i X_i + \beta_{ii} X^2 i + \Sigma\beta_{ij} X_i X_j$$

Where Y represents the experimental response, β_0, β_i, β_{ii} and β_{ij} are constants and regression coefficients of the model, and X_i and X_j are uncoded values of independent variables. The experimental design variables and responses are represented in Table 1. The responses obtained from the experimental design were subjected to multiple nonlinear regressions using the software Design-Expert 9.0 (State-Ease, MN, USA) to obtain the coefficients of the second polynominal model.

Table 1. Box & Behnken's experimental design and responses

	Parameters*			Responses	
Test	X_1 (°C)	X_2 (Min.)	X_3 (ml:mg)	Oil yield (%)	Omega-3-EPA (%)
A_1	25	60	10	63.92	16.18
A_2	25	30	20	65.36	16.25
A_3	25	60	30	65.85	16.09
A_4	25	90	20	66.14	15.94
A_5	45	90	30	71.54	15.08
A_6	45	30	30	68.65	15.77
A_7	45	60	20	69.42	15.63
A_8	45	60	20	69.61	15.58
A_9	45	30	10	66.43	15.86
A_{10}	45	60	20	71.16	15.47
A_{11}	45	90	10	71.45	15.32
A_{12}	65	30	20	73.18	15.02
A_{13}	65	90	20	75.60	14.11
A_{14}	65	60	30	74.15	14.45
A_{15}	65	60	10	73.67	14.58

*X_1:Extraction temperature, X_2: Extraction time, X_3: Solvent::microalgae ratio

Analyses

Oil content and oil yield analyses
Total lipid content analysis was performed according to method of Bligh and Dyer (1959). Lipid yield was calculated as below:

LY (%): (L_1 x 100)/L_0)

Where LY represents the lipid yield, L_0 is total lipid content of algal and L_1 is lipid content of algae extracts.

Fatty acid composition analysis
Methyl esters were prepared by transmethylation using 2 M KOH in methanol and n-hexane, according to the method of (Özogul and Özogul, 2007). The fatty acid composition was analyzed by a gas chromatography device (Clarus 500 Perkin-Elmer, USA) equipped with a flame ionization detector and a fused silica capillary SGE column (30 m x 0.32 mm ID x 0.25 µm BP20 0.25 UM, USA). The fatty acid composition analyses were performed in triplicate and the results were given in chromatography area % as mean values.

Optical microscopy
Optical microscopy images were taken from surface of algae extract residue using stereo microscope.

Statistical analysis
Data were subjected to multiple nonlinear regression using the software Design-Expert 9.0 (State-Ease, Inc., Minneapolis, USA), to obtain the coefficients of the second polynomial model.

RESULTS AND DISCUSSIONS

The effect of variables on the extraction of lipids from N. oculata biomass
Total lipid content of *N. oculata* dried biomass was 31.07 g/100 g. Table 1 shows the experimental conditions and the results of lipid extractions according to design. The extracted total lipid yield of alga biomass ranged between 63.9 and 75.6%. Maximum lipid yield (75.6%) was determined under the experimental parameters of extraction temperature of 65 °C, extraction time of 90 min and solvent:alga ratio of 20:1 (ml:mg). The statistical analysis revealed that extraction temperature and extraction time significantly affected total lipid yield (P<0.05), while solvent:alga ratio had no effect on the lipid yield.

The effect of variables on the omega-3 EPA content of N. oculata.
The omega-3 EPA content of lipids extracted from *N. oculata* biomass ranged between 14.1 and 16.3% of algal lipid. Maximum omega-3 EPA content was found under the experimental parameters of extraction temperature of 25°C, extraction time of 30 min. and solvent:alga ratio of 20:1 (ml:mg) (Table 1). The extraction temperature and extraction time significantly affected omega-3 EPA content of algal biomass (P<0.05), while solvent:alga ratio had no effect on the EPA content of algal lipid. Fig. 1 shows the effect of extraction temperature and extraction time on the omega-3 EPA content of extracted algal lipid. Decreasing extraction temperature and time at the moderate solvent:alga ratio (20:1, ml:mg) significantly increased the omega-3 EPA content of extracted algal oil.

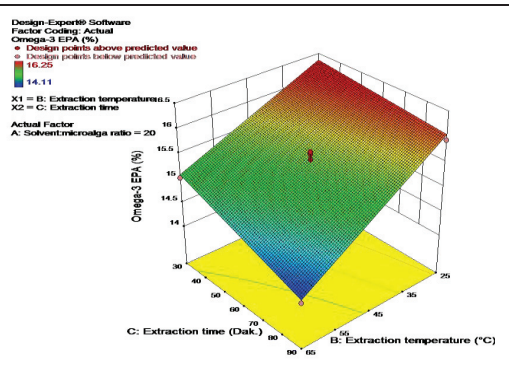

Figure 1. Effect of extraction temperature and extraction time on the omega-3 EPA content.

Fig. 2 shows the effect of extraction temperature and solvent:microalga ratio on the omega-3 EPA content of extracted algal lipid. Decreasing of extraction temperature at moderate extraction time (60 min) and solvent ratio (20:1, ml:mg) significantly increased omega-3 EPA content, whereas increasing of solvent:alga ratio did not increased during extraction (Fig. 2).

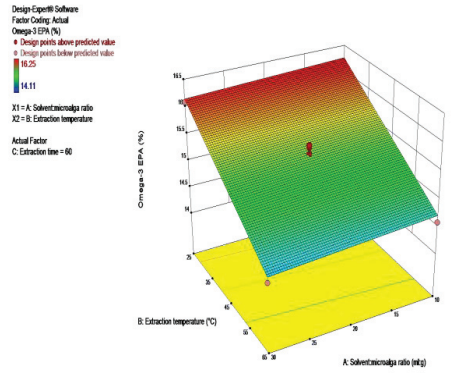

Figure 2. Effect of extraction temperature and solvent:alga ratio on the omega-3 EPA content.

Fig. 3 shows the effect of extraction time and solvent:microalga ratio on the omega-3 EPA content of extracted algal lipid. Both increasing of extraction time and solvent:alga ratio did not change the omega-3 EPA content of extracted lipid of *N. oculata* (Fig.3).

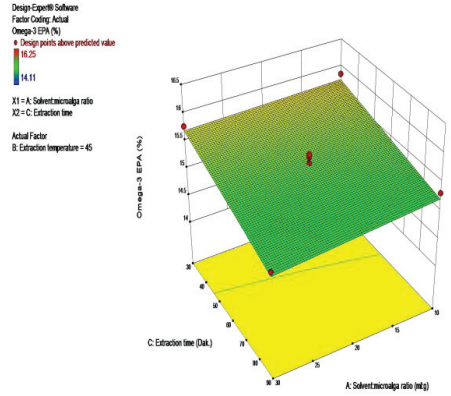

Figure 3. Effect of extraction time and solvent:alga ratio on the omega-3 EPA content.

Optical microscopy images

As clearly seen in optical microscopy images given in Fig. 4, freeze dried extraction residue kept its green color after the *extraction* (Fig. 4a). Drying of concentrated *N. oculata* biomass with heat treatment turned its green color to yellowish (Fig. 4b) and dark brown color (Fig. 4c). Drying of algal biomass in conventional oven made dried algal cell more solid and fragile with irregular shape.

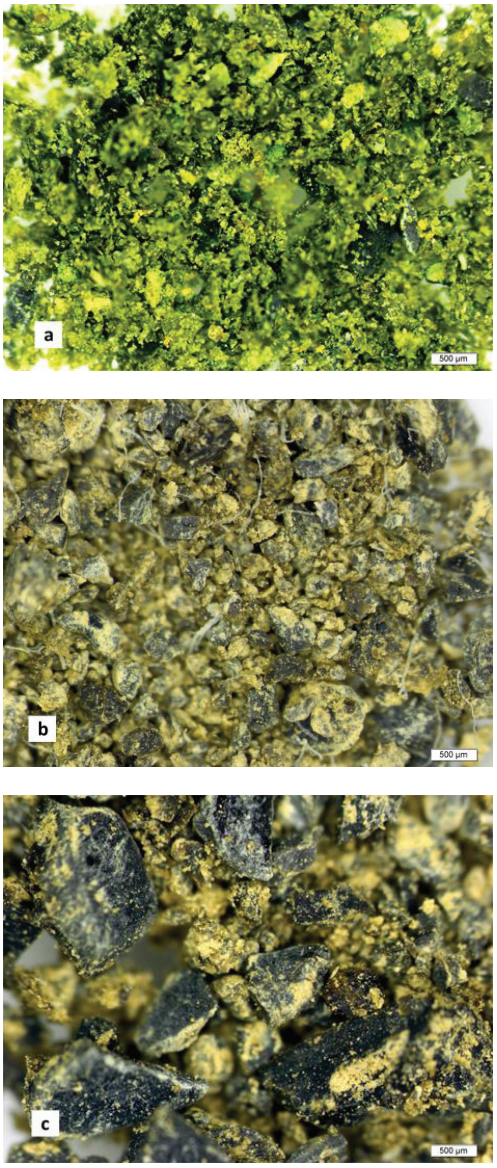

Figure 4. Optical microscopy images of dried and lipid extracted algal biomass

Optimum lipid extraction conditions

The optimum extraction conditions were determined and used for calculating the predicted values of response variables using the prediction equations derived by response surface methodology. Verification experiments performed at the predicted conditions derived

from ridge analysis of RSM demonstrated that experimental values were reasonably close to the predicted values confirming the validity and adequacy of the predicted models. The optimum conditions obtained using the model for maximum omega-3 EPA content of *N. oculata* lipid was follows: extraction temperature, 27.67°C; extraction time, 34 min and solvent alga ratio, 21.3:1 (Table 2). Under these conditions, the model predicted maximum extraction yield and omega-3 EPA content were 62.85 of 16.25%, respectively. To verify the predicted result with the practical value and compare to biomass drying methods including, conventional oven drying (ı), fluidized bed drying (ıı) and freeze drying (ııı), new extractions at optimal conditions were performed. Results of experimental extractions carried out in optimum conditions were given in Table 2. Highest omega-3 EPA content and lowest extraction yield were determined in freeze dried samples (Table 2). Both extraction yield and omega-3 EPA content of samples were significantly (P<0.05) affected biomass drying methods. Highest extraction yield of BD sample could be stemmed from fluidized bed drying methods which allows heat treatment in a short time. However, omega-3 EPA content of BD samples was at a moderate level in comparison with FD and OD samples. If both freeze and fluidized bed drying methods are important in alga biomass drying, a combination of fluidized bed drying and freeze drying treatments might be more effective than conventional oven treatment.

Table 2. Optimum conditions for omega-3 EPA rich algal lipid extractions from *N. oculata* microalga

Optimum conditions*			Responses**					
X_1 (°C)	X_2 (Min.)	X_3 (ml:mg)	Extraction yield (%)			Omega-3 EPA content (%)		
			FD	BD	OD	FD	BD	OD
27.67	34.00	21.34	63.17±0.35^{C}	68.49±0.55^{A}	65.82±0.87^{B}	17.28±0.21^{A}	16.75±0.29^{B}	16.19±0.32^{C}

*X_1:Extraction temperature, X_2: Extraction time, X_3: Solvent::microalgae ratio
**FD: Freeze dried biomass, BD: Fluidized bed dried biomass, OD: Oven dried biomass. Means±standard deviation (n:3). Means with different capital (A, B, C) in rows are significantly different (P<0.05).

CONCLUSIONS

The ultrasound-assisted extraction of omega-3 EPA rich lipid from dried *N. oculata* biomass was performed with a three-variable, three levels Box-Behnken design based on the RSM. The experimental results showed that the extraction temperature and extraction time were the major contributing factor to extraction of algal lipids from *N. oculata*.

The drying of algal biomass by freeze dryer improved the green color intensity and omega-3 EPA content.

Although the drying of biomass by fluidized bed dryer maximized the extraction yield, its omega-3 EPA content was lower than its freeze dried. Considering industrial demand for 'minimal processed' products, freeze drying and fluidized bed drying methods could be served as an effective biomass drying methods.

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REFERENCES

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology, 37*(8), 911-917.

Box, G. E., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics, 2*(4), 455-475.

Chaturvedi, R., Uppalapati, S. R., Alamsjah, M. A., & Fujita, Y. (2004). Isolation of quizalofop-resistant mutants of Nannochloropsis oculata (Eustigmatophyceae) with high eicosapentaenoic acid following N-methyl-N-nitrosourea-induced random mutagenesis. *Journal of Applied Phycology, 16*(2), 135-144.

dos Santos, R. R., Moreira, D. M., Kunigami, C. N., Aranda, D. A. G., & Teixeira, C. M. L. L. (2015). Comparison between several methods of total lipid extraction from Chlorella vulgaris biomass. *Ultrasonics Sonochemistry, 22*, 95-99.

Gogus, U., & Smith, C. (2010). n-3 Omega fatty acids: a review of current knowledge. *International Journal of Food Science and Technology, 45*(3), 417-436.

Guillard, R. R. (1975). Culture of phytoplankton for feeding marine invertebrates. In *Culture of marine invertebrate animals*, (pp. 29-60): Springer.

Khozin-Goldberg, I., & Boussiba, S. (2011). Concerns over the reporting of inconsistent data on fatty acid composition for microalgae of the genus Nannochloropsis (Eustigmatophyceae). *Journal of Applied Phycology, 23*(5), 933-934.

Kirschbaum, J., Krause, C., & Brückner, H. (2006). Liquid chromatographic quantification of synthetic colorants in fish roe and caviar.

European Food Research and Technology, 222(5-6), 572-579.

Lee, Y. L., Chuang, Y. C., Su, H. M., & Wu, F. S. (2013). Freeze-dried microalgae of Nannochloropsis oculata improve soybean oil's oxidative stability. *Applied Microbiology and Biotechnology, 97*(22), 9675-9683.

Özogul, Y., & Özogul, F. (2007). Fatty acid profiles of commercially important fish species from the Mediterranean, Aegean and Black Seas. *Food Chemistry, 100*(4), 1634-1638.

Ryckebosch, E., Bruneel, C., Termote-Verhalle, R., Goiris, K., Muylaert, K., & Foubert, I. (2014). Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. *Food Chemistry, 160*, 393-400.

Stodołnik, L., Salacki, M., & Rogozińska, E. (1992). Changes in sheath properties of rainbow trout eggs during cold and frozen storage. *International journal of refrigeration, 15*(5), 270-275.

Topuz, O. K., Gokoglu, N., Yerlikaya, P., Ucak, I., & Gumus, B. (2015). Optimisation of antioxidant activity and phenolic compound extraction conditions from red seaweed (Laurencia obtuse). *Journal of Aquatic Food Product Technology*(just-accepted).

Yusop, S. M., O'Sullivan, M. G., Preuss, M., Weber, H., Kerry, J. F., & Kerry, J. P. (2012). Assessment of nanoparticle paprika oleoresin on marinating performance and sensory acceptance of poultry meat. *Lwt-Food Science and Technology, 46*(1), 349-355.

Zhou, C., Zhu, C., & Ren, Y. (2013). Optimization of ultrasound-assisted extraction of single cell oil from mortierella isabellina. *Separation Science and Technology, 48*(14), 2188-2195.

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ALGAL OIL: A NOVEL SOURCE OF OMEGA-3 FATTY ACIDS FOR HUMAN NUTRITION

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Abstract

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) a polyunsaturated fatty acid (PUFA) that belongs to the omega-3 fatty acids group. In recent years, omega-3 fatty acids rich oil has attracted much attention because of its recognized beneficial effect on human health. At present, fish oil is the major source of omega-3 fatty acids, but omega-3 fatty acids rich oils can be produced by microalgae with additional commercial benefits. Consumption of omega-3 rich fish oil has been steadily increasing for decades due to their health benefits. Microalgal oil might be the most promising alternative to fish oil, since they are the primary producers of omega-3 fatty acids. Recent advances regarding production of omega-3 fatty acid rich oil from microalgae have been reviewed with an emphasis on the scientific data. At first, description of the omega-3 fatty acids, biosynthesis pathways and their role in the human health is presented. Microalgae are the initial source of omega-3 fatty acids. Microalgae species intensively used in omega-3 fatty acids rich algal oil production and their culturing conditions were reviewed in this paper. The algal oil extraction and refining process are also presented. We present here a review of the most recent advances regarding the production of omega-3 fatty acid rich algal oil from the marine origin microalgae.

Key words*: Omega-3 fatty acids, docosahexaenoic acid, eicosapentaenoic acid, microalgae, algal oil.*

INTRODUCTION

Polyunsaturated fatty acids (PUFA), specifically docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been linked to several health benefits (Armenta & Valentine, 2013; Covington, 2004). Several studies have suggested there is positive health effects consumption of DHA and EPA, such as lowering the risk of vision loss due to eye macular degeneration, reduction of blood vessel stiffness, and relief of anxiety and inflammation (Ho, van Leeuwen, Witteman, van Duijn, Uitterlinden, Hofman, et al., 2011; Kiecolt-Glaser, Belury, Andridge, Malarkey, & Glaser, 2011; Pase, Grima, & Sarris, 2011). DHA consumption has been associated with reducing risk of colds in babies from moms that took this dietary supplement (Imhoff-Kunsch, Stein, Martorell, Parra-Cabrera, Romieu, & Ramakrishnan, 2011). All the latter, plus numerous studies that suggest there are health effects for the heart and brain, and many other benefits for treating several illnesses and diseases including asthma, rheumatoid arthritis, schizophrenia, depression, multiple sclerosis, migraine headaches, etc (Gray, 2011). Even low DHA levels have been associated to suicide risk (Armenta & Valentine, 2013; Lewis, Hibbeln, Johnson, Lin, Hyun, & Loewke, 2011).

The major sources of EPA and DHA in food and dietary supplements were found in fatty fish, fish products, marine oils, and certain algae oils (Andız & Ünlüsayın 2015). At present, the major resource of omega-3 fatty acids for human nutrition is marine fish oils. However, their reserves are dwindling, and supplies via wild fish fisheries are limited. Moreover, omega-3 fatty acid content of oil from wild-caught fish varies with species, location, water depth and temperature, seasonal climatic conditions, and availability and type of primary food chain (Khozin-Goldberg, Iskandarov, & Cohen, 2011). A variety of fish species such as herring, mackerel, sardine and salmon are regarded as good sources of omega-3 fatty acids. Due to the many shortcomings of fish-derived oil including undesirable taste and odour, diminishing supplies, objections by vegetarians, its chemical processing methods, and the presence of contaminants such as

mercury, dioxins and polychlorinated biphenyls (Certik & Shimizu, 1999; Hooper, Thompson, Harrison, Summerbell, Ness, Moore, et al., 2006), research has been diverted towards the exploitation of other marine species for the development of a suitable and sustainable alternatives (Gupta, Barrow, & Puri, 2012). Microalgae offer a promising non-polluted resource for biotechnology and bioengineering of omega-3 fatty acid oil production, as an alternative to fish oil. Compared to terrestrial crop plants, microalgae present a few advantages as omega-3 fatty acids sources, such as commonly occurring genes for the biosynthesis of these nutrients, simpler acid profiles and higher growth rates (D. A. Martins, Custodio, Barreira, Pereira, Ben-Hamadou, Varela, et al., 2013). Most of oil producing microalgae studied within the past decade have been eukaryotes; and have been found worldwide, both along coastlines and in the open ocean. Using microalgae to produce omega-3 fatty acid rich algal oil is still a relatively new field, and research on this area has been growing significantly within the last few years (Armenta & Valentine, 2013). Studied microalgae include *Nannochloropsis oculata (Pal, Khozin-Goldberg, Cohen, & Boussiba, 2011) Pavlova lutheri*, *Odontella aurita* (Guihéneuf, Fouqueray, Mimouni, Ulmann, Jacquette, & Tremblin, 2010), *Schizochytrium* sp. (I. Fedorova-Dahms, P. A. Marone, M. Bauter, & A. S. Ryan, 2011), *Crypthecodinium cohnii* (Mendes, Reis, Vasconcelos, Guerra, & da Silva, 2009), *Ulkenia* sp. (Quilodrán, Hinzpeter, Hormazabal, Quiroz, & Shene, 2010). This review focus on recent advances made in biotechnological production of omega-3 fatty acids rich oil from microalgae alternative to fish oil.

Omega-3 fatty acids

Polyunsaturated fatty acids (PUFAs) constitute a large group of fatty acids containing long chain carbonic molecules that include ω-3 and ω-6 fatty acids. Omega 'ω' is the position of the first double bond when counted from the methyl end and the number '3' refers to the number of carbon atoms at that position from the methyl end. The molecular structure of the fatty acids consists of an even number of carbon atoms (4 to 24) with diverse saturations (0 to 6 double bonds) (Gupta, Barrow, & Puri, 2012). Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are two members of the ω-3 family (Figure 1).

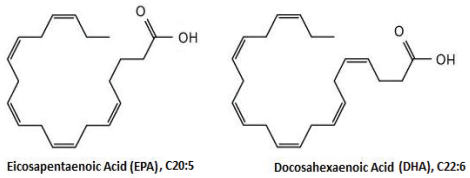

Figure 1. Structure of common omega-3 fatty acids: DHA and EPA.

Biosynthesis pathways of EPA and DHA are shown in Figure 2. The prefixes 'docosa' and 'eicosa' are of Greek descent, meaning the 22 and 20 C atoms present in DHA and EPA, which contain 6 and 5 cis-double bonds respectively (Lozac'h, 1986). PUFAs rich in omega-6 fatty acids originate from terrestrial plants are consumed in higher abundance than those from fish, so that plant origin ω-6 fatty acids are consumed in higher quantities than ω-3 fatty acids (Newton, 1998). This over consumption of ω-6 relative to ω-3 oil has been linked to increased risk of cancer, diabetes, cardiovascular and neurodegenerative diseases (A. Simopoulos, 2006). To restore a balance, consumption of ω-3 fatty acids should be increased compared to ω-6 fatty acids. ω-6 to ω-3 PUFA ratios of between 5:1 and 3:1 have been suggested as optimum for human consumption (A. P. Simopoulos, 2008).

Omega-3 fatty acids rich microalgae

In the marine food system, polyunsaturated fatty acids are primarily formed by phytoplankton and transferred on to herbivorous zooplankton, hence affecting food quality for organism at higher trophic levels. Various photoautotrophic and heterotrophic marine species from different classes produce EPA and DHA omega-3 fatty acids.

According to recent reviews of total lipid extracts, *Bacillariophyceae* (diatoms) and *Chrysophyceae* species may be rich sources of EPA and DHA; *Cryptophyceae, Prasinophyceae, Rhodophyceae, Xanthophyceae, Glaucophyceae* and *Eustigmatophyceae* can represent interesting

EPA sources, whereas DHA is found in significant amounts mostly in *Dinophyceae*, *Prymnesiophyceae*, and *Euglenophyceae* (Lang, Hodac, Friedl, & Feussner, 2011; D. A. Martins, et al., 2013).

These organisms offer a promising vegetative and non-polluted resource for biotechnology and bioengineering of omega-3 fatty acids rich algal oil production as an alternative to fish oil.

Currently, the production of omega-3 fatty acids by marine microorganisms is the subject of intensive research and increasing commercial attention (L. Sijtsma & M. E. de Swaaf, 2004). Several photosynthetic and heterotrophic marine microalgae are considered as a good source of omega-3 fatty acids (EPA and DHA) for the commercial production of algal oil (Table 1).

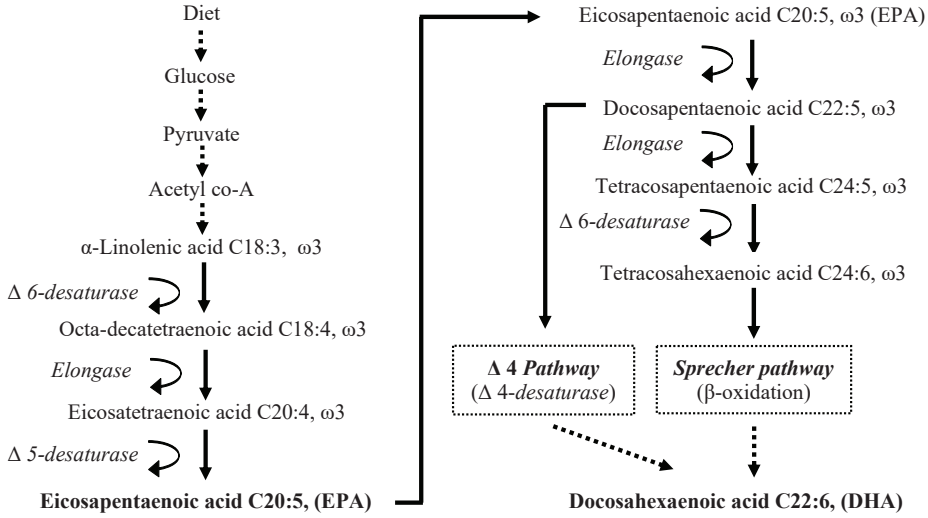

Figure 2. Biosynthesis pathways of EPA and DHA ω-3 fatty acids.

Photoautotrophic microalgae

It is generally thought that photosynthetic microalgae tend to produce higher levels of EPA than heterotrophs. *Nannochloropsis* sp. Hibberd, *Phaeodactylum* Bohlin, *Nitzschia* Hassall and *Porphyridium* Nägeli can present elevated levels of EPA in total fatty acids, although relatively low cell lipid contents tend to result in small EPA amounts in the biomass (D. A. Martins, et al., 2013) (Table 1). *Pavlova lutheri* and *Nannochloropsis occulata* are marine microalgae commonly used in aquaculture, is a well-known source of n-3 LC-PUFA, such as EPA and DHA under specific conditions such as during exponential growth using low light (Guihéneuf, Mimouni, Tremblin, & Ulmann, 2015; Winwood, 2013).

Heterotrophic microalgae

Currently, the most common micro-algae used for the production of DHA rich algal oil are from the marine members of the families *Thraustochytriaceae* and *Crypthecodiniaceae*.

The Thraustochytrids include the genera *Schizochytrium* sp. and *Ulkenia* sp., whereas *Crypthecodinium* is a genus of the family Crypthecodiniaceae (Table 1). Thraustochytrids, which includes the genera Schizochytrium and Thraustochytrium, are among the most promising microorganisms for producing omega-3 fatty acids, with reported oil contents of >50 % (dry basis) and more than 30 % DHA within the total oil produced (Burja, Radianingtyas, Windust, & Barrow, 2006). Among the heterotrophic marine microalgae, *Crypthecodinium cohnii* was identified as a prolific producer of DHA. The *C. cohnii* is extraordinary in that it produces no other PUFAs other than DHA in its cell lipid in any significant amount (Mendes, Reis, Vasconcelos, Guerra, & da Silva, 2009; Van Pelt, Huang, Tschanz, & Brenna, 1999).

Table 1. Microalgae species intensively used in omega-3 fatty acids rich algal oil production

Microalgae	Culture condition	Omega-3 fatty acids	References
Schizochytrium sp.	Heterotrophic	24-45 % DHA, 10 % EPA	(I. Fedorova-Dahms, P. Marone, M. Bauter, & A. Ryan, 2011; Hammond, Mayhew, Naylor, Ruecker, Mast, & Sander, 2001)
Ulkenia sp.	Heterotrophic	35-40 % DHA	(Dulce Alves Martins, Custódio, Barreira, Pereira, Ben-Hamadou, Varela, et al., 2013)
Crypthecodinium cohnii	Heterotrophic	25-60 % DHA	(De Swaaf, Sijtsma, & Pronk, 2003; L. Sijtsma & M. De Swaaf, 2004)
Thraustochytrium striatum	Heterotrophic	37 % DHA, 23 EPA	(Fan, Chen, Jones, & Vrijmoed, 2001)
Aurantiochytrium sp.	Heterotrophic	40 % DHA	(Hong, Rairakhwada, Seo, Park, Hur, Kim, et al., 2011)
Pavlova lutheri	Phototrophic	12-18 % DHA, 22-28 % EPA	(Guihéneuf, Mimouni, Ulmann, & Tremblin, 2009)
Nannochloropsis sp.	Phototrophic	38-39% EPA	(Chaturvedi & Fujita, 2006)
Phaeodactylum tricornutum	Phototrophic	40-57 % EPA	(Fernández, Pérez, Sevilla, Camacho, & Grima, 2000)
Nitzschia laevis	Phototrophic	25-33 % EPA	(Xiao-Hong, Song-Yao, WANG, & Mei-Fang, 2007)
Porphyridium cruentum	Phototrophic	25 % EPA	(Durmaz, Monteiro, Bandarra, Gökpinar, & Işik, 2007)

Extraction and purification of algal oil

After growth and harvesting of microbial biomass, oil must be gently extracted. Nonetheless, there is no universal method that will yield the best oil recovery for algae. In some algae cells, cell walls are particularly thick and cell disruption method must be employed for recovery process. Extraction of algal oil for the production of omega 3 fatty acids is difficult process because as soon as the algal cell walls are ruptured, these PUFAs are exposed to potential oxidation. Once these highly unsaturated fatty acids have reacted with oxidized radicals, an unstoppable chain reaction begins which leads to the production of rancid, highly odorous oil which is unsuitable for human consumption. Hence, so far as possible, all sources of materials that can initiate the oxidation process should be eliminated during the extraction and storage period (Winwood, 2013). Crude vegetable oils, including algal oils, require refining to improve color, clarity, odour and remove any particulate material and chemical contaminants. There are wide ranges of impurities in the crude oil that can be removed by the refining process, including: free fatty acids, phosphatides (i.e. lecithin), pigments (i.e. carotenoids, chlorophyll), trace metals, sterols (i.e. cholesterol), waxes, mono acyl and diacyl glycerides, waxes, oxidation products and trace contaminants. Therefore, oil refining needs to be performed to remove non-triglyceride, colorants, smelly and toxic compounds in the production of edible oil (Rubio-Rodríguez et al., 2010). The conventional oil refining in industry is usually made by chemical methods, which include several steps as degumming, to separate phospholipids; neutralization or de-acidification, to clear free fatty acids and decrease oil acidity; bleaching to absorb pigments or contaminants and deodorization to remove smelly compounds. Supercritical fluid technology, together with membrane and enzymatic processes, is one of the most recent technologies proposed as alternative to oil refining with chemical products or high temperatures (Čmolík & Pokorný, 2000).

CONCLUSIONS

Scientists have a great opportunity to discover and exploit many as yet unidentified marine microbes capable of producing higher levels of omega-3 fatty acids and other valuable products. One of the main challenges is developing optimum culture conditions for rapidly growing marine microbes that produce high levels of omega-3 fatty acids, as compared to the limited number of commercially useful

species presently available in the international depository. The application of metabolic engineering techniques to improve the wild-type strain is in an early stage of development. The bioengineering can further improve yields of omega-3 fatty acids from algae. Consumers are aware of the importance of an adequate provision of these nutrients and several properties of microalgal oils are particularly appealing, such as their sustainability, high purity and quality, "vegetarian" origin, and improved organoleptic qualities when compared to fish oils. Although genetically modified crops will likely serve as omega-3 sources in the future, microalgae oils have a great potential to present purer profiles, which are highly advantageous during processing and may address differentiated purposes in the market.

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REFERENCES

Armenta, R. E., Valentine, M. C. 2013. Single-Cell Oils as a Source of Omega-3 Fatty Acids: An Overview of Recent Advances. Journal of the American Oil Chemists Society, 90 (2), 167-182.

Burja, A. M., Radianingtyas, H., Windust, A., Barrow, C. J. 2006. Isolation and characterization of polyunsaturated fatty acid producing Thraustochytrium species: screening of strains and optimization of omega-3 production. Applied Microbiology and Biotechnology, 72 (6), 1161-1169.

Certik, M., Shimizu, S. 1999. Biosynthesis and regulation of microbial polyunsaturated fatty acid production. Journal of Bioscience and Bioengineering, 87 (1), 1-14.

Chaturvedi, R., Fujita, Y. 2006. Isolation of enhanced eicosapentaenoic acid producing mutants of Nannochloropsis oculata ST-6 using ethyl methane sulfonate induced mutagenesis techniques and their characterization at mRNA transcript level. Phycological Research, 54 (3), 208-219.

Covington, M. B. 2004. Omega-3 fatty acids. American Family Physician, 70 (1), 133-140.

De Swaaf, M. E., Sijtsma, L., Pronk, J. T. 2003. High-cell-density fed-batch cultivation of the docosahexaenoic acid producing marine alga Crypthecodinium cohnii. Biotechnology and Bioengineering, 81 (6), 666-672.

Durmaz, Y., Monteiro, M., Bandarra, N., Gökpinar, Ş., Işik, O. 2007. The effect of low temperature on fatty acid composition and tocopherols of the red microalga, Porphyridium cruentum. Journal of Applied Phycology, 19(3), 223-227.

Fan, K., Chen, F., Jones, E. B., Vrijmoed, L. L. 2001. Eicosapentaenoic and docosahexaenoic acids production by and okara-utilizing potential of thraustochytrids. Journal of Industrial Microbiology and Biotechnology, 27(4), 199-202.

Fedorova-Dahms, I., Marone, P., Bauter, M., Ryan, A. 2011. Safety evaluation of DHA-rich Algal Oil from Schizochytrium sp. Food and Chemical Toxicology, 49 (12), 3310-3318.

Fernández, F., Pérez, J., Sevilla, J., Camacho, F. G., Grima, E. M. 2000. Modeling of eicosapentaenoic acid (EPA) production from Phaeodactylum tricornutum cultures in tubular photobioreactors. Effects of dilution rate, tube diameter, and solar irradiance. Biotechnology and Bioengineering, 68 (2), 173-183.

Gray, N. 2011. Beyond the heart and brain: emerging benefits of omega-3. In): NutraIngredients-USA.

Guihéneuf, F., Fouqueray, M., Mimouni, V., Ulmann, L., Jacquette, B., Tremblin, G. 2010. Effect of UV stress on the fatty acid and lipid class composition in two marine microalgae Pavlova lutheri (Pavlovophyceae) and Odontella aurita (Bacillariophyceae). Journal of Applied Phycology, 22 (5), 629-638.

Guihéneuf, F., Mimouni, V., Tremblin, G., & Ulmann, L. 2015. Light intensity regulates LC-PUFA incorporation into lipids of Pavlova lutheri and the final desaturase and elongase activities involved in their biosynthesis. Journal of Agricultural and Food Chemistry, 63 (4), 1261-1267.

Gupta, A., Barrow, C. J., Puri, M. 2012. Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils. Biotechnology advances, 30(6), 1733-1745.

Hammond, B. G., Mayhew, D. A., Naylor, M. W., Ruecker, F. A., Mast, R. W., & Sander, W. J. 2001. Safety assessment of DHA-Rich microalgae from Schizochytrium sp.: I. Subchronic rat feeding study. Regulatory Toxicology and Pharmacology, 33(2), 192-204.

Ho, L., van Leeuwen, R., Witteman, J. C., van Duijn, C. M., Uitterlinden, A. G., Hofman, A., de Jong, P. T., Vingerling, J. R., Klaver, C. C. 2011. Reducing the genetic risk of age-related macular degeneration with dietary antioxidants, zinc, and ω-3 fatty acids: the Rotterdam study. Archives of ophthalmology, 129(6), 758-766.

Hong, W.-K., Rairakhwada, D., Seo, P.-S., Park, S.-Y., Hur, B.-K., Kim, C. H., & Seo, J.-W. 2011. Production of lipids containing high levels of docosahexaenoic acid by a newly isolated microalga, Aurantiochytrium sp. KRS101. Applied Biochemistry and Biotechnology, 164(8), 1468-1480.

Hooper, L., Thompson, R. L., Harrison, R. A., Summerbell, C. D., Ness, A. R., Moore, H. J., Worthington, H. V., Durrington, P. N., Higgins, J. P.,

Capps, N. E. 2006. Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. Bmj, 332(7544), 752-760.

Imhoff-Kunsch, B., Stein, A. D., Martorell, R., Parra-Cabrera, S., Romieu, I., & Ramakrishnan, U. 2011. Prenatal docosahexaenoic acid supplementation and infant morbidity: randomized controlled trial. Pediatrics, 128(3), e505-e512.

Khozin-Goldberg, I., Iskandarov, U., & Cohen, Z. 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Applied Microbiology and Biotechnology, 91(4), 905-915.

Kiecolt-Glaser, J. K., Belury, M. A., Andridge, R., Malarkey, W. B., & Glaser, R. 2011. Omega-3 supplementation lowers inflammation and anxiety in medical students: a randomized controlled trial. Brain, behavior, and immunity, 25(8), 1725-1734.

Lang, I., Hodac, L., Friedl, T., & Feussner, I. 2011. Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. BMC plant biology, 11(1), 124.

Lewis, M. D., Hibbeln, J. R., Johnson, J. E., Lin, Y. H., Hyun, D. Y., & Loewke, J. D. 2011. Suicide deaths of active duty US military and omega-3 fatty acid

clinical psychiatry, 72(12), 1585.

Lozac'h, N. 1986. Extension of Rules A-1.1 and A-2.5 concerning numerical terms used in organic chemical nomenclature (Recommendations 1986). Pure and Applied Chemistry, 58(12), 1693-1696.

Martins, D. A., Custodio, L., Barreira, L., Pereira, H., Ben-Hamadou, R., Varela, J., & Abu-Salah, K. M. 2013. Alternative Sources of n-3 Long-Chain Polyunsaturated Fatty Acids in Marine Microalgae. Marine Drugs, 11(7), 2259-2281.

Mendes, A., Reis, A., Vasconcelos, R., Guerra, P., & da Silva, T. L. (2009). Crypthecodinium cohnii with emphasis on DHA production: a review. Journal of Applied Phycology, 21(2), 199-214.

Newton, I. 1998. Long-chain polyunsaturated fatty acids—the new frontier in nutrition. Lipid technol, 10, 77-81.

Pal, D., Khozin-Goldberg, I., Cohen, Z., & Boussiba, S. (2011). The effect of light, salinity, and nitrogen availability on lipid production by Nannochloropsis sp. Applied Microbiology and Biotechnology, 90(4), 1429-1441.

Pase, M. P., Grima, N. A., & Sarris, J. 2011. Do long-chain n-3 fatty acids reduce arterial stiffness? A meta-analysis of randomised controlled trials. British Journal of Nutrition, 106(07), 974-980.

Quilodrán, B., Hinzpeter, I., Hormazabal, E., Quiroz, A., & Shene, C. 2010. Docosahexaenoic acid (C22: 6n−3, DHA) and astaxanthin production by Thraustochytriidae sp. AS4-A1 a native strain with high similitude to Ulkenia sp.: Evaluation of liquid residues from food industry as nutrient sources. Enzyme and Microbial Technology, 47(1), 24-30.

Sijtsma, L., De Swaaf, M. 2004. Biotechnological production and applications of the ω-3 polyunsaturated fatty acid docosahexaenoic acid. Applied Microbiology and Biotechnology, 64(2), 146-153.

Simopoulos, A. P. (2008). The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Experimental Biology and Medicine, 233(6), 674-688.

Van Pelt, C. K., Huang, M. C., Tschanz, C. L., & Brenna, J. T. 1999. An octaene fatty acid, 4,7,10,13,16,19,22,25-octacosaoctaenoic acid (28 : 8n-3), found in marine oils. Journal of Lipid Research, 40(8), 1501-1505.

Winwood, R. J. 2013. Recent developments in the commercial production of DHA and EPA rich oils from micro-algae. OCL, 20(6), D604.

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STUDIES ON THE CHROMATIC CHARACTERISTICS OF SOME ROMANIAN RED WINES

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Abstract

The chromatic profile of red wines is formed by the participation of various phenolic compounds: anthocyanin, tannins, flavones and phenolic acids; anthocyanin are polyphenolic substances with the most important role in the color of young wines. The evolution of red wine leads to changes of structures and chromatic properties of wine due to polymerization reactions, condensation and oxidation. Also the red wine color is influenced by region of origin, the wine grape variety and vintage year. There were studied chromatic characteristics of Romanianred wines as Pinot noir and Feteasca Neagră from two vineyards with different ecopedoclimatic conditions , Sâmbureşti and Ceptura (in southern Romania), the 2011 year harvest. The polyphenolic composition of wines was assessedby the content in total polyphenols, tannins and anthocyanin. A wine tannin structure was analysed by their concentration in condensed tannins (HCL index), astringent tannins (gelatine index) and tannin-polysaccharide complex (ethanol index). Analyses have been carried out in the wine by UV-VIS spectrometry techniques. Total content of polyphenols have been determined by the DO_{280} index. Tannins have been determined by the Ribereau-Gayon method, tannin structure after Glories method; anthocyanins were determined by the discoloration technique with SO_2. The study on color of red wines analysed during their evolution referred to the study of chromatic parameters, the content of anthocyanin monomers and polymers (Glories method).

Key words: *red wines, anthocyanin monomers, condensed tannins.*

INTRODUCTION

Phenolic compounds are essential compounds for the quality of wines and particularly red wines; also they have a very important role in health and food hygiene of the grape and wine consumers.With a powerful antioxidant effect, polyphenols represent an important class of chemical compounds; red wine is a rich product in polyphenols, although its content in these substances vary, sometimes in wider limits in relation with: variety,viticultural area, vintage year etc.(Mazue F., 2001, Paixao N., 2007, Vişan L., 2012).

The polyphenolic compounds have an important role in the evolution of wines during maturation.The evolution of red wines leads to the modification of the structure and chromatic proprieties bypolymerization, condensation and oxidation. The content of wines in polyphenolic compounds depends of many factors, among them, the most important are:the variety, the year of harvest and region (Landrault N, 2001, Vişan L., 2012).

Our studies concern the influence of the viticultural region (with different ecopedoclimatics conditions) and grape variety about the chromatic characteristics of two red quality wines, *Pinot noir* and *Fetească Neagră,* in the climatic conditions of the same harvest year 2011.Also, was studied the chromatic profile evolution of wines through the modification of anthocyanin structure during maturation process .

MATERIALS AND METHODS

Pinot noir and *Fetească Neagră* wines, from two different Romanian vineyards Sâmbureşti and Ceptura, the 2011 year harvest, were analysed in terms of physico-chemical: alcoholic strength (vol% alcohol), total acidity (g/L tartaric acid), volatile acidity (g/L acetic acid), non-reducing extract (g/L) and glycerol (g/L)analyses were performed by standard methods (O.I.V.): ebulliometricmethod, for alcoholic strength; titrimetricmethod for total acidity; distillation method Saunier-Cazenave

for volatile acidity; Tabarié method for dry extract and volumetric method for glycerol.
The polyphenolic composition of wines was assessed by the total content in polyphenols, tannins and anthocyanin. Analyses have been carried out in the wine by UV-VIS spectrometry techniques (Giusti M., 2001). Total content of polyphenols have been determined by DO_{280} index(Ribereau-Gayon J., 1978). Tannins have been determined by the Ribereau-Gayon method (1996) and tannins structure after Glories method (1978), based on the following indicators: gelatine index (for astringenttannins); HCl index (for condensed tannins); ethanol index (for the macromolecular associations tannins-polysaccharides).These indicators was determined by spectrophotometric method at λ = 280 nm (Glories Y, 1984). The anthocyanins were determined by the discoloration technique with SO_2 (Dallas C., 1994). The study on color of red wines analysed during their evolution referred to the study of chromatic parameters, the content of anthocyanin monomers and polymers (Glories method).
Wines have been noted: PN_1 – *Pinot noir* Sâmbureşti region; PN_2 – *Pinot noir*Ceptura region; FN_1 – *Feteasca neagră*Sâmbureşti region; FN_2 – *Feteasca neagră*Ceptura region.

RESULTS AND DISCUSSIONS

In Romania, the year 2011 was characterized by optimum temperature and precipitations for vines, both in winter and spring. The prolonged drought of summer-autumn period has positively influenced the accumulation of sugars in the grapes, but also the concentration in phenolic compounds, especially anthocyanin.The two varieties of vine *Pinot noir* and *Feteasca Neagră* behaved well in 2011, in both studied regions, the larger accumulation of sugars leads to obtain some higher alcoholic wines.
Of the two varieties, the *Feteasca Neagră* variety has accumulated the largest amount of sugars:240 g/L in Ceptura region, respectively 233.6 g/L in Sâmbureşti region. Both vines have recorded higher values of the non-reducing extract and glycerol content in both regions, although slightly higher, in both cases of vines in Ceptura region.

Although normally,the two varieties accumulate normal amounts of organic acids, in the study year, the total acidity was low, especially for *Pinot noir* variety. *Pinot noir* wines, especially in Ceptura region had total acidity very low, requiring corrections.

Table1. Chemical parameters of red wines *Pinot noir*and *Feteasca neagră*

Wines	Chemical parameters of red wines				
	alcoholic strength (vol % alcohol)	total acidity (g/l tartaric acid)	volatile acidity (mg/l CH_3COOH)	non-reducing extract (g/l)	glycerol (g/l)
PN_1	13.7	5.9	0.3	29.5	8.4
PN_2	13.5	4.3	0.3	30.9	8.38
FN_1	13.9	6.3	0.45	27.2	8.42
FN_2	14.5	4.9	0.55	31.1	9.4

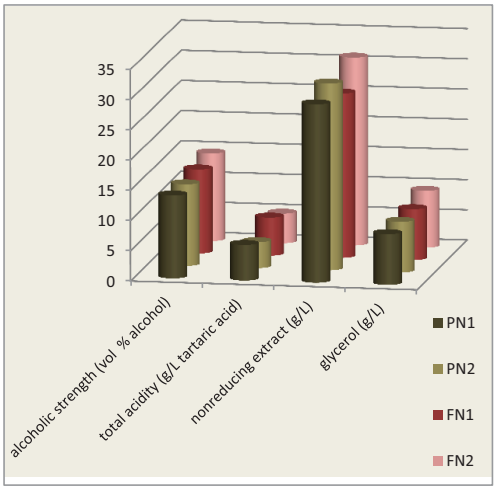

Fig 1. The main chemical parameters in red wines (*Pinot noir* and *Feteasca neagră)*

The accumulations of phenolic compounds in grapes were high, as in the sugar case, leading to higher concentrations of polyphenols in wine.Influence of ecopedoclimatic conditions on the accumulation of phenolic compounds is visible, in both varieties recorded higher concentrations in the Ceptura region. The results show a greater sensitivity of *Pinot noir* variety at the climatic and edaphic factors of the culture region.Regarding the influence of

variety, we can see that *Fetească Neagră* variety had higher accumulation of polyphenols compared with *Pinot noir*.The wine concentration in tannins varied, as well as total polyphenol content, both by region and variety. In tannin case, both varieties recorded slightly higher values in Sâmbureşti region.

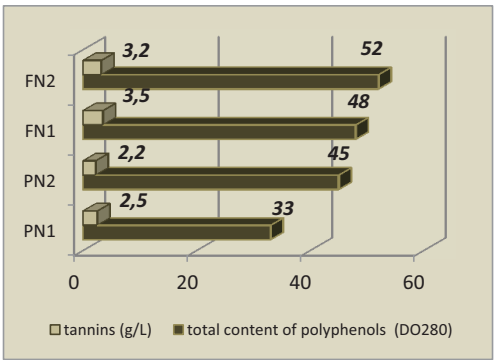

Fig 2. Total content of polyphenols and tannins in red wines (*Pinot noir* and *Fetească neagră)*

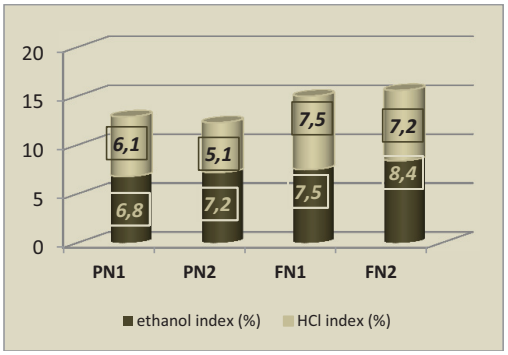

Fig. 3. Ethanol index and HCl index

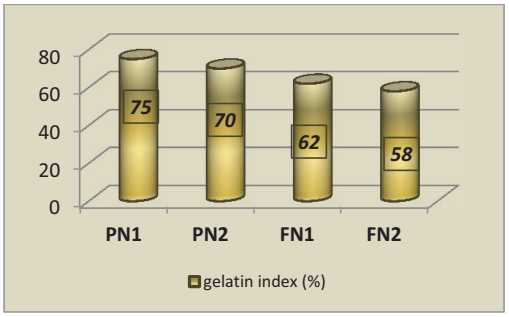

Fig. 4. Gelatine index (%)

The tannins in wines were characterized based on three indices, with their help has determined the share of different types of tannins in wines; the ethanol index, which sets the share of macromolecular associations of polysaccharide; the HCL index, which were determined the condensed tannins and the gelatine index, for determination of tannin percentage with astringent properties.

Regarding the tannins structure of wines,the results show that the *Fetească neagră* wine has a grater ethanol index; the tannins proportion from the tannins-polysaccharides complex is higher than FN_2(Ceptura region).

In both varieties, the ethanol index has a higher value in Ceptura region. The percentage of condensed tannins, determined using HCI index was also higher in the *Fetească Neagră* wine.

In terms of the tannins proportion less condensed, with astringent properties, this was higher in *Pinot noir* wine; on regions, the obtained wines in Sâmbureşti had presented a higher percentage of astringent tannins, in both varieties.

The wine content in anthocyanin is different by variety, a larger amount being in *Fetească Neagră* wine. Differences occur in the anthocyanin accumulation, depending on ecopedoclimatic conditions of culture area. Results showed a higher accumulation of anthocyanin in Ceptura region, for both varieties.

The polyphenols compounds structure is change during the wine maturation due to polymerization reactions, condensation and oxidation, leading to the change of the chromatic proprieties. As regarding anthocyanin, at the red wines color participate the anthocyaninmonomers, polymersand co-pigmented, they change during wine maturation. Therefore, the anthocyanin monomers turn into the polymeric form.

In fig. 6 and 7 shows the percentage of the 3 types of anthocyanin in young wines, of *Fetească Neagră* and *Pinot noir*, immediately analysed after the alcoholic fermentation.

The evolution of the three types of anthocyanin can be see from fig. 8 and 9, wines being analysed at 3, 6 and 12 months after completion of alcoholic fermentation. The percentage of monomeric anthocyanin, higher in *Fetească Neagră* wine, decreases along maturation of wines, in favor of the polymer shape of anthocyanin.

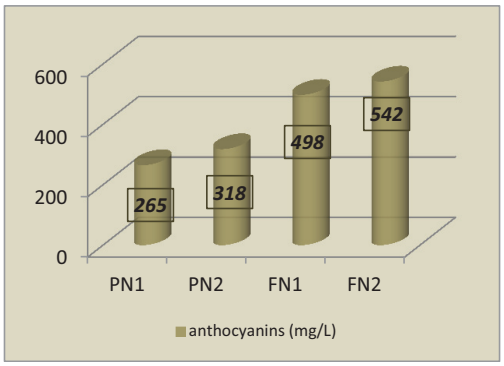

Fig. 5. Anthocyanincontent (mg/L) of red wines

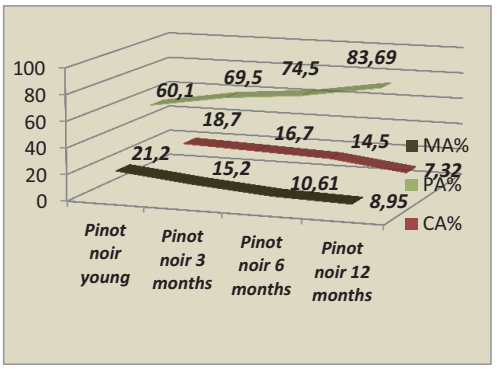

Fig. 8. Evolution of anthocyanin(%) during the *Pinot noir* wine maturation

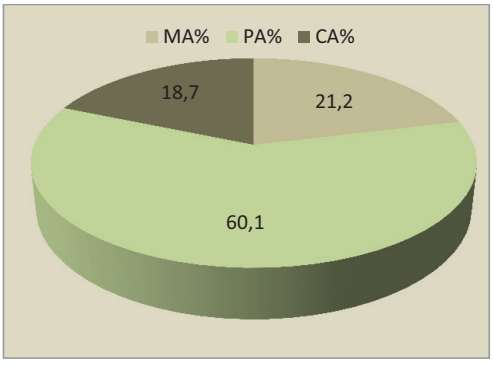

Fig. 6. Percentage of anthocyanin monomers(MA%), polymers (PA%) and co-pigmented (CA%) in young wine *Pinot noir*

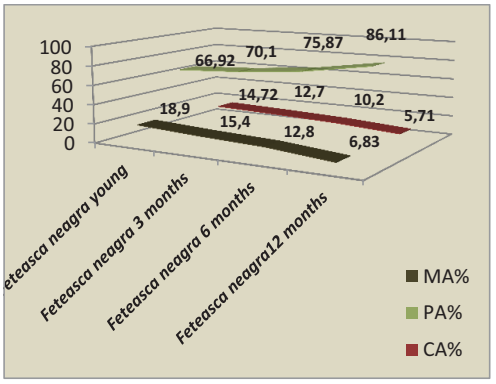

Fig. 9. Evolution of anthocyanin(%) during the *Fetească neagră* wine maturation

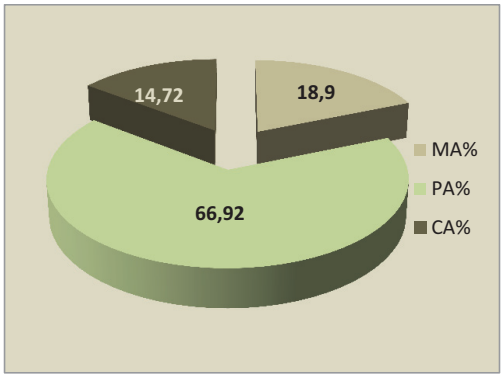

Fig. 7. Percentage ofanthocyaninmonomers (MA%), polymers (PA%) and co-pigmented (CA%) in young *Fetească neagră* wine

CONCLUSIONS

Fetească Neagră and *Pinot noir* varieties give high quality wines, especially in the south area of Romania (including the Ceptura and Sâmbureşti regions). Obtained wines have a strong typicality specific of the variety, complex flavours and well structured. However, the two varieties have reacted to the difference of ecopedoclimatic conditions specific of the two regions, the *Pinot noir* variety is more sensitive and also the differences between wines are visible.

The accumulations of sugars were elevated in both varieties, in both regions, although *Fetească Neagră* presented higher values, especially in Ceptura region. The non-reducing extract and glycerol content, two important markers of quality of wine, showed higher

values in the *Fetească Neagră* wine in Ceptura region.

The accumulations of phenolic compounds in grapes were raised, which led to high concentrations of polyphenols in wine. The influence on the accumulation of polyphenols from the region is visible, in both varieties recorded higher concentrations in Ceptura region. Results show a greater sensitivity of *Pinot noir* variety at the influence of the climatic and edaphic factors of region.

The wine content in tannins varies, also with variety, *Fetească Neagră* wines are richer in tannins compared to those of *Pinot noir*. The results show differences determined by the culture region, wines from the both studied varieties being more tannin in the Sâmbureşti region.

In terms of structure of tannins, results show that *Fetească Neagră* wine has a higher percentage of good tannins and a lower percentage of astringent tannins compared with *Pinot noir*, superior values in Ceptura region.

The anthocyanin content varies with growing area and variety, a higher content presenting the *Fetească Neagră* wine in Ceptura region.

The percentage of anthocyanin monomers decreases along maturation of wines, in favor of their polymer form.

REFERENCES

1. Dallas C., 1994. Effect of SO_2 on the extraction of anthocyanins. Vitis 33, 42-51.
2. Giusti M, Wrolstad R.E., 2001. Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy. Current Protocols in Food Analytical Chemistry.
3. Glories Y., 1984. La couler des vins rouges, Connaisance Vigne Vin, 18, 253-271.
4. Landrault N., Poucheret P., Ravel P., Gasc F., Cros G., Teissedre P.L., 2001. Antioxidant capacities and phenolic levels of French wines from different varieties and vintages, *J. Agric. Food Chem.,* 49(7), 3341-3348.
5. Mazue F., 2001. Effets des polyphenols de vin rouge sur la proliferation cellulaire et sur le metabolisme du rezveratrol. These Pour l'obtention du grade de Docteur de l'Université de Bourgogne.
6. Paixao N., Perestrelo R., Marques J., Caˆmara J., 2007. Relationship between antioxidant capacity and total phenolic content of red, rose and white wines. Science Direct Food Chemistry 105 (2007) 204–214.
7. Vişan L., Dobrinoiu R.V., Dumbravă M, 2012. Study of chemical and aromatic composition in a Romanian wine *Cabernet Sauvignon,*Romanian Biotechnological Letters, 17 (1), 6855-6861.
8. Recueil des methodes internationales d'analyse des vins et des mouts edition, 2011. Organisation internationale de la vigne et du vin.

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ADVANTAGES AND DISADVANTAGES OF ACTIVE CARBON IN QuEChERS SAMPLE PREPARATION METHOD FOR PESTICIDE RESIDUES

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Abstract

QuEChERS is an analytical method which simplifies the the sample preparation for pesticide residues. It is the result of the necessity to save the time for sample preparation, to reduce the amount of toxic organic solvents and thereby to contribute to the preservation of the environment. In order to obtain a higher recovery, thus making the analysis results more precise and representative, it is necessary during the extraction to pay particular attention to matrix components which can significantly affect the investigation results. In order to decrease the effect of the present components it is necessary to use the adequate substances – sorbents and to examine how their presence affects the validation parameter. The sour cherry extract, as an exceptionally pigmented matrix, needs the use of a sorbent with a strong affinity towards planar molecules thus causing its discoloration by removing the pigments from the extract. The most frequently used sorbents in QuEChERS method of extract purification are primary secondary amine (PSA), graphitized black carbon sorbent (GCB), C18 and Z-Sep sorbent (silicon dioxide coated by zirconium). The paper deals with a possibility of using active carbon (AC) as a possible sorbent which presents a form of carbon obtained in controlled oxidation processes having a porous structure with spacious active surface which enables it to adsorb a wide range of compounds and pollutants from the extract. By use of AC and QuChERS methods an exceptionally purified colorless extract is obtained. The obtained validation parameters point at a very low recovery of the studied pesticides which is a serious disadvantage of this sorbent while high correlation coefficients (R^2>0.99) are obtained with irrelevant matrix effect in the linearity investigation process.

Key words*: AC sorbent, QuEChERS, validation, pesticide residues, LC-MS/MS.*

INTRODUCTION

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) is an acronym for the analytical method which, to a great extent, simplifies the analysis of pesticide residue determination in the samples of various origin. It is the result of the necessity to save time for sample preparation, decrease the amount of toxic organic solvents and to contribute to the preservation of the environment (Anastasiades et al., 2003). QuEChERS method in its basic form comprises two steps stages: liquid micro extraction by use of acetonitrile as a solvent with the addition of salts aimed at a better phase separation line and the adjustment of pH values; matrix purification using sorbents.

The sour cherry extract, as an exceptionally pigmented matrix, needs the use of a sorbent with a strong affinity towards planar molecules thus causing its discoloration by removing the pigments from the extract (Bursić et al., 2013).

The extraction of pesticides from plant tissues is a complex task. The process of proving and determining particular pesticides depends on the present compounds in the matrix which can interfere with their separation. Acetonitrile (MeCN) is the most frequently used solvent in the extraction. In the QuEChERS method MeCN is used together with the combination of salts NaCl and $MgSO_4$ which help with better phase separation and contribute to the recovery without dilution. In the phase of extract purification the most frequently used sorbents are PSA (primary secondary amine), AC (active carbon), GCB (graphitized black carbon sorbent), C_{18} sorbent, Z-Sep sorbent (silicon dioxide coated by zirconium) and as the latest EMR-Lipid (Bursić et al., 2016). Their function is to remove fatty acids, sugars and lipids from

the matrix that makes them quite suitable for the purification of various matrices.

In order to reduce the matrix influence on the detection of pesticide residues at LC-MS/ MS sour cherry analysis the substances which have a strong affinity towards planar molecules and efficiently remove the extract pigments are used. The objective of the study is to check the linearity and recovery of pesticides from sour cherries by use of liquid chromatography with tandem mass spectrometry (LC-MS/MS) in case when for the extract purification, within the QuChERS method, AC is uses as a sorbent.

AC is a form of carbon obtained in carefully controlled oxidation processes resulting in its porous structure. There are three different pore groups: macro-pores (radius>25nm); mezzo-pores (radius 1-25 nm) and micro-pores (radius<1nm). The most numerous are macro-pores which attract small gas molecules and mezzo-pores which attract larger molecules like color molecules (Momčilović, 2012). The functional groups on the surface of active carbon make it amorphous so that, depending on the pH values of the solution and the adsorbents, the mechanism can be based on the dipole-dipole interactions, hydrogen and covalent binding or ion exchange. The basic units of the active carbon composition are micro crystals consisting of planes of joint hexagonal carbon rings. The functional groups of heteroatoms on the edges of aromatic layers disturb the parallel orientation of planes characteristic of graphite and provide so-called turbostratic structure typical of active carbon. The most frequent heteroatoms in the structure are hydrogen, nitrogen, sulphur and phosphorus and the functional group which they make determine the reactivity in adsorbent, catalytic and electrochemical processes. The given AC structure presents a large active surface that enables it to adsorb a wide range of compounds and pollutants from particular environments. The active carbon can be obtained from various raw materials which contain a high percentage of carbon such as wood, coal, coconut shells. The thermic decomposition of these raw materials is performed at the temperature of 900 °C combined with water vapour:

$C + H_2O \rightarrow CO + H_2 - 175.440$ kJ/mol

The reaction is exothermic and the temperature is sustained by partial burn of the products of carbon-monoxideand hydrogen.

$2CO + O_2 \rightarrow 2CO_2 + 393.790$ kJ/mol

$2H_2 + O_2 \rightarrow 2H_2O + 396.650$ kJ/mol

The air is proportionally added to the system in order to burn the gases without burning the carbon.

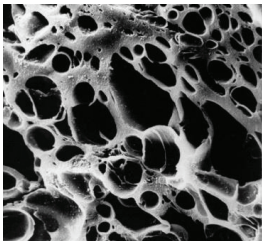

Figure 1. AC porous structure

The objective of the paper is to study the effect of AC, as a sorbent, in the process of extract purification by QuChERS method to basic validation parameters of multi- residual LC-MS MS method for the determination of pesticide residues in sour cherries using carbofuran-D3 as an internal standard.

MATERIALS AND METHODS

Chemicals: The analytical standards of all the studied pesticides are the products by Dr. Ehrenstorfer. The stock and the working solution were prepared by dissolving pesticides in methanol (HPLC purity, J.T. Baker).

Validation parameters: The recovery was checked for the levels of 0.05 and 0.1 µg/ml in three replications. To 10 g of the control sample 100 µg/ml of internal standard and 100 (50) µl of working solution of mass concentration was added so that final pesticide concentration in the enriched sample was 0.1 (0.05) µg/ml. The linearity of the detector response was performed for the concentration of 0.2; 0.1; 0.05 and 0.02 µg/ml by adding 1 ml of the already prepared calibration standards to the evaporated extract. The matrix effect (ME) was calculated based on the slopes of the calibration curves in matrix and solvent (SANCO/12571/2013).

Instrument: An Agilent 1200 (Agilent Technologies, USA) system with a binary pump and autosampler was used. This was equipped with a reversed-phase C18 analytical

column of 50×4.6 mm and 1.8 μm particle size (Agilent Zorbax Eclipse XDBC18). Mobile phases were 0.1% formic acid in methanol (solvent A) and 0.1% formic acid in Milli-Q water (solvent B). The gradient was 0 min (80% B), 10 min (50% B), 20 min (5% B), 24 min (0% B), 25 min (80% B), with the flow rate 0.4 ml/min. For the mass spectrometric analysis, an Agilent 6410B Triple-Quad LC/MS system was applied. Agilent MassHunter software was used for method development, acquisition and data processing.

RESULTS AND DISCUSSIONS

Before the calibration and quantification of pesticides it is necessary to set the acquisition parameters of mass spectrometer- to determine the reactions for ion monitoring (MRM), to find the energy of collision cell (CE) and the fragmentation energy (Frag) where the response of the studied pesticide will be the highest for the given conditions (Table 1). The recovery of the studied pesticides was checked for the levels of 0.05 and 0.01 μg/kg. The control of the detector response linearity was carried out for a series of mass concentrations: 0.02; 0.05; 0.1 and 0.2 μg/ml.

The average values of the recovery from sour cherry matrix as well as the correlation coefficients (R^2) with the ME are shown in Table 1.

By use of AC in the purification process of sour cherry extracts the exceptionally clear extracts are obtained with the high R^2 values (>0.99) which entails the insignificant ME on all the studied pesticides. Namely, according to SANCO/12571/2013 regulation the assessment of ME on the studied pesticides i.e. their ionization in the ion source can be regarded as being of no influence in case ME is within the interval from 0 to ±15%, if the values range from ±15 to 25% the influence is slight and if the values exceed ±25% ME is significantly prominent.

Table 1. Pesticides analyzed by LC-MS/MS and some of their MS and analytical performance characteristics

Pesticide	MW[1]	MRM transition (m/z)	Frag.[2] (V)	CE[3] (V)	Rt (min)[4]	R^2 [5]	ME (%)[6]	Average Rec (%)	RSD[7] (%)
Carbendazim	191.20	192.1→160.1 192.1→132	104 104	18 34	7.869	0.9999	-0.42	16.6	4.27
Pyrimetanil	199.25	200.1→107.1 200.1→82.1	136 136	26 30	15.050	0.9989	-0.52	32.2	5.31
Azoxystrobin	403.39	404.1→372.1 404.1→344.1	100 100	9 25	15.964	0.9991	-0.13	26.0	7.34
Metoxyfenozide	368.47	369.2→149.1 369.2→313.2	100 90	12 15	16.599	0.9994	0.17	20.0	2.95
Myclobutanil	288.78	289→125.1 289→79.2	150 150	20 15	16.669	0.9999	11.09	32.6	3.58
Cyprodinil	225.29	226.1→93 226.1→108	120 120	30 40	16.678	0.9974	1.49	20.6	7.94
Triadimenol	295.76	296.2 →227 296.2 →70.2	60 60	5 10	16.887	0.9988	11.86	30.2	6.07
Fenhexamide	302.20	302.1→97.1 302.1→55.1	110 110	25 30	16.983	0.9982	-12.64	17.1	9.31
Krezoixm-methyl	313.35	336.2→246.2 336.2→229.2	120 120	15 15	17.644	0.9999	2.72	15.6	3.71
Penconazol	284.18	284.1 →158.9 284.1→70.1	80 100	30 20	17.632	0.9999	14.48	29.2	6.23
Tebuconazole	307.80	308.1 →125 308.1→70.0	100 100	25 25	17.686	0.9999	-0.05	24.5	6.94
Pyraclostrobin	387.82	388.1→194 388.1→163	100 100	10 10	17.923	0.9969	2.47	16.9	4.95
Indoxacarb	527.80	528.1→203 528.1→150	120 120	36 16	18.135	0.9954	3.15	16.0	5.37
Difenoconazole	406.27	406→337 406→251	100 100	20 20	18.202	0.9994	1.16	26.0	4.08
Quinoxifen	308.13	308→197 308→272	135 135	30 32	19.365	0.9937	11.62	13.5	7.35
Chlorpyrifos	350.59	349.9→197.9 349.9→97.0	130 130	15 41	19.469	0.9999	0.1	16.2	3.57

[1]MW – Molecular weight, [2]Frag. - Fragmentor, [3]CE - collision energy, [4]Rt – Retention time, [5]R^2 - linearity, [6]ME – Matrix effect, [7]RSD - Relative standard deviation.

The effect of matrix below 1% was recorded with carbendazim, pyrimethanil, tebuconazole azoxystrobin, metoxyfenozide and chlorpyrifos (Figure 2).

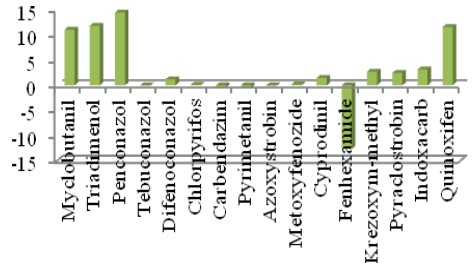

Figure 2.Matrix effect on investigated pesticides (%)

With multi-residual methods for the determination of pesticide residues the recommended interval of the recovery in the validation is from 70 to 120% (SANCO/12571/2013). By use of AC the obtained recoveries do not exceed 33% (Figure 3) which are extremely low values and point to the conclusion that AC cannot be used as a sorbent in QuEChERS method.

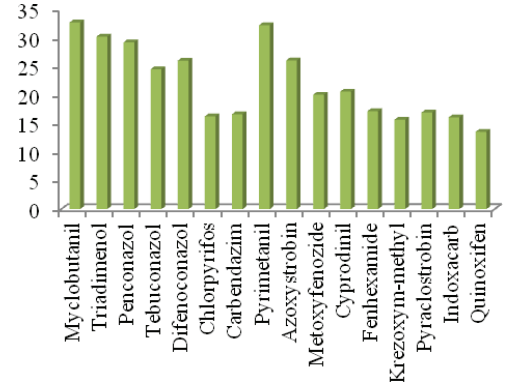

Figure 3.Average recovery of investigated pesticides (%)

CONCLUSIONS

By use of active carbon as a sorbent aimed at the removal of planar molecules, which can affect the pesticide detection itself and their ionization in QuChERS method, exceptionally clear and colourless sour cherry extracts were obtained. AC was excellent in reducing the matrix effect simultaneously having high R^2 values (>0.99) at the pesticide calibration.

However, this sorbent, besides being excellent at removing the impurities from the extract binds the pesticides which resulted in very low recoveries (below 33%). The obtained RSD values were below 20% which is in accordance with the valid EU regulations.

The obtained results show that the active carbon cannot be used as a sorbent in the QuChERS method and that in such a case it must be replaced by other sorbents as follows PSA, C18, GCB or Z-Sep.

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REFERENCES

Michelangelo Anastassiades, Steven J. Lehotay, Derek Stajnbaher, Frank J. Schenck (2003). Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction“ for the determination of pesticide residues in produce, Journal of AOAC, 86(2):412-31.

Vojislava Bursić, Gorica Vuković, Bojana Špirović, Sanja Lazić, Mira Pucarević, Tijana Zeremski (2013). QuEChERS method for determination of pesticide residues in cherries, Agriculture and Forestry, Vol. 59, 3: 91-100.

Vojislava Bursić, Mirjana Ljubojević, Gorica Vuković, Bojana Špirović-Trifunović, Nebojša Vuković (2016). Influence of EMR during QuEChERS pesticide analyses on recoveries in sour cherries, 11th European Pesticide Residue, Workshop, Limassol, Cyprus, 24-27.05.2016., Programme and Book of abstracts, 187.

Milan Momčilović (2012). Kinetic and equilibrium parameters of adsorption processes under removal of certain harmful cationic ingrediants from aqueous solutions using activated carbons derived by thermochemical treatment of chestnut kernel and Black pine, PhD thesis, Faculty of Science, University of Niš, Serbia.

SANCO/12571/2013, Method validation and quality control procedures for pesticide rasidues analysis in food and feed.

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THE EVALUATION OF TRANSFER LEVEL FOR PESTICIDE RESIDUES, IN CASE OF PROCESSING BY SOLVENT EXTRACTION OF MEDICINAL PLANTS

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Abstract

There are multiple ways in which pesticide contamination can take place, depending on the method used for obtaining the raw material: by conventional agriculture or harvest from wild flora. Assessing the transfer level of residues in extracts, in controlled conditions of preparation and analysis, contributes to determining the risk level for the consumer, as well as making processing choices that lead to a low concentration of contaminants. We used an adapted QuEChERS extraction method for liquid samples to compare the level of pesticides in plant extracts obtained by ethylic alcohol and acetone extraction. This was done by extraction in the presence of MgSO4 and NaCl and cleanup by dispersion with PSA, active charcoal and Mg SO4. To achieve the appropriate level of sensitivity, the injection module of the equipment was used in Programmed Temperature Vaporization (PTV) mode, injecting 3 µl of sample. Calibration was performed by the standard addition technique, in which case the correlations were linear. Our results show that ethylic alcohol extraction, commonly used for obtaining tinctures, leads to different residue transfer levels, depending on their chemical structure and solubility (between 15 and 76,8 % recovery, for quintozene and terbuthylazine respectively). On the other hand, acetone extraction is selective for pesticides, and the crystallization of the resulting bioactive compounds leads to significantly reduced pesticide levels.

Key words*: extracts, medicinal plants, pesticides residues, QuEChERS.*

INTRODUCTION

The presence of pesticide residues in plant products is a prevailing problem, and their identification and quantification in the fresh or processed plant products is of great importance. MADR (Ministry of Agriculture and Rural Development), is concerned with tracking the levels of pesticide residues in plants but not so much in processed products (e.g. plant extracts). Their 2015 official report shows that 28.84% of vegetable samples had residue values lower than MRL (maximum residue limits), while 1.8% of samples exceeded MRL. The highest level of residues, in over 50% of the samples, was found in green salad, dill, lovage, scallion, parsley and celery. In fruits, the highest percentage of residues identifications (70%) was found in grapes, followed by strawberries (68%) and apples (67%). The most frequently identified pesticides were carbendazim and tebuconazol (MADR report). Organochlorurate pesticides residues are also frequently detected in fruits and vegetables, sometimes exceeding the accepted limit (Crentsil, 2011; Akan, 2014; Nsikak, 2011; Łozowicka, 2016, Dobrinas, 2011). Studies concerning the levels of pesticide residues during different processing steps show that residues can concentrate in the finite product (in the case of drying fruits or extracting fatty or volatile oils or other compounds) (Cortés, 2009), or can decrease (through washing, peeling, chopping, grinding, juicing or thermal preparation) (Elpiniki, 2011; Mekonen, 2015).

In medicinal plants, the presence of residues is the result of inadequate agricultural techniques, remanence of residues in the soil and/or contamination during the processing stages. Most studies were concerned with method validation and residue determination (LOQ = limit of quantitation < MRL), and in some instances the pesticide residues exceeded the approved levels (Hua, 2012; Sadowska, 2012; Amirahmadi, 2013; Brahushi, 2014; Agbeve1, 2014; Al-Othman, 2015). Consumers, oftentimes vulnerable segments of the population expect these products to be obtained in controlled conditions, following the GAP (Good Agricultural Practice) and GMP (Good Manufacturing Practice) rules. There is

therefore an intense concern with the evaluation of residue transfers from raw materials to products such as infusions (Rodrigues, 2005) tinctures (Kong, 2016) decocts, oils (Dugo, 2002; Garland, 2004) and concentrated fractions (Zuin, 2000).
In the European Pharmacopoeia, Ed. 8.0, for the pesticide residues parameter (2.8.13) there is a differentiation between residues in the raw plant and in processed products, and the accepted limit is adjusted with the following formulas:

If DER ≤ 10, then

$$MRLprep = MRL_{HD} \times DER$$

If DER>10, then

$$MRLprep = \frac{ADI \times M}{MDD_{HP} \times 100}$$

where: DER= drug/extraction ratio (ratio between the quantity of herbal drug used in manufacture and the quantity of herbal drug preparation obtained, $MRL_{prep.}$= officially accepted limit for pesticide residues in herbal drug preparation (mg/kg), MRL_{HD}= officially accepted limit for pesticide residues in raw material, ADI=acceptable daily intake (mg/kg), M=body mass (kg), MDD_{HP}=daily dose of herbal drug preparation (kg).
The present study is concerned with evaluating the transfer level of pesticide residues in extracts of plants initially fortified with pesticides. The QuEChERS technique was initially validated on vegetable and fruit matrices, and later tested and applied on various types of samples, such as cereals (Kolberg, 2010), fatty matrices (Wilkowska, 2011), water and soil (Brondi, 2011), medicinal plants (Sadowska-Rociek, 2013; Huebschmann, 2012; Amirahmadi, 2013), juicies and other liquid products (Kong, 2016; Cherta, 2013) or food supplements (Thomas, 2010; Dominguez, 2014). In the present study the QuEChERS method was adapted for liquid samples.

MATERIALS AND METHODS

Dried ivy leaf (*Hedera helix*) and dried sage leaf (*Salvia officinalis*) were fortified with pesticide solutions of known concentrations, prepared in the lab from individual and mixed standards bought from dr Ehrenstorfer. Their selection was made taking into account the real chance of contamination, both with pesticides used for plant protection and with banned pesticides that are still present in the environment due to their persistence. The ivy leaves were subjected to ethylic alcohol extraction 70% (ratio 1/30), concentrated 20:1 in the rotary evaporator. The final extract was conditioned in propylene glycol. The pesticide levels were assessed in the alcoholic phase and in the final propylene glycol conditioned phase. The sage leaves were extracted with acetone and the bioactive compound (BAC) was crystallized by solvent evaporation. The pesticide concentration was determined both in the acetone phase and in the crystallized substance. The analytical extraction of pesticides from plant extracts was performed with a modified QuEChERS protocol, for alcoholic, acetonic and propylene glycolic liquid samples and for solid BAC samples. The parameters for the analysis steps are in table 1.

Table 1. Extraction and cleanup methods

Type of sample	Extraction method	Cleanup method
EA 70%	4 ml extract, solvent evap. and, ACN solvent exchange 1: 1	PSA-25mg, CA-10 mg, MgSO4-250 mg, all for 1ml extract; filtration 0,22 µm PTFE
AC	4 ml extract without prep.	
PG	1 g sample, 10 ml ACN extraction, with NACl (0.5 g and Mg SO4 (2g)	PSA-25mg, CA-10 mg, MgSO4-150 mg, all for 1ml extract, filtration 0,22 µm PTFE
BAC	0.2 g, 5 ml ACN extraction	PSA-25 mg, Mg SO4-150 mg, all for 1ml extract

where: EA-ethylic alcohol, AC-acetone, PG-propylene glycol, BAC-bioactive compound. The analysis was performed using an Agilent GC-MS equipment (7890A-5975C)–SIM mode. The acquisition parameters were HP-5MS column, 60 m x 0.25 mm, 0.25 µm, MMI Inlet with PTV solvent vent: 60^{O} C (0.35 min) → 600 ^{O}C/ min to 325 ^{O}C (5 min) , then 20 ^{O}C/min to 220 ^{O}C; 3µL injection volume; Oven profile: 50^{o}C=ct, 1 min, 25^{o}C/ min→ 190^{o}C, 0 min, 3^{o}C/min→202^{o}C, 0min, 1,5^{o}C/min→240^{o}C, 0 min, 5^{o}C/min→250^{o}C, 0 min, 2^{o}C/min→ 266^{o}C, 8^{o}C/min→290^{o}C, 15.2

min, run time = 62.5 min; solvent delay: 9 min.

Quantitation was performing through standard method, by Chemstation software using individual parameters for peak integration. The method is validated for 100 compounds, and the representative compounds for this study are shown in table 2, with the specific quantitation ions in the SIM mode and the initial plant concentrations.

Table 2. Pesticides monitored in the study

Pesticide	Activity	Quant Ion	Initial concentration in plant (mg/kg)
Chlorprofam	plant growth regulator	213	0.47
Terbutilazin	herbicide	173	0.02
Quintozene	fungicide	237	0.21
Diazinon	fungicide	304	0.27
Prothiofos	insecticide	267	0.24
γ HCH	persistent OCl	217	0.20
Tolclofos methyl	fungicide	265	0.33
Malathion	insecticide	285	0.67
Chlorpirifos	insecticide	199	0.09
Fenthion	insecticide	278	0.23
Dieldrin	persistent OCl	263	0.20
Deltametrin	insecticid	181	2.27

RESULTS AND DISCUSSIONS

A partial chromatogram, with the monitored compounds is shown in Figure 1, Figure 2 and 3 show overlapping chromatograms of pesticides with standard addition and the calibration curve for diazinon.

The transfer ratio between the initial level of pesticides in the samples and the alcoholic extract is between 15 and 76.8 %, for quintozene and terbuthylazyne, respectively.

The transfer ratio between the initial pesticide level in samples and the PG extract is between 6.8 and 55.8 %, for quintozen and dieldrin, respectively.

The acetone extraction and further concentration in preparation for BAC is a selective method, both increasing the BAC purity and decreasing the level of pesticides in the final product by a significant percentage (transfer ratio: 0.05-0.7 % in the final product).

The residues concentrations found in the different preparation methods are shown in table 3.

Fig. 2. Overlaping 304 ion for diazinon

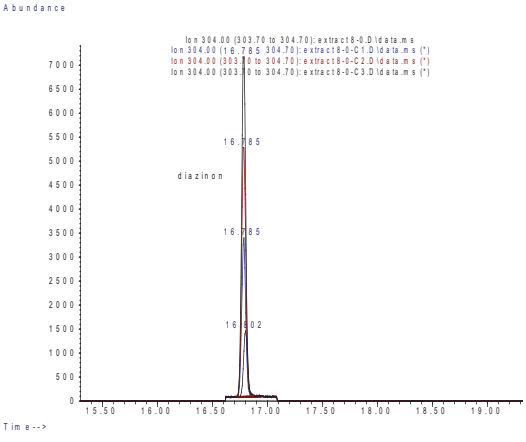

Fig. 3. Graphical representation of chromatographic responses using the standard addition method

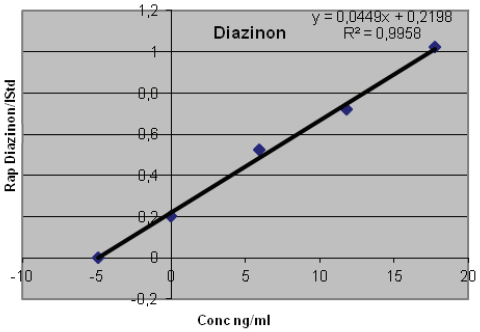

Fig.1 Section from the chromatogram with the monitored pesticides

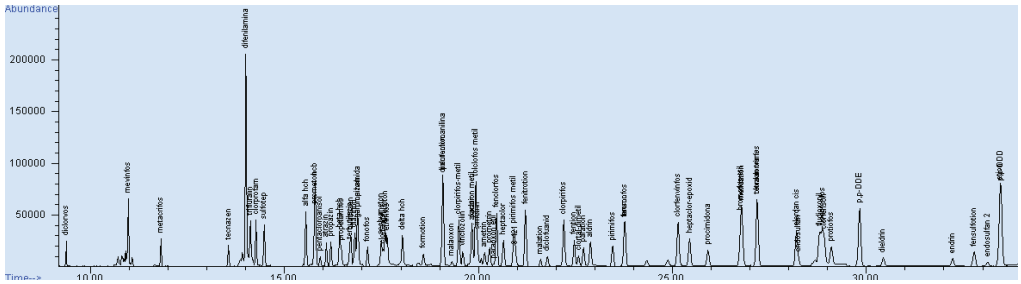

Table 3. The residues concentrations for different types of extraction

Pesticide	Recovery, % from initial quantity			
	AE extract	PG extract	acetonic extract	solid extract (BAC)
Chlorprofam	52	40	44	0.23
Terbutilazin	76.8	52	32	0.11
Quintozene	15	6.8	69	0.7
Diazinon	57.4	44	62	0.33
Prothiofos	28.6	15.5	69.5	0.44
γ HCH	75	50	72	0.5
Tolclofos methyl	51.6	20.73	80	0.3
Malathion	65	51	65	0.2
Chlorpirifos	50.2	30	99.5	0.55
Fenthion	27	17.7	41.5	0.05
Dieldrin	68.8	55.8	72	0.7
Deltametrin	45	25	80	0.1

CONCLUSIONS

The proposed analysis method is selective, and the peak separation in the SIM analysis conditions is appropriately used.

The quantification method through standard addition is adequate, as there is linearity between signals of aliquots with pesticides added in the final stage of analysis and the aliquot extracted without pesticide addition.

It is important to determine the ratio in which existing pesticides in the raw material transferred to extracts; extraction with alcohol allows retrieval of appreciable quantities of residues that remain at significant levels even if conditioning in propylene glycol is performed.

Acetone extraction followed by BAC crystallization was proved to be a very successful preparation method, as it leads to very low levels of residues in the solid extract for all pesticides (less than 1% of the initial concentration).

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REFERENCES

1. http://www.madr.ro/reziduuri-de-pesticide-in-plante-si-produse-vegetale/raport-de-monitorizare-a-reziduurilor-de-pesticide.html
2. Crentsil Kofi Bempah, Archibold Buah-Kwofie, Dzifa Denutsui, Jacob Asomaning and Anita Osei Tutu, 2011, Monitoring of Pesticide Residues in Fruits and Vegetables and Related Health Risk Assessment in Kumasi Metropolis, Ghana, Research Journal of Environmental and Earth Sciences 3(6): 761-771
3. Joseph Clement Akan, Lami Jafiya, Zaynab Muhammad Chellube, Zakari Mohammed, Fanna Inna Abdulrahman, 2014, Determination of Some Organochlorine Pesticide Residues in Vegetable and Soil Samples from Alau Dam and Gongulong Agricultural Sites, Borno State, North Eastern Nigeria, World Academy of Science, Engineering and TechnologyInternational Journal of Chemical, Molecular, Nuclear, Materials and Metallurgical Engineering Vol:8, No:4
4. Nsikak U. Benson* and Aruwajoye I. Olufunke, 2011, Assessment of contamination by organochlorine pesticides in *Solanum lycopersicum* L. and *Capsicum annuum* L.: A market survey in Nigeria, African Journal of Environmental Science and Technology Vol. 5(6), pp. 437-442
5. Bożena Łozowicka, Piotr Kaczyński, Elżbieta Wolejko, Janina Piekutin, Abay Sagitov, Kazbek Toleubayev, Gulmira Isenova&Elmira Abzeitova, 2016, Evaluation of organochlorine pesticide residues in soil and plants from East Europe and Central Asia, Desalination and Water Treatment , Volume 57, Issue 3
6. Simona Dobrinas, Gabriela Stanciu, Alina Soceanu and Adriana Culea, 2011, Analysis of organochlorine and pyrethroid pesticide residues in baby food samples, Ovidius University Annals of Chemistry Volume 22 Number 2, pp.107-112,
7. Elpiniki G. Amvrazi (2011). Fate of Pesticide Residues on Raw Agricultural Crops after Postharvest Storage and Food Processing to Edible Portions, Pesticides - Formulations, Effects, Fate, Prof. Margarita Stoytcheva (Ed.), ISBN: 978-953-307-532-7, InTech, DOI: 10.5772/13988. Available from: http://www.intechopen.com/books/pesticides-formulations-effects-fate/fate-of-pesticide-residues-on-raw-agricultural-crops-after-postharvest-storage-and-food-processing-t
8. Yichen Hua,LiWana, JinmingZhanga, FangYanga, JiliangCao, 2012, Rapid determination of pesticide residues in Chinese materia medica using QuEChERS sample preparation followed by gas chromatography–mass spectrometry, Acta Pharmaceutica Sinica B 2012;2(3):286–293
9. Anna Sadowska-Rociek, Magdalena Surma, Ewa Cieslik, 2012, Application of QuEChERS Method for Simultaneous Determination of Pesticide Residues and PAHs in Fresh Herbs, Bull Environ Contam Toxicol DOI 10.1007/s00128-012-0951-x
10. Maryam Amirahmadi, Shahram Shoeibi, Mehdi Abdollahi, Hossein Rastegar, Roya Khosrokhavar and

Morteza Pirali Hamedani, 2013, Monitoring of some pesticides residue in consumed tea in Tehran market, *Iranian Journal of Environmental Health Science & Engineering* 2013, 10:9

11. Ferdi Brahushi, Endrit Kullaj, 2014, The Assessment of Pesticides Residues in Some Organic Cultivated and Wild-Collected Medicinal Plants in Albania,*Albanian j. agric. sci. 2014*
12. S. K. Agbeve1, P. Osei-Fosu and D. Carboo, 2014, Levels of organochlorine pesticide residues in *Mondia whitei*, a medicinal plant used in traditional medicine for erectile dysfunction in Ghana, International Journal of Research in Environmental Studies
13. Al-Othman, Sherif H. Abd-Alrahman, and Nasser Al-Daghri, 2015, Application of QuEChERS Pesticide Multiresidue Method in Traditional Saudi Medicine and Analysis by Gas Chromatography Mass Spectrometry, *International Journal of Chemical Engineering and Applications, Vol. 6, No. 5, October 2015*
14. Wei-Jun Kong,Qiu-Tao Liu, , Dan-Dan Kong, Qian-Zhen Liu, , Xin-Ping MaMei-Hua Yang, 2016, Trace analysis of multi-class pesticide residues in Chinese medicinal health wines using gas chromatography with electron capture detection, Sci Rep. 2016 Feb 17;6:21558. doi: 10.1038/srep21558.
15. Seblework Mekonen, Argaw Ambelu, and Pieter Spanoghe, 2015, Effect of Household Coffee Processing on Pesticide Residues as a Means of Ensuring Consumers' Safety, *J. Agric. Food Chem.*, 2015, 63 (38), pp 8568–8573
16. Cortés, J. M.; Vázquez, A.; Santa-María, G.; Blanch, G. P.; Vill, J, 2009, Pesticide residue analysis by RPLC-GC in lycopene and other carotenoids obtained from tomatoes by supercritical fluid extraction, Food ChemistryVolume 113, Issue 1, 1 March 2009, Pages 280–284
17. Vania G. Zuin and Janete H.Y. Vilegas, 2000, Pesticide residues in medicinal plants and phytomedicines, Phytoterapy Research, 14, 73-88
18. M. V. N. Rodrigues, , F. G. R. Reyes, V. L. G. Rehder, S. Rath, 2005, An SPME–GC–MS Method for Determination of Organochlorine Pesticide Residues in Medicinal Plant Infusions, Chromatographia, March 2005, Volume 61, Issue 5, pp 291-297
19. Giacomo Dugo and Juseppa Di Bella, 2002, Citrus, the genus Citrus, cap: Contaminants in citrus essential oil, pag 518-520, Taylor and Francis
20. Sandra M. Garland, Prof. Robert C. Menary NW Davies, Garth S. Oliver, 2004,Practical approaches to the analyses for pesticide residues in essential oils, A report for the Rural Industries Research and Development Corporation –Australian Guvernment
21. European Pharmacopoeia 8th edition
22. Diana I. Kolberg, Osmar D. Prestes, Martha B. Adaime, Renato Zanella, 2010, Development of a fast multiresidue method for the determination of pesticides in dry samples (wheat grains, flour and bran) using QuEChERS based method and GC–MS, Food Chemistry, doi:10.1016/j.foodchem.2010.10.041
23. Angelika Wilkowska, Marek Biziuk, 2011, Determination of pesticide residues in food matrices using the QuEChERS Methodology, Food Chemistry 125 (2011) 803–812
24. S. H. G. Brondi, A. N. de Macedo, G. H. L. Vicente, and A. R. A. Nogueira, 2011, Evaluation of the QuEChERS Method and Gas Chromatography–Mass Spectrometry for the Analysis Pesticide Residues in Water and Sediment, Bull Environ Contam Toxicol. 2011 January; 86(1): 18–22
25. Anna Sadowska-Rociek,Magdalena Surma, and Ewa Cieślik, 2013, Application of QuEChERS Method for Simultaneous Determination of Pesticide Residues and PAHs in Fresh Herbs, Bull Environ Contam Toxicol. 2013 Apr; 90(4): 508–513
26. Hans-Joachim Huebschmann, Joachim Gummersbach, 2012, Multi-Residue Pesticide Analysis in Herbal Products Using Accelerated Solvent Extraction with a Triple Quadrupole GC-MS/MS System, Thermo Fisher Application Note 52291
27. Maryam Amirahmadi, Shahram Shoeibi, Mehdi Abdollahi, Hossein Rastegar, Roya Khosrokhavar and Morteza Pirali Hamedani, 2013, Monitoring of some pesticides residue in consumed tea in Tehran market, *Iranian Journal of Environmental Health Science & Engineering* 2013, 10:9
28. Jason Thomas, Julie Kowalski, Jack Cochran, Michelle Misselwitz, 2010, Evaluation of QuECheRS, Cartridge SPE Cleanup, and Gas Chromatography Time-of-Flight Mass Spectrometry for the Analysis of Pesticides in Dietary Supplements, LC-GC-Chromatography on line
29. Martínez-Domínguez G, Plaza-Bolaños P, Romero-González R, Garrido-Frenich A., 2014, Analytical approaches for the determination of pesticide residues in nutraceutical products and related matrices by chromatographic techniques coupled to mass spectrometry, doi: 10.1016/j.talanta.2013.10.006
30. Laura Cherta, Joaquim Beltran, Elena Pitarch, Félix Hernández, 2013, Comparison of Simple and Rapid Extraction Procedures for the Determination of Pesticide Residues in Fruit Juices by Fast Gas Chromatography–Mass Spectrometry, Food Analytical Methods, December 2013, Volume 6, Issue 6, pp 1671-1684

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THE INFLUENCE OF PHYSICAL PARAMETERS OF MALT IN THE QUALITY OF WORT FOR BEER

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Abstract

Decisive to produce a good beer is the production of qualitative wort. The process of wort production requires coordination of operations (temperature / time / pH) in order that from the raw materials used, to handle and to extract the maximum capacity of the production of wort for kg malt. To obtain the optimum potential from malt we should know and interpret very well its parameters. Physical characteristics of malt are very important and affect the technological process of brewing. In this paper are presented the results of physical analyses performed on samples of maltand also is studied the impact of these results in the quality of wort. Each base malt should have mealy contentof 90%. If we will use the infusion method the mealy content must be at least 95%. The bigger are the size of barley grains the higher is their efficiency. The uniformity of malt grains shows how uniform will be its fragmentation. Good malt should have about 90% of its grains uniform in terms of size. Malt is classified by how much percent of its grains are glassy. Therefore it is checked if grains are mealy, half glassy, glassy ends and glassy. If the endosperm is not more than 25% glassy then the malt is called mealy. The malt grains are half glassy if 25-75% of endosperm is glassy. When the endosperm is over 75% glassy the malt is called glassy. Glassy malt produces less extract because the grains are not fragmented as well and hydrolysis occurs with difficulties in mashing process.

Key words: *glassy, grains, malt, physical analyse.*

INTRODUCTION

Barley (*Hordeum vulgare*) is the cereal grain most often malted. Wheat (*Triticum aestivum*) and sorghum(*Sorghum vulgare*) are also malted in notable quantities (the latter in Africa), but small amounts of rye (*Secale cereale*), oats (*Avena sativum*) and millets (various spp.) are also used. The barley grain or corn has a complex structure (Briggs, 1978, 1998), and is a single-seeded fruit (a caryopsis). Barley varieties differ in their suitability for malting. Grains vary in size, shape and chemical composition. It is important to understand that malts consist of mixtures of grains with differing properties. Before malting, grain is screened and aspirated to remove large and small impurities and thin corns. To initiate malting it is hydrated. This is achieved by "steeping", immersing the grain in water or "steep liquor". Later, the moisture content may be increased by spraying or "sprinkling" the grain (Briggs et al., 2004).

A malt analysis will typically list three types of data: physical analysis, wort analysis and chemical analysis. While each attribute uniquely impacts the brewing process or finished beer, some have a greater impact and significance than others. The physical analysis include: glassy of malt, rate of crystallization, friability and size of malt grains. These tests are very important because through them we know the physical characteristics of malt, which will be used for wort production. Data from the analysis conducted with different malt samples, we see that the quality of wort take from them varies depending on physical characteristics. The physical parameters of malt affect the efficiency of wort, the milling of malt and the method that should be used for mashing.

MATERIALS AND METHODS

The data on which this paper is performed are obtained from the analyses that are made for different samples of malt. The period analysed

in this study was 2014-2015. The physical Analyses made are: the determination of glassy, of friability and the size of grains.

Glassy of malt

It is the opposite of mealy character of malt. The experiments proceed by taking a small quantity of malt grains. Marked with: 1 glassy grain, 0.5 half glassy grains, 0.25 the grains that have endosperm with glassy ends and 0 the mealy grains. Then calculate the quantity and the average. The observation was subjective and random therefore the grassy and mealy character of malt may be discussed.

Friability

Friability indicate if malt is easy milled and is related with mealy character of grains. In this test which indicates the level of modification, malt is crushed using a friability instrument. The friability is the percentage (by weight) of material that passes through the sieve. Investigation of material remaining on the sieve can be informative and can indicate if the malt corns generally contain unmodified material or if a substantial proportion of wholly unmodified grains are present.We weighed 50 grams malt that is placed in the drum of sieve in the form of a net. During a fixed time (8 min) cereal is pressed against a rotating metal net through the pressure force of a rubber roller. The crushed malt (particles fragmented easily) falls through the sieve in a container, while the glassy grains (the strong part) remain inside the drum. After testing will be possible the following fractions:

- The content of the drum, the glassy part.
- The content of the contanier is not important for evaluation and may be removed.

We weigh glassy part exactly after test them with an accuracy of 0.1 g.

$$M = 100 - 2 \times A$$

M = friability in % ; A = weigh in g of the fraction remaining inside the drum.

Grain size

The grain size is determined by sieve. Sortimat is an instrument used to measure the size of grains. Grain size was measured according to the methodology described previously (Fox et al. 2006), where in the upper sieve we put a sample of 100g clean grain. During the registered time the grain is selected depending of their size in four (five or six) fractions, as a result of the movement front-back of the sieve. By leaning the sieves with 90º, by opening the sliding plan and by pulling the cleaner of the sieves, the grain will fall into the vesseles which are placed in the bottom. Then we weigh the selectionated malt according to the grain size, respectively:

- Grains with size >2.8mm
- Grains with size 2.5-2.8mm
- Grains with size 2.2-2.5mm
- Grains with size > 2.2mm
- Half grains
- Damaged grains and impurity.

RESULTS AND DISCUSSIONS

Analyses for determining of mealy character of malt

Definition of mealy character of grains is done by taking samples with a weigh of 50 g. Malt samples were taken during the 2014-2015 in the different furnishing malt of beer factory. The separation of malt is made in two categories: glassy malt and mealy malt through friability instrument. After categorization of malt were done the weighing and by the above formula was calculated the friability. The rezults of analysis for various samples are summarized in the following table:

Table 1.The results of analysis for various samples for determination of mealy character of malts.

Number of sample	Weigh of mealy malt (g)	Weigh of glassy malt (g)	Friability (%)
1.	41.4	8.5	83
2.	43.4	5.7	88
3.	44.7	4.7	90.6
4.	36.66	12.84	74.32
5.	40.64	9.22	81.56
6.	41.5	8.3	83.4
7.	30.76	18.6	62.8

A similar evolution of cattle and dairy cow Friability shows if malt mill easly and is recomended a friability about 80%. From the results obtained see that samples 1,2,3,5 and 6 have a friability above 80%, so that these malts can mill easily. Samples 4 and 7 have friability under 80%, so that these malts mills with difficulties. Friability affects the quality of wort having a direct impact on the amount of the extract. After milling, malt mixed with water

and formed the mash. The efficiency of mashing is often estimated by comparing the extract recovered in the brewery with that obtained in laboratory mashes when the hot water extract (HWE) of the grist is determined. The mash is wormed, to dissoved the important substances in the water, obtaining the sweet wort.The purpose of this process is to obtain a production of the extract as high as possible from the milling malt. If we use a common procces of wort production would deal only 10-15% of the extract, the amount of the extract would be 10-15%. We determined in laboratory for each sample of malt the quantity of extract obtained.

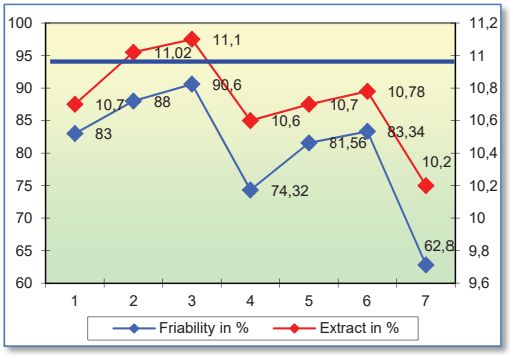

Figure 1.Friability performance for different samples of malts.

From this chart (Figure 1.) it is clear that the values of friability for different sample of malt are below the thick blu line. Since friability of malt is below 95% is not recommended to work with the infusion method. Friability walks in proportional to extract of wort.The chart shows that the much higher friability is, much higher extract of wine is produced. The above results in the wort production is the same and is working in relatively constant conditions.

Analysis for determinate the grain size of malt

In parallel with the analyses for determinate the friability were conducted the analyses for determinate the grain size for the same malts that furnished the beer factory. In this case were taken more samples of malt with a weigh of 100g. Samples were taken after the cleaning process. Through sortimatit the malt grains are divided depending on their size according to the method explained above. The results obtained from these tests are summarized in the following table (Table 2).

The experiments confirm that malt grains in general, 98% have greater size than 2.2 mm. Different samples of malt used for analyses have a constant performance in terms of size. Malt with grains size less than 2.2 mm should be returned because it meant that their grains have not made the modification. The larger are the grains of barley, the higher is their productivity.

Table 2.The results of analysis for various samples for determination of grains size of malts

Number of sample	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Weigh of grains with size >2,8mm (g)	77.98	89.90	89.90	74.08	69.28	87.84	69.70	82.02	72.32	89.52
Weigh of grains with size 2,8-2,5mm (g)	16.92	7.1	8.4	19.64	21.36	10.56	22.1	14.42	21.7	8.20
Weigh of grains with size 2,5-2,2mm (g)	3.54	1.3	1.0	4.4	5.70	0.98	5.3	2.28	4.46	0.9
Weigh of grains with size <2,2mm (g)	0.64	0.4	0.3	0.86	2.06	0.16	1.6	1.04	0.66	0.28
Weigh of half grains (g)	0.78	0.8	0.38	0.84	1.22	0.38	1.1	1.32	0.80	1.10
Weigh of damaged grains and impurity (g)	0.1	0.3	0.1	0.1	0.38	0.06	0.2	0.58	0.12	0.0

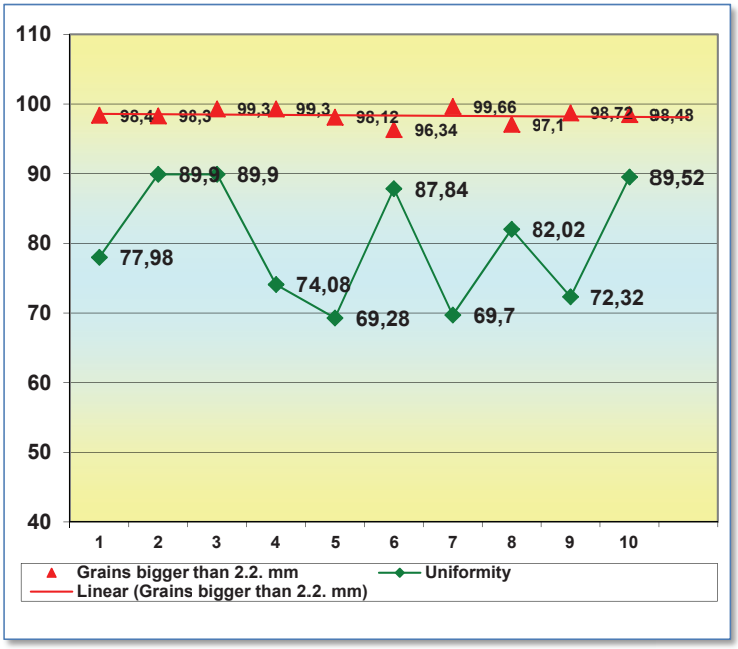

Figure 2.Grains size and uniformity performance for different samples of malts

However except this quality malt must complete the criteria of uniformity. Uniformity of malt grains shows how uniform will be its milling.Malt completes the standards, if the uniformity of its grains is 90%. As evident from the chart (Figure 2), in our case there is no sample that have a uniformity higher than 90%.

CONCLUSIONS

Good malt must have e mealy content. Every base malt should have a mealy content of 90%. If we have to use the infusion method to produce the wort, the mealy content must be at least 95%. For base malt where their composition is expressed through the proportion, mealy/half glassy/glassy, the ratio should be 92% / 7% / 1% for decoction method and 95% / 4% / 1% for infusion method.

Friability is the characteristic that is related with the mealy character of barley grains. Malt should have a friability about 80%. When we use the infusion method, malt should have a friability at least 95% or higher.

The size of the malt grains is physical parameter very important that is determined through Sortimati. In Europe generally used malt with grains size 2.2 mm. Malt with grains size less than 2.2 mm should be returned because it meant that their grains have not made the modification. The larger are the grains of barley, the higher is their productivity. Good malt should have about 90% of its grains uniform in terms of size.

REFERENCES

Briggs, D. E., 1978.Barley.Chapman & Hall, London.612 pp.

Briggs, D. E., 1998.Malts & Malting. Blackie Academic & Professional, London. 796 pp.

Brissart R., Brauninger U., Haydon S., Morand R., Palmer G., Sauvage R., Seward B., 2000. European Brewery Convention Manual of Good Practice.Malting Technology.Fachverlag Hans Carl. NuÈrnberg.224 pp.

Briggs D.E., Boulton C.A., Brookes P.A., Stevens R., 2004. Brewing Science and practice. Published by Woodhead Publishing Limited, Abington Hall, Abington

Fox G.P., Kelly A.M., Poulsen D.M.E., Inkerman P.A., Henry R.J., 2006. Genetic and environmental effects on selecting improved barley grain size in dry environments. Journal of Cereal Science 43.

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INVESTIGATION OF SOME EXTRACTION METHODS FOR THE RECOVERY OF PEANUT PROTEINS FROM OILS AND FATS

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Abstract

Food allergies have a considerable impact on modern society. There is no known cure. As a result, consumers can only avoid offending foods and use pharmacological agents. Some of the most severe allergic reactions occur when peanuts and peanut derivatives as peanut oils are consumed. The food industry will have to comply with requirements set forth by law for all packaged foods sold in the European Union. At times, it can be difficult to measure allergenic proteins in a wide variety of foods. Yet the food matrix can sequester allergens, inhibiting their detection, without significantly affecting allergenicity. The studies about allergenicity of edible oils and related to peanut oils are few and enough controversies. Some studies showed the presence of peanut allergens some not. It has to be emphasised that different studies used different methods for extraction, concentration and detection the peanut traces so the results had difficult been compared. In this context we investigated some extraction and concentration methods for the recovery of proteins from oils and fats derived from, or containing, peanut. The recovery of total protein and peanut allergens are very different for each method. Our result show how much the results depend on the method used to extract or/and concentrate the proteins from different matrices. The influence of solvent plays an important role in that process. Interactions with lipids of protein may alter the possibility to detect and quantify them by a hiding allergen/protein effect.

Key words*: peanut allergens, oil, extraction methods, concentration methods.*

INTRODUCTION

Peanut and nut allergens represent nowadays a challenge for health and food manufacturers both. The threat of an adverse reaction can be present for sensitive people everywhere in food.

Peanuts are one of the 8 most common allergenic foods and a large proportion of peanut-allergic individuals have severe reactions, some to minimal exposure. Specific protein constituents in the peanuts are the cause of the allergic reactions in sensitized individuals who ingest the peanuts.

Peanut seeds are rich in oil (40 – 50 %) and as a consequence are used like an excellent source of oil. It seems that peanut oilseeds production is in a slightly increment in the last years.

Refined peanut oil is usually labelled as vegetable oil but according with the European legislation now it is mandatory to specify on label when peanut is used.

The allergenicity of refined peanut oils is not so clear (Moneret-Vautrin, Hatahet et al., 1991; Moneret-Vautrin, Hatahet et al., 1994; Hourihane, Bedwani et al., 1997; Peeters, Knulst et al., 2004).

Some studies showed the allergenicity, some not, of edible peanut oils. However it is obviously that the refining process may affect the allergenicity and the thresholds for adverse reaction vary according to sensitive patient.

The studies about allergenicity of edible oils and related to peanut oils are few and enough controversies.

Edible oils undergo usually extensive processing which removes virtually all the protein from the oil. Scientific studies showed that refined oils don't contain allergic protein in detectable amounts (Hefle, 1999). But some studies showed that vegetable oils/fats, crude or even refined, can contain proteins - in peanut allergens case - even that these were hot-pressed processed (Klurfeld and Kritchevsky,

1987; Hoffman and Collins-Williams, 1994; Teuber, Brown et al., 1997; Koppelman, Bruijnzeel-Koomen, et al., 1999; Zitouni, Errahali et al., 2000; Hidalgo, Alaiz et al., 2001; Hidalgo, 2006). Usually by mechanical or cold press the allergenic proteins are not removed in totality, have been considered impurities. These oils aren't used domestically but are often found in healthy food, with increased nutritional value, or in gourmet food stores. Therefore the restaurants and food service facilities have to specify what kind of oil was used. In the meantime vegetable oils, and obviously the oil from peanut, are used for preparing margarine and spreads, and if oils used contained allergenic protein the product would contain it too. Obviously, if some ingredients contain protein from the source material, they are likely to be allergenic to consumers who are allergic to the source foods (Taylor and Hefle, 2001).

Nowadays are known 17 peanut allergens, Ara h1 - Ara h17.

Major peanut allergens are Ara h1, Ara h2, Ara h3 with Ara h4 as isoallergen of Ara h3 and Ara h6. Minor allergens are Ara h5, and Ara h7 to Ara h17. Belong to these proteins there is Ara h agglutinin whose role is not yet clearly understood.(Olszewski, Pons et al., 1998; Besler, Steinhart et al., 2001; Enrique, Utz et al., 2006; Agrawal et al., 2010; Mueller et al., 2014; Offerman et al., 2015; Schwager et al., 2015).

The published peanut proteins/allergens content of edible peanut oils fluctuate widely being dependent on source of the oil as well as the methodology used for extraction, concentration and analysis. Each manufacturer uses own protein extract method, content determination method and standards. The manufacturing processes are very different and the residual protein content too.

To avoid accidental ingestion of peanut-contaminated food, methods of analysis for the determination of the allergenic proteins in foods are important tools. Such methods could help identify foods inadvertently contaminated with peanuts, thereby reducing the incidence of allergic reactions to peanuts. Commercial immunoassay kits are available but need study for method performance, which requires reference materials for within- and between-laboratory validations. Alternative methods are necessary too in aim to have a better analysis of allergen proteins.

This study will report a comparison and assessment of some peanut allergens extraction methods.

MATERIALS AND METHODS

The test samples were obtained spiking pasteurized fresh margarine with peanut reference material 481 (IRMM). Butter was spiked with peanut grinded and peanut extract in Tepnel buffer without gelatine for 10.000 ppm, 1000ppm, 100ppm 10 ppm and 1ppm concentrations.

The test samples were obtained using Unirea Original margarine, 60% fat, produced by SC Orkla Foods Romania SA.

Figure 1. Unirea Original margarine

The reference material containing the peanut allergens, denoted 481, was obtained from the Institute for Reference Materials and Methods (IRMM), Belgium.

The samples for testing were obtained by homogenizing the reference material, which has a content of 1000000 ppm Ara h1, in appropriate amounts to give concentrations of 10 000 ppm, 1000 ppm, 100 ppm, 10 ppm and 1ppm. For homogenization was used a Braun mixer.

For immunochemical analyses of peanut allergens we used BioKit-sandwich Cat ELISA. No.902048Q. The kit was purchased from R-Biopharm, Darmstadt Germania.

Stirrers centrifuge and ultracentrifuge, water baths are necessary to prepare the samples and extracts.

For allergens analysis was used a plaque reading spectrometer Model 3200, serial number 2100, from Awareness Technology Inc. US.

Extraction methods:

Method I: 1.0 g (± 0.1 g) of each sample was weighed and extracted with 10 ml Tris-HCl buffer (0.6 % Tris, 1.17 % NaCl and 10 % gelatine; pH 8.2) for 15 min at 60 ºC in a water bath with continuous shaking. The extracts were centrifuged at 1730 g for 20 min at 4ºC. The supernatant was collected and used in the analyses. This method is Tepnel kit extraction procedure.

Method II: 1.0 g (± 0.1 g) of each sample was weighed, melted at 40°C and CMC solution was added into the same beaker to a total weight of 8g. The mixtures were homogenized until homogenise emulsions were obtained and the samples were stored in a fridge until using.

Method III: 1.0 g (± 0.1 g) of each sample was weighed and extracted with 0.8 ml of 0.2mol/L ammonium bicarbonate, pH=7.8, for 48 hours at room temperature (20–22^{o}C) using a rotative stirrer. The extraction tubes were centrifuged, 3800g/30min/4°C and the clear aqueous layers were collected by suction with syringes after a part of fat layer is removed.

Extraction plus Concentration methods:

Method IV: 1 g samples + 5 mL ammonium bicarbonate 0.1 M were shacked overnight at 60°C. 5 ml of hexane was added to each sample; samples were vortexes until they formed an emulsion and then centrifuged at 3800g/30min/4°C. The hexane layer was removed and other 10 ml of hexane were added following the above procedure (2 times). The aqueous layer was removed carefully with a syringe. 2 mL aqueous layer was treated with 20 mL cold 10% TCA in acetone; samples were incubated at 20^{o}C overnight. Samples were centrifuged at 18000g/15min/4^{o}C. Precipitates were dissolved in Tepnel buffer (without gelatine) until 0.75 ml each.

Method V: 1 g sample (before add the extraction buffer I melted the samples at 45^{o}C for 10 min) + 20mL 20% ethanol in TBS was vortexes then put in the ultrasonic bath for 20 min/4°C. Centrifugation was made at 9300g/30 min/4°C. Were taken 10 ml aqueous layer without disturbing the lipid layer and put in a clean tuba; the rest of solution was discarded. Were added 40 ml 10% TCA in acetone, mixed well with Turax and then incubated on ice 15 min. The samples were centrifuged at 9300/30min/4°C. The supernatant was removed and then added 1 ml mili Q water and vortexes 15 sec. 10 mL cold acetone were added and vortexes until all pellets were dispersed; incubated at -20°C overnight. The samples were centrifuged at 9300/30min/4°C. The supernatant was carefully removed and the pellets dissolved in 1.5 ml Tepnel buffer (without gelatine) and kept the product at freezer.

RESULTS AND DISCUSSIONS

The extractable proteins consist of both the allergenic proteins and non-allergenic proteins. The proportion of which may vary from product to product and of the matrix composition.

Taking in account the mainly methods used by different laboratories to extract and concentrate the oil/fat proteins we followed the core ideas of some of its. So we extracted by 3 methods and extracted and concentrated the oil/fat proteins by 2 methods.

To evaluate the capacity of extraction and concentration of the methods used we processed and analyzed the same spiked peanut and we reported the results to the same starting quantity 1g margarine spiked with peanut even if the quantities of sample used by various methods were different. So in aim to compare the efficiency of each method to report all results to 1 g original sample it is the best choice.

The general averages of peanut allergens from each extracted sample resulted by extraction methods only are presented in Table 1.

Analyzing the results from the table above we see that in the method II case the allergen proteins detected are less than those detected by the reference method I (Tepnel method). Taking in account the fact that by method II we used more sample (corresponding to 1.25 g margarine spiked processed) following the same procedure as in Tepnel method but without a supplementary buffer extraction, the peanut allergens content obtained showed actually a dilution and the CMC solution even if realizes a better homogeneity and stability in time is not a very good buffer for extraction.

Table 1. Averages of peanut allergen content in samples extracted by different methods

Sample	Method I		Method II		Method III	
	ppm	Recovery, %	ppm	Recovery, %	ppm	Recovery, %
1	0.5	53.0	1.7	177.3	7.7	777.5
10	7.5	75.2	5.23	52.3	93.5	935.2
100	95.2	95.2	37.1	37.2	1242.8	1242.8
1000	1131.3	113.1	561.2	56.1	12465.9	1246.5
10000	13057.5	130.6	11822.3	118.2	127834.3	1278.3

Table 2. Averages of peanut allergen content in samples extracted and then concentrated by different methods

Sample	Method I		Method IV		Method V	
	ppm	Recovery, %	ppm	Recovery, %	ppm	Recovery, %
1	0.5	53.0	0.10	10.0	0	0
10	7.5	75.2	1.7	17.3	0	0
100	95.2	95.2	5.2	5.2	4.6	4.6
1000	1131.4	113.1	34.4	3.4	22.4	2.3
10000	13057.6	130.6	205.2	2.1	240.6	2.5

Method III seems that realizes a concentration in the meantime with the extraction. The explanation is very simple. The quantity of buffer extraction was only 0.8 ml per 1g sample so we obtained a solution 12.5 time concentrated. If we take in account this factor of concentration and calculate the real quantity detected using Tepnel procedure we find that the peanut allergens recovered are less than by method I (1:0.62; 10:7.5; 100:99.4; 1000:997.3; 10000:10226.7). But even so this method has the advantage to concentrate the sample just by extraction in a small quantity of buffer.

The general averages of peanut allergens from each extracted sample resulted by extraction and then concentration methods only are presented in Table 2.

The above results show how much the different preparation method of a sample presumed that contain protein allergens might lead to unreal values, to the loss of allergens. The capacity of recovery it seems that is not depend on starting concentration being enough similar for all kind of sample in the same method.

Considering the quantity of allergen which was added and the quantity which we found in each sample we calculate the concentration factor.

In Table 3 are presented the concentration factor calculated against the theoretical quantity which was added (CFa) and the concentration factor against the quantity which we found in the original samples (margarine spiked with peanut) (CFb) for each method. As we presumed the methods III have the biggest concentration factor. The large variability between results for the same method can be explained mainly by the strong influence of matrix but by the difficulty of each method too. In the method III case the lower ratio between sample quantity and the extraction buffer (1 g/ 0.8 mL) determined a bigger extraction of allergens/proteins. Nonetheless this method doesn't offer a complete extraction of peanut allergic proteins.

Table 3. Concentration factor of peanut allergens

Sample	Method I	Method II		Method III		Method IV		Method V	
	CFa	CFa	CFb	CFa	CFb	CFa	CFb	CFa	CFb
1	0.50	1.77	3.54	7.78	15.56	0.100	0.200	0.000	0.000
10	0.75	0.52	0.70	9.35	12.47	0.170	0.227	0.000	0.000
100	0.95	0.37	0.39	12.43	13.05	0.052	0.055	0.046	0.048
1000	1.13	0.56	0.50	12.47	11.02	0.034	0.030	0.022	0.020
10000	1.31	1.18	0.91	12.78	9.79	0.021	0.016	0.024	0.018

Taking in account the recovery capacity and concentration factor the best method is III and the weakest VII: III > II > IV > V.

Our results show how much the results depend on the method used to extract or/and concentrate the proteins from different matrices, the influence of solvents plays an important role in that process and that some proteins/peptides are soluble in lipid matrixes. In addition fatty acids which are present in dairy products and industrially hydrogenated vegetables (such as margarine) affect the quantity determination of protein content.

Finally, the lowest observed adverse effect levels of allergenic proteins in edible oils should be determined and simple methodologies developed for their analysis. All these advances will contribute to development of naturally nutritionally enhanced and safer edible oils.

CONCLUSIONS

As a general conclusion to evaluate samples regarding peanut protein included into a mass with a large quantity of an outsider protein it is a difficult task because that protein can hidden the protein what we looking for. There had been some speculations that total extractable proteins were not correlated to their allergenicity or allergen contents.

However our results like other presented in different articles show that between total proteins an allergen proteins exist a significant correlation.

Complex and time consuming protocols cause wastage of proteins, materials and time too.

We proposed simplified method to extract and concentrate the proteins/allergens from oils or vegetable fats.

The use of validated analytical methodologies for extraction/concentration and for establishing proteins/allergens content of oil are required to compare the data obtained by different laboratories. The development of more simple methodologies to extract or/and concentrate oils proteins it is necessary in aim to be applied routinely in research laboratories and industrial plants.

The lack of use of appropriate and validated methodology for protein content determination still pose questions touching the validity of oil proteins data from different published studies.

The lowest observed adverse effect levels of allergenic proteins in edible oils should be determined and simple methodologies developed for their analysis. All these advances will contribute to development of naturally nutritionally enhanced and safer edible oils.

REFERENCES

Agrawal P., Kumar S., Das H.R., 2010. Mass spectrometric characterization of isoform variants of peanut (Arachis hypogaea) stem lectin (SL-I). Journal of Proteomics, 73(6): 1573-1586.

Besler M., Steinhart H., et al., 2001. Stability of food allergens and allergenicity of processed foods. Journal of Chromatography B: Biomedical Sciences and Application, l 756(1-2): 207-28.

Enrique E., Utz M., et al., 2006. Allergy to lipid transfer proteins: cross-reactivity among pomegranate, hazelnut, and peanut. Annals Allergy, Asthma and Immunology, 96(1): 122-3.

Hefle S., Taylor S.L., 1999. Allergenicity of edible oils. Food technology, 53(2): 62-70.

Hidalgo F., Zamora R., 2006. Peptides and proteins in edible oils: Stability, allergenicity, and new processing trends." Trends in Food Science & Technology, 17: 56-63.

Hidalgo F.J., Alaiz M., et al., 2001. Determination of peptides and proteins in fats and oils. Analytical Chemistry, 73(3): 698-702.

Hoffman D.R., Collins-Williams C., 1994. Cold-pressed peanut oils may contain peanut allergen. The Journal of Allergy and Clinical Immunology, 93(4): 801-802.

Hourihane, J.O., Bedwani S.J., et al., 1997. Randomised, double blind, crossover challenge study of allergenicity of peanut oils in subjects allergic to peanuts. BMJ, 314(7087): 1084-8.

Klurfeld D.M., Kritchevsky D., 1987. Isolation and quantitation of lectins from vegetable oils. Lipids. 22(9): 667-668.

Koppelman S. J.,. Bruijnzeel-Koomen C.A, et al., 1999. Heat-induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. The Journal of Biological Chemistry, 274(8): 4770-4777.

Moneret-Vautrin D.A., Hatahet R., et al., 1994. Risks of milk formulas containing peanut oil contaminated with peanut allergens in infants with atopic dermatitis. Pediatric Allergy Immunology, 5(3): 184-188.

Moneret-Vautrin D.A., Hatahet R., et al., 1991. Allergenic peanut oil in milk formulas. The Lancet, 338(8775): 1149.

Mueller G.A., Maleki S.J., Pedersen L.C., 2014. The molecular basis of peanut allergy. Current Allergy and Asthma Reports, 14(5): 429.

Offermann, L., Perdue M., He J., Hurlburt B., Maleki S., Chruszcz M., 2015. Structural Biology of Peanut Allergens. Journal of Contemporary Immunology, 2(1): 1-26.

Olszewski A., Pons L., Et Al., 1998. Isolation and characterization of proteic allergens in refined peanut oil Clinical & Experimental Allergy, 28(7): 850-859.

Peeters, K.A., Knulst A.C., et al., 2004. . Peanut allergy: sensitization by peanut oil-containing local therapeutics seems unlikely. The Journal of Allergy and Clinical Immunology, 113(5): 1000-1001.

Schwager C., Kull1 S., Krause1 S., Schocker F., Petersen A., Becker, W-M., Jappe, U., 2015. Development of a Novel Strategy to Isolate Lipophilic Allergens (Oleosins) from Peanuts. PLoS ONE, 10(4):e0123419.

Taylor S.L. and. Hefle S.L., 2001. Ingredient and labeling issues associated with allergenic foods. Allergy, 56 (67): 64-69.

Teuber S.S., Brown R.L., et al., 1997. Allergenicity of gourmet nut oils processed by different methods. The Journal of Allergy and Clinical Immunology, 99(4): 502-507.

Zitouni N., Errahali Y., et al., 2000. Influence of refining steps on trace allergenic protein content in sunflower oil. The Journal of Allergy and Clinical Immunology, 106(5): 962-967.

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VEGETABLE OIL CONVERSION INTO CORE-SHELL BIOPRODUCTS FOR STORED GRAIN PROTECTION

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Abstract
The paper presented a possibility for the eco-efficient valorisation of vegetable oils, a precious renewable natural resource from Romanian wild and cultivated flora, cheap and abundant raw materials for conversion into value added bioproducts for plant protection in organic agriculture systems and great importance for bioeconomy. An original technical solution was proposed for the stored grains protection against pests and diseases transferred from agricultural field to warehouses after the harvest time. Based on the validated properties of insecticidal diatomaceous earth and antimicrobial essential oils extracted from aromatic plants, an eco-friendly bioproduct was conceived and produced using a clean technology of green chemistry inspired by cold saponification process of natural fats followed by the microencapsulation of the essential oil in the soft potassium soap. One variant of the plant protection bioproduct was obtained by conversion of cold pressed rapeseed oil and essential thyme oil into a core-shell formulation obtained by granulation of the concentrated oil emulsion with fine powdered diatomaceous earth thus improving the controlled release of bioactive principles from the organomineral structure. The significant repellence potential against Sitophilus granaries insect adults and strong fungicidal action of thyme essential oil coupled with insecticidal effects and mycotoxin absorbent capacity of diatomite recommended the new product with a wide spectrum of action for a long preventive protection against biological contamination of warehouses.

Key words*: vegetable oils, essential oils, stored grain protection, cold saponification.*

INTRODUCTION

The most important risk factors regarding the contamination of stored grains are the pests and diseases transferred from agricultural field to storage at the time of harvest. Insects hatching inside the stored cereal grains release metabolites which promote microbial growth in favorable conditions of temperature and humidity. The most dangerous pathogens are fungal strains producing mycotoxins, metabolic products which reduce the quality of grains contaminating agro-products for feed or food processing and seriously affecting the health of animals and humans. Most agrochemicals currently used to control pests and diseases of stored grains are organophosphoric fumigants very soon under ban due to their high toxicity for mammals and environment.

Present tendency of scientific research to discover and develop environmental friendly products, with high efficiency and low toxicity for users, led to the reconsideration of renewable natural resources, rich in bioactive principles for the protection of stored grains against pests and diseases.

Diatomaceous earth used as mineral vehicle for solid agrochemicals was considered the most effective mechanical insecticide for controlling insects damaging cereal seeds, the mechanisms of action being the rapid dehydration of the cuticle by contact and probably blocking the digestive system after ingestion (Lupu C., Manole T., 2015). Diatomite was also used to limit the contamination produced by micotoxigenic fungi growing on cereals, acting as an absorbent for mycotoxins.

Several essential oils extracted from wild or cultivated aromatic plants with antioxidant and antimicrobial effects have been used against fungal toxigenic growth on grains during storage. Essential oils extracted from leaves of eucalyptus and cupressus by hydrodistillation proved an excellent insecticidal and repellent action against stored grain insects when applied by fumigation (Bett Philip K. & al., 2016).

Volatile oils of thuya unpeeled fruit, eucalyptus and peppermint have proven fumigant effect against adult *Sitophilus granaries* in the storage of wheat (Hamza Ali F. & al., 2015).
Bio-based plant protection products should be formulated by microencapsulation for stabilization and controlled release of bioactive principles. In order to reduce the use of chemical pesticides, seed treatment should become the preferred application technique of protection within integrated management systems (Matyjaszczyk E., Pieczyńska A., 2015). Bioproducts based on essential oils are relatively non-toxic to vertebrates, fulfills the criterion for low-risk pesticides and should be included in organic farming (Krimer Malešević V. & al., 2016). They are valuable alternatives to synthetic pesticides applicable in agriculture, beekeeping, food and medicine, offering limitless opportunity for scientific research to find new directions and possibilities of application in the future (Sparagano O. & al., 2016).
Present work proposed a technical solution for the simultaneously protection of stored grains against pests and diseases with an eco-friendly product obtained through a simple clean technology of "green chemistry" (Popescu M., Oancea F., Desliu-Avram M., 2015).

MATERIALS AND METHODS

The eco-friendly product proposed for stored grain protection was made using the following materials: a vegetable oil, potassium hydroxide, one or more bioactive essential oils, powdered diatomite, acetic acid, water.
Nonvolatile oil can be any type of cooking oil, waste oil from fast-food or restaurants, waste oil from industrial processing of vegetable oils, preferable vegetable oils extracted from seeds of oilseed plants such as rapeseed, camelina, castor, and mustard containing biofumigant active principles. Volatile essential oils were selected based on fungicide and insecticide bioassays specific for pests and diseases of warehouses, from essential oils of thyme, thuja, sage, basil, oregano, rosemary, cloves, cinnamon, coriander, pine.
The first experimental sample was prepared using cold pressed rapeseed oil (Luna Sola Romania), KOH (89.3 % purity scales, produced by Lach - Ner, Czech Republic), essential oil of thyme (Solaris, Romania) by more than 42 % thymol active substance (Figure 1), and diatomaceous earth (Figure 2).

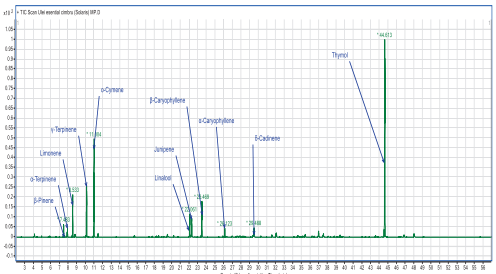

Figure 1. Gas chromatogram of essential thyme oil

Diatomite rocks purchased from Romanian holdings Adamclisi Urluia, Pătârlagele and Adamclisi Fabrica were finely ground, dried and tested to establish the water activity value (Aw) with a portable device LabSwift-aw (Novasina). The relative humidity RH (%) = w x 100 was under 17% for products resistant to fungal contamination in stored grains or feed.


Figure 2. Diatomaceous earth from Romanian holdings Adamclisi Urluia, Pătârlagele and Adamclisi Fabrica

The process for preparing the oil-based product involved several experimental steps: a) cold saponification of vegetable oil with potassium hydroxide in aqueous solution with stirring at 52ºC; b) neutralization the excess of potassium hydroxide with acetic acid to pH=9; c) encapsulation of the volatile oil into the soft soap texture; d) entrapping the oily core in diatomite coating using a rotating drum or a fluid bed granulator; e) drying the granules to air under ambient temperature and pressure; f) shifting the granules on different size meshes to packaging. Essential oil retention in the core was determined by extracting the crushed granules with methanol and identifying the major constituents of essential oils using a

7000 TripleQuad Agilent Technologies GC-MS by NIST library.

RESULTS AND DISCUSSIONS

Experimental for active ingredient selection

The most suitable type of diatomite was selected as function of their special features.

1.Patârlagele: 14,4% rh (26,9°C)

2.Adamclisi Urloaia.: 15,9% rh (27,2°C)

3.Adamclisi Fabrica.: 15,8% rh (27,4°C)

Diatomaceuos earth have the relative density of 320-430 kg/m^3, specific surface area 10-30 m^2/g, Mohs hardness 4.5-5, and liquid absorption capacity of at least 150 %.

The dose of 900 ppm (900 g/ton seeds) of diatomaceous earth used to control the attack of *S. granarius* in the cereal storage was effective after 21 days of exposure, as compared with an untreated control and a standard diatomaceous earth supplemented with a natural pyrethroid. The mortality of individuals of harmful population was significant after 14 days of application, recording values between 83.33% (source Urloaia and Adamclisi) and 100% for diatomite from Pătârlagele which blocked the development of insect reproduction after 60 days from the application (Lupu C., Manole T., Chiriloaie A., 2016).

A preliminary study conducted in laboratory conditions had been tested insect *S. granarius* behavior to thyme, thuya and oregano oils, individuals of this species showing a strong repellent reaction to *Thymus vulgaris* oil (Manole T., Fatu V., 2016). Administered in small doses in cereal stocks, thyme essential oil induced to harmful individuals a changing of feeding rhythm and disturbance of motility and mobility, allowing closer and faster contact with nearby diatomaceous earth.

Experimental cold saponification

A laboratory system composed of glass flask of 1 liter capacity, fitted with reflux cooler, mechanical stirrer, thermometer and dropping funnel (Figure 3) was loaded with 150 grams of cold pressed rapeseed oil which was heated at 55-60ºC, then 75 ml aqueous KOH 25% was added dropwise with stirring and saponification reaction was completed after 2.5 hours. The pH of the resulting mixture was adjusted to 8.5-9 with acetic acid. A portion of 15 g of thyme essential oil were added dropwise with stirring, and more mixing for another 30 minutes obtaining a concentrated emulsion.

Figure 3. Cold saponification of vegetable oils using a laboratory glass installation

Experimental formulation and packaging

A 70 grams portion of the fluid obtained from the concentrated oil emulsion were added dropwise into a laboratory rotating metal drum (Figure 4), loaded with a fine powder obtained from ground diatomaceous earth and 120 grams were retained on the surface of the granules.

Figure 4. Vegetable oil-based product formulation using a rotating drum granulator

Maintaining about 24 hours the obtained wet granules in open air at ambient temperature and pressure, 160 grams of dry product were obtained and sieved to yield granules with 2 mm, 1.6 mm and 1.02 mm grains (Figure 4).

Characterization of final product

The vegetable oil-based product for stored grain protection against pests and diseases resulted as gray-brown granules (Figure 5), with porous aspect, pleasant smell, and chemical composition: 15-25 % of potassium soap of rapeseed oil, 3-5 % potassium acetate, 1.5-3 % glycerin, 1-5% thyme oil, 40-65% of diatomaceous earth, 1.5-2% unsaponifiable matter and water up to 100%. The ability of the granules to retain the volatile oil by internal pores of diatomaceous earth and by oily core

(about 65 % thymol) was shown by GC-MS analysis (Figure 5).

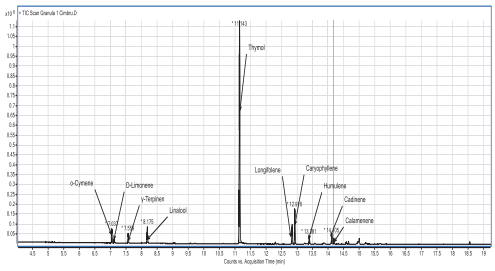

Figure 5. Composition of thyme oil retained in granulated core-shell product

When a similar composition was formulated using a fluidized bed granulator, the efficacy of essential oil encapsulation was ten times lower than with the rotating drum, due to the volatile oil drive with the air flow (Figure 6).

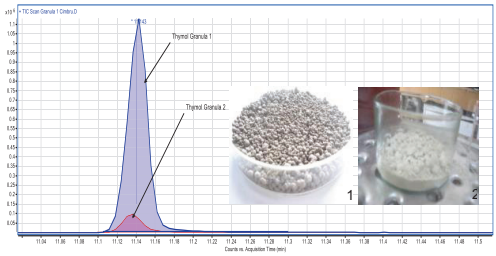

Figure 6. Comparative GC assay of thyme oil retained in oil-based product using rotating drum (1) and fluidized bed granulator (2) expressed as thymol concentration

After our knowledge, there is no scientific evidence regarding such an innovative (micro) encapsulated vegetable oil-based product into bioactive mineral core-shell granules conceived to ensure slow-release of active ingredients for simultaneous protection against storage insects and mycotoxigenic fungi.

CONCLUSIONS

Bio-based products for stored grain protection should be considered one of the most important means for agri-food chain decontamination and health safety areas of bioeconomy. Vegetable oils are one of the most available, cheap and versatile raw materials from natural renewable resources. Microencapsulation of antimicrobial essential oils into insecticidal salts of fatty acids from special plant oils and formulation as biodegradable granules into bioactive mineral shell represent modern and clean technologies for slow-release plant protection products against pests and diseases easy to obtain with a simple rotating granulator and use by organic farmers for stored grain long term preserving.

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REFERENCES

Bett P. K., Deng A. L., Ogendo J. O., Kariuki S. T., Kamatenesi-Mugisha M., Mihale J. M., Torto B., 2016. Chemical composition of Cupressus lusitanica and Eucalyptus saligna leaf essential oils and bioactivity against major insect pests of stored food grains. Industrial Crops and Products, Vol. 82, 51-62.

Hamza A. F., El-Orabi M. N., Gharieb O.H., El-Saeady A. A., Hussein A.E., 2015. Response of Sitophilus granarius L. to fumigant toxicity of some plant volatile oils. Journal of Radiation Research and Applied Sciences, In Press.

Krimer Malešević V., Vaštag V., Radulović-Popović L., Mađarev-Popovič S., Peričin-Starčević I., 2016. Chapter 12 - Microencapsulation Technology and Essential Oil Pesticides for Food Plant Production. Essential Oils in Food Preservation, Flavor and Safety, 123-129.

Lupu C., Manole T., Chiriloaie A., 2016. Effectiveness of main sources of diatomaceous earth from Romania on populations of S*itophilus granarius* l. (Coleoptera: curculionidae) under controlled conditions. Romanian Journal of Plant Protection, In Press.

Manole T., Fatu V., 2016. Behaviour of the insect species S*itophilus granarius* l. (Coleoptera: curculionidae) in the presence of volatile thyme oil. Romanian Journal of Plant Protection, In Press.

Matyjaszczyk E., Pieczyńska A., 2015. The Use of an Active Substance Depending on the Application Method of Plant Protection Products: Seed Dressing Versus Foliar Treatment. Agriculture and Agricultural Science Procedia, Vol. 7, 165-169.

Popescu M., Oancea F., Desliu-Avram M., 2015. Produs ecologic pentru tratamentul depozitelor de cereale si procedeu de obtinere. Pattent appl. A00817/2015

Sparagano O., Pritchard J., David G., 2016. The Future of Essential Oils as a Pest Biocontrol Method. Bio-Based Plant Oil Polymers and Composites, 207-211.

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ESTIMATION OF QUALITY OF 4 MONOFLORAL HONEY SAMPLES: ACACIA HONEY, LIME HONEY, OILSEED RAPE HONEY AND RASPBERRY HONEY

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Abstract

Honey is a natural, very complex product that can be adulterated using different methods. A reliable determination of honey quality includes complex physico-chemical analyses, organoleptic evaluation as well as microscopic identification of the pollengrains. From the large offer of different honey types made in Romania that are commercially available today, ten brands of monofloral honey bought in Bucharest were analyzed in our lab and the results were compared to the honey standards and other relevant information found in the literature. Simple indicators of quality such as fragrance, appearance and consistency, and the identification of the most frequently occurring pollen helped estimate the accurate labeling of the product. The present study includes the results of the analysis for the samples of 4 monofloral honey types, namely acacia honey, lime honey, oilseed rape honey and raspberry honey.

Key words*: monofloral honey, honey quality, pollen analysis.*

INTRODUCTION

Adulteration of honey can be done directly by using different substances that replace, cover, preserve or change the original product, or indirectly, by feeding the bees with diverse unnatural products (Mencinicopschi, 2010; Colţa, 2012). The consumer preference for some monofloral honey types may lead to an increase in price (Ianovici et al., 2008), therefore the control of the correct labeling of monofloral honey is necessary.

Microscopic pollen analysis is among the lab tests that are carried out to determine the quality of honey since it helps to establish the floral source used for its production. Bees bring pollen to the hives along with the nectar from the flowers that they have visited. Since vegetation is specific to each geographical area, the pollen in honey can also help tracing the honey's region of origin. Although theoretically specific pollen could be added to honey, in practice it is difficult to add all types of pollen found in one area and to obtain the right qualitative and quantitative combination of a pollen spectrum found in natural honey (Vorwohl, 1971).The analysis of pollen in honey can be done with only an optical microscope and a centrifuge as apparatus, but it requires a lot of experience and skill regarding the identification of pollen grains and their combinations in different types of honey (Vorwohl, 1971).

At present, international organizations that set food safety standards consider necessary the presence of the pollen in honey and suggest the use of filtering devices with meshes not smaller than 200µm, thus allowing 95% of pollen in the original honey to remain in the product (Food Safety News, 2011).

In Romania most honey comes from false acacia trees, lime trees, sunflower, forage crops or grass land vegetation, but there is also a smaller production of numerous other varieties. Table 1 presents some examples of honey types produced in Romania.

Previous studies of melissopalynological analysis of monofloral acacia and lime honey samples from markets in Timişoara were done by Ianovici et al. (2008) who found a polifloral profile in a preliminary study, while problems regarding the establishment of standards for monofloral honey were presented by Prepelicean&Teuşan who analyzed acacia honey from markets in Iaşi. Dobre (2012) determined the pollinic profiles of 8 types of Romanian honey, including acacia honey, lime honey and oilseed rape honey.

In the present study the correct labeling of 4 monofloral honey types was estimated using specific indicators of quality (fragrance, consistency, appearance) and an orienting microscopic analysis that consisted in the identification of the most frequently occurring pollen type.

Table 1. Some examples of honey types produced in Romania

Types of honey	Sources of honey
Acacia honey	False acacia trees from forests (*Robinia pseudacacia*, Fam. *Fabaceae*)
Chestnut honey	Chestnut trees from forests (*Castanea sativa*, Fam. *Fagaceae*)
Clover honey	Cultures of forage crop varieties of white clover (*Trifolium repens,* Fam. *Fabaceae*)
Coriander honey	Cultivated coriander(*Coriandrum sativum*, Fam. *Apiaceae*)
Honeydew honey	The sweet secretions of some insects (for example aphids) that feed on tree sap
Linden honey	Linden/lime trees from forests (*Tilia* sp., Fam. *Tiliaceae*)
Meadow honey	Grassland vegetation, the predominant source may be cornflower (*Centaurea cyanus,* Fam. *Asteraceae*)
Mint honey	Mint in cultivation or mint from the spontaneous vegetation in certain areas (*Mentha* sp., Fam. *Lamiaceae*)
Oilseed rape honey	Oilseed rape cultures (*Brassica napus* var. *oleifera*, Fam. *Brassicaceae*)
Raspberry honey	Raspberry in the forest area (*Rubusidaeus*, Fam. *Rosaceae*)
Spring honey (May honey)	Plants that flourish in spring (fruit trees, alfalfa, rape, acacia)
Sunflower honey	Cultures of sunflower (*Helianthus annuus*, Fam. *Asteraceae*)
Thyme honey	Wild thyme (*Thymus serpyllum*, Fam. *Lamiaceae*)
Danube Delta honey	Wild plants from the Danube Delta (yellow melilot, mint)
Wildflower honey	Plants from spontaneous vegetation
Yellow melilot honey	Yellow melilot (*Melilotus officinalis*, Fam. *Fabaceae*) from the spontaneous vegetation

MATERIALS AND METHODS

The honey was obtained commercially and was brought to the Biology Labat the Faculty of Biotechnology(The University of Agronomic Sciences and Veterinary Medicine of Bucharest) where it was analyzed. Indicators of quality such as fragrance, appearance and consistency were noted (Gonnet & Vache, 1989) and the results were compared to the descriptions found in the literature (Popescu & Meica, 1997; Asociaţia Crescătorilor de Albine din România, 2007).

Honey samples were dissolved in water and were concentrated by centrifugation(10 min at 2500 r/min), then the sediment was analyzed in bright field microscopy without further treatment (Louveaux et al., 1978).An orienting microscopic analysis was carried out by photographing the pollen grains on the slides with a digital still camera (Sony Cyber-shot®, Carl Zeiss Vario-Tessar 5× zoom lens) and using the pictures for later identification of the most frequently occurring particles (Louveaux et al., 1978). Pollen types were determined by comparisons with pollen descriptions and images found in the volumes of Tarnavschiet al. (1981, 1987, 1990, 1994).

RESULTS AND DISCUSSIONS

Organoleptic characteristics noted for the four types of honey that were analyzed are given in Table 2. Results of the orienting microscopic analysis of the pollen are presented in Table 3. A selection of microscopic images of the pollen grains that were identified in the present study is presented in Figures 1-4.

The identification of pollen types was based on shape, morphological characteristics and size of the pollen grains. In the case of *Brassica napus* for example, the length of the polar axis of the pollen indicate that the pollen is from cultivated oilseed rape since this variety has the largest pollen.

In the linden honey, various other pollen types were found, many were from sunflower.

Oilseed rape honey contains mostly oilseed rape pollen and crystalizes fast, so that its creamy consistency is similar to that of a sorbet.

One of the best indicators of quality of honey is its specific fragrance which reflects the plant source and is hard to imitate, for example the fresh flavor of raspberry honey. Honey natural flavor can be lost due to improper processing (heating).

Table 2. Organoleptic characteristics noted for the four types of monofloral honey that were analyzed in the present study

Type of honey	Organoleptic characteristics		
	Fragrance	Color	Consistency
Acacia honey	Characteristic smell of acacia tree flowers	Light golden yellow „Extra white" (18 mm Pfund Scale)*	Fluid
Linden honey	Intense smell of lime tree flowers	Orange „Light amber" (80 mm Pfund Scale)*	Fluid
Oilseed rape honey	Specific smell of oilseed rape flowers	Milky white „Water white" (0 mm Pfund Scale)*	Creamy (set honey with fine texture)
Raspberry honey	Delicate smell of the raspberry fruit	Peach yellow „Extra light amber" (34 mm Pfund Scale)*	Viscous, shows a tendency to crystallize

*The Oxford Honey Company, 2015

Table 3. The most frequently occurring pollen grains in samples of acacia honey, linden honey, oilseed rape honey and raspberry honey determined in the present study (no. of pollen grains > 100) and comparison to the description found in the literature

Honey sample	Most frequently occurring pollen grains	Percent of pollen needed to classify honey as monofloral
Acacia honey	*Robinia* pollen (27%)	*Robinia* pollen ≥5 % (Dobre, 2012)
Linden honey	*Tilia* pollen (27%) and *Helianthus* type pollen (27%)	*Tilia* pollen ≥29 % (Dobre, 2012)
Oilseed rape honey	Pollen of *Brassica napus* var. *oleifera* (85%)	*Brassica napus*> 45 % (Dobre, 2012)
Raspberry honey	Raspberry pollen (61%)	Raspberry pollen > 25 % (Popescu & Meica, 1997)

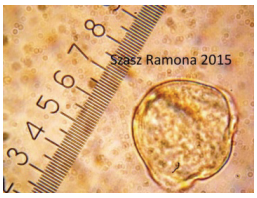
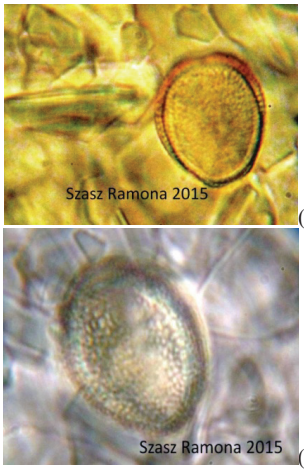


Figure 1. Rounded-triangular tricolpate pollen of *Robinia pseudacacia*in polar view, ~ 30µm size, in a sample of acacia honey analyzed in the present study (ob. 100×)

Figure 2. Apical view of a *Tilia* sp.pollen grain in a sample of linden honey analyzed in the present study (ob. 100×), thicken edexine can be seen in the colpi area



(a)

(b)

Figure 3. (a) Apical view of pollen from *Brassica napus* var. *oleifera* in a sample of oilseed rape honey analyzed in the present study; many small crystals are present(ob. 100×); (b) detail – the reticulate exine

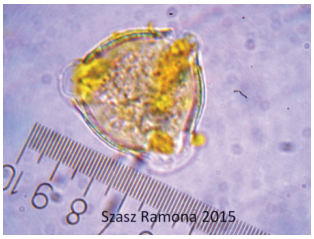


Figure 4. Polar view of *Rubus* type pollen in a sample of raspberry honeyanalyzed in the present study (ob. 100×), size ~30µm

CONCLUSIONS

The results of the orienting microscopic analysis of the pollen carried out in the present study suggest that the honey comes from the indicated floral source in all the 4 samples of monofloral honey that were analyzed. The determination of the floral origin and honey quality should be confirmed further through a complete microscopic analysis.

REFERENCES

Asociaţia Crescătorilor de Albine din România (Bucureşti) (autori: Alexandru V., Barac I., Drăgan M., Fota G., Grosu E., MateescuC., et.al), 2007. Manualul Apicultorului. Ed. a 9-a. Editura LVS Crepuscul, Ploieşti, Prahova.

Colţa T., 2012. Calitatea produselor apicole. Capitol în: Asociaţia Crescătorilor de Albine din România, Institutul de Cercetare-Dezvoltare pentru Apicultură: Apicultura. Manualul cursantului. Ediţia I. Editura LVS Crepuscul, Ploieşti, Prahova.

Dobre I., 2012. Studii privind autentificarea şi caracterizarea prin tehnici moderne a mierii de albine din România. Teză de doctorat, Universitatea Dunărea de Jos, Galaţi, Facultatea de Ştiinţa şi Ingineria alimentelor.

Food Safety News,2011. Top pollen detective finds honey a sticky business.

Gonnet M., Vache G., 1989. Tehnica de degustare a mierilor şi punerea la punct a unui sistem de notaţie şi de clasificare obiectivă pentru a aprecia calitatea lor prin analiză organoleptică. În Produsele stupului hrană, sănătate, frumuseţe. Editura Apimondia.

Ianovici N., Ionuti A., Zbîrcea S., Crașovan G.,2008. Preliminary contribution to the characterization of commercial unifloral honey samples by melissopalynology analysis. Annals of West University of Timișoara, ser. Biology, vol. XI, pp. 85-94.

Louveaux J., Maurizio A., Vorwohl G., 1978. Methods of melissopalynology. Bee World 59, 139-157.

Mencinicopschi G., 2010. Şi noi ce mai mâncăm? Vol. I. Coreus Publishing, pp. 201-206.

Popescu N., Meica S., 1997. Produsele apicole si analiza lor chimica (Mierea, ceara, laptisorul de matca, polenul, pastura, propolisul si veninul). Editura Diacon Coresi.

Prelipcean A.A., Teuşan A., 2007. Melisopalinological aspects regarding the commercial *Robinia pseudoacacia* natural honey. Lucrări Ştiinţifice – Seria Zootehnie, U.S.A.M.V. Iaşi 52 (14).

Tarnavschi I.T., Şerbănescu-Jitariu G., Mitroiu-Rădulescu N., Rădulescu D., 1981, 1987, 1990, 1994. Monografia polenului florei din România. Vol. I, II, III, IV. Editura Academiei Române, Bucureşti.

The Oxford Honey Company, 2015. Pfund Colour Scale. http://www.oxfordhoney.uk/pfund-colour-scale/.

Vorwohl G., 1971. Significance and aim of pollen analysis of honey. Apiacta2

.

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PHYSICO-CHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF WHEAT FLOURS ON ROMANIAN MARKET IN RELATION TO THE SHELF LIFE

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Abstract

This paper aims to assess the quality and microbiological parameters of a range of wheat flours for domestic consumption, purchased on the Romanian market. In this regard there were purchased 20 samples of flour from 13 manufacturers. Quality parameters analyzed were: Moisture (%), Protein content (%), Ash content (%), Water absorbtion (%), Total *combined* Yeasts and Molds count *(CFU/g) and the number of days until the deadline of the shelf life. The analyzed flours were characterized by the following variation ranges of the parameters: Moisture (%) 10.9 - 14.4, Protein content (%) 10.0 - 15.7, Ash content (%) 0.40 - 1.59, Water absorbtion (%) 57.0 - 61.9, Yeast and molds (CFU/g): 10-410, and number of days until the deadline of the shelf life: 9-326. The results showed that there are no significant correlations between analyzed parameters and the content of yeasts and molds. This suggests that the dynamics of yeasts and molds population in packed flours is dependent primarily on the processing conditions and less on the factors that act during the lifetime of the product on the shelf.*

Key words*: molds, quality parameters, wheat flour, yeast.*

INTRODUCTION

Romania is one of the countries where consumption of milling and bakery products recorded a value above the EU average. In the year 2013 this consumption was estimated at 101.4 kg /person [5]. This value is approximately 30% higher than the European average, estimated at about 75-80 kg/year [4]. The total quantity of flour used in households was 7.3 kg/person/year. Romanian wheat crops are characterized by a large annual variability of the main quality parameters [3]. This fact is reflected in significant variations of the main flour quality parameters. Grinding traditional technologies, common to most mills in Romania, do not provide specific process steps to reduce microbial load. Reduction of microbial load in raw material to finished products is a natural consequence of processes like cleaning and conditioning the feedstock, followed by grinding and separating the outer layers of wheat grain. It is estimated that the degree of reduction of microbial load is about 10-100 times (1-2 log) in the process of grinding [1, 2].

Romanian legislation does not provide obligatory criteria on flours microbial load. The National Sanitary-Veterinary Agency for Food Safety ordinance no. 27/2011 recommend a maximum of 1000 CFU/g yeasts and molds for white flour, but introduces a validation criteria for batches of flour (three of the five samples must be below 100 CFU/g) [6]. In this context, we characterized different types of flour used for household consumption, in terms of physico-chemical and microbiological aspects, in relation to the shelf life.

MATERIALS AND METHODS

20 samples of flour were purchased in November 2015, from several supermarkets in Bucharest. Flour samples were processed by the following manufacturers: Farinsan S.A. (2 samples), Sano Vita (1 sample), Bio Logistic Romania (1 sample), Good Mills Romania (5 samples), Boromir (2 samples), Pivetti Italy (1

sample), Pambac (2 samples), Arpis (1 sample), 7 spice (2 samples), Liszt Agro Monar (1 sample) and Băneasa SA (2 samples).
From a technological viewpoint the 20 samples were part of the following categories: whole flour 2 samples, black flour 1250 - 2 samples, flour type 00 - 1 sample, flour type 650 -6 samples, flour type 480 - 1 sample, flour type 000 - 7 samples and flour type 550 - 1 sample.
For each of the samples were determined the following parameters: Moisture (M, %; using KERN MLB 50-3thermobalance, at 130^0C), Ash content (Ash, %; SR ISO 2171/2009), Protein content (P, %; ICC 159-95 - NIR method, PertenInframatic 8600), Water absorbtion (WA, %; SR ISO 5530-1/2007, Brabender Farinograph E), Total combined Yeasts and Molds count (TYMC/CFU) (ISO 21527-2 SR / 2009).
The period of time until the deadline ofthe shelf life (SL, days) was calculated according to the data inscribed by the manufacturer on the package, starting from the day of microbiological analysis effectuation.

RESULTS AND DISCUSSIONS

The results from the analyzes performed on 20 samples of flour are shown in Table 1.

Table 1.The determined values and the variability estimates of quality parameters for 20 types of flour

Sample no.	M(%)	P (%)	Ash (%)	WA (%)	TYMC (CFU/g)	SL (days)
1. (Whole flour)	11.8	10.9	**1.21**	59.5	10	119
2. (Whole flour, eco)	11.8	10.9	1.59	61.9	170	250
3. Black wheat flour type 1250	11.9	15.7	1.08	60.2	150	89
4. Black wheat flour	11.7	13.8	**0.96**	59.3	110	29
5. Type 00 flour	10.9	12.2	**0.57**	60.7	20	287
6. Type 650 wheat flour	12.6	10.6	0.62	57.8	80	188
7.Type 650 wheat flour	11.4	12.2	0.62	59.2	140	9
8. Type 650 wheat flour	13.2	11.6	0.63	58.2	150	119
9. Type 650 wheat flour	14.4	10.7	**0.71**	57.1	400	128
10. Type 650 wheat flour	13.3	10.8	0.63	57.4	20	201
11. Type 650 wheat flour	12.9	10.1	**0.51**	58.0	298	108
12. Type 480 wheat flour	13.3	10	**0.53**	57.0	30	188
13. Type 000 wheat flour	12.9	10.9	0.4	59.7	10	174
14. Type 000 wheat flour	13.4	10.6	**0.59**	57.5	120	326
15. Type 000 wheat flour	14.4	11	**0.51**	57.9	50	122
16. Type 000 wheat flour	11.7	10.5	0.46	58.3	10	69
17. Type 000 wheat flour	12.5	12.2	**0.58**	59.4	30	103
18. Type 000 wheat flour	13.1	11.6	**0.53**	58.6	20	189
19. Type 000 wheat flour	13	10.45	0.48	58.8	410	113
20. Type 550 wheat flour	13	13.3	0.55	61.2	160	110
Descriptive statistics						
Mean	12.660	11.502	0.688	58.885	119.40	146.050
Standard deviation	0.942	1.415	0.297	1.372	123.45	80.117
Coeff. of variation (%)	7.441	12.302	43.169	2.328	103.39	54.856

We can see in table 1, that the abnormal values of the quality parameters in relation to the flour type were highlighted (bold). We observed that 50% of the flour samples did not comply the characteristic values of Ash parameter. Thus, sample 1 (whole flour) had a value of 1.21% ash, to a minimum permitted of 1,4 - 1,5% that is a characteristic value for the whole flour. Sample 4 (black flour) had a smaller value of ash content (0.96%), compared to a minimum

permitted of 1.2%. The flours type 00, type 000 or 480 (samples 5, 12, 14, 15, 17, 18) exceeded the value of 0.5%, that is accepted for these flour types. Flour sample no. 9 had an ash value of 0.71% compared to a max. of 0.67% characteristic for 650 flour type. Regarding sample no 11, 650 flour type, it looks like the ash value was far below the minimum characteristic for this type of flour (0.51% toward 0.63%).

There is therefore a major tendency to overestimate 000 and 480 flour type, sold on the Romanian market, because two thirds of the samples did not respect the criteria of ash value, specific to the type of flour on the package. The phenomenon is less extensive in the case of higher extraction flours (type 650 and above), although in the case of sample 1, a black flour is sold as a whole flour.

In table 1 it is observed that analyzed flours were characterized by low Moisture variability (mean 12.66% ± 0.94), in the range of 10.9 - 14.4%.The Protein content had a medium degree of variation (mean 11.5% ± 1.41), in the range of 10.0 - 15.7%.The Ash content was the quality parameter that reflected the best the heterogeneity of the sample, being characterized by an average of 0.69% ± 0.30 (in the range 0.40-1.59%). The Water absorbtion of the flours (mean 58.9% ± 1.37) was the parameter with the lowest variability in the analyzed samples, between57.0 and 61.9%. Total combined Yeasts and Molds count was characterized by significant variability, ranging between 10-410. None of the samples exceeded the limit of 1000 CFU/g recommended by legislation and only 10 samples exceeded the limit of 100 CFU/g. Concerning the number of days until the deadline of the shelf life of the flours, it ranged between 9 and 326.This parameter average was 146 days.

Table 2 presents the linear correlation coefficient values between the analyzed parameters of the flour samples.

We have observed in table 2 that the only quality parameters of flours that correlated with each other, where Water absorbtion - Moisture (r = -0.62**), Water absorbtion - Protein content (r = 0.53 *) and Water absorbtion - Ash (r = 0.51*). This is a natural relationship between parameters, given that the ability of flour to bind water is the higher the degree of hydration of the flour components is lower and the concentration of elements capable of binding water (protein, fiber) is also higher.

Table 2. The correlation coefficients between the quality parameters

	M (%)	P (%)	Ash (%)	WA (%)	TYMC (CFU/g)	SL (days)
M (%)	1.00					
P (%)	-0.40	1.00				
Ash (%)	-0.39	0.29	1.00			
WA (%)	**-0.62****	**0.53***	**0.51***	1.00		
YM (CFU/g)	0.30	-0.07	0.05	-0.09	1.00	
SL (days)	0.13	-0.36	0.04	-0.00	-0.20	1.00

*p<0.5, semnificative; **P<0.01, distinct semnificative

We see that there are no significant correlations between the number of yeasts and molds and the analyzed quality parameters.No flours Moisture (r = 0.30 ns), nor their Ash content (r = 0.05 ns) did not significantly affect their microbial load with yeasts and molds. It was noted that the number of days until the deadline of the shelf life does not correlate with the degree of microbial load, the correlation coefficient between the two parameters being negative and low (r = -0.2 ns).

These results suggest that the dynamics of the microbial population in flours, rather depends on specific conditions of grinding process and depends less on the factorsthat act on the packaged flours during their shelf life.

CONCLUSIONS

Our results show a significant tendency to overestimate flours type 000 or 480, sold on the Romanian market, two thirds of them do not comply the limitations regarding ash, specific to the type of flour on the package. The phenomenon is less visible to higher extraction flours (type 650 and above), although in our experiment I saw that a black flour was sold as whole flour.

There were no significant correlations between the content of flours yeast and molds and quality parameters, nor between yeasts and molds content of flours and number of days until the deadline of the shelf life. Our results lead to the conclusion that the dynamics of microbial population in flour is depending on grinding, generally on technological conditions

for obtaining flour and less on factors acting during their shelf life.

REFERENCES

1. Mardare Ş, Cristea S., Gâdea M., Tamba-Berehoiu R., 2015, The influence of some abiotic factors on the development of Alternaria spp. pathogen ("in vitro"), Romanian Biotechnological Letters, Vol. 20, No. 5, 10880-10884.
2. Mortimore S., Wallace C., 2013, HACCP A practical approach, Third Edition, Ed. Springer, New York.
3. Popa N.C., Tamba-Berehoiu R., Popescu S., 2008, Main quality parameters' evolution of the wheat cultivated in Romania during the last years, Roumanian Biotechnological Letters Vol. 13, No. 1, p. 3557-3566.
4. Zanirato S., 2013, Wheat flour standars in European Union, TFIF International Grain Congress on "Wheat, Flour, Climatic Changes and New Trends, 7 – 10 march Antalya.

5 *** Guvernul României, Ministerul Finanţelor Publice, 2014, Evaluare Ex-Post a efectelor macroeconomice ale reducerii TVA la făină, pâine şi specialităţi de panificaţie, septembrie, Bucureşti.

6 ***ANSVSA Order no. 27/2011 regarding approval of microbiological and hygiene criteria which apply to food products, other than the ones mentioned in Regulation (CE) no. 2073/2005 regarding the microbiological criteria for food products

7 *** ISO 21527-2 SR / 2009 - Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of yeasts and moulds -- Part 2: Colony count technique in products with water activity less than or equal to 0,95.

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IDENTIFICATION OF THE MOST RELEVANT QUALITY PARAMETERS FOR BERRIES - A REVIEW

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Abstract

Fresh fruit jointly to vegetables are an essential component of a healthy diet, able to decrease the risk of cardiovascular diseases and cancer. In the last years, their consumption has continued to grow rapidly linked to the increased public awareness of their health benefits, even if it remains below the recommended daily intake in many countries, due to barriers such as complacency and lack of willpower to change the diet. The attributes of berries, like chemical-physical and nutritional characteristics, microbial contamination, chemical contaminants as well as sensorial properties represent some very important quality parameters that must be determined in order to establish the quality of berries after ripening and during storage, until they reach their final destination (consumer). The aim of this study was to perform a literature review in order to determine the most relevant quality parameters of berries and to describe methods for their determination.

Key words*: berries, determination methods, quality parameters.*

INTRODUCTION

Fruits, especially berries, have been found to possess pharmacological and biochemical properties that are caused mainly by the anti-oxidant activity of their diversified compositions (Jia et al., 2012). Berry fruits have been widely recognized as an excellent source of bioactive phenolic compounds including flavonoids, phenolic acids, and tannins, that both individually and synergistically may help protect against cardiovascular disease, cancer, inflammation, obesity, diabetes, and other chronic diseases (Wu et al., 2010).

Mulberry is grown wild or cultivated in many countries for its foliage, which is a primary source of food for silkworms. Mulberry fruit is rich in carotene, vitamins B1, B2 and C, glucose, sucrose, morin, tartaric acid and succinic acid (Wang et al., 2013). Mulberry fruit is a highly perishable fruit, with short shelf life due to its soft texture and high sensitivity to fungal attack (Wang et al., 2013).

Strawberry is a popular and attractive fruit due to its high visual appeal and desirable flavour (Aday & Caner, 2014). Strawberries are rich in phytonutrients (amino acids, vitamins, and anthocyanins), high visual appeal, and desirable flavour, but are highly perishable and have relatively high physiological activity after harvest. Such behaviour results in a rapid deterioration in quality such as softening and shrinkage, discoloration, off-flavours, and finally fungal decay, resulting in short storage life (Wang et al., 2014; Wang & Gao, 2013).

Blueberries are recognized for their contribution to a healthy diet with different beneficial bioactive compounds such as flavonoids, anthocyanins, and others, which helps to avoid important diseases including different cancers (Concha-Meyer et al., 2015). Fresh berries are highly valued for their high antioxidant and vitamin content. Many bioactive compounds in berries have been shown to provide significant health benefits (Huang and Chen, 2014).

Raspberries are a high-value crop due to their unique flavour, exacting climatic requirements, high costs of production and perishability. Raspberry fruits contain small amounts of vitamins; only vitamin C is present at a significant level (Oduse and Cullen, 2012).

Cranberry is highly valued for its nutritional and medicinal properties. It prevents many aliments, which include scurvy and bladder

infections in elderly women. Bringing this high-value crop to market is plagued by fruit rot, which is caused by a number of fungal and bacterial microorganisms (Palanimuthu et al., 2009).

Black currant have a high anthocyanin content. Many studies have demonstrated the excellent antioxidant activity of black currant extract (BCE) and its health benefits, including anticarcinogenic activity (Jia et al., 2012). They are considered to be a rich source of ascorbic acid, citric acid, malic and tartaric acids with plenty minerals, such as potassium, calcium and magnesium. Moreover, currants contain polyphenolic compounds such as anthocyanins, vanillic acid, caffeic, gallic and p-coumaric acids and quercetin (Kostarelou et al., 2014).

Blackberry is an aggregate fruit, composed of small drupelets, belonging to the *Rosaceae* family. They are rich in functional components, which are mainly represented by polyphenols such as anthocyanins and flavonoids, which are strong natural antioxidants (Azofeifa et al., 2015).

Goji berry grows in China, Tibet and other parts of Asia and its fruits are 1-2 cm-long, bright orange-red ellipsoid berries. Concentrated extracts and infusions prepared from the berries have a history of use as ingredients in various soft or alcoholic drinks that were marketed for their benefits to anti-aging, vision, kidney and liver functions cytoprotection (Amagase and Farnsworth, 2011; Donno et al., 2015a).

Seabuckthorn has been recognised as a versatile nutraceutical crop with diverse uses, from controlling soil erosion to being a source of horse fodder, nutritious foods, drugs and skin-care products. All parts of this plant are considered to be a good source of a large number of bioactive compounds, including carotenoids, tocopherols, sterols, flavonoids, lipids, vitamins, tannins, minerals etc. which contribute to its wide usage as a natural antioxidant (Maheshwari et al., 2011; Kumar et al., 2013).

Gooseberry has many cultivars from different regions and countries and is differentiated by size, colour, taste, flower shape, plant height and plant size (Bravo and Osorio, 2016). Gooseberries are popular fruits known for their organoleptic properties (flavour, odour, and colour), nutritional value (vitamins A and C, potassium, phosphorous, and calcium), and health benefits (Vasquez-Parra et al., 2013).

The fruits of **European elder** are a rich source of bioactive compounds like anthocyanins. Elderberries contain a high phenolic content and antioxidant activity when compared with other fruits and even with other berries (Seabra et al., 2010).

Black chokeberry (*Aronia melanocarpa*) belongs to the *Rosaceae* family, which is native to North America. The health beneficial effects of chokeberry have been suggested to be attributed to polyphenols, as the chokeberry contains a large amount of polyphenols (Lee et al., 2014).

QUALITY PARAMETERS AND METHODS OF THEIR DETERMINATION

1. Physical-chemical analysis methods

1.1. pH determination

In general, the pH is determined using specific instruments, like pH-meters. For this determination the glass electrode is connected at the apparatus and it is washed with distilled water before being introduced into the sample. The electrode is introduced into the sample in vertical position, such as the membrane glass electrode to be entirely in contact with the sample and kept until stabilization of the pH value on the screen. This method was used in this research to determine the pH of mulberries (Jiang and Nie, 2015), strawberries (Kartal et al., 2012; Aday and Caner, 2013), cranberries (Caminiti et al., 2011), blackberries (Wu et al., 2010), seabuckthorn (Gunenc et al., 2016) or goji berry (Donno et al., 2015a).

1.2. Determination of total titratable acidity

Total acidity is the sum of organic acids and their salts, titratable acid neutralization determined by their parties titratable acid with an alkaline solution (usually 0.1 NaOH). Determination of total acidity can be done by the following methods: by potentiometric titration method or electro titrimetric: the titration method in the presence of indicators such as phenolphthalein and bromothymol blue, which are inserted into the glass titration instead of phenol red by drops put on a white tile which paraffin is turn control. The result is expressed conventionally prevailing in the product acid (malic acid, tartaric acid or citric

acid).This method was used in this research to determine the total titratable acidity mulberries (Jiang and Nie, 2015), strawberries (Wang et al., 2014; Ozkaya et al., 2009), raspberries (Stavang et al., 2015),blackberries (Wu et al., 2010), seabuckthorn (Gunenc et al., 2016), or gooseberries (Wójcik and Filipczak, 2015).

1.3. Determination of dry soluble matter (Brix)

Using this method is evaluated the content of reducing and non-reducing sugars (total sugar) of the samples by measuring the percentage of the solutes or index refractor. In general, the refractive index is measured with a refractometer and correlated to the amount of soluble solids (expressed as the concentration of sucrose), using the conversion table by direct reading on the scale of the refractometer. This method was used within the researches for determination of dry soluble matter of mulberries (Jiang and Nie, 2015), strawberries (Wang et al., 2014; Ozkaya et al., 2009; Aday and Caner, 2013), blueberries (Diaz et al., 2011), raspberry (Stavang et al., 2015; Giovanelli et al., 2014), cranberries (Caminiti et al., 2011), currants (Pantelidis et al., 2007; Jensen et al., 2010), blackberries (Wu et al., 2010), goji berry (Donno et al., 2015a) or gooseberries (Pantelidis et al., 2007; Wójcik and Filipczak, 2015).

1.4. Determination of water activity (a_w)

The index a_w s a measure of the energy state of the water in the system, showing how the water is bound tightly, structurally or chemically, into a substance. It is the relative humidity in equilibrium with a sample in a closed measuring chamber. The concept of water activity is of particular importance in determining the quality and safety of food. The index a_w influences the colour, aroma, texture and shelf life of food. In addition, based on the values of a_w, can evaluate the safety and stability of food in conjunction with the microbial growth, the speed of the chemical and biochemical reactions, and with the physical properties.

1.5. Determination of total dry matter (D.M.%)

Determination of dry matter using thermo balance is a quick and reliable method for determining the moisture content using the thermo gravimetric principle. Thermo gravimetry consists in weighing the sample before and after heating it, to determine the moisture content by difference. Conventional oven-drying technique works on the same principle, but the measurements takes more time.

2. Methods for analyzing nutritional properties

2.1. Determination of vitamin C

To determine the content of vitamin C is usually used titrimetric 2.6 diclorfenolindofenol method. This method was used to determine the level of vitamin C of cranberries (Rudy et al., 2015) or currants (Pantelidis et al., 2007).

Jiang and Nie (2015) used this method for determination of vitamin C content of mulberries, using the following working protocol: the EDTA solution, acetic acid solution, and fast blue B salt solution were respectively added into homogenised samples and diluted with water. The mixture was placed at room temperature for 3 min and detected at 420 nm using a UV spectrophotometer. The content of ascorbic acid was calculated according to the ascorbic acid standard curve (Jiang and Nie, 2015). This method was used also for the determination of ascorbic acid content of gooseberries (Pantelidis et al., 2007; Vasquez-Parra et al., 2013).Another method used to determine the content of vitamin C in the berries is using HPLC analysis of samples. Giovanelli et al. (2014) described this method for the determination of vitamin C content of raspberry as it follows: 4 g of homogenate were extracted with 16 mL of diluted meta-phosphoric acid (0.001%), which was prepared daily. The mixture was stirred for 20 min and centrifuged at 11,000 × g for 10 min at 10°C. The clear supernatant was injected in the HPLC apparatus and analyzed (Giovanelli et al., 2014; Mikulic-Petkovsek et al., 2013).

2.2. Determination of total phenolic compounds

To determine the total phenolic content, the most used method is the method of Folin-Ciocalteu. Therefore, for the extraction of polyphenolic compounds, samples were placed in 50 ml test tubes, and 25 ml of extraction solution was subsequently added to the

weighed samples; after 60 min in the dark, the extracts were homogenized for about 1 min and then centrifuged for 15 min. This is based on Folin–Ciocalteu phenol reagent and spectrophotometric determination at 765 nm. The standard calibration curve was plotted using gallic acid at concentrations of 0.02–0.1 mg•ml^{-1}. The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW) (Donno et al., 2015b). This method is frequently found in the literature for the determination of total phenolic content from mulberry (Donno et al., 2015b; Sánchez-Salcedo et al., 2015), blueberries (Pertuzatti et al., 2014; Ketata et al., 2013), raspberry (Cekic and Ozgen, 2010; Jin et al., 2012; Bobinaite et al., 2016; Chanjirakul et al., 2006; Giovanelli et al., 2014; Zhang et al., 2010), cranberry (Chiang et al., 2014; Chen et al., 2015a; Chen et al., 2015b; Vu et al., 2012), gooseberry (Pantelidis et al., 2007; Chiou et al., 2014; Vagiri et al., 2015; Mikulic-Petkovsek et al., 2013), blackberries (Ramos-Solano et al., 2015; Wu et al., 2010; Azofeifa et al., 2015; Barba et al., 2015; Da Fonseca Machado et al., 2014), goji berry (Donno et al, 2015a), seabuckthorn (Saggu et al., 2007; Kumar et al., 2013; Maheshwari et al., 2011), gooseberry (Pantelidis et al., 2007; Bochi et al., 2014; Vega-Gálvez et al., 2014, Vega-Gálvez et al., 2016), elderberry (Seabra et al., 2010; Duymus et al., 2014),aronia (Cujic et al., 2016; Jakobek et al., 2012; Samoticha et al., 2016; d'Alessandro et al., 2012).

2.3. Total anthocyanins content

The total anthocyanin content (TAC) in the fruit extracts is usually directly determined using the pH-differential method. The extracts for TAC analysis were prepared using the method described for quantification of total polyphenols. Anthocyanins demonstrate maximum absorbance at 515 nm at pH 1.0 and also at 700 nm at pH 4.5. The coloured oxonium form of anthocyanin predominates at pH 1.0, and the colourless hemiketal form at pH 4.5. The pH-differential method is based on the reaction producing oxonium forms. This method allows an accurate and rapid measurement of the total monomeric anthocyanins. Absorbance was measured at 515 and 700 nm and the results, considered as the monomeric anthocyanin pigment, was expressed as milligrams of cyanidin-3-O-glucoside (C3G) (Donno et al., 2015b). This method was used for the determination of total anthocyanin content of mulberries (Chen et al., 2016; Jiang and Nie, 2015), blueberries (Pertuzatti et al., 2014;Ketata et al., 2013), raspberries (Zhang et al., 2010; Bobinaite et al., 2016; Cekic and Ozgen, 2010; Jin et al., 2012; Chanjirakul et al., 2006; Giovanelli et al., 2014), cranberries (Caminiti et al., 2011; Rudy et al., 2015), currants (Chiou et al., 2014; Jia et al., 2012; Pantelidis et al., 2007; Bakowska-Barczak and Kolodziejczyk, 2011), blackberries (Wu et al., 2010; Barba et al., 2015; Da Fonseca Machado et al., 2014), gooseberries(Pantelidis et al., 2007; Bochi et al., 2014), elderberry(Duymus et al., 2014), aronia (Cujic et al., 2016).

2.4. Determination of antioxidant capacity

The determination of the antioxidant capacity of berries can be performed by different methods such as:

A. *DPPH radical scavenging activity*

To apply this method, different samples are dissolved in deionised water to obtain various concentrations. Then the DPPH is mixed in ethanol with the sample (in various concentrations). The mixture is then shacked and kept in the dark for 30 min at room temperature and absorbance is measured at 517 nm. This method was applied for the determination of antioxidant activity of mulberry (Chen et al., 2015; Sanchez-Salcedo et al., 2015), raspberry (Bobinaite et al., 2016; Jin et al., 2012; Zhang et al., 2010), cranberry (Chen et al., 2015a), currants (Chiou et al., 2007; Chiou et al., 2014; Jia et al., 2012; Bakowska-Barczak and Kolodziejczyk, 2011), blackberry (Azofeifa et al., 2015; Da Fonseca Machado et al., 2014; Wu et al., 2010), goji berry (Florino et al., 2016), seabuckthorn (Kumar et al., 2011; Kumar et al., 2013; Gunenc et al., 2016; Ting et al., 2011), gooseberry (Vega-Gálvez et al., 2014; Vega-Gálvez et al., 2016), elderberry (Seabra et al., 2010; Duymus et al., 2014), aronia (Lee et al., 2014; Jakobek et al., 2012; Gironés-Vilaplana et al., 2012; d'Alessandro et al., 2012).

B. *Ferric reducing antioxidant power (FRAP)*
This method is based on the reduction of the ferric (Fe^{3+}) TPTZ (2,4,6-tripyridyl-S-triazine) complex to its ferrous form (Fe^{2+}). Absorbance at 595 nm is recorded with a UV/Vis spectrophotometer. The standard curve can be obtained using $FeSO_4 \bullet 7H_2O$ (concentration range: 100–1000 μmol•L^{-1}), and results are expressed as millimoles of Fe^{2+} equivalents per kilogram (solid food) of FW. This method was applied for the determination of antioxidant activity of mulberry (Donno et al., 2015a), blueberry (Pertuzatti et al., 2014), raspberry (Cekic and Ozgen, 2010; Giovanelli et al., 2014), cranberry (Chen et al., 2015b), currants (Jia et al., 2012; Pantelidis et al., 2007), blackberry (Wu et al., 2010), goji berry (Donno et al., 2015b), seabuckthorn (Kumar et al., 2011; Kumar et al., 2013; Ting et al., 2011), gooseberry (Pantelidis et al., 2007; Vega-Gálvez et al., 2014; Vega-Gálvez et al., 2016).

C. *Hydroxyl radical scavenging activity (OH; HOSC)*
Briefly, the solution of $FeSO_4$, together with H_2O_2, salicylic acid and the tested sample in different concentrations are mixed well and incubated together at 37°C for 1 h. The absorbance of the mixture is then measured at 562 nm, while using ascorbic acid as positive control. This method can be adapted depending on the analyzed sample (different concentrations, different wavelengths, different control). This method was applied for the determination of antioxidant activity of mulberry (Chen et al., 2015), strawberry (Wang and Gao, 2013), raspberry (Jin et al., 2012).

D. *Oxygen radical absorbance capacity (ORAC)*
Sample solution is diluted with phosphate buffer (pH 7.4). Then the sample is mixed with Trolox standard at different concentration, followed by the addition of fluoresce in sodium salt. The mixture is shaken for 10 s and preincubated for 25 min at 37°C. Finally, the fluorescence intensity is measured at excitation of 485 nm and emission of 538 nm. Final ORAC value is expressed as mean μMol Trolox equivalent (TE) per g of dry weight (DW).This method was applied for the determination of antioxidant activity of mulberry (Chen et al., 2015), blueberry (Pertuzatti et al., 2014), raspberry (Jin et al., 2012; Chanjirakul et al., 2006; Zhang et al., 2010), cranberry (Chen et al., 2015a; Chen et al., 2015b), blackberry (Wu et al., 2010; Azofeifa et al., 2015), seabuckthorn (Gunenc et al., 2016), gooseberries (Vega-Gálvez et al., 2014), elderberry (Duymus et al., 2014).

E. *Free radical capture (ABTS)*
A stock solution of ABTS in potassium sulphate is realized and it is stored refrigerated in the dark. Prior to doing the analyses, this was diluted in ethanol until the absorbance at 734 nm was 0.70 ± 0.02. Then the tested sample is mixed with the realized solution and it is incubated at 30 °C for 25 minutes. Than the absorbance was read and compared to that of Trolox. Results are expressed as Trolox equivalents per g of dry weight, or TE/g DW.This method was applied for the determination of antioxidant activity of blueberry (Pertuzatti et al., 2014), raspberry (Cekic and Ozgen, 2010), currants (Jia et al., 2012; Bakowska-Barczak and Kolodziejczyk, 2011), blackberry (Sanchez et al., 2014; Da Fonseca Machado et al., 2014),aronia (Jakobek et al., 2012).

F. *Nitric oxide-scavenging activity (NO)*
Nitric oxide (NO) was generated from sodium nitroprusside. Then Griess reagent is added, the absorbance was read at 540 nm and compared to the absorbance of standard solutions of sodium nitrite. This method was described and used for determination of antioxidant properties of blackberries by Azofeifa et al. (2015) and seabuckthorn (Kumar et al., 2013).

3. Methods for determination of berries contaminants

3.1. Microbial contaminants (yeasts, moulds, bacteria)

A. *Determination of total mesophilic aerobic count*
Mesophilic aerobic total germ can be determined according to the standard SR EN ISO 4833:2003. From each sample are taken 10 g and introduced into Erlenmeyer glasses with 90 ml of sterile distilled water. The samples obtained are taken into 9 ml sterile distilled

water, thereby producing for each sample dilution 1. From these solutions, dilutions have been realized by the decimal dilutions method, the number of dilutions depending on the sample. From each dilution 1 ml is seeded in duplicate on nutrient agar plates. Petri dishes are then incubated aerobically for 72 hours at 30 °C and then the grown colonies were counted on each plate.

B. *Determination of yeasts and moulds*
The number of yeasts and moulds can be determined according to SR ISO 21527-1:2009. The analysis method comprises the following steps: From each sample are taken 10 g and introduced into Erlenmeyer glasses with 90 ml of sterile distilled water. The samples obtained are taken into 9 ml sterile distilled water, thereby producing for each sample dilution 1. From these solutions, dilutions have been realized by the decimal dilutions method, the number of dilutions depending on the sample. From each dilution 1 ml is seeded in duplicate on nutrient agar plates. Petri dishes are then incubated at 25 °C. After 3 days yeast colonies are counted and after 5 days the moulds colonies are counted.
In Table 1 is presented the situation of the frequency with which various yeasts and moulds are meet on berries.

Table 1. The frequency with which various yeasts and moulds are meet on berries (Tournas and Katsoudas, 2005)

Microorganism	Contaminated samples (%)	The level of contamination * (area)
Blackberries		
Botrytis cinerea	78	0-100
Cladosporium	33	0-80
Fusarium	22	0-100
Penicillium	22	0-50
Rhizopus	11	0-50
Blueberries		
Botrytis cinerea	55	0-100
Alternaria	46	0-75
Fusarium	13	0-25
Penicillium	9	0-50
Aureobasidium pullulans	5	0-40
Cladosporium	5	0-20
Trichoderma	5	0-30
Yeasts	5	0-60
Raspberry		
Botrytis cinerea	75	0-100
Fusarium	25	0-50
Cladosporium	20	0-65
Penicillium	15	0-50
Rhizopus	10	0-90
Yeasts	5	0-65
Strawberries		
Botrytis cinerea	77	0-100
Rhizopus	23	0-100
Penicillium	10	0-67
Fusarium	8	0-75
Alternaria	8	0-67
Cladosporium	5	0-60
Trichoderma	3	0-50
Yeasts	3	0-75

* Percentage of contaminated products (per sample)

3.2. Chemical contaminants (mycotoxins)
As moulds grow in a commodity, it does not create the putrefactive degradation associated with bacteria, and therefore the foods is sometimes eaten even though infected, which can result in ingestion of toxins. The fungi

themselves are not toxic, but their secondary metabolites can sometimes be hazardous substances. These are mycotoxins such as aflatoxins, ochratoxin A,penitrem A, sterigmatocystein, roquefortin C, PR toxin and cyclopiazonic acid. Yeasts are not known to produce mycotoxins. There are hundreds of known mycotoxins produced by a large number of mould species. For production of toxins the demands on the substrate, as well as on the environmental factors, is different than for growth. Toxin production often requires a higher aw than growth, as well as more available oxygen. Less favourable conditions can also result in less potent or stable toxins, or limited production. The chemistry of the substrate can also affect production of toxins. For example production of aflatoxins is stimulated by the presence of fatty acids, specific amino acids and zinc. Other microorganisms can also inhibit growth and formation of toxins (Eklöf, 2013).

The mycotoxins most commonly found in fruits and their processed products are aflatoxins, ochratoxin A, patulin and *Alternaria* toxins (Fernández-Cruz et al., 2010).

Aflatoxins (AF) are a group of closely related metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. They are difuranocoumarin derivatives and the main components of this group are aflatoxin B1, B2, G1 and G2, based on their fluorescence under UV light (blue or green) and their relative chromatographic mobility. Aflatoxins are classified by the International Agency for Research on Cancer (IARC) as being carcinogenic to humans (group 1).

Alternaria fungi are commonly parasitic on plants and may cause spoilage of fruits and vegetables during transport and storage. *Alternaria alternata* produces a number of mycotoxins, including the dibenzo-pyrones alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT), altertoxin I and II (ATX-I and -II) and tenuazonic acid (TeA) a tetramic acid.

Ochratoxin A (OTA) was originally isolated from Aspergillus ochraceus in 1965. Several different ochratoxins exist, but ochratoxin A is the most common.

Patulin (PAT) is a toxic metabolite produced by several species of *Penicillium* and *Aspergillus*. The most important producer of PAT is the apple-rotting fungus *Penicillium expansum*. The IARC has classified PAT as category 3, not classifiable regarding its carcinogenicity to humans.

In Table 2 is presented the occurrence of mycotoxins in fruits and their processed products.

4. Sensory analysis

Sensory analysis involves assessing the sensory quality of food, using previously checked senses (sight, taste, smell, sound, touch), using methods and qualified people in this field, under certain conditions that ensure objectivity, fairness and the opportunity to reproduce the outcomes (Miteluț et al., 2007). To determine the quality of berries at different times after harvesting, sensory analysis was performed with the help of expert groups (panellists) for mulberries (Wang et al., 2013), strawberries (Wang et al., 2014; Aday and Caner, 2013), raspberry (Stavang et al., 2015; Bobinaite et al., 2016; Junqueira-Goncalves et al., 2016), cranberries (Caminiti et al., 2011).

5. Determination of colour of berries

From the literature, the most widely used method for determining the colour of both fresh fruit and those subjected to various processes of preservation (like refrigeration, freezing, freeze-drying), is the colorimetric method, resulting in the three critical factors L * (lightness), a * (chromaticity on an axis of the green (-) to red (+)) and b * (chromaticity on an axis of blue (-) to yellow (+)).Therefore, many researchers have studied the original colour and its evolution over time or after subjecting the fruit to various technological processes, of mulberries (Wang et al., 2013), strawberries (Ozakaya et al., 2009; Kartal et al., 2012; Aday and Caner, 2013; Wang et al., 2014), blueberries (Yemmireddy et al., 2013), raspberry (Bobinaite et al., 2016; Giovanelli et al., 2014), cranberries (Rudy et al., 2015), gooseberries (Vasquez-Parra et al., 2013; Vega-Galvez et al., 2014) and aronia (Samoticha et al., 2016).

Table 2. The occurrence of mycotoxins in fruits and their processed products (Fernández-Cruz et al., 2010)

Commodities	Positives/Total	Toxins	Maximum concentration	Concentration range
Oranges	8/25	AFB1/AF	52/120 µg/kg	-
Apple rotten areas	30/30	AF	350 µg/kg	-
Apple remainders	0/30	-	-	-
Apple juice	5/5	B1, G1	-	µg/L
Musts	19/47	AF B1	-	0.01 – 0.46 µg/L
Dried raisins	-	AF	-	Max. 2 – 550 µg/kg
Dried figs	-	AF	-	Max. 10 – 325 µg/kg
	7/8	AOH	59000 µg/kg	-
	8/8	AME	2300 µg/kg	-
Rotten apples	8/8	TEA	500 µg/kg	-
Apples	1/22	AOH	160 µg/kg	-
	1/22	AME	250 µg/kg	-
Rotten mandarins	2/2	AOH	-	1000 – 5200 µg/kg
	-	AME	-	500 – 1400 µg/kg
	-	TEA	-	21000 – 87200 µg/kg
Tangerine flavedo	6/8	AOH	-	2.5 – 17.4 µg/kg
	-	AME	-	0.9 – 3.5 µg/kg
Apple juice concentrate	17/32	AOH	-	1.35 – 5.42 µg/L
	1/32	AME	1.71 µg/L	-
Apple juice	11/11	AOH	-	0.04 – 2.40 µg/L
	10/11	AME	-	0.03 – 0.43 µg/L
Red grape juices	5/10	AOH	-	0.03 – 0.46 µg/L
	-	AME	-	0.01 – 39.5 µg/L
Red wine	20/25	AOH	-	0.03 – 7.41 µg/L
	-	AME	-	0.01 – 0.23 µg/L
Peaches	21/56	OTA	-	0.21 µg/kg
Cherries	6/6	OTA	-	2.71 µg/kg
Strawberry	4/10	OTA	-	1.44 µg/kg
Apple	2/4	OTA	-	0.41 µg/kg
Red wine	40 – 87 %	OTA	Average 0.30 µg/L	0.01 – 15.6 µg/kg
White wine	10 %	OTA	Average 0.18 µg/L	0.05 – 1.13 µg/L
Special wines	20 – 45 %	OTA	Average 4.47 µg/L	0.09 – 15.25 µg/L
Grape juice	29 – 85 %	OTA	Average 0.15 – 0.48 µg/L	0.010 – 5.3 µg/L
Vinegar	50 – 100 %	OTA	-	0.22 – 6.4 µg/L
Raisins	60 – 98 %	OTA	Average 1.4 – 9.2 µg/kg	Max 26 – 250 µg/kg
Dried figs	3 – 100 %	OTA	Average< 0.12 µg/kg	< 0.12 - 6900 µg/kg
Apple rotten areas	30/30	PAT	1000 µg/kg	2 – 11,3000 µg/kg
Apples, remainders	30/30	PAT	300 µg/kg	-
Blueberries	1/12	PAT	21 µg/kg	-
Cherries	9/10	PAT	113 µg/kg	-
Strawberries	8/10	PAT	145 µg/kg	-
Raspberry	3/5	PAT	746 µg/kg	-
Apple juice	3 – 100 %	PAT	Average 1 – 140 µg/L	0.5 – 1150 µg/L
Apple juice concentrated	78 – 100 %	PAT	-	7 – 376 µg/L
Cider mills	19 %	PAT	36.9 µg/L	4.6 – 467.4 µg/L
Retail cider	28 %	PAT	24.2 µg/L	15.3 – 35.2 µg/L
Apple puree	4/8	PAT	Average 63.2 µg/kg	4 – 221 µg/kg
Apple marmalade	6/26	PAT	Average 8.4 µg/kg	3 – 39 µg/kg
Pear marmalade	1/6	PAT	Average 4.8 µg/kg	2 – 25 µg/kg

6. Determination of texture of berries

The texture is a basic quality of fresh berries. Thus, it can be determined by means of laboratory apparatus generally called texturometre; this method being applied for the determination of texture of strawberries (Ozakaya et al., 2009; Wang et al., 2014; Kartal et al., 2012; Aday and Caner, 2013),blueberries (Yemmireddy et al., 2013; Zielinska et al., 2015; Diaz et al., 2011) and raspberry (Giovanelli et al., 2014).

CONCLUSIONS

After the literature review, a series of parameters that are determined in order to establish the quality of berries resulted. These parameters are presented in the table below (Table3).

Table 3. Quality parameters determined for berries

No.	Quality parameters
Chemical-physical parameters	
1.	pH
2.	total titratable acidity
3.	soluble solids (Brix)
Nutritional parameters	
4.	the content of ascorbic acid (vitamin C) (titrimetric method, HPLC)
5.	total phenolic content
6.	total anthocyanin content
7.	antioxidant capacity (DPPH, FRAP, HOSC, ORAC, ABTS, NO)
Microbial contamination	
8.	yeasts and moulds
9.	mesophilic aerobic total germ
Chemical contaminants	
10.	aflatoxins (AF)
11.	toxins produced by *Alternariasp*.
12.	ochratoxin A (OTA)
13.	patulin (PAT)
Sensorial analysis	
14.	sensory attributes (panel - taste, aroma, texture, color, appearance)
15.	colour - colorimetric
16.	texture - texturometre

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REFERENCES

Aday M.S., Caner C., 2013. The shelf life extension of fresh strawberries using an oxygen absorber in the biobased package, LWT - Food Science and Technology, 52, 102-109.

Aday M.S., Caner C., 2014. Individual and combined effects of ultrasound, ozone and chlorine dioxide on strawberry storage life, LWT - Food Science and Technology, 57, 344-351.

Amagase H., Farnsworth N.R., 2011. A review of botanical characteristics, phytochemistry, clinical relevance in efficacy and safety of *Lycium barbarum* fruit (Goji), Food Research International, 44, 1702–1717.

Azofeifa G., Quesada S., Perez A.M., Vaillant F., Michel A., 2015. Pasteurization of blackberry juice preserves polyphenol-dependent inhibition for lipid peroxidation and intracellular radicals, Journal of Food Composition and Analysis, 42, 56–62.

Bakowska-Barczak A.M., Kolodziejczyk P.P., 2011. Black currant polyphenols: Their storage stability and microencapsulation, Industrial Crops and Products, 34, 1301– 1309.

Barba F.J., Galanakis C.M., Esteve M.J., Frigola A., Vorobiev E., 2015. Potential use of pulsed electric technologies and ultrasounds to improve the recovery of high-added value compounds from blackberries, Journal of Food Engineering, 167, 38–44.

Bobinaite R., Viskelis P., Bobinas C., Miezeliene A., Alencikiene G., Venskutonis P.R., 2016. Raspberry marc extracts increase antioxidative potential, ellagic acid, ellagitannin and anthocyanin concentrations in fruit purees, LWT - Food Science and Technology, 66, 460-467

Bochi V.C., Barcia M.T., Rodrigues D., Speroni C.S., Giusti M.M., Godoy H.T., 2014. Polyphenol extraction optimisation from Ceylon gooseberry (*Dovyalis hebecarpa*) pulp, Food Chemistry, 164, 347–354.

Bravo K., Osorio E., 2016. Characterization of polyphenol oxidase from Cape gooseberry (*Physalis peruviana* L.) fruit, Food Chemistry, 197, 185–190.

Caminiti I.M., Noci F., Muñoz A., Whyte P., Morgan D.J., Cronin D.A., Lyng J.G., 2011. Impact of selected combinations of non-thermal processing technologies on the quality of an apple and cranberry juice blend, Food Chemistry, 124, 1387–1392.

Cekic C., Ozgen M., 2010. Comparison of antioxidant capacity and phytochemical properties of wild and cultivated red raspberries (*Rubus idaeus* L.), Journal of Food Composition and Analysis, 23, 540–544.

Chanjirakul K., Wang S.Y., Wang C.Y., Siriphanich J., 2006. Effect of natural volatile compounds on antioxidant capacity and antioxidant enzymes in raspberries, Postharvest Biology and Technology, 40, 106–115.

Chen P.X., Tang Y., Marcone M.F., Pauls P.K., Zhang B., Liu R., Tsao R., 2015a. Characterization of free, conjugated and bound phenolics and lipophilic antioxidants in regular- and non-darkening cranberry beans (*Phaseolus vulgaris* L.), Food Chemistry, 185, 298–308.

Chen P.X., Bozzo G.G., Freixas-Coutin J.A., Marcone M.F., Pauls P.K., Tang Y., Zhang B., Liu R., Tsao R., 2015b. Free and conjugated phenolic compounds and their antioxidant activities in regular and non-darkening cranberry bean (*Phaseolus vulgaris* L.) seed coats, journal of functional foods, 18, 1047–1056

Chen C., You L.J., Abbasi A.M., Fu X., Liu R.H., 2015. Optimization for ultrasound extraction of polysaccharides from mulberry fruits with antioxidant and hyperglycemic activity *in vitro*, Carbohydrate Polymers, 130, 122–132.

Chen Y., Zhang W., Zhao T., Li F., Zhang M., Li J., Zou Y., Wang W., Cobbina S.J., Wu X., Yang L., 2016. Adsorption properties of macroporous adsorbent resins for separation of anthocyanins from mulberry, Food Chemistry, 194, 712–722.

Chiang Y.C., Chen C.L., Jeng T.L., Lin T.C., Sung J.M., 2014. Bioavailability of cranberry bean hydroalcoholic extract and its inhibitory effect against starch hydrolysis following in vitro gastrointestinal digestion, Food Research International, 64, 939–945.

Chiou A., Karathanos V.T., Mylona A., Salta F.N., Preventi F., Andrikopoulos N.K., 2007. Currants (*Vitis vinifera* L.) content of simple phenolics and antioxidant activity, Food Chemistry, 102, 516–522

Chiou A., Panagopoulou E.A., Gatzali F., De Marchi S., Karathanos V.T., 2014. Anthocyanins content and antioxidant capacity of Corinthian currants (*Vitis vinifera* L., var. *Apyrena*), Food Chemistry, 146, 157–165.

Concha-Meyer A., Eifert J.D., Williams R.C., Marcy J.E., Welbaum G.E., 2015. Shelf Life Determination of Fresh Blueberries (*Vaccinium corymbosum*) Stored under Controlled Atmosphere and Ozone, International Journal of Food Science, 1-9.

Cujic N., Šavikin K., Jankovic T., Pljevljakušic D., Zdunic G., Ibric S., 2016. Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique, Food Chemistry, 194, 135–142.

d'Alessandro L.G., Kriaa K., Nikov I., Dimitrov K., 2012. Ultrasound assisted extraction of polyphenols from black chokeberry, Separation and Purification Technology, 93, 42–47.

Da Fonseca Machado A.P., Pasquel-Reátegui J.L., Barbero G.F., Martínez J., 2014. Pressurized liquid extraction of bioactive compounds from blackberry (*Rubus fruticosus* L.) residues: a comparison with conventional methods, Food Research International, xxx (2014) xxx–xxx.

Díaz P., Henríquez O., Enrione J., Matiacevich S., 2011. Thermal transitions of pulp and cuticle of blueberries, Thermochimica Acta, 525, 56– 61.

Donno D., Beccaro G.L., Mellano M.G., Cerutti A.K., Bounous G., 2015a. Goji berry fruit (*Lycium* spp.): antioxidant compound fingerprint and bioactivity evaluation, Journal of functional foods, 18, 1070–1085.

Donno D., Cerutti A.K., Prgomet I., Mellano M.G., Beccaro G.L., 2015b. Foodomics for mulberry fruit (*Morus* spp.): Analytical fingerprint as antioxidants' and health properties' determination tool, Food Research International, 69, 179–188.

Duymus H.G., Göger F., Can Baser K.H., 2014. In vitro antioxidant properties and anthocyanin compositions of elderberry extracts, Food Chemistry, 155, 112–119.

Eklöf D., 2013. Survey of mycotoxin producing fungi in goji berries, oil seeds and walnuts on the Swedish market, Biology Education Centre , Uppsala University, and the National Food Agency.

Fernández-Cruz M.L., Mansilla M.L., Tadeo J.L., 2010. Mycotoxins in fruits and their processed products: Analysis, occurrence and health implications, Journal of Advanced Research 1, 113–122.

Forino M., Tartaglione L., Dell'Aversano C., Ciminiello P., 2016. NMR-based identification of the phenolic profile of fruits of *Lyciumbarbarum* (goji berries). Isolation and structural determination of a novelN-feruloyl tyramine dimer as the most abundant antioxidant polyphenolof goji berries, Food Chemistry, 194, 1254–1259

Giovanelli G., Limbo S., Buratti S., 2014. Effects of new packaging solutions on physico-chemical, nutritionaland aromatic characteristics of red raspberries (Rubus idaeus L.) inpostharvest storage, Postharvest Biology and Technology, 98, 72–81.

Gironés-Vilaplana A., Valentão P., Andrade P.B., Ferreres F., Moreno D.A., García-Viguera C., Phytochemical profile of a blend of black chokeberry and lemon juice with cholinesterase inhibitory effect and antioxidant potential, Food Chemistry, 134, 2090–2096.

Gunenc A., Khoury C., Legault C., Mirrashed H., Rijke J., Hosseinian F., 2016. Seabuckthorn as a novel prebiotic source improves probiotic viability in yogurt, LWT - Food Science and Technology, 66, 490-495.

Huang Y., Chen H., 2014. A novel water-assisted pulsed light processing for decontamination of blueberries, Food Microbiology, 40, 1-8.

Jakobek L., Drenjancevic M., Jukic V., Seruga M., 2012. Phenolic acids, flavonols, anthocyanins and antiradical activity of "Nero", "Viking", "Galicianka" and wild chokeberries, Scientia Horticulturae, 147, 56–63.

Jensen M.B., Christensen K.V., Andrésen R., Søtoft L.F., Norddahl B., 2011. A model of direct contact membrane distillation for black currant juice, Journal of Food Engineering, 107, 405–414.

Jia N., Kong B., Liu Q., Diao X., Xia X., 2012. Antioxidant activity of black currant (*Ribes nigrum L.*) extract and its inhibitory effect on lipid and protein oxidation of pork patties during chilled storage, Meat Science, 91, 533–539.

Jiang Y., Nie W.J., 2015. Chemical properties in fruits of mulberry species from the Xinjiang province of China, Food Chemistry, 174, 460–466.

Jin P., Wang S.Y., Gao H., Chen H., Zheng Y., Wang C.Y., 2012. Effect of cultural system and essential oil treatment on antioxidant capacity in raspberries, Food Chemistry, 132, 399–405.

Junqueira-Gonçalves M.P., Alarcón E., Niranjan K., 2016. The efficacy of potassium sorbate-coated packaging to control postharvest gray mold in raspberries, blackberries and blueberries, Postharvest Biology and Technology, 111, 205–208.

Kartal S., Aday M.S., Caner C., 2012. Use of microperforated films and oxygen scavengers to maintain storage stability of fresh strawberries, Postharvest Biology and Technology, 71, 32–40.

Ketata M., Desjardins Y., Ratti C., 2013. Effect of liquid nitrogen pretreatments on osmotic dehydration of blueberries, Journal of Food Engineering, 116, 202–212.

Kostarelou P., Kanapitsas A., Pyrri I., Kapsanaki-Gotsi E., Markaki P., 2014. Aflatoxin B1 production by *Aspergillus parasiticus* and strains of *Aspergillus* section *Nigri* in currants of Greek origin, Food Control, 43, 121-128.

Kumar M.S.Y., Dutta R., Prasad D., Misra K., 2011. Subcritical water extraction of antioxidant compounds from Seabuckthorn (*Hippophae rhamnoides*) leaves for the comparative evaluation of antioxidant activity, Food Chemistry, 127, 1309–1316

Kumar M.S.Y., Tirpude R.J., Maheshwari D.T., Bansal A., Misra K., 2013. Antioxidant and antimicrobial properties of phenolic rich fraction of Seabuckthorn (*Hippophae rhamnoides* L.) leaves *in vitro*, Food Chemistry, 141, 3443–3450.

Lee J.E., Kim G.S., Park S., Kim Y.H., Kim M.B., Lee W.S., Jeong S.W., Lee S.J., Jin J.S., Shin S.C., 2014. Determination of chokeberry (*Aronia melanocarpa*) polyphenol components using liquid chromatography–tandem mass spectrometry: Overall contribution to antioxidant activity, Food Chemistry, 146, 1–5.

Maheshwari D.T., Kumar M.S.Y., Verma S.K., Singh V.K., Singh S.N., 2011. Antioxidant and hepatoprotective activities of phenolic rich fraction of Seabuckthorn (*Hippophae rhamnoides* L.) leaves, Food and Chemical Toxicology, 49, 2422–2428.

Mikulic-Petkovseka M., Slatnar A., Schmitzer V., Stampar F., Veberic R., Koron D., 2013. Chemical profile of black currant fruit modified by different degree of infection with black currant leaf spot, Scientia Horticulturae,150, 399–409.

Miteluț A., Niculiță P., Nediță G., Popa M., Turtoi M., Ghiduruș M., 2007. Siguranță alimentară și analiză senzorială, Editura Printech, București.

Oduse K.A., Cullen D., 2012. An Investigation Into The Fruit Firmness Properties of Some Progeny and Cultivars of Red Raspberry (*Rubus Idaeus*), IOSR Journal Of Environmental Science, Toxicology And Food Technology (IOSR-JESTFT), 1(6), 04-12.

Ozkaya O., Dundar O., Scovazzo G.C., Volpe G., 2009. Evaluation of quality parameters of strawberry fruits in modified atmosphere packaging during storage, African Journal of Biotechnology, 8 (5), 789-793.

Palanimuthu V., Rajkumar P., Orsat V., Gariépy Y., Raghavan G.S.V., 2009. Improving cranberry shelf-life using high voltage electric field treatment, Journal of Food Engineering 90, 365–371.

Pantelidis G.E., Vasilakakis M., Manganaris G.A., Diamantidis G., 2007. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries, Food Chemistry, 102, 777–783.

Pertuzatti P.B., Barcia M.T., Rodrigues D., Nogueira da Cruz P., Hermosín-Gutiérrez I., Smith R., Godoy H.T., 2014. Antioxidant activity of hydrophilic and lipophilic extracts of Brazilian blueberries, Food Chemistry 164, 81–88.

Ramos-Solano B., Algar E., Gutierrez-Manero F.J., Bonilla A., Lucas J.A., García-Seco D., 2015. Bacterial bioeffectors delay postharvest fungal growth and modify total phenolics, flavonoids and anthocyanins in blackberries, LWT - Food Science and Technology, 61, 437-443.

Rudy S., Dziki D., Krzykowski A., Gawlik-Dziki U., Polak R., Rozylo R., Kulig R., 2015. Influence of pre-treatments and freeze-drying temperature on the process kinetics and selected physico-chemical properties of cranberries (*Vaccinium macrocarpon* Ait.), LWT - Food Science and Technology, 63, 497-503.

Seabra I.J., Braga M.E.M., Batista M.T., de Sousa H.C., 2010. Effect of solvent (CO_2/ethanol/H_2O) on the fractionated enhanced solvent extraction of anthocyanins from elderberry pomace, J. of Supercritical Fluids, 54, 145–152.

Saggu S., Divekar H.M., Gupta V., Sawhney R.C., Banerjee P.K., Kumar R., Adaptogenic and safety evaluation of seabuckthorn (*Hippophae rhamnoides*) leaf extract: A dose dependent study, Food and Chemical Toxicology, 45, 609–617

Samoticha J., Wojdyło A., Lech K., 2016. The influence of different the drying methods on chemical composition and antioxidant activity in chokeberries, LWT - Food Science and Technology, 66, 484-489.

Sánchez L.C.A., Real C.P.V., Perez Y.B., 2014. Effect of an edible crosslinked coating and two types of packaging on antioxidant capacity of castilla blackberries, Food Science and Technology, 34(2), 281-286.

Sánchez-Salcedo E.M., Mena P., García-Viguera C., Martínez J.J., Hernández F., 2015. Phytochemical evaluation of white (*Morus alba L.*) and black (*Morus nigra L.)* mulberry fruits, a starting point for the assessment of their beneficial properties, Journal of functional foods, 12, 399–408.

Stavang J.A., Freitag S., Foito A., Verrall S., Heide O.M., Stewart D., Sønsteby A., 2015. Raspberry fruit quality changes during ripening and storage as assessed by colour, sensory evaluation and chemical analyses, Scientia Horticulturae, 195, 216–225.

Ting H.C., Hsu Y.W., Tsai C.F., Lu F.J., Chou M.C., Chen W.K., 2011. The *in vitro* and *in vivo* antioxidant properties of seabuckthorn (*Hippophae rhamnoides* L.) seed oil, Food Chemistry, 125, 652–659.

Tournas V.H., Katsoudas E., 2005. Mould and yeast flora in fresh berries, grapes and citrus fruits, International Journal of Food Microbiology, 105, 11 – 17.

Vásquez C., Díaz-Calderón P., Enrione J., Matiacevich S., 2013. State diagram, sorption isotherm and color of blueberries as a function of water content, Thermochimica Acta, 570, 8-15.

Vásquez-Parra J.E., Ochoa-Martínez C.I., Bustos-Parra M., 2013. Effect of chemical and physical pretreatments on the convective drying of cape gooseberry fruits (*Physalis peruviana*), Journal of Food Engineering, 119, 648–654.

Vagiri M., Ekholm A., Johansson E., Andersson S.C., Rumpunen K., 2015. Major phenolic compounds in black currant (*Ribes nigrum* L.) buds: Variation due to genotype, ontogenetic stage and location, LWT - Food Science and Technology, 63, 1274-1280

Veberic R., Jakopic J., Stampar F., Schmitzer V., 2009. European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols, Food Chemistry, 114, 511–515.

Vega-Gálvez A., López J., Torres-Ossandón M.J., Galotto M.J., Puente-Díaz L., Quispe-Fuentes I., Di Scala K., 2014. High hydrostatic pressure effect on chemical composition, color, phenolic acids and antioxidant capacity of Cape gooseberry pulp (*Physalis peruviana* L.), LWT - Food Science and Technology, 58, 519-526.

Vega-Gálveza A., Díaz R., López J., Galotto M.J., Reyes J.E., Perez-Won M., Puente-Díaz L., Di Scala K., 2016. Assessment of quality parameters and microbial characteristics of Cape gooseberry pulp (*Physalis peruviana* L.) subjected to high hydrostatic pressure treatment, food and bioproducts processing, 97, 30–40.

Vu K.D., Carlettini H., Bouvet J., Côté J., Doyon G., Sylvain J.F., Lacroix M., 2012. Effect of different cranberry extracts and juices during cranberry juice processing on the antiproliferative activity against two colon cancer cell lines, Food Chemistry, 132, 959–967.

Wang R., Satyanarayan R.S.D., Vijaya G.S.R., Gariépy Y., 2013. Improving mulberry shelf-life using PEAK fresh package in cold environment, Journal of Food Research and Technology, 1 (2), 73-79.

Wang S.Y., Gao H., 2013. Effect of chitosan-based edible coating on antioxidants, antioxidant enzyme system, and postharvest fruit quality of strawberries (*Fragaria* x *aranassa* Duch), LWT - Food Science and Technology, 52, 71-79.

Wang Z., Narciso J., Biotteau A., Plotto A., Baldwin E., Bai J., 2014. Improving Storability of Fresh Strawberries with Controlled Release Chlorine Dioxide in Perforated Clamshell Packaging, Food Bioprocess Technology, 7, 3516–3524.

Wójcik P., Filipczak J., 2015. Response of 'White Smith' gooseberry to boron fertilisation under conditions of low soil boron availability, Scientia Horticulturae xxx (2015) xxx–xxx

Wu R., Frei B., Kennedy J.A., Zhao Y., 2010. Effects of refrigerated storage and processing technologies on the bioactive compounds and antioxidant capacities of 'Marion' and 'Evergreen' blackberries, LWT - Food Science and Technology, 43, 1253-1264.

Yemmireddy V.K., Chinnan M.S., Kerr W.L., Hung Y.C., 2013. Effect of drying method on drying time and physico-chemical properties of dried rabbiteye blueberries, LWT - Food Science and Technology 50, 739-745.

Zhang L., Li J., Hogan S., Chung H., Welbaum G.E., Zhou K., 2010. Inhibitory effect of raspberries on starch digestive enzyme and their antioxidant properties and phenolic composition, Food Chemistry, 119, 592–599.

Zielinska M., Sadowski P., Błaszczak W., 2015. Freezing/thawing and microwave-assisted drying of blueberries (*Vaccinium corymbosum* L.), LWT - Food Science and Technology, 62, 555-563.

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INDOOR CULTIVATION OF SELECTED OIL – CONTAINING CONSORTIA OF PHOTOSYNTHETIC MICROORGANISMS FOR FURTHER BIODIESEL PRODUCTION; PRELIMINARY FINANCIAL EVALUATION

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Abstract

This paper presents results concerning the indoor cultivation of selected mixed populations of photosynthetic microorganisms using BG_{11}medium as well as a cheaper medium based on chemical fertilizers and residual glycerol. Preliminary financial evaluations are also presented in order to focus on the economical constraints. The growth of selected oil- containing consortia of photosynthetic microorganisms in 90L of BG_{11}(prepared in spring water) produced in 14 days 285 grams of dry weight biomass containing 28% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass means 83.3 lei; whereas the same consortium grown in 30L of alternative medium, prepared with agricultural fertilizers, residual glycerol and spring water produced in 14 days 150g grams of dry weight biomass containing 20% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass is 3.2 lei.

Key words*: photosynthetic microorganisms, biomass, lipids, agricultural fertilizers.*

INTRODUCTION

Biodiesel is produced by the transesterification of different origins triglyceride with monohydric alcohols such as methanol. One main advantage of biodiesel it that it can be blended in any proportion with fossil based diesel which is not possible with bioethanol. Classically, rapeseed, canola, sunflower, soybean oils, beef tallow and many other oils have been used for the production of bio-diesel esters. The ability of photosynthetic microorganisms, both prokaryotes and eukaryotes, to accumulate lipids inside the cell is very well known from more than a century, but a national program, in USA, was funded for the production of biodiesel from high lipid-content algae (Sheehan et al., 1998) only in 1978. Over the almost two decades of this program (1978-1996), tremendous advances were made in the science of manipulating the metabolism of algae and the engineering of microalgae algae production systems (Sheehan et al., 1998). Mainly there is a huge increase practically all over the globe for the use of photosynthetic microorganisms in the last two decades, both prokaryotes and eukaryotes, as sources of lipids for biodiesel (Thomas et., 1983; Chisti, 2007; Li et al., 2008; Brune et al., 2009; Griffiths and Harrison., 2009; Vijayaraghavan and Hemanathan K., 2009; Liang et al., 2009; Thurmond, 2009; Demirbas, 2010; Huang, 2010; Mata et al., 2010; Brennan and Owende, 2010; Amaro et al.,; 2011; Santibáñez et al., 2011; Kaiwan-arporn 2012; Schuhmann et al., 2012; Borowitzka, 2013; Rawat et al., 2013; Velea et al., 2014) as well as for other bio(nano)-tehnogical application (Borowitzka, 2013; Ardelean and Zarnea, 1998; Ardelean, 2006; Ardelean 2015a and b, and references herein).

This interest is based mainly on the followings (for more details see Demirbas, 2010; Amaro et al.; 2011; Rawat et al., 2013, and references herein):

Microalgae have rapid growth rates, thus they can to double their weight with respect to biomass within 24 h, some of them being able to grown strictly photoautotrophic but also mixotrophic or even heterotrophic (into darkness);

Photosynthetic microorganism which converts sunlight, water and CO_2 to sugars, from which macromolecules such as lipids and

triacylglycerides (TAGs) can be obtained which are promising and sustainable feedstock for biodiesel production;
Many micro algae have the ability to produce substantial amounts (20-50%) of TAGs as a storage lipid under photooxidative stress or other adverse environmental conditions;
Microalgae require less freshwater for cultivation than terrestrial plants (but water is still a problem for true economically applications!)
Lipids produced are generally neutral lipids that have a high level of saturation making it a suitable feedstock for biodiesel production; the chemical and physical properties of biodiesel produced from microalgae reach the international standard for cars (EN14214).
During their growth microalgae can use N and P in the form of phosphates and nitrates from wastewater and CO_2, even residual one, which is a major component of flue gases.
Apart of lipids, some microalgae produce other valuable substances such as proteins, pigments, biopolymers and carotenoids including antioxidant substances for commercial or pharmaceutical purpose.
However, so far there are some drawbacks (for more details see Demirbas, 2010; Amaro et al.; 2011; Rawat et al., 2013, and references herein): Recovery of microalgae from their growth media is seen as one of the major challenges of using microalgae for the production of biodiesel owing to their small size and planktonic distribution;
Cell wall breaking is difficult and leads to unspecific mixtures thus large scale extraction techniques for microalgal lipids are complex and expensive;
Most metabolic pathways in microalgae are not fully known, thus, biochemical optimization is difficult;
The knowledge of regulatory basis of metabolic networks in microalgae is incipient, so rational control of endocellular reactions is hard to accomplish;
Microalgae grown in open pond systems are prone to contamination, bacteria being strong competitors for nutrients, including organic ones (in the case of mixotrophic or heterotrophic growth of photosynthetic microorganisms);
Open systems are also susceptible to grazers in the form of protozoa and zooplankton which can devastate algal concentration in 2–3 days;
There is a huge need of nitrogen to sustain the intensive growth of photosynthetic microorganisms, many specialist thinking that the only true answer for that is the use of residual waters rich in inorganic nitrogen;
The conversion of lipids to biodiesel produce wastes, including residual glycerol whose destruction is an expensive step; alternatively, it could be used as row carbon source for growing other useful microorganisms, including lipid accumulation one.
The aim of this paper is to present original results concerning the in- door cultivation of selected mixed populations of photosynthetic microorganisms using BG_{11}medium as well as a cheaper medium based on chemical fertilizers and residual glycerol, in connection with the amount of biomass and oil produced. Preliminary financial evaluations are also presented in order to focus on the economical constraints.

MATERIALS AND METHODS

The consortia's selection of photosynthetic microorganisms relatively rich in lipids was done taking into account the fluorescence signal in the presence of the fluorochrome Nile red (Ardelean et al., manuscript in preparation)
Microscopic investigation of lipid content. The photosynthetic microorganisms consortia were treated with Nile red(9-(Diethylamino)-5H benzo [∞] phenoxazin- 5), one of the selective fluorescence markers for lipids (Greenspan et al., 1985; Chen et al., 2009). The microbial populations were incubated for 30-120 minutes in the presence of Nile red in order to allow as much as possible the penetration of cell wall and cell membrane; then, the microbiological samples were inspected using an epifluorescence microscop, with respect to fluorescence signal in the red region as well as in the green region of the spectrum.
The growth of photosynthetic populations. In agreement with the literature, BG_{11} was used to cultivate the mixtures of photosynthetic microorganisms in 20L PET bottles at 28-32^{o} C with natural 12 hours sunlight illumination (7am-7pm) and 12 hours (7pm-7am) artificial lighting (2400 luxes ?), air bubbling (240 L/hour), the pH varying between 8.0 and 9.0. The growth of photosynthetic populations was carried out also in alternative medium containing residual glycerol and chemicals

found in commercially available fertilizers for agriculture, thus decreasing the cost of the growing medium, in agreement with the proposals in the literature (Santibáñez et al., 2011). The growth conditions were as those for the growth in BG_{11}. Cell harvesting can be done very well at laboratory level by centrifugation; however, for larger volumes centrifugation is very costly; thus, in the experiments reported in this paper the harvesting was done by flocculation using $Al_2(SO_4)_3x18H_2O$ (SR EN 878/2004 A) (Teodosiu, 2001; Mâşu and Zamfiroiu, 2007) the stock solution (20g/L) was diluted 10 times with the microbial culture and in short time (5-10 minutes) the cells are gravitationally separated from the clear liquid supernatant (Figure 1).

Figure 1. The separation of microbial cell by flocculation.

Lipid extraction was done by Bligh and Dyer method (Bligh and Dyer 1959) and ultrasonication. For that microalgal biomass were collected by centrifuging the cells at 4000 *g* for 10 minutes. The cells were washed with distilled water, dried (48 hours at 85°C) and weighted. The known amount of biomass (100 mg) was then homogenized with chloroform: methanol 1:2 at 35°C and sonicated for 60 min. The extract was centrifuged for 7 minutes at 10,000 g and supernatant was collected in a separating funnel. The residue was further homogenized with chloroform and again centrifuged (10,000 g) to collect the supernatant. Now 0.9% NaCl solution was added to the filtrate and washed, lower layer of chloroform was separated and treated with anhydrous Na_2SO4 to remove the traces of water. The lipid content was determined gravimetrically and expressed as dry weight % after evaporating the chloroform calculated using the following equation: Y(%) = WL/WDA, where WL and WDA were the weights of the extracted lipid and the dry algae biomass, respectively.

RESULTS AND DISCUSSIONS

In Figure 2 one can see the PET bottles after 14 days of indoor cultivation.

Figure 2. PET bottles with selected consortia of photosynthetic microorganisms, both prokaryotes and eukaryotes, after 14 days of autotrophic cultivation (see Materials and methods).

In Figure 3 there are presented microscopic images both in bright field and in epifluorescence microscopy (Nile red labeling of lipid droplets- when present) of different microscopic fields; each microscopic field has three images; one in bright field and two fluorescent images: red and green portion of the fluorescence emission spectrum of the Nile red.

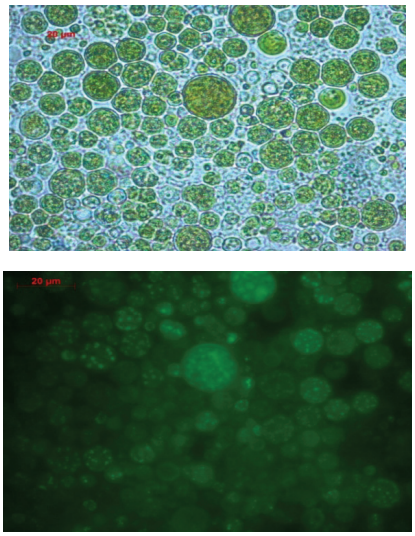

Figure 3. Bright filed and pifluorescence images (red and green filters, respectively) of different populations of photosynthetic microorganisms presented in the A9 mixture of populations

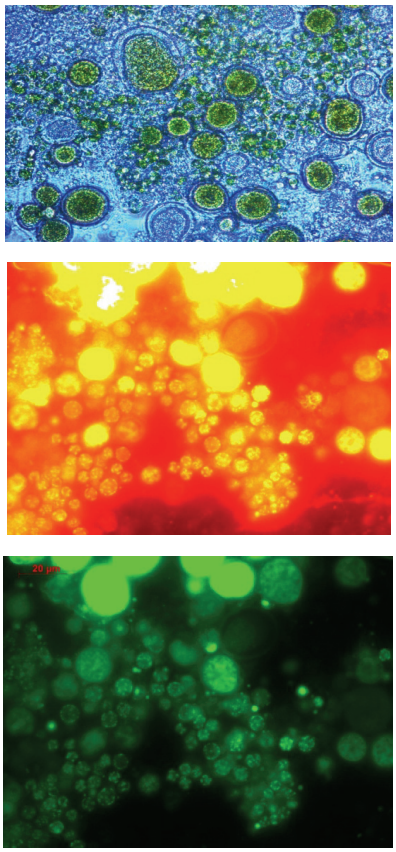

Figure 4. Bright filed and epifluorescence images (red and green filters, respectively) of different populations of photosynthetic microorganisms presented in the A3 mixture of populations

The lipid intracellular inclusions (lipids droplets) can be seen in some cells of photosynthetic microorganisms as red or green surfaces; the size and the intensity of the fluorescence varies from cell to cell. The size and the intensity emission are higher for cells containing larger quantities of lipid inclusions. These images show the diversity of the mixture of populations of photosynthetic populations with respect to lipid intracellular inclusions, strongly arguing for the necessity to further isolate in pure cultures the strains with high lipid content.

In Table 1 there are presented the synthetic results concerning the dry biomass of photosynthetic microorganisms obtained after 14 days of indoor cultivation either in BG_{11} prepared with tap water or in alternative medium, containing chemicals found in commercially available fertilizers for agriculture, and residual glycerol.

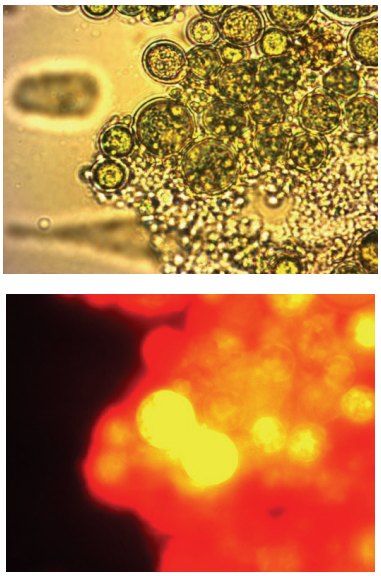

Figure 5. Bright filed and epifluorescence (red and green filters, respectively) images of different populations of photosynthetic microorganisms presented in the A7 mixture of populations

Table 1. The results concerning dry biomass and lipid content of selected consortia of photosynthetic microorganisms grown in $BG_{11}$and in alternative medium (with spring water and residual glycerol)

Growing medium	Volume of culture	Dry biomass	Lipid content %
BG_{11} (with spring water)	95 L	285 g	28%
Alternative medium	30 L	150 g	20 %

As one can see from Table 1, the results obtained in indoor cultivation are rather modest with respect to lipid content, probably because of the use of mixture of populations, containing strains with rather high lipid content (as can be seen during microscopic inspection- see Figure 3) and strain with practically no lipid deposits (as can be seen during microscopic inspection- see Figure 3). The use of alternative medium allows a higher dry biomass per liter, as compared with standard medium prepared in tap water, which seems to be promising for the future research. The cost of BG_{11} prepared in distilled water is 1.25 lei /L and prepared with

spring water 0.25 lei/L whereas the cost of alternative medium, prepared with agricultural fertilizers, residual glycerol and spring water is 0.0163 lei /L. Taking into account the biomass obtained by growing these populations of photosynthetic microorganisms in BG_{11} prepared in spring water the cost of 1 Kg of dry biomass is 83.3 lei whereas the use of alternative medium reduce the cost significantly down to 3.2 lei. We have to remember that the calculation concerns only cost of water and chemical ingredients, all other costs (illumination, continuous air-bubbling, flocculation, drying, chemical extraction – including chemicals and ultrasonication, labor etc.) were not take into account for the time being. It has to be said that the price of 83.3 lei/kg dry biomass of photosynthetic microorganisms cannot commercially compete with the price of oleaginous plants, which is 100 times lower!

CONCLUSIONS

The indoor cultivation of selected oil- containing consortia of photosynthetic microorganisms in 90L of BG_{11} (prepared in spring water) produced in 14 days 285 grams of dry weight biomas containing 28% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass means 83.3 lei;
The indoor cultivation of selected oil-containing consortia of photosynthetic microorganisms in 30L of alternative medium, prepared with agricultural fertilizers, residual glycerol and spring water produced in 14 days 150g grams of dry weight biomas containing 20% of lipids; the cost (taking into account only chemicals and water) of 1 Kg of dry biomass is 3.2 lei.
The future prospects for this research are the followings:
Selection and isolation of strains able to store lipids in high proportions and to rapidly grow in standardized media (BG_{11}, for example);
Further selection of the above (1) isolates but able to grow in cheaper media containing agricultural fertilizers or, better, in different types of waste waters;
The growth of the selected strain(s) in large volumes (1,000-10,000 L) only under solar irradiation, extraction of oil and its conversion to biodiesel
Cost calculations for the scaled up processes, and future improvements.

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REFERENCES

Ardelean I.I., Zarnea G., 1998. Biosensors with intact cyanobacteria for environmental protection. In: Subramanian G., Kaushik D., Venkataraman G.S. (Eds.) Cyanobacterial Biotechnology Publishers M/S Oxford IBH Publishing House, New Dehli, 341-346.

Ardelean I.I., 2006. Biosensors with Cyanobacteria and Algae In: Tewari A., (Ed.) Recent Advances on Applied Aspects of Indian Marine Algae with reference to Global Scenario. Vol II Central Salt & Marine Chemicals Research Institute, 87-103.

Ardelean I. I., 2015. Metallic Nanoparticle Synthesis by Cyanobacteria: Fundamentals and Applications. In: Sahoo D., Seckbach J., (Eds.) The Algae World, Springer Netherlands, **Series Title**, Cellular Origin, Life in Extreme Habitats and Astrobiology, volume 26, pp. 429-448.

Ardelean I.I., 2015. The Involvement of Cyanobacteria in Petroleum Hydrocarbons Degradation: Fundamentals, Applications and Perspectives. In: Davison D. (Ed) Cyanobacteria: Ecological Importance, Biotechnological, Uses and Risk Management .Nova Science Publishers pp.41-61.

Amaro H.M., Guedes A.C., Malcata F.X., 2011. Advances and perspectives in using microalgae to produce biodiesel. Appl Energy, 88: 3402–3410.

Bligh E.G., Dyer W.J., 1959.A rapid method of total lipid extraction and purification [J]. Canadian Journal of Biochemistry and Physiology, 37(8): 911−917.

Borowitzka M.A., 2013. High-value products from microalgae-their development and commercialisation. J. Appl.Phycol. 25: 743–756.

Brennan L., Owende P., 2010. Biofuels from microalgae – a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sustain Energy Rev, 14: 557–577.

Brune D.E., Lundquist T.J., Benemann J.R., 2009. Microalgal biomass for greenhouse gas reductions: Potential for replacement of fossil fuels and animal feeds [J]. Journal of Environmental Engineering—Asce, 135(11): 1136−1144.

Chen W., Zhang C., Song L., Sommerfeld M., Hu Q., 2009.A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods, 77: 41–47.

Chisti Y., 2007. Biodiesel from microalgae.Biotechnol. Adv. 25: 294–306.

Demirbas M.F., 2010. Microalgae as a feedstock for biodiesel [J]. Energy Education Science and Technology Part A, 25(1−2): 31−43.

Greenspan P., Mayer E.P., Fowler S.D., 1985. Nile red—A selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100: 965–973.

Griffiths M.J., Harrison S.T.L., 2009.Lipid productivity as a key characteristic for choosing algal species for biodiesel production [J]. J ApplPhycol, 21(5): 493−507.

Huang G., Chen F., Wei D., Zhang X., Chen G., 2010. Biodiesel production by microalgal biotechnology. Appl Energy. Elsevier Ltd, 87(1): 38–46.

Huang G.H., Chen F., Wei D., Zhang X.W., Chen G., 2010. Biodiesel production by microalgal biotechnology. Appl Energy, 87: 38–46.

Kaiwan-arporn P., Hai P.D., Thu N.T., Annachhatre A.P., 2012. Cultivation of cyanobacteria for extraction of lipids.Biomass and Bioenergy. 44: 142–149.

Li Y., Horsman M., Wang B., Wu N., Lan C.Q., 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochlorisoleoabundans.* Appl. Microbiol Biotechnol.; 81(4): 629–636.

Liang Y.N., Sarkany N., Cui Y., 2009. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett, 31: 1043–1049.

Mata T.M., Martins A.A., Caetano N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy Rev. 14: 217–232.

Mâşu S., Zamfiroiu E., 2007. Coagularea cu adaos de reactivi-procedeu de tratare şi eliminarea impurităţilor din ape cu ajutorul sărurilor hidrolizare. Editura EUROBIT, Timişoara.

Rawat I.R., Ranjith Kumar T., Mutanda F., 2013. Bux Biodiesel from microalgae: A critical evaluation from laboratory to large scale production Applied Energy, 103: 444–446.

Santibáñez C., Varnero M.T., Bustamante M., 2011 Residual Glycerol From Biodisel Manufacturing, Waste Or Potential Source Of Bioenergy: A Review Chilean Journal Of Agricultural Research 71(3): July-September, 469-475.

Schuhmann H., Lim D.K.Y., Schenk P.M., 2012. Perspectives on metabolic engineering for increased lipid contents in microalgae. Biofuels 3: 71–86.

Sheehan J., Dunahay T., Benemann J., Roessler P., 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae; National Renewable Energy.

Teodosiu C., 2001. Tehnologia apei potabile şi industrial. Editura Matrix Rom, Bucureşti.

Thomas W.H., Tornabene T.G., Weissman J., 1983. Screening for lipid yielding microalgae: activities for SERI/STR-231-2207.

Thurmond W., 2009. Algae 2020: advanced biofuel markets and commercialization outlook. 1st ed. 460.

Velea S., Ilie L., Stepan E., Chiurtu R., 2014. New photobioreactor design for enhancing the photosynthetic Productivity of *Chlorellahomosphaera* culture Revista de Chimie (Bucharest) 65, 1, pp 56-60

Vijayaraghavan K., Hemanathan K., 2009. Biodiesel production from freshwater algae [J]. Energy Fuels, 23: 5448−5453.

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HPTLC PHENOLIC COMPOUNDS FINGERPRINT AND ANTIOXIDANT ACTIVITY OF *SAMBUCUS EBULUS* LEAVES AND FRUIT

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Abstract

Phenolic compounds fingerprint of the 50% (v/v) ethanol extracts obtained from leaves (LE) and fruits (FE) of Sambucus ebulus (dwarf elder), fam Adoxaceae, was obtained by high performance thin layer chromatography (HPTLC), in order to evaluated its qualitative chemical composition and antioxidant activity (by DPPH and TAC assays). Quantitative evaluation of total polyphenolic compounds was made by Folin Ciocalteu assay. The obtained fingerprints showed that both extracts are characterized by the presence of flavonoid glycosides and phenol carboxylic acids. The presence of anthocyanins was revealed only in fruit extract Total phenol content was 19.5 mg GAE/g dry material for LE and 52,5 mg GAE/g dry material for FE. Both extracts have important antioxidant activity in a concentration-dependent manner, fruits extract exhibiting a higher one.

Key words*: HPTLC profile, Sambucus ebulus, leaves, fruit, antioxidant activity.*

INTRODUCTION

Medicinal plants are important natural renewable resources. Superior valorification of natural resources is important in the context of the development of new and innovative products as food supplement and cosmetics that can contribute in a positive manner to the living standards of the population. Antioxidants compounds become a major research subject due to their important biological activity. They can protect the human body against damage produces by reactive oxygen species (ROS). Our bodies natural antioxidant system is composed of enzymes (SOD -superoxide dismutase and GPx – glutathione peroxidase) and low molecular weight antioxidants (lipid soluble antioxidants – tocopherol, carotenoids, quinones and some polyphenols and water soluble antioxidants (vitamin C, uric acid, and polyphenols). Results of the research studies suggest that polyphenols may protect cells against oxidative stress. Free radicals play important role in the pathogenesis of diseases as degenerative diseases such as atherosclerosis, diabetes, ischemia /reperfusion (l/R) injury, inflammatory diseases (rheumatoid arthritis, inflammatory bowel diseases and pancreatitis), cancer, neurological diseases, hypertension etc (Yoshihara, et al., 2010; Kumar, 2011; Opara, 2006).

Sambucus ebulus L. (dwarf elder, elderberry or danewort), is a perennial plant, belonging to *Adoxaceae* family. The species is widespread in most part of the Europe. Leaves, fruits, flowers and root are used for therapeutic purpose. In Romania, in south Transylvania, the fruits are used for wine and jam. In traditional medicine, the species is used for diuretic, laxative and purgative, diaphoretic, depurative properties. The species is also used as toothache analgesic, antispasmodic, anti-allergic and anti-inflammatory against insect bites (Parvu, 1997). Current pharmaceutical studies have revealed antioxidant, anti-inflammatory, antinociceptive, antimicrobial, antiarthritic effects of the species. According to recent studies, this species shows anticancer proprieties (Tasinov et al., 2013). Depending on the part of the plant used (leaves, fruits, flowers, aerial parts, leafy stems, roots, whole plant), the chemical composition is different. Thus, the leaves contain essential oil, polyphenolic compounds, proteins; flowers – essential oils, sugars, cyanogenic glycosides; fruits – anthocyanins, sugars, valerianic, malic and tartaric acids, tannins, pectin's, resin, vitamin C; roots - tannins, saponins, anthocyanins, terpenoids (Shokrzadeh and

Saeedi Saravi, 2010; Feizbakhsh et al., 2014; Pieri et al., 2009; Pribela et al., 1992; Tasinov et al., 2013; Popescu et al., 2014).

The present paper presents the polyphenolic compounds content analysed by qualitative determination (HPTLC – high-performance thin layer chromatography) and quantitative determination (Folin- Ciocalteu assay) and the antioxidant activity (DPPH and TAC assays) for 50% (v/v) ethanolic extracts of *Sambucus ebulus* leaves and fruits.

MATERIALS AND METHODS

Raw material – Sambucus ebulus L.- leaves and fruit. The fruit samples were harvested at the end of August 2015 at their optimum fruit maturity (Southern Romania). A voucher specimen is deposited in INCDCF-ICCF Plant Material Storing Room.

Sample preparation: Leaves (LE) and fruits (FE) samples were prepared by extraction with 50% (v/v) ethanol, 1/10 plant material/solvent ratio, at boiling temperature, for 30 minutes The solutions were filtered and kept frozen until analysis.

Phenols HPTLC Analysis:

The densitometric analysis (HPTLC) was made according to TLC Atlas - Plant Drug Analyses (Wagner and Balt, 1997) and the characteristic fingerprint profile for phenolic compounds was determined. 3-3.5µl of the samples and 1-3µl of references substances (10^{-3}M rutin, hyperoside, chlorogenic acid, caffeic acid-Sigma-Aldrich) were loaded as 10mm band length in the 20 x 10 Silica gel 60F254 TLC plate using Hamilton- Bonaduz, Schweiz syringe and CAMAG LINOMAT 5 instrument. The mobile phase consisted in 100:11:11:27 (v/v/v/v) ethyl acetate-acetic acid-formic acid-water. The TLC twin chamber was pre-saturated with mobile phase for 30 min at ~20°C. The plate was developed in the mobile phase up to 90mm. After development, plates were dried and derivatized in Natural Product followed by PEG4000 reagent. The fingerprints were evaluated at UV with a WinCats and VideoScan software. Anthocyanins VIS detection was made without chemical treatment.

Total phenol content- Total phenol content was determined according to Folin – Ciocalteu method (European Pharmacopoeia 6,0). Briefly, 1ml of the extract was transferred to a 25ml volumetric flask, 10ml of water and 1ml of Folin Ciocalteu reagent was added. The volume was made to 25ml with 5% sodium carbonate (w/v). The blend was left at room temperature for 30 minutes. Then the absorbance of the samples was read at 760nm with a UV/VIS spectrophotometer (Helios λ, Thermo Electron Corporation). Distilled water was used as blank. Total phenol content was determined from the extrapolation of the calibration curve (*$y=0.0525x-0.020$, $R_2 = 0.992$)*, which was obtained for gallic acid (Sigma Chemical Co., St. Louis, USA) The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dried material.

Anthocyanins spectrophotometric determination was made according to European Pharmacopoeia 6.0. 0.1ml of extract was transferred to a 10ml volumetric flask. The volume was made to10 ml with 85:15 (v/v) ethanol: HCl 1.5M solution. The absorbance of the samples was determined at 546 nm with a UV/VIS spectrophotometer (Helios λ, Thermo Electron Corporation). The etanolic solution was used as blank. Anthocyanin content was determined from the extrapolation of the calibration curve (*$y=0.1976x-0.270$, $R_2 = 0.97$)*, which was obtained for cyanidin chloride (Sigma Chemical Co., St. Louis, USA) The results was expressed as miligrams of cyanidin chloride equivalents (CCE) per 100 grams of dried material.

*Free radical scavenging assay- w*as evaluated using the Sanchez-Moreno et al. (1998) assay. The extracts concentration was 0,1%, in methanol. 50µl aliquots of the extract were mixed with 2950µl of the DPPH methanolic solution (0.025g/l). The radical scavenging activity of the extracts against 2,2-diphenyl-1-picryl hydrazyl radical (Sigma-Aldrich) was determined by measuring UV absorbance at 517nm. A blank solution was prepared containing the same amount of methanol and DPPH, and measured after keeping at room temperature 30 minutes. The radical scavenging activity (RSA) was calculated using the following formula:

% inhibition = {(AB – AA)/AB} x 100.

Where AB is the absorption of blank sample and AA is the absorption of tested extract solution.

Total antioxidant capacity assay

Was assessed by phosphomolybdenum method, according to Prieto et al. 1999. To 0.3ml ethanolic solution of the sample (concentration 0.1mg/ml)was added 2.7ml of reagent solution (0.6M sulfuric acid, 28mM sodium molybdate, and 4mM ammonium phosphate). The mixtures were incubated at 95^0C for 90 minutes. After cooling the samples to room temperature, their extinction was measured at 695nm with UV-VIS spectrophotometer. Ethanol was used as negative control. The antioxidant capacity was expressed as ascorbic acid equivalent to 1mg of active substance. The calibration curve is linear for ascorbic acid in the range of 0.001 to 1mg/ml, n= 6, r^2=0.999.

RESULTS AND DISCUSSIONS

HPTLC analysis

Figure 1 shows the phenolic profiles of the *Sambucus ebulus* 50% (v/v) ethanolic fruit extract (FE) - track T1, T2, - duplicate sample, 50% (v/v) ethanolic leaves extract (LE) – track T7, T8- duplicate sample, comparatively with references substances caffeic acid T3, chlorogenic acid - T4, rutin - T5, hyperoside – T6.

Chromatographic profile of fruits extract (T2) have four proeminent yellow-orange (z1, z2, z5, z6) and one yellow – green flavonoid glycosides zones (z7), including rutin (z2 – Rf~0.42) and hyperoside (z6-Rf~0.66) accompanied by three blue florescent zones as phenol carboxylic acids with chlorogenic acid and caffeic acid as z3- Rf~0.49, respectively z8- Rf~0.96 spots. Track 1 reveals two clearly defined violet- blue pigment zones as anthocyanins.

The leaves extract fingerprint is characterised by a six yellow-orange (z11, z12, z13, z16, z17, z19) and one green (z18) flavonoid glycosides zones, two blue fluorescence zones (z14, z15) including rutin, hyperoside, and chlorogenic acid.

Figure 2 presents the comparison between extracts LE and FE and references substances hyperoside and chlorogenic acid fingerprints.

Phenol content

Table 1 shows the total phenol and anthocyanins content of the extracts expressed as gallic (GAE) acid equivalents per g of dry material, respectively as cyanidin chloride equivalents (CCE) per 100 grams of dried material. The results obtained show that the fruits have a higher content in total phenolic compounds and also, contain anthocyanins.

Table 1 shows the total phenol and anthocyanins content of the extracts expressed as gallic (GAE) acid equivalents per g of dry material, respectively as cyanidin chloride equivalents (CCE) per 100 grams of dried material. The results obtained show that the fruits have a higher content in total phenolic compounds and also, contain anthocyanins.

Table 1. Phenol content of the extracts

No	Extract	mg (GAE)/g	mg (CCE)/100g
1	LE	19.25	-
2	FE	52.5	118

Our results are in agreement with the literature data, methanolic and water extracts obtained from fruits, were reported to have a content in total phenol compounds as 41.59 ± 0.25 and 27.37 ± 0.18 mg (GAE)/g of extract powder, respectively (Ebrahimzadeh et al., 2009).

Antioxidant activity

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical with an unpaired electron. The color change of the solution from violet to yellow was the measure of the ability to scavenge DPPH free radical. This action is considered radical scavenging properties (Brighente et al., 2007; Ionita, 2005).

TAC -total antioxidant capacity assay shows that the tested antioxidant has the ability to donate an electron, reducing the radicals. In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green colored phosphomolybdenum V complex (Wan et al., 2011).

Table 2 presents the antioxidant activity of the extracts obtained by the two methods DPPH and TAC.

Table 2. Antioxidant activity

No	Extract	DPPH radical scavenging activity (%)	TAC (mg ascorbic acidequivalents)
1	FE	80.32 ±0.35	158 ±0.84
2	LE	72.14±1.21	123.03±2.35

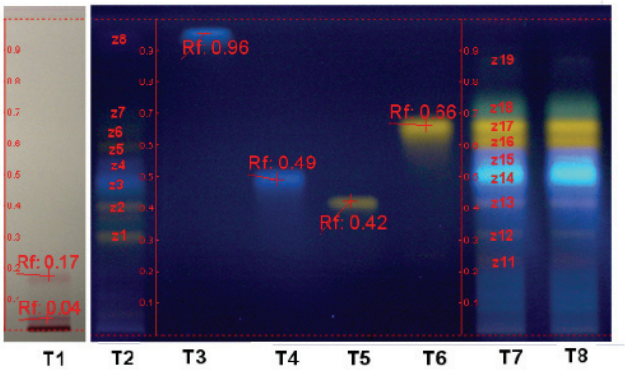

Figure 1. Phenolic profiles of fruit and leaves extracts of *Sambucus ebulus* comparative with references substances

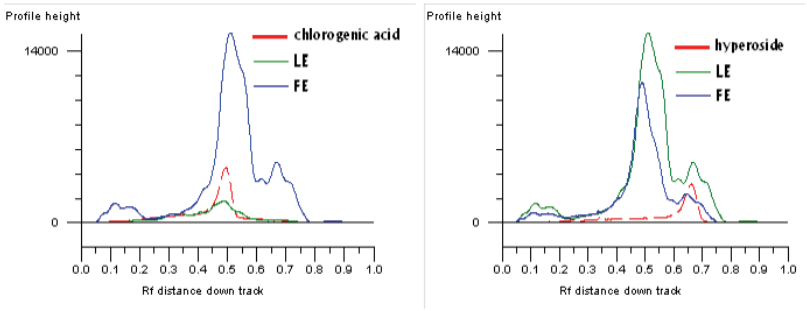

Figure 2. Profile comparison between *Sambucus ebulus* fruit and leaves extracts and references substances fingerprints

The results indicated that all the extracts have antioxidant activity in a concentration-dependent manner. According to both methods testing the antioxidant activity of the fruit extract is higher than the one of the leaves extract. Acidified methanol (0.3% v/v HCl) extract of the fruit have demonstrated neutralizing activity of DPPH – 83.17% (Anton AM et al., 2013).

CONCLUSIONS

This study shows that *Sambucus ebulus* is an important source for antioxidants compounds. Due to its antioxidant activity and to the scientific results regarding the therapeutic potential, the species is an important resource for bio-products with benefits for human health.

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REFERENCES

Anton A.M., Pintea A.M., Rugină D.O., Sconța Z.M., Hanganu D., Vlase L., Benedec D. (2013), Preliminary studies on the chemical characterization and antioxidant capacity of polyphenols from *Sambucus* sp. Digest Journal of Nanomaterials and Biostructures, 8 (3): 973 – 980.

Brighente I.M.C., Dias M., Verdi L.G., Pizzolatti M.G., (2007), Antioxidant Activity and Total Phenolic Content of Some Brazilian Species. Pharmaceutical Biology, 45: 156.

European Pharmacopoeia 6,0.

Ebrahimzadeh M.A., Somayyeh E., Bahman E. (2009) *Sambucus ebulus elburensis* fruits: A good source for antioxidants. Pharmacognosy Magazine, 5 (19): 213-218.

Feizbakhsh A., Haniyeh P., Vahid M. and Ebrahimzadeh M.A. (2014), Effect of Phytohormones on the Composition of *Sambucus ebulus* Leaf Essential Oil Tropical. Journal of Pharmaceutical Research; 13 (4): 581-586.

Ionita P., 2005, Is DPPH StableFree Radical a GoodScavenger for Oxygen Active Species? Chem. Pap. 59 (1): 11−16.

Kumar S. (2011), Free Radicals and Antioxidants: Human and Food System Advances. Applied Science Research, 2 (1): 129-135.

Opara C.E. (2006), Oxidative stress. Nutrition in Inflammatory Bowel Disease, Part II Disease-a-Mounth, 52 (5):183-198.

Parvu C. (1997), Universul plantelor Mica enciclopedie, Ed. Enciclopedica , Bucuresti.

Pieri V, S., Schwaiger E. P. Ellmerer H. S. (2009), Iridoid glycosides from the leaves of *Sambucus ebulus*. J. Natural Products, 72 (10): 1798-1803.

Pribela A. J., Durcanska J., Piry J. K. (1992) Volatile substances of dwarf elder (*Sambucus ebulus*) fruits. Biologia, Ser. C., 47 (3): 225-230.

Popescu R., Chirigiu L., Bubulica M.V, Averis L.M.E. (2014), Qualitative screening of secondary metabolites from *Sambucus ebulus* roots by multiple analytical technique . Rev. Chim. (Bucharest), 65(2): 148-151.

Prieto P., Pineda M., Aquilar M. (1999), Spectrophotometric qantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical Biochemistry, 269(2): 337-41.

Sanchez-Moreno, C. Larrauri, J.A., Saura-Calixto, F., (1998) A procedure to measure the antiradical efficiency of polyphenols. Journal of Agricultural and Food Chemistry, 76: 270-276.

Shokrzadeh M. and Saeedi Saravi S.S.(2010), The chemistry, pharmacology and clinical properties of *Sambucus ebulus*: A review. Journal of Medicinal Plants Research, 4(2): 095-103.

Tasinov O., Kiselova-Kaneva Y., Ivanova D. (2013), *Sambucus ebulus* – from traditional medicine to recent studies. Scripta Scientifica Medica, 45(2): 36-42.

Wagner H., Bladt S., (1996), Plant Drug Analysis, Second Edition, Springer.

Wan C., Yu Y., Zhou S., Liu W., Tian S., Cao S. (2011), Antioxidant activity and free radical-scavenging capacity of *Gynuradi varicata* leaf extracts at different temperatures. Pharmacognosy Magazine, 7(25): 40-45.

Yoshihara D., Fujiwara N., Suzuki K. (2010), Antioxidants: benefits and risk for long- term health. Maturitas, 67: 103-107.

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IN VITRO BIODEGRADATION OF KERATINIZED SUBSTRATES BY KERATINOPHILIC FUNGI

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Abstract
Keratinophylic fungi are present in the environment with a variable distribution, being influenced by human and animal presence and playing an important role in the biodegradation of keratinized substrates (skin, hair shaft, nails, claws, horns, wool and feathers). These fungi are geophilic, zoophilic and anthropofilic. Keratinophylic fungi have the ability to degrade keratinized materials, using the keratin as the sole source of carbon and nitrogen. This behaviour is based on the activity of keratinases, enzymes belonging to the group of proteases that can specifically degrade keratin. The aim of the present study was to evaluate the biodegradative ability of some keratinophylic fungal strains, clinical and geophilic isolates. The tested keratinized substrates were represented by animal hair strands. The rate of keratinized substrates biodegradation was expressed as weight loss over three weeks of incubation in minimal liquid medium in an orbital incubator. The morphological changes of hair samples were observed by light microscopy and scanning electron microscopy (SEM).

Key words*: Keratinophylic fungi, biodegradation, keratinases.*

INTRODUCTION

Keratinophilic fungi are present in the environment with a variable distribution, being influenced by human and animal presence and playing an important role in the biodegradation of keratinized residues and bioremediation of environment (Geetanjali et al., 2014; Khan et al., 2015; Mini et al., 2012; Sharma et al., 2010**;** Sharma and Choudhary, 2014). These fungi are geophilic, zoophilic and anthropofilic from ecological poit of view (Maruthi et al, 2012). The keratinophilic fungi have the ability to use keratin from keratinized materials (superficial layers of the skin, hair shaft and nails in humans and claws, horns, wool in animals) as the unique source of carbon and nitrogen (Ganaie et al., 2010; Narula et al, 2011). Keratin is found predominantly in feathers, hair, nails, horns, hooves, furs, claws, bird beaks, skin and consists of two type of keratin: α (alpha) and β (beta)-keratin; α-keratin (soft) is usually found in hair, wool, horns, nails, claws and hooves, whereas β-keratin (harder) is found in bird feathers, beaks, and claws (Gopinath et. al., 2015). Keratins are also basic or acidic (Bragulla and Homberger, 2009).

The keratinophilic fungi can cause superficial mycosis both in humans and animals (Sarkar, 2014). They include a variety of taxonomic groups of filamentous fungi, one of them being the dermatophytes fungi (Gopinath, 2015; Jain and Sharma, 2012; Maruthi, 2012).

The keratinophilic fungi can produce a specific enzyme named keratinase that is responsible for keratin degradation. Keratinases can be serine proteases or metalloproteases (Kumar and Kushwaha, 2014).

The aim of the present study was to evaluate the biodegradative ability of some keratinophylic fungal strains from clinical and geophilic isolates.

MATERIALS AND METHODS

Microbial strains

The tests were carried out with the following keratinophilic fungal strains: *Trichophyton mentagrophytes*, *Fusarium* sp., *Cladosporium sp.* and *Trichoderma* sp. The tested strains were grown and maintained on potato dextrose agar (PDA) slants at 4^oC.

Keratin substrate

Horse hair strands were washed thoroughly under running water, dried and sterilized with 3% ethanol. The hair strands were cut into pieces of about 2 cm in length, divided into portions of 50 mg weight and autoclaved at 121°C for 20 min.

Conditions of fungal cultivation

The minimal liquid culture medium had the following composition (g/L): 0.1, KH_2PO_4; 0.1, $CaCl_2$; 0.1, $FeSO_4 \cdot 7H_2O$; 0.005, $ZnSO_4 \cdot 7H_20$; pH 7.0. In each Erlenmayer flask 50 ml of liquid medium and 50 mg of horse hair strands (cut into 2 cm fragments length) were added. The flasks were inoculated with a piece of mycelium from each strain. The flasks were incubated in an orbital incubator Heidolph Unimax 1010 at 27°C and 80 rpm, for three weeks (Figure 1).

Figure 1. Flasks in an orbital incubator Heidolph Unimax 1010

The tests include the following control flasks: microbiological control with the pure microbial culture on nutrient medium, flasks with only the nutrient medium, and the nutrient medium with the keratin substrates (horse hair strands) without the microorganism. The experiments were performed in triplicate. The broth culture was filtered through Whatman filter paper, washed carefully with distillated water. The fungal mycelium was removed gently from the horse hair strands, dried at 75°C for 48 hours and weighted. The biodegradation rate of keratinized substrates was expressed as weight loss over periods of incubation in experimental conditions. All tests were performed in the ICECHIM laboratories.

Morphological aspects of the horse hair strands

The fungal growth was observed by light microscopy with Olympus BX51 and by SEM on a FEI-QUANTA 200 instrument. All investigations were carried out in the ICECHIM laboratories.

RESULTS AND DISCUSSIONS

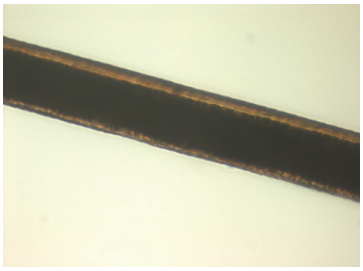
The test results are presented in Figures 2, 3 and Table 1.

The morphological examination of hair strands at different resolutions, with light microscopy as well as with SEM offer important data on the real capacity of a fungal strain to degrade keratin substrates. According to certain scientific reports, a model of the morphological expression of keratinolysis has identified two types of fungal attack, surface erosion and radial penetration (Marchisio et al., 1994; Marchisio, 2000). The authors considered that surface erosion is the hair keratin destruction from the exterior along the length of hair or in certain zones producing extensive “pockets”. The radial penetration is a random attack by so-called “boring hyphae” acting at right angles with respect to the hair surface. The principles of this model are used in the present paper to discuss the images obtained from light microscopy and SEM.

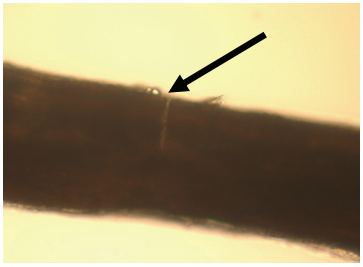
As it can be observed in Figure 2a, the hair strand aspect was not changed by incubation in liquid culture medium in the absence of microorganism. The cuticle and the inner medullar channel of the control hair strand are preserved.

Trichophyton mentagrophytes develops “boring hyphae” specialized as perforating organs (Marchisio et al., 1994; Marchisio, 2000). (Figures 2b and 2c). Also, the lifting of the cuticle (Figure 2d) and modifications in the medullar channel (Figure 2e) can be observed. The images from optical microscopy obtained

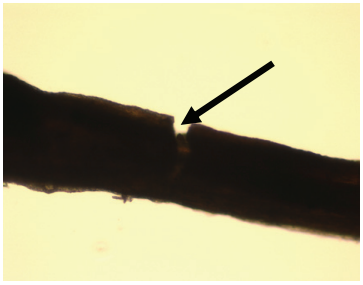
for *Fusarium* sp. (Figure 2f), *Trichoderma* sp. (Figure 2h) and *Cladosporium* sp. (Figure 2j) show a normal network of hyphae grown around hair strand. Further growing of fungal network may be responsible for the surface erosion of hair strand.



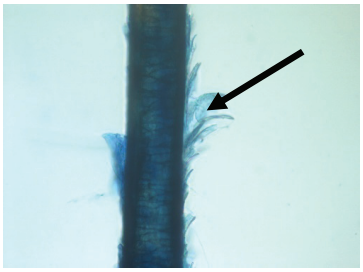
a) Control-horse hair strand (20x)



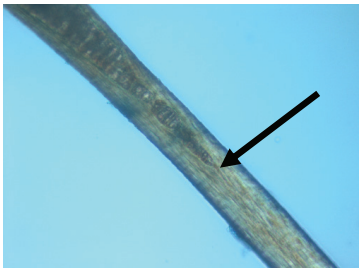
b) *Trichophyton mentagrophytes*
(20x; pointed arrow - boring hyphae; 1 week)



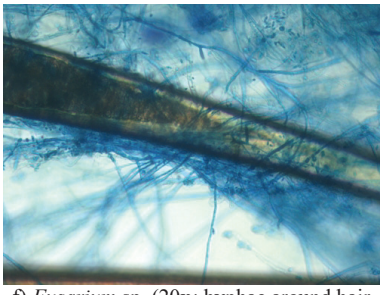
c) *Trichophyton mentagrophytes*
(20x; arrow - perforating organ; 1 week)



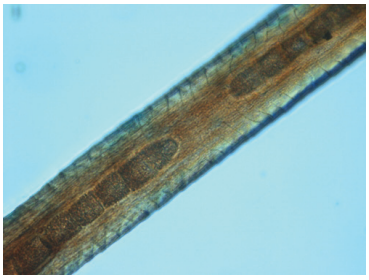
d) *Trichophyton mentagrophytes*
(20x; arrow - lifting of cuticle;
lactophenol cotton blue; 3 weeks)



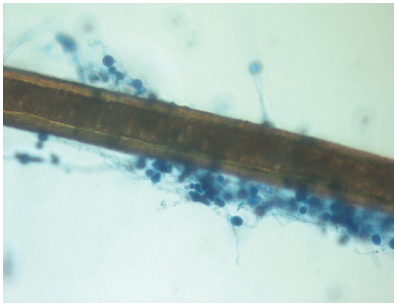
e) *Trichophyton mentagrophytes*(20x;
arrow - modification in medullar channel;
lactophenol cotton blue; 3 weeks)



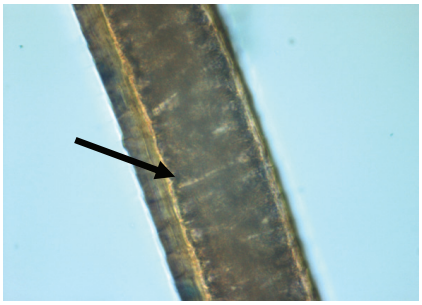
f) *Fusarium* sp. (20x*;* hyphae around hair
strand; interrupted medullar channel;
lactophenol cotton blue; 3 weeks)



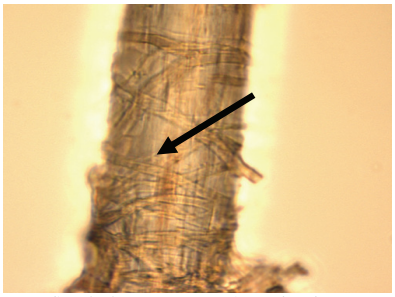
g) *Fusarium* sp. (40x*;* interrupted and
narrowed medullar channel;
lactophenol cotton blue; 3 weeks)



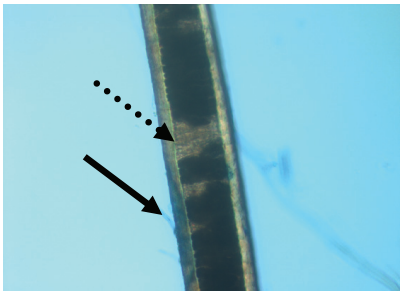
h) *Trichoderma* sp.(20x; hyphae and spores around hair strand; lactophenol cotton blue; 3 weeks)



i*) Trichoderma* sp.(20x; arrow - slight boring hyphae in medullar channel; lactophenol cotton blue; 3 weeks)

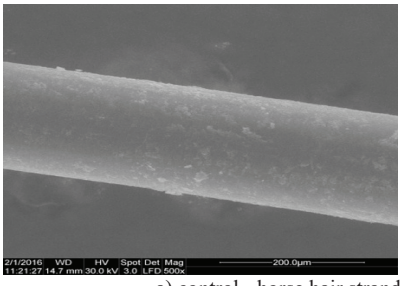


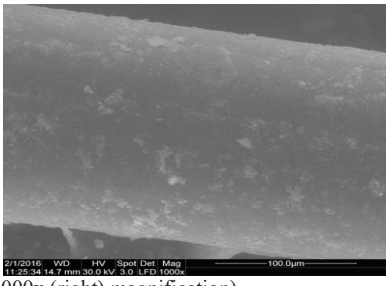
j) *Cladosporium* sp. (40x; hyphae surrounding the hair strand; 3 weeks)



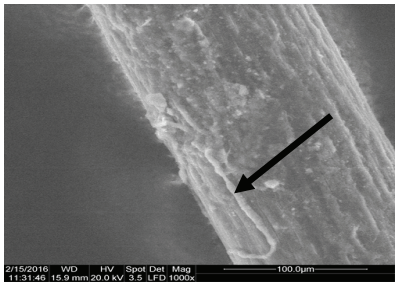
k) *Cladosporium* sp. (20x; dotted arrow – interrupted medullar channel; arrow - slight boring hyphae in medullar channel; lactophenol cotton blue; 3 weeks)

Figure 2. Light microscopy of entire fungus-hair units

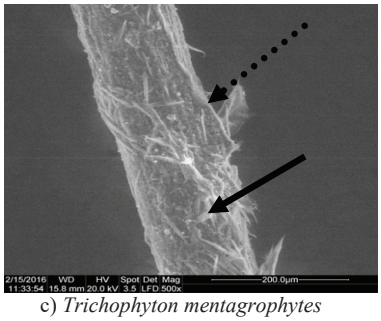




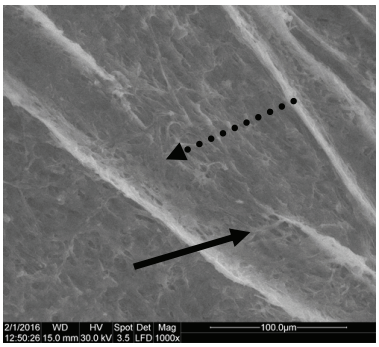
a) control - horse hair strand (500x (left); 1000x (right) magnification)



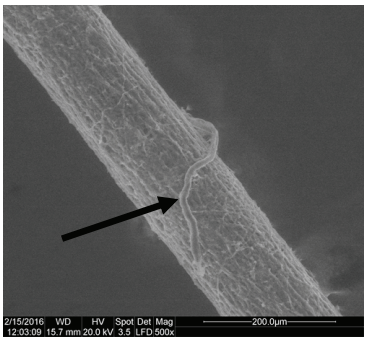
b) *Trichophyton mentagrophytes* (1000x; arrow - fungal hyphae attached to hair strand)



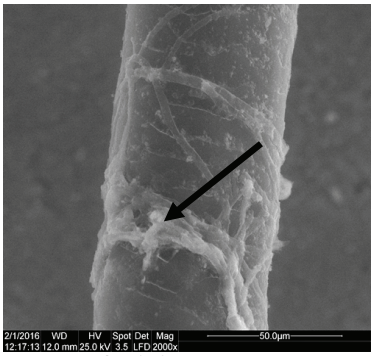
c) *Trichophyton mentagrophytes* (1000x; dotted arrow – lifting of cuticle; arrow – fungal hyphae attached to hair strand)



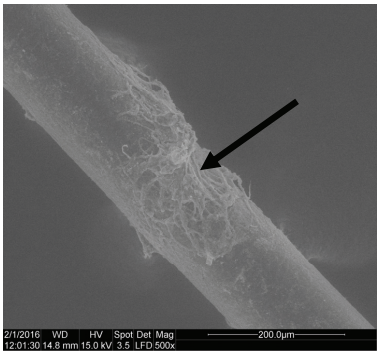
d) *Fusarium* sp.
(1000x; dotted arrow – "pocket" as surface erosion of hair strand; arrow - fungal hyphae attached to hair strand)



e*) Fusarium* sp.
(1000x; arrow - fungal hyphae attached to hair strand)



f) *Cladosporium* sp.
(2000x; arrow – network fungal hyphae attached to hair strand)



g) *Cladosporium* sp.
(500x; arrow – network fungal hyphae attached to hair strand)

Figure 3. SEM images of fungus-hair units after 3 weeks of incubation

The SEM micrographs offer more detailed information about the morphological modifications due to microbial contact (Figure 3).

In the case of *Trichophyton mentagrophytes*, hyphae network on the surface (Figure 3b, c) and cuticle lifting (Figure 3c) can be seen. The strain of *Fusarium* sp. produces surface erosion in an "extensive pocket" after 21 days of incubation (Figure 3d).

Attachment of fungal hyphae to the hair surface is observable also for *Fusarium* sp. (Figure 3e) and for *Cladosporium* sp. (Figure 3f and 3g). For *Trichoderma* sp. no significant changes in hair morphology were revealed by SEM images (data not shown).

The keratin substrates presented different rates of degradation according to the specific activity of the fungi under investigation (Table 1).

Table 1. Weight loss of keratin substrates after incubation with fungal strains

Fungal species	Weight loss (%; w/w)		
	1st week	2nd week	3rd week
Trichophyton mentagrophytes	15	49	75
Fusarium sp.	13	22	62
Trichoderma sp.	12	22	54
Cladosporium sp.	15	27	56

After the first week of incubation, there were minor differences between the weight loss values for the tested strains. The longer period of incubation facilitated the weight loss of keratinized substrates.

A significant difference between the weight loss values was observed after 2 weeks of

incubation, the highest value (49%) being obtained for *Trichophyton mentagrophytes.*
As regarding the others strains, *Cladosporium sp*. reached 27% weight loss, followed by *Fusarium* sp. and *Trichoderma* sp. with 22% weight loss each. The trend of the values was similar after 3 weeks of incubation. The weight loss values decreased in the following order: 75% for *Trichophyton mentagrophytes*> 62% for *Fusarium* sp.> 56% for *Cladosporium* sp. > 54% for *Trichoderma* sp.

CONCLUSIONS

The keratinophilic fungi play an important ecological role in the biodegradation of keratin substrates and can help in the environment protection. Our study provides useful information related to microbial degradation of keratin. According to our results, the *Trichophyton mentagrophytes* strain showed o good biodegradative activity. After 3 weeks of fungal contact, the hair strand lost 75% of initial weight and morphological changes were observed by microscopical analysis. The strain was selected for further studies dedicated to keratin biodegradation and isolation of keratinases.

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REFERENCES

Bragulla H. H., Homberger D. G., 2009. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. Journal of Anatomy, 214, 516–559.

Ganaie M. A., Sood S., Rizvi G., Khan T. A., 2010. Isolation and identification of keratinophilic fungi from different soil samples in Jhansi city (India), Plant Pathology Journal., 9(4), 194-197.

Geetanjali R., Kumar J. S., 2014. Occurrence of Keratinophilic Fungi from Soils of Ujjain (HolyCity), India. International Research Journal of Biological Sciences, 3(10), 28-31.

Gopinath, S. C. B., Anbu P., Lakshmipriya T., Tang, T.-H., Chen Y, Hashim U., Ruslinda Rahim A., Arshad M. K. Md., 2015. Biotechnological Aspects and Perspective of Microbial Keratinase Production. BioMed Research International, 1-10.

Jain N., Sharma M, 2012. A descriptive study of keratinophilic fungal flora of animal and bird habitat, Jaipur, Rajasthan. African Journal of Microbiology Research., 6(42), 6973-6977.

Khan A. M., Bhadauria S., 2015. A Review On chemical and Molecular Characterization of Keratinophilic Fungi. International Journal of Scientific Research, 4(1), 420-423.

Kumar J., Kushwaha R. K. S, 2014. Screening of fungi efficient in feather degradation and keratinase production. Archives of Applied Science Research, 6 (1):73-78.

Marchisio V. F., Fusconi A., Rigo S., 1994. Keratinolysis and its morphological expression in hair digestion by airborne fungi, Mycopathologia, 127: 103-115.

Marchisio V. F., 2000. Keratinophilic fungi: Their role in nature and degradation of keratinic substrates. Revista Iberoamericana de Micología, Apdo. 699, E-48080 Bilbao (Spain), 86-92.

Maruthi Y. A., Hossain K., Hari Priya D., Tejaswi B, 2012. Prevalence of Keratinophilic fungi from Sewage Sludge at Some Wastewater out lets along the coast of Visakhapatnam: A case study. Advances in Applied Science Research, 3(1), 605-610.

Mini K. D., Mini K. P., Jyothis M., 2012, Screening of fungi isolated from poultry farm soil for keratinolytic activity. Advances in Applied Science Research., 3(4), 2073-2077.

Narula N., Sareen S, 2011. Effect of natural antifungals on keratinophilic fungi isolated from soil. Journal of Soil Science, 1(1), 12-15.

Sarkar A. K, Rai V., Gupta A. K., 2014. Incidence of keratinophilic fungi in areas of Raipur City, Chhattisgarh region, India. African Journal of Microbiology Research., 8(3), 264-269.

Sharma M.., Sharma M., Mohan V. R., 2011. In vitro biodegradation of keratin by dermatophytes and some soil keratinophiles. African Journal of Biochemistry Research, 5(1),1-6.

Sharma R, Choudhary N., 2014. A study on role of keratinophilic fungi in nature: a review. Biolife, 2(2), 690-701.

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EVALUATION OF THE CAPACITY OF DIFFERENT MICROORGANISMS TO SOLUBILIZE SEVERAL COMPOUNDS OF PHOSPHOROUS AND ZINC

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Abstract

Microorganisms are the main responsible for bioremediation processes. They have the capacity to convert toxic substances in lesser or untoxic ones and also can solubilize compounds in order to make them available to plants. This article emphasizes the capacity of several microorganisms, isolated from different sources, to solubilize P_{in}, P_{org} and Zn based compounds. The screening methods comprised the use of several culture media (PVK, NBRIP, PSM and Bunt&Rovira) with added dyes used as pH markers (bromphenol blue and bromcresol purple). The used bacteria were three strains of Bacillus amyloliquefaciens (BIR, BW, OS15, OS17), four strains of Bacillus subtilis (BPA, ICPA, ATCC6633, 10), two strains of Bacillus spp. (B3, B4), one strain of Kluyvera ascorbata(35) and one unindentified strain isolated from soil contaminated with swines manure. After 1 to 7 days of incubation, the halos formed around the colonies showed the bacteria capacity to degrade the compounds of interest. Depending on the screening assay it was determined the eventual mechanisms involved, such as organic acids production, phytase or phosphatases synthesis, etc. Bacteria which had the capacity to solublize P_{in} based compounds were B3, B4, OS15, BPA, BIR, BW, OS17, ATCC6633, 35 and 10. The ones which were able to degrade phytate (P_{org}) were BIR, BPA, ICPC, OS15, OS17, BW, 10 and 35. The strains that solublized ZnO were BPA, B4, ATCC 6633, 32 and 35. The isolation sources of bacteria were mainly different types of soil, fact that strengthens the teory that soil bacteria are able to secret metabolites which could be involved in bioremediation and crop protection because they are already adapted to soil contaminants from environment.

Key words*: Bacillus spp., bioremediation, phytase, phytic acid.*

INTRODUCTION

The development of intensive agriculture and of industries determined the growth of xenobiotics concentration in water, soil and air. Shukla and Sharma (2010), stated that the most advanced technology for the remediation of the damages produced by polluting agents is bioremediation. Bioremediation, according to Kamaludeen et al. (2003), consists in using the biological agents, mostly microorganisms, in order to remediate the sources which are essential for life maintaining. The degradation of pollution agents requires the actual contact between microorganisms and contaminants, which is relatively difficult to happen because of the uneven spreading in natural conditions at soil level of both microorganisms and pollutants. However some bacteria are mobile, having a chemotactic response which is responsible for their capacity to move toward contaminant. Although some plants can be used in bioremediation processes, microorganisms have a lot more potential because of the shorter time required for their development, the adaptative mutagenesis necessary for pollutant biodegradation, the specific degradation of some pollutants or degradation of various pollutants all at once. Also different microorganisms species cand be used in clusters and, by symbiosis, are able to bioremediate more efficiently a polluted site and also to enhance the vegetation development on that site, this leading to the raising of the phytoremediation efficiency.

Phosphorus, along with potassium and calcium, is one of the macronutrients which are essential for the development, functioning and health of

all living creatures. Phosphorus, at cellular level, exists under the form of orto- and pyrophosphoric acids. It is found in nucleic acids, phospholipids, coenzymes, enzymes and hormones. Organic phosphorus (P_{org}) represents 30-50% from the entire soil phosphorus (Dai et al. 2011). Although phosphorus is one of the most important elements on Earth which ensure the development of living creatures, only 5% from the global amount is available in order to be used by plants (Maksimov et al., 2011). Phytic acid (mioinositol hexakisphosphate– PA – phytic acid (IUPAC-IUB 1978) and his derivatives, phytates, (phytic acid salts) are the phosphorus most common forms that are met at soil level. The international abbreviation PA is used both for phytic acid and phytates. PA being an inositol derivative, it has six molecules of phosphoric acid bound to six hidroxyl groups which can be assimilate by the animal body and microorganisms, but not by plants (Bohn et al., 2008). This is why the most common form met in nature is phosphoric acid molecules.

Once complete dissociation occurs, the six phosphate groups of phytic acid carry 12 negative charges. These charges will bond different bivalent or trivalent cathions (ex. Ca, Mg, Fe, Zn, Cu, Mn) in low acidic or neutral pH and form a stable complex (Frank, 2013). Due to its molecular structure, phytic acid has an anti-nutritive effect on certain living beings.

Interactions between phytic acid and proteins have also been reported. According to Munir and Maqsood (2013), in conditions of acidity, phytic acid will negatively influence the solubility of proteins by way of ionic bonding between phosphate groups of phytic acid and protonated amino acids (lysyl, histidyl and arginyl residues).

Zinc appears to be the element whose bioavailability is most influenced by phytic acid. Research done by Flanagan (1984) demonstrates that phytic acid not only reduces the bioavailability of zinc in alimentation, but also greatly reduces the intestinal reabsorption of endogenous zinc. Moreover, high concentrations of calcium increase the anti-nutritive effects of PA on zinc's bioavailability through the formation of Ca-Zn-PA insoluble complexes. A molar report of PA x Ca/Zn > 3.5 is regarded as a critical determining factor of using Zn (Munir & Maqsood, 2013).

According to Ritnbach et al. (2008), iron is difficult to assimilate by living beings because of its irreversible chelation in the digestive tract by certain fibers, polyphenols, as well as phytic acid. However, phytic acid inhibits the absorption of iron in its different forms via a high level of phosphorylation, such as IP5 and IP6, compared to IP1, IP2, IP3 and IP4.

Modern agriculture implies not using fertilizer supplementation, and focuses on the exploitation of soil resources with the help of microorganisms. One of the most important roles of microorganisms involved in development of crops is their capacity to solubilize phosphorus. The process consists of synthesizing metabolites, capable of chelating cations, such as calcium, from the structure of insoluble compounds. These phosphorous-based compounds release phosphorous molecules, which can be assimilated by plants (Vassilev et al. 2012).

Microbial phytases are actively secreted in the soil, where they participate in both the decay of vegetal detritus, as well as in releasing phosphorous from organic compounds found in soil. This makes microbial phytases the key enzymes of the organic phosphorous cycle in the soil. This enzyme is not secreted in the rhizosphere by the plant; consequently, it is incapable of assimilating the phosphorous which is bound to phytates in the soil. Thus, microorganisms play a unique role in degrading these compounds (Guimaraes et al. 2006). Of the many different types of bacteria which have the capacity to synthesize these enzymes, we mention: *Pseudomonas*, *Bacillus*, *Raoultella* and *Enterobacter* (Simon et al. 2002). Phytases produced by species of the genus *Bacillus*, which present optimal enzymatic activity in conditions of neutral pH, are substrate-specific with regards to calcium phytate and have a high thermic stability, according to Fu et al. 2008.

In its metallic form, zinc does not influence the environment because of the lack of its bioavailability. However, Radhika et al. (2006) claim that zinc can react with other elements, the result being toxic reaction products, which have a negative impact on ecosystems.

According to Perpetuo et al. (2011), there is a competition between zinc and cadmium for the

same cellular sites, which determines replication errors, mutagenesis, the destabilization of cellular structures, etc. The *Bacillus* spp. is recognized for its resistance in sites contaminated with heavy metals, including zinc (Krishna et al. 2013). The species of the *Bacillus* spp. have the capacity of assimilating, not only zinc, but other heavy metals such as copper, lead and cadmium, at the soil level (Issazadeh et al. 2011).

Specialty literature mentions that numerous microorganisms, capable of solubilizing otherwise insolvable forms of phosphate (or of other chemical elements), accomplish this through the generation of organic acids, which vary in type and quantity, according to the microbial species. Among the most frequent organic acids produced by bacteria which solubilize phosphate we find gluconic, ketogluconic, lactic, succinic, formic, malic, citric, oxalic, fumaric, tartaric, propionic, acetic, izobutiric, izovaleric, valeric and izocaproic acids (Khan et al. 2014). Also, the solubilization of otherwise insolvable forms of phosphate can be realized through biosynthesis of some specific enzymes, such as phosphatases and phytases. Phosphatases (acidic and alkaline) are eliminated outside of the cells as exo-enzymes. They utilize P_{org} as a substrate in order to transform it in its inorganic form, accessible to plants. Phytases have a more specific behavior, determining the liberation of phosphorous.

Having in mind these aspects, within the present experiments it was detected the capacity of several bacteria from various sources to solubilize different insoluble compounds.

MATERIALS AND METHODS

The used microorganisms

The evaluated microorganisms were mostly from the *Bacillus* genus, as follows in the Table 1:

The screening assay for detection of bacteria which have the capacity to solubilize the anorganic phosphorus based compounds

In order to identify the capacity of bacteria to synthesize organic acids involved in phosphate solubilisation, were used the following culture media: PVK and NBRIP, where the phosphorus source is tricalcic phosphate ($Ca_3(PO_4)_2$).

Table 1. The used microorganisms

No	Notation	Species	Isolation source
1	BIR	*B.amyloliquefaciens*	Soil (Microorganisms Collection of Biotechnologies Faculty USAMVB)
2	BPA	*B. subtilis*	Soil (M. C. of B. F. USAMVB)
3	Icpc	*B. subtilis*	M. C. of B. F. USAMVB
4	ATCC 6633	*B. subtilis* ATCC6633	M. C. of B. F. USAMVB
5	OS17	*B.amyloliquefaciens*	Onion ryzoshpere (Sicuia et al., 2012)
6	OS15	*B.amyloliquefaciens*	
7	BW	*B.amyloliquefaciens*	Soil (Sicuia et al, 2012)
8	B3	*Bacillus sp.*	Compost (M. C. of B. F. USAMVB)
9	B4	*Bacillus sp.*	
10	10	*Bacillus subtilis ss. subtilis*	*Microorganisms isolated from soils on which is deposited pig manure. The microorganisms are preservated in the Microbiology Laboratory of the Biotechnologies Department, NIRDBS*
11	32	*Unindentified*	
12	35	*Kluyvera ascorbata*	

The PVK ingredients for 1000 ml are: 10g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 0.0001 g $FeSO_4 \cdot 7H_2O$, 0.0001g $MnSO_4 \cdot H_2O$, 15 g agar, 5 g $Ca_3(PO_4)_2$, pH 7 (Kaur and Reddy, 2013). The NBRIP ingredients for 1000 ml are: 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \bullet 6H_20$, 0.25 g $MgSO_4 \bullet 7H_20$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, pH 7.

To these media were added dyes as markers in order to detect the pH variations of culture media which, in case of acidification would semnalize the presence of organic acids synthesized by bacteria. The color markers used were blue bromphenol (0.1 g/L(Gupta et al., 1994) and bromcrezol purple 0.1 g/L (Agrawal et al., 2015).

The presence of the hydrolysis halos generated by the interaction bacteria - stained culture media and the colour migration from blue to yellow (in case of blue bromphenol) and from purple to yellow-orange (in case of bromcrezol purple) highlights the decrease of pH value from 7 to 6 or less. This fact shows the biosynthesis of organic acid by the bacteria, in

order to solubilize the anorganic phosphorus-base compunds.

The screening assay for detection of bacteria which have the capacity to solubilize the organic phosphorus based compounds

For emphasis of the involvement of organic acids in solubilisation of P_{org} (phytate), the bacteria strains were cultivated on PSM with or without the dyes mentioned before. The PSM ingredients for 1000 ml are: 15 g glucose 15 g, 5 g $(NH_4)_2SO_4$, 0.5 g KCl, 0.1g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2.2H_2O$, 0.01 g $FeSO_4$, 0.01 g $MnSO_4$, 15 g agar, 5 g sodium phytate, pH 6.5 (Bae et al., 1999; Singh et al., 2013; Tungala et al., 2013). The observations were made after 48 h.

The screening assay for evaluation of bacteria's ability to solubilize certain zinc compounds

The cultivation medium used is Bunt & Rovira. The ingredients for 1000 ml are the following: 20 g glucose, 1 g peptone, 1 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.4 g K_2HPO_4, 0.1 g $MgCl_2$, 0.01 g
$FeCl_3$, 250ml soil extract, ZnO 0.1% in 750 ml water, pH 6.6.-7.0. Zinc oxide (Abaid-Ullah et al. 2015), as well as the aforementioned dyes (Kumar et al. 2012) have been added to the medium. The bacteria have been incubated for 7 days.

RESULTS AND DISCUSSIONS

Detection of bacteria which have the capacity to solubilize the anorganic phosphorus based compounds

By using the PVK medium without dyes, the solubilisation halo was detected at the following bacterias: B3, B4, OS15, BIR and 10 (Figure.1).
Regarding the screening using PVK medium + bromcresol purple the cultivated bacteria determined the pH variation, by decreasing it, after 24 h (Figure 2).
When bromphenol blue was used as pH marker, the color intensity generated on PVK media was much lower then within the experiments when was used bromcresol purple for the same purpose. Slightly color modifications were observed after 24h from inoculation and as well after 48h.

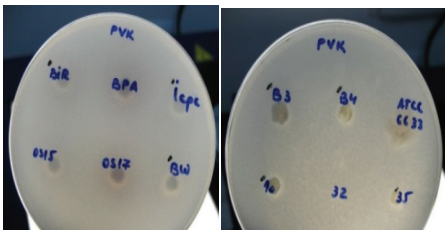

Figure 1. Highlighting hydrolysis halos on medium PVK agar

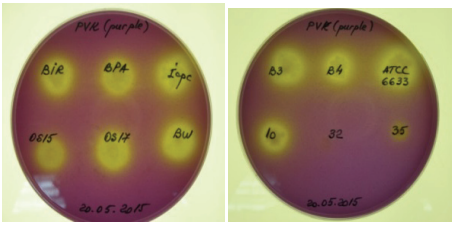

Figure 2. pH modification on PVK + bromocresol purple after 24 h from inoculation

After 48 h of cultivation, the decreasing of pH value was even more obvious and it was generated by all bacteria (Figure 3). This fact showed the microorganisms capacity to synthesize the organic acids required for phosphate solubilization.

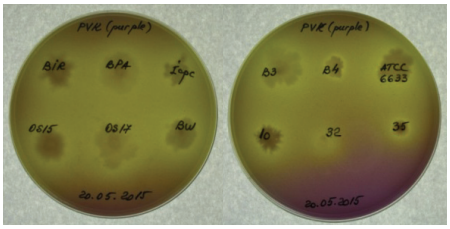

Figure 3. pH modification on PVK + bromocresol purple at 48 h after inoculation

After 24h, the bacteria that formed thin halos which showed a pH decrease were the following: BIR, BPA, ICPC, OS15, OS17, BW, B3, B4, ATCC 6633 and 10 (Figure 4).
Also, as in the previous case, when it was used, as pH marker, bromcresol purple, the strain 35 is the one who produces the smallest halo, showing its low capacity to synthesize organic acids (Figure 5).

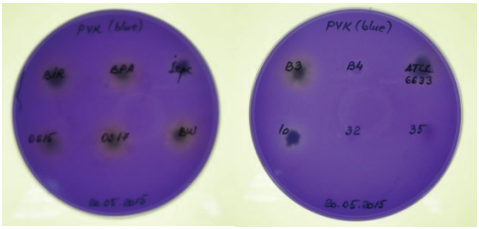

Figure 4. pH modification on PVK + bromphenol blue at 24 hours

By comparing the data obtained after using these two types of dyes as pH markers, it can be stated that the most bacteria were able to produce organic acids which modified the pH value and which also could be involved in the solubilisation of anorganic phosphorus.
Although, it needs to be mentioned that the pH decrease wasn't significant; most probably the bacteria strains synthesize organic acids which determine a pH decrease to the value of aprox. 5.0.

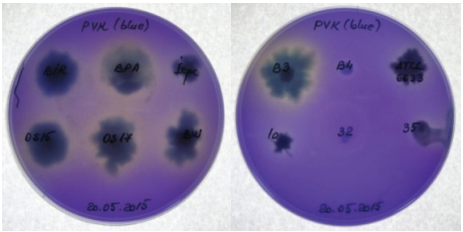

Figure 5. pH modification on PVK + bromphenol blue at 48 h

By concluding, it can be stated that the screening in which it was used PVK agar + bromcrezol purple it is the best assay for detecting the solubilisation of tricalcium phosphate by bacteria which synthesize organic acids for this purpose.
In order to validate the results obtained with this assay, there were realized similar experiments with other culture medium, NBRIP (National Botanical Research Institute's Phosphate growth medium). This medium is also used for the evaluation of microorganisms capacity to solubilize anorganic phosphate based compounds (Singh et al., 2014). The experiments comprised by using NBRIP within this article validated the observations made on PVK medium.
Because of the lack of contrast, the halos are slightly difficult to measure, same thing had happen also before, when PVK media was used. Although, by comparing the observations made on the two media (NBRIP and PVK), the NBRIP media showed greater halos (Figure 6).

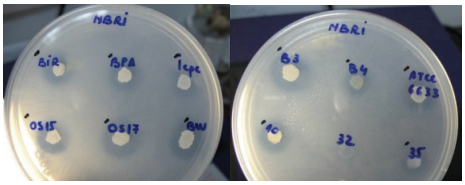

Figure 6. Highlighting hydrolysis halos on medium NBRIP

In order to detect the organic acids production which is associated to the bacteria ability to solubilize the phosphate, were added the same dyes as before in the NBRIP composition, but the results were different. Comparing with the results obtained on NBRIP, the observations made on NBRIP + bromcrezol purple lead to registration of a weak decrease of culture medium pH value. This fact suggests that, at least on this medium, the solubilisation of tricalcic acid is realized not only by organic acids production, but by other mechanisms, perhaps enzymatic ones (Figure 7).

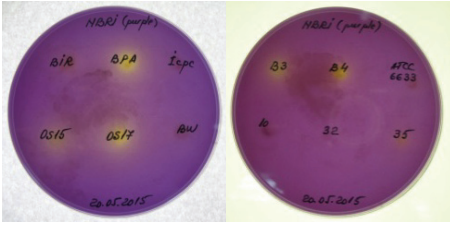

Figure 7. Highlighting hydrolysis halos on medium NBRIP agar + bromocresol purple

It needs to be mentioned the fact that the halos produced on NBRIP + bromcrezol purple are smaller than the ones generated on PVK + bromcrezol purple. NBRIP + bromphenol blue wasn't able to indicate the pH variations, although the bacteria developed optimaly.
The obtained results suggest that the concentration of organic acids produced by bacteria seems to be lower than the one produced by bacteria on PVK + pH markers. This fact could happen because of the media different composition. Also, the observations made are in conformity with the observations made by other researchers which stated that the

utilization of bromphenol blue as pH marker is not an efficiently way to highlight the solubilisation of some compounds (Gadagi and Sa, 2012).

Detection of bacteria which have the capacity to solubilize the organic phosphorus based compounds

On PSM culture medium the production of organic acids is very low (according to stained culture medium). Thus, on PSM + bromcresol purple, the strain 10 is the one that decrease the most the culture medium pH (figure 8). The strains 35, B3 și ATCC 6633 generated clear halos around their colonies, but with a color of intense violet, which is correlate with a basic pH value. This fact could be explained by the phytase and/or phosphatase synthesis which hydrolized the sodium phytate and did not decrease the pH value of the culture medium.

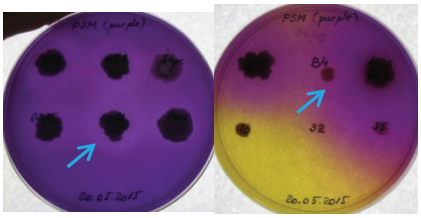

Figure 8. Solubilization of sodium phytate on PSM agar + bromocresol purple after 48 h of cultivation

Similar observations were made for BIR, BPA, ICPC, OS15, OS17 și BW (figure 8). For these ones were observed halos which weren't colored in yellow-orange, leading to the conclusion that the pH wasn't modified. Also, when blue bromphenol was used as pH marker, the results were similar. The phytate was solubilized without occuring a pH variation (Figure 9). Overall, the halos had a more intense color, suggesting an eventual increase of pH value. These increase is specific to *Bacillus* spp. The strain 10 cultivated on PSM + blue bromphenol did not decreased the culture medium pH, this leading to the conclusion that the bacteria did not biosynthesize organic acids on this culture medium. The obtained results confirmed the fact that on PSM, which contains sodium phytate, the phosphorus solubilisation is mainly realized by enzymatic mechanisms (phytase or phosphatases biosynthesis).

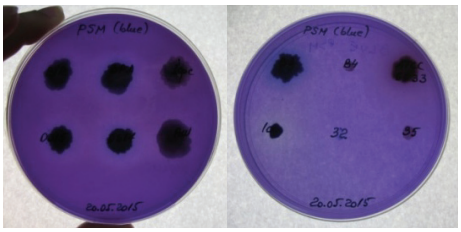

Figure 9. Highlighting the halos on PSM agar + bromphenol blue after 48 h from inoculation

By comparing the results obtained after using anorganic phosphorus or organic phosphorus it can be concluded that the solubilisation of phosphate requires the production of organic acids. Also, for phytate solubilisation are necessary enzymatic mechanisms. The data obtained emphasize that at least several strains have the capacity to solubilize phosphorus based compounds regardless its form. These bacteria have the potential to be used in bioremediation, or crop protection.

Evaluation of bacteria's ability to solubilize certain zinc compounds

Bacteria's involvement in the solubilization process of zinc on the ground level is an important attribute. This is due to the conversion of zinc-based compounds in compounds available for plants, zinc being an essential metal for their metabolic activities (Abaid-Ullah et al. 2015). Since previous experiments highlighted the capacity of certain bacterial strains to solubilize phosphate compounds, the following experiments are concerned with testing the capacity of the same bacteria to solubilize insolvable zinc compounds as well (ZnO).
After completing the experiments, the lack in development of certain bacteria was observed; this was due to a cultivation medium which was poor in nutrients, as well as the presence of zinc oxide in high concentrations. Also, we have noticed the development of strains B4, ATCC6633, 32, 35 and BPA, which generated a halo of clarification of the medium, around colonies (figure 10).

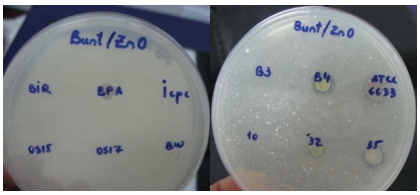

Figure 10. Evaluation of bacteria on medium Bunt& Rovira with ZnO

It should be noted that out of the 12 bacterial strains subjected to experiments, the B4 strain manifested the highest capacity to solubilize zinc oxide, the size of the clarification halo of the medium having grown constantly over the 7-day cultivation period (Figure11).

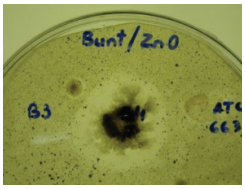

Figure 11. The ability of the strain B4 to solubilize the zinc from ZnO

Considering the fact that the B4 strain is isolated from compost, its capacity to solubilize both phosphorous from its insolvable compounds, as well as zinc oxide with a high yield, represents an important advantage with regards to its uses in different practical applications. With regards to the mechanisms involved in the zinc solubilization process, and taking into account the previous results obtained during phosphorous solubilization, the capacity to produce organic acids was also tested. After having used a purple bromcrezol dye as a pH indicator, we noticed a weak acidification of the medium, generated by strains BPA, B4 and 35 (Figure 12).

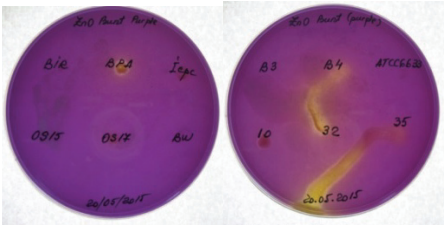

Figure 12. Evaluation of bacteria on medium Bunt and Rovira + bromcresol purple

After correlating these results with the ones obtained by using bromfenol blue (figure 13), we can conclude that the solubilization of zinc by the bacteria mentioned above is mainly due to the production of organic acids, even though the pH value of the medium does not decrease very much (~ 6.0). Moreover, the fact that the clarification halo of the medium has maintained on the plates inoculated with the bacteria of interest, even after a period of 1 month of storage at room temperature, suggests the possible implication of other mechanisms in the bio-solubilization process.

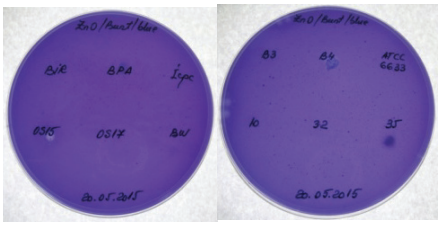

Figure 13. Evaluation of bacteria on Bunt&Rovira medium + bromphenol blue

The bacterial strains, identified as being potentially able to solubilize zinc, have different origins: compost - the *Bacillus* sp. B4 strain, a collection strain - *B. subtilis* ATCC6633, soil - *B. subtilis* BPA and soil contaminated with manure from swines - strain 32 (unidentified) and strain *Kluyvera ascorbata* 35. Considering these results, we can remark that soil represents a rich source for isolating microorganisms capable of solubilizing phosphate and zinc compounds, useful in the development process of plants. At the same time, these strains can be used in bio-remediation of contaminated sites (Issazadeh et al. 2011). The experiments were realized with a culture medium pH of 6.6 – 7, which confirms the study made by Hazarika et al. (2015), which claims that the solubilization of zinc-based compounds by bacteria was optimal in conditions of neutral pH.

CONCLUSIONS

During experiments performed on PVK and NBRIP media, the B3, B4, OS15, BPA, BIR, BW, OS17, ATCC6633, 35 and 10 strains were noted for generating specific halos, which emphasize their capacity to solubilize inorganic phosphorous.

The use of pH-indicator dyes (bromcresol purple and blue bromfenol) allowed us to highlight that solubilization of inorganic phosphorous can be done, in most cases, by the production of organic acids.

Clear solubilization halos of organic phosphorous have been highlighted on the PSM selective medium in the case of BIR, BPA, ICPC, OS15, OS17, BW, 10 and 35 strains, which suggests the production of enzymes.

Adding dyes to the PSM medium demonstrated that the mechanism for solubilization of organic phosphorous is, first of all, enzymatic, through the production of phytases; this, however, does not exclude the phosphatase synthesis (at least for some of the tested bacterial strains).

Equally, the production of organic acids can be associated with the solubilization of organic phosphorous but in a greatly reduced manner.

The solubilization of zinc from insolvable compounds (ZnO) was clearly highlighted in the case of BPA, B4, ATCC 6633, 32 and 35 strains. The use of pH-indicator dyes led to the conclusion that this solubilization ability can be largely due to the production of organic acids.

Taking the origin of the bacterial strains with phosphorous and zinc solubilization abilities into account we can appreciate that the soil represents a rich source of microorganisms, which, through their properties, can offer plants easy to assimilate nutritive compounds.

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REFERENCES

Abaid-Ullah, M., Hassan, M., N., Jamil, M., Brader, G., Shah, M., K., N., Sessitsch, A., Hafeez, F., Y., 2015, Plant growth promoting rhizobacteria: an alternate way to improve yield and quality of wheat (Triticum aestivum), Int. J. Agric. Biol., vol. 17, pp. 51 – 60

Agrawal, T., Kotasthane, A., S., Kushwah, R., 2015, Genotypic and phenotypic diversity of polyhydroxybutyrate (PHB) producing Pseudomonas putida isolates of Chhattisgarh region and assessment of its phosphate solubilizing ability, 3 Biotech, vol. 5, pp. 45 – 60

Bae, H., D., Yanke, L., J., Cheng, K., J., Selinger, L., B., 1999, *A novel staining method for detecting phytase activity*, J Microbiol Methods, vol. 39, nr. 1, pp. 17 – 22

Bohn, L., Meyer, A., S., Rasmussen, S., K., 2008, Phytate: Impact on Environment and Human Nutrition, A Challenge for Molecular Breeding, vol. 9, nr. 3, pp. 165 – 191

Dai, F., Qiu, L., Ye, L., Wu, D., Zhou, M., Zhang, G., 2011, *Identification of a Phytase Gene in Barley (Hordeum vulgare L.)*, PLoS One, vol. 6, nr. 4, pp. 18 – 29

Flanagan, P., R., 1984, *A model to produce pure zinc deficiency in rats and its use to demonstrate that dietary pliytace increases the excretion of endogenous zinc*. The Joumal of Nutrition, vol. 114, pp. 493 – 502

Frank, A., W., 2013, Chemistry of Plant Phosphorus Compounds, ed. Elsevier, New York, pp. 16 – 17

Fu, S., Sun, J., Qian L., Li, Z., 2008, *Bacillus Phytases: Present Scenario and Future Perspectives*, Appl Biochem Biotechnol, vol. 151, pp.1 – 8

Gadagi, R., S., Sa, T., 2002, *New Isolation Method for Microorganisms Solubilizing Iron and Aluminum Phosphates Using Dyes*, Soil Sci. Plant Nutr., vol. 48, nr. 4, pp. 615 - 618

Guimaraes, L., H., S., Peixoto-Nogueira, S., C., Michelin, M., Rizzatti, A., C., S., Sandrim, V., C., Zanoelo, F., F., Aquino, A., C., Junior, A., B., Polizeli, M., 2006, *Screening of Filamentous Fungi for Production of Enzymes of Biotechnological Interest*, Brazil. J. Microbiol., vol. 37, pp. 474 – 480

Gupta, R., Mahapatra, H., 2003, Microbial biomass: An economical alternative for removal of heavy metals from waste water, Indian Journal of Experimental Biology, vol. 41, pp. 945 – 966

Hazarika, S., I., Paramasivam, S., Talukdar, S., Dutta, K., 2015, *Natural bioremediation of nickel (Ni) and zinc (Zn) in coal mine effluent using Bacillus megaterium and Pseudomonas aeruginosa*, International Journal of Emerging Technology & Research, vol. 2, nr. 2, pp. 5 - 17

IUPAC-IUB, 1978, *Nomenclature of phosphorus-containing compounds of biochemical importance. (Recommendations 1976). lUPAC-IUB Commission on Biochemical Nomenclature*, The Biochemical Joumal, vol. 7, pp. 1 – 19

Issazadeh, K., Pahlaviani, M., R., M., K., Massiha, A., 2011, bioremediation of toxic heavy metals pollutants by Bacillus spp. isolated from guilan bay sediments, North of Iran, International Conference on Biotechnology and Environment Management IPCBEE vol.18, ed. IACSIT, Singapore

Kamaludeen, S.,P.,B.,K., Arunkumar, K.,R., Avudainayagam ,S., Ramasamy, K., 2003, Bioremediation of chromium contaminated environments. Ind. J. Exp Bio., vol. 41, pp. 972 – 985

Kaur, G., Reddy, M., S., 2013, *Phosphate solubilizing rhizobacteria from an organic farm and their influence on the growth and yield of maize (Zea mays L.)*, J. Gen. Appl. Microbiol., vol. 59, pp. 295 - 303

Khan,M.D., Zaidi,A., Ahmad,E., 2014, Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate-Solubilizing Microorganisms, în M.S. Khan et al. (eds.), Phosphate Solubilizing Microorganisms, Springer International Publishing Switzerland, p.31-62

Krishna, M,. P., Varghese, R., Babu, V., A., Jyothy, S., Hatha, A., A., M., 2013, *Bioremediation of Zinc Using Bacillus sp. Isolated from Metal-Contaminated Industrial Zone*, Prospects in Bioscience: Addressing the Issues, ed. Springer, New Delhi, pp. 11 – 18

Kumar, P., Dubey, R., C., Maheshwari, D., K., 2012, Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens, Microbiological Research, vol. 167, pp. 493 – 499

Maksimov, I., V., Abizgil'dina, R., R., Pusenkova, L., I., 2011, Plant Growth Promoting Rhizobacteria as Alternative to Chemical Crop Protectors from Pathogens (Review), Applied Biochemistry and Microbiology,vol. 47, nr. 4, pp. 333 – 345

Munir, K., Maqsood, S., 2013, *A review on role of exogenous enzyme supplementation in poultry production*, Emir. J. Food Agric., vol. 25, nr. 1, pp. 66 – 80

Perpetuo, E., A., Souza, C., B., Nascimento, C., A., O., 2011, Engineering Bacteria for Bioremediation, Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications, Prof. Angelo Carpi (Ed.), ISBN: 978-953-307-268-5, InTech, http://www.intechopen.com/books/progress-in-molecular-and-environmental-bioengineering-fromanalysis-and-modeling-to-technology-applications/engineering-bacteria-for-bioremediation

Radhika, V., Subramanian, S., Natarajan, K., A., 2006, Bioremediation of zinc using Desulfotomaculum nigrificans: Bioprecipitation and characterization studies, Water Research, vol. 40, pp. 3628– 3636

Ritnbach, G., Pallauf, J., Moehring, J., Kraemer, K., Minihane, A.,M., 2008, *Effect of dietary phytate and microbial phytase on mineral and the element bioavailability - a literature review*, Current Topics in Nutraceutical Research, vol. 6, nr. 3, pp. 131 - 144

Shukla, K., P., Singh, N., K., Sharma S., 2010, *Bioremediation: Developments, Current Practices and Perspectives,* Genetic Engineering and Biotechnology Journal, 2010, pp. 1 – 20, http://astonjournals.com/manuscripts/Vol2010/GEBJ-3_Vol2010.pdf

Sicuia, O., A., Oancea, F., Constantinescu, F., Dinu, S., Cornea, C., P., 2012, *Bacillus strains useful in improving vegetal mulch techology trough bio-activation*, Rom.Biotechnol. Letters, vol. 17, nr. 5, 7610 – 7619

Simon, O., Igbasan, F., 2002, *In vitro properties of phytases from various microbial origins*, Int. J. Food Sci. Technol., vol. 37, pp. 813 – 822

Singh, N., K., Joshi, D., K., Gupta, R., K., 2013, *Isolation of Phytase Producing Bacteria and Optimization of Phytase Production Parameters*, Jundishapur Journal of Microbiology, vol. 6, nr. 5, pp. 6419 – 6424

Tungala, A., Narayanan, K., A., Muthuraman, M., S., 2013, Izolation of phytase producing bacteria from poultry faeces and optimization of culture conditions for enhanced phytase production, International Journal of Pharmacy and Pharmaceutical Sciences, vol. 5, nr. 4, pp. 264 - 269

Vassilev, N., Eichler-Löbermann, B., Vassileva, M., 2012, *Stress-tolerant P-solubilizing microorganisms*, Appl Microbiol Biotechnol, vol. 95, pp. 851 – 859

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OBTAINING GROWTH CURVES FOR *Scheffersomyces stipitis* STRAINS AND THEIR MODELING

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Abstract

Growth curves are used in a wide range of applications such as crop science and biotechnology. Besides, mathematical models for fermentation can provide more information about kinetic of cell growth, and also promote the control and optimization of cell growth during fermentation. The main objectives of this study were undertaken not only plotted the cell growth curves, determined the specific growth equations, and calculated the kinetic parameters belong to Scheffersomyces stipitis strains (ATCC 58784 and 58785) but also to modelled their cell growths by using modified logistic and modified Richards models. The results indicated that the specific growth curves of ATCC 58784 in glucose and xylose mediums were $y=0.3047\times Abs_{600}-0.2656$ and $y=0.2322\times Abs_{600}+0.4329$, respectively. For ATCC 58785, they were $y=0.2639\times Abs_{600}+0.0282$ and $y=0.2323\times Abs_{600}+0.6211$, respectively. Furthermore, for ATCC 58784, maximum growth rate and doubling time values in glucose and xylose media were 0.23, 0.11 g/L/h and 2.45, 8.58 h, respectively. For ATCC 58785, they were 0.33, 0.11 g/L/h and 2.51, 7.66 h, respectively. In addition, modified logistic and modified Richards models were tested in order to describe cell growth profiles during fermentation by S. stipitis strains. Results indicated that these models can serve as a universal equation to fit cell growth. Moreover, validation of these models demonstrated that cell growth was all predicted accurately (slope=0.96 and 0.97, R^2=0.998 and 0.998 for ATCC 58784 in xylose media by modified logistic and modified Richards models, respectively; slope=1.01 and 1.01, R^2=0.995 and 0.995 for ATCC 58785 in xylose media by modified logistic and modified Richards models, respectively).

Key words: *cell growth curve, Scheffersomyces stipitis, modified logistic, modified Richards.*

INTRODUCTION

In biotechnology, batch culture is a sealed system without any inlet or outlet streams since the nutrients are prepared in a volume-stable liquid media. Then the inoculum prepared under certain conditions for each microorganism is aseptically transferred in the media, which slowly grow and reproduce. Nutrients are exhausted and end products are formed as well as cell propagates. The biomass concentration is one of the major determinants to define the stage of cell growth, which is essential to understand the checkpoints of the cell growth. There are numerous methods to predict cell growth by direct or indirect measurements such as dry cell weight (DCW), optical cell density (OD), cell turbidity, cell respiration, metabolic rate, and metabolites, which are fairly favorable for analyzing cell growth. However, DCW and OD are the most used approaches to determine the microbial growth (Flickinger, 2013; Najafpour, 2015).

Kinetic modeling of value-added products' production by microbial fermentation is important since simplify the control and optimization of cell growth as well as product formation at different fermentation conditions such as pH, temperature, medium content, agitation, aeration, etc. To create kinetic model, cell growth has to be accounted and modeled. In general, cell growth indicates a phase containing lag, exponential, stationary, and death phases. In this phase, specific growth rate begins along with inoculation and maximum growth rate reaches to the highest value for a length of time, which leads to determine of lag time. Furthermore, while cell growth stops in stationary phase and dx/dt is zero, in death phase, the growth rate reaches zero by decreasing, therefore an asymptote is reached. When the cell growth curve is described as the logarithm of cell number plotted versus time, which leads to a sigmoidal curve containing lag phase, exponential phase, and stationary phase as mentioned above (Zwietering et al., 1990).

In order to identify a cell growth curve and to decrease measured data, several cell growth models such as modified logistic and modified Richards are developed, which are describe not only the cell growth, but also to define the substrate consumption and product formation (Zwietering et al., 1990). In this paper, the cell growth curves for *S. stipitis* strains (ATCC 58784 and ATCC 58785) were obtained in glucose and xylose media and also some kinetic parameters related to cell growth were calculated. Besides, cell growth curves were modelled by using modified logistic and modified Richards models. However, the substrate level and product concentration are not of interest in our study since the objective of this study is also only to monitor the cell growth of *S. stipitis* strains.

MATERIALS AND METHODS

Microorganisms and mediums

The yeasts used to obtain cell growth curves were *Scheffersomyces stipitis* (formerly *Pichia stipitis*) strains ATCC 58784 and ATCC 58785, which were obtained from American Type Culture Collection (Manassas, VA, USA). *S. stipitis* ATCC 58784 was grown at 30ºC for 48 h in a yeast extract-malt (YM) medium containing 10 g of glucose, 3 g of yeast extract, 3 g of malt extract, and 5 g of peptone per liter of deionized water. The pH was adjusted to 6.2 with 4 N NaOH and HCl. *S. stipitis* ATCC 58785 was grown at 30ºC for 48 h in a yeast extract-peptone (YPD) medium containing 20 g of glucose, 10 g of yeast extract and 20 g of peptone per liter of deionized water. The medium pH was adjusted to 5.6 with 4 N NaOH and HCI. The cultures were stored at 4ºC and sub-cultured bi-monthly in order to maintain viability. For a long-term storage, stock cultures were maintained in 20% glycerol at -80ºC. *S. stipitis* strains were grown in 250 mL flasks containing 100 mL of YM or YPD at 30ºC and 150 rpm for 24 h for inoculation (Lee et al., 2011; Zhu et al., 2014).

Fermentations

For cell growth curves, fermentations were carried out in a shaking incubator (CERTOMAT® IS, Goettingen, Germany) with 250 ml flasks containing 150 ml of YM and YPD. Temperature was maintained at 30ºC, agitation rate was set to 150 rpm, and 1% (v/v) of inoculum was used for fermentation. The pH of mediums were initially adjusted to 6.2 and 5.6 for the strain *S. stipitis* ATCC 58784 and ATCC 58785, respectively. Sampling (2 ml) was performed at every hour.

DCW and OD

DCW in a fermentation broth was measured by drying in an oven. Firstly, one milliliter of fermentation broth was transferred into tared eppendorf tubes (total volume of 2 ml) and centrifuged at 14000 rpm for 10 min. Then supernatant was removed and the cells were washed twice with deionized water. Finally, the cells were dried at 85ºC to constant weight (Koch, 2013). The OD of the fermentation broth was measured using a spectrophotometer (ThermoScientific 201 UV-Visible Evolution, Shanghai, China) at 600 nm. Uninoculated fermentation broth was used as a blank (Lee et al., 2011; Zhu et al., 2011).

Mathematical models

The modified logistic and modified Richards models were used to fit data related to cell growth curves.

Modified logistic model

The modified logistic function was used to describe the cell growth. Zwietering et al. (1990) modified the logistic equation (Pearl and Reed, 1920) to include parameters with biological meaning yielding the modified logistic equation.

$$X_t = \frac{X_m}{1 + \exp\left(\frac{4Q_X \times (\lambda - t) + 2 \times X_m}{X_m}\right)}$$

Modified Richards model

The modified Richards function was utilized to define the cell growth. Zwietering et al. (1990) modified the Richards equation (Richards, 1959) to involve parameters with biological meaning yielding the modified Richards equation. The symbols used in equation were given in Table 1.

$$X_t = X_m \times \left\{ 1 + v \times \exp(1+v) \times \exp\left(\frac{Q_X \times (1+v)^{\left(1+\frac{1}{v}\right)} \times (\lambda - t)}{X_m} \right) \right\}^{\left(\frac{-1}{v}\right)}$$

Table 1. The kinetic parameters belong to cell growth and mathematical models

Kinetic parameters	*S. stipitis* ATCC 58784		*S. stipitis* ATCC 58785	
	Glucose media	Xylose media	Glucose media	Xylose media
Biomass production (*X*, g/L)	4.10	3.86	4.30	3.49
Maximum biomass concentration (X_{max}, g/L)	4.21	4.38	4.47	4.19
Maximum growth rate (Q_X, g/L/h)	0.23	0.11	0.33	0.11
Shape parameter for modified Richards model (*v*)	0.28	0.08	0.28	0.09
Doubling time (t_d, h)	2.45	8.58	2.51	7.66
Lag time (λ, h)	3.90	0.50	2.80	0.00

The shape parameter (*v*) was calculated with non-linear least squares regression procedure in order to the least error value and the nearest R^2 value to 1.

RESULTS AND DISCUSSIONS

The current study was designed not only to obtain the cell growth curves for *S. stipitis* strains using different medium compositions in point of carbon sources and to calculate some kinetic parameters such as Q_X and t_d but also to model mathematically the obtained growth curves by using modified logistic and modified Richards models.

Determination of cell growth curves for *S. stipitis* strains

The growth curves in terms of absorbans and weight values were initially plotted versus time. Then, specific growth equations for *S. stipitis* strains were obtained with weighed weight values plotted versus the OD values at the same times. R^2 values and the specific cell growth equations were achieved along with the addition of trendlines (Madigan, 2005).

Scheffersomyces stipitis ATCC 58784

The growth curves for *S. stipitis* ATCC 58784 by using both glucose and xylose mediums were obtained as can be seen in Figure 1. According to results, in glucose medium (Figure 1A-B), cell growth equation was found to be $y=0.3047\times Abs_{600}-0.2656$, where *y* is cell concentration (g/L), while R^2 value was calculated as 0.9985. In xylose medium (Figure 1C-D), cell growth equation was obtained to be $y=0.2322\times Abs_{600}+0.4329$, where *y* is cell concentration (g/L), while R^2 value was estimated to be 0.9960. Therefore, these equations were completely represented the growth curves of *S. stipitis* ATCC 58784. On the other hand, the kinetic parameters with related to cell growth such as *X*, X_{max}, Q_X, t_d, and λ were also computed, as can be seen in Table 1. In glucose medium, the kinetic parameters were determined as 4.10 g/L, 4.21 g/L, 0.23 g/L/h, 2.45 h, and 3.9 h, respectively. Furthermore, in xylose medium, they were found as 3.86 g/L, 4.38 g/L, 0.11 g/L/h, 8.58 h, and 0.5 h, respectively. In conclusion, *S. stipitis* ATCC 58784 was successfully grown in both mediums, but its t_d value was too long in xylose medium compared to glucose media due to lower specific growth rate, while the lag time was too short in xylose medium compared to glucose media.

Scheffersomyces stipitis ATCC 58785

The growth curves for *S. stipitis* ATCC 58785 by using both glucose and xylose mediums were obtained as can be seen in Figure 2. According to results, in glucose medium (Figure 2A-B), specific growth equation was found to be $y=0.2639\times Abs_{600}+0.0282$, where *y* is cell concentration (g/L), while R^2 value was calculated as 0.9954. In xylose medium (Figure 2C-D), specific growth equation was obtained to be $y=0.2323\times Abs_{600}+0.6211$, where *y* is cell concentration (g/L), while R^2 value was estimated to be 0.9946. Therefore, these equations were completely represented the growth curves of *S. stipitis* ATCC 58785. On the other hand, the kinetic parameters with related to cell growth as above were also computed, as can be seen in Table 1.

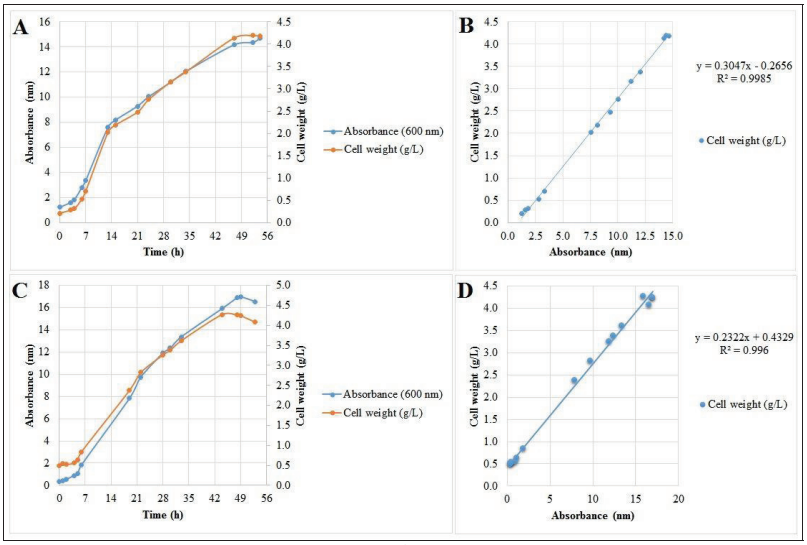

Figure 1. A: *Cell growth curve in point of absorbance and cell weight for* S. stipitis *ATCC 58784 in glucose medium,* ***B:*** *Specific growth equation for* S. stipitis *ATCC 58784 in glucose medium,* ***C:*** *Cell growth curve in point of absorbance and cell weight for* S. stipitis *ATCC 58784 in xylose medium,* ***D:*** *Specific growth equation for* S. stipitis *ATCC 58784 in xylose medium.*

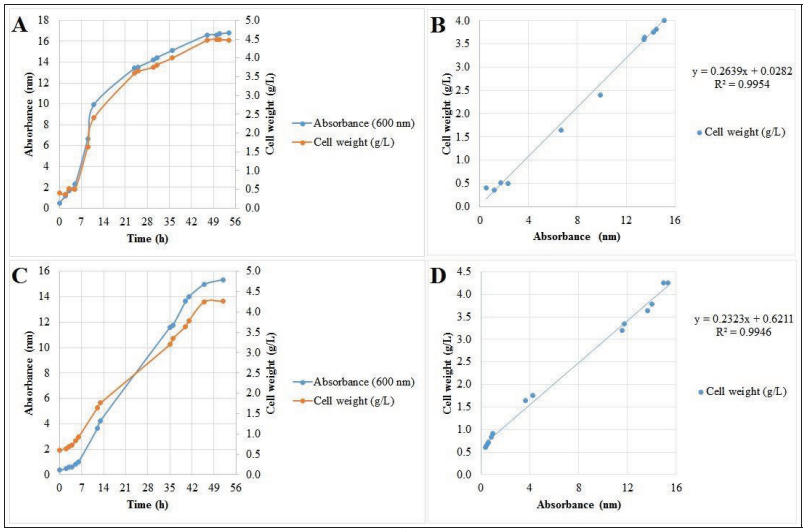

Figure 2. A: *Cell growth curve in point of absorbance and cell weight for S. stipitis ATCC 58785 in glucose medium,* ***B:*** *Specific growth equation for S. stipitis ATCC 58785 in glucose medium,* ***C:*** *Cell growth curve in point of absorbance and cell weight for S. stipitis ATCC 58785 in xylose medium,* ***D:*** *Specific growth equation for S. stipitis ATCC 58785 in xylose medium.*

In glucose medium, the kinetic parameters were determined as 4.10 g/L, 4.21 g/L, 0.23 g/L/h, 2.45 h, and 3.9 h, respectively. Furthermore, in xylose medium, they were found as 3.86 g/L, 4.38 g/L, 0.11 g/L/h, 8.58 h, and 0 h, respectively. Consequently, *S. stipitis* ATCC 58785 was successfully grown in both mediums, but its t_d value was too long in xylose medium compared to glucose media due to lower specific growth rate, while the lag time was too short in xylose medium compared to glucose media.

Modeling of cell growth

The kinetic parameters obtained from fermentations (Table 1) were applied to

modified logistic and modified Richards equations for cell growth. Afterwards, an independent set of fermentation date was utilized to validate the constructed model. The modified logistic and modified Richards equations were applied to fit cell growth. Subjective comparisons of the actual cell growth curve with modified logistic and modified Richards models were carried out by plotting both the experimental and the predicted values obtained from the models (Figure 3A-D). The modified logistic and modified Richards models sufficiently fitted the experimental data of all cell growth curves. Further validation was obtained via regression thorough origin of experimental data and predicted values obtained via modified logistic and modified Richards models. Results were shown in Table 2.

For *S. stipitis* ATCC 58784, R^2 values in glucose and xylose mediums were 0.954 and 0.998 and slops were 1.10 and 0.96 by using modified logistic model, respectively. In addition, R^2 values of glucose and xylose mediums were 0.963 and 0.998 and slopes were 1.13 and 0.97 by using modified Richards model, respectively (Table 2). The results demonstrated that the modified logistic and modified Richards models can sufficiently define the experimental data (Figure 3). The values of RMSE in glucose and xylose mediums were 0.53 and 0.11 g/L by using modified logistic model while they were 0.47 and 0.22 g/L by using modified Richards model, respectively. Besides, the values of MAE in glucose and xylose mediums were 0.38 and 0.09 g/L by using modified logistic model while they were 0.31 and 0.19 g/L by using modified Richards model, respectively (Table 2). Results showing that the usage of modified logistic and modified Richards models can obviously represent the cell growth curves of *S. stipitis* ATCC 58784 in glucose and xylose mediums.

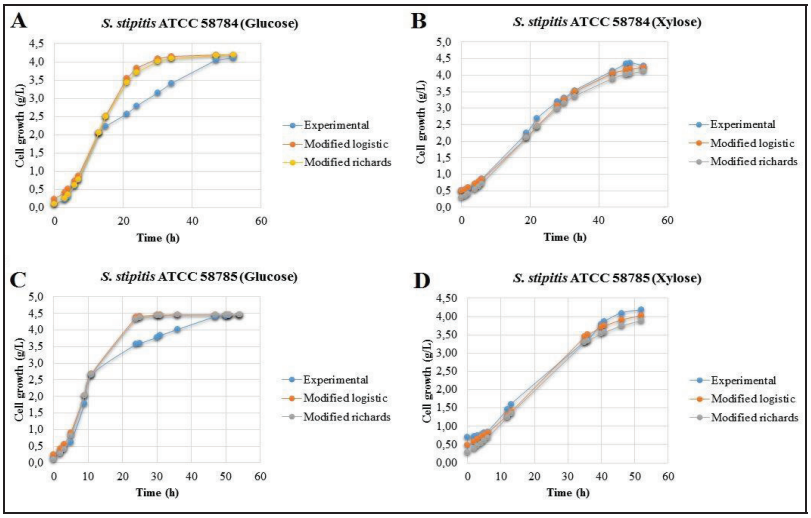

Figure 3. *Experimental and predicted cell growth profiles by S. stipitis strains.*

Table 2. Validation of models for biomass production.

Model	Microorganism and media	RMSE (g/L)	MAE (g/L)	R^2	Slope
Modified logistic	*S. stipitis* ATCC 58784 (Glucose media)	0.53	0.38	0.954	1.10
	S. stipitis ATCC 58784 (Xylose media)	0.11	0.09	0.998	0.96
	S. stipitis ATCC 58785 (Glucose media)	0.41	0.29	0.972	1.04
	S. stipitis ATCC 58785 (Xylose media)	0.14	0.13	0.995	1.01
Modified Richards	*S. stipitis* ATCC 58784 (Glucose media)	0.47	0.31	0.963	1.13
	S. stipitis ATCC 58784 (Xylose media)	0.22	0.19	0.998	0.97
	S. stipitis ATCC 58785 (Glucose media)	0.42	0.27	0.975	1.08
	S. stipitis ATCC 58785 (Xylose media)	0.26	0.24	0.995	1.01

RMSE: Root-mean-square errors. **MAE:** Mean absolute error.

For *S. stipitis* ATCC 58785, while R^2 values in glucose and xylose mediums were 0.972 and 0.995 and slops were 1.04 and 1.01 by using modified logistic model, they were 0.975 and 0.995 and slopes were 1.08 and 1.01 by using modified Richards model, respectively (Table 2). The results demonstrated that the modified logistic and modified Richards models can adequately define the data (Figure 3). The values of RMSE in glucose and xylose mediums were 0.41 and 0.14 g/L by using modified logistic model while they were 0.42 and 0.26 g/L by using modified Richards model, respectively. On the other hand, while the values of MAE in glucose and xylose mediums were 0.29 and 0.13 g/L by using modified logistic model, they were 0.27 and 0.24 g/L by using modified Richards model, respectively (Table 2). Results indicating that the usage of modified logistic and Richards models can evidently represent the cell growth curves of *S. stipitis* ATCC 58785 in glucose and xylose mediums (Figure 3).

CONCLUSIONS

In this study, the cell growth curves belong to *S. stipitis* strains (ATCC 58784 and ATCC 58785) were determined and their specific growth equations were obtained. In addition, the kinetic parameters belong to cell growth of *S. stipitis* strains were also calculated. Besides, modeling of fermentations towards cell growth of *S. stipitis* strains in media was also investigated and achieved. According to results, the specific growth equation of ATCC 58784 in glucose medium was $y=0.3047\times Abs_{600}-0.2656$. In xylose medium, it was $y=0.2322\times Abs_{600}+0.4329$. For ATCC 58785, the specific growth equation was $y=0.2639\times Abs_{600}+0.0282$ in glucose media while it was $y=0.2323\times Abs_{600}+0.6211$ in xylose medium. Moreover, for *S. stipitis* ATCC 58784, Q_X and t_d values in glucose and xylose media were 0.23, 0.11 g/L/h and 2.45, 8.58 h, respectively. For *S. stipitis* ATCC 58785, they were 0.33, 0.11 g/L/h and 2.51, 7.66 h, respectively. We also modelled the cell growth curves of *S. stipitis* strains by using modified logistic and modified Richards models. Modified logistic and modified Richards models in glucose and xylose mediums (slop=1.10 and 1.13, R^2=0.954 and 0.963; slop=0.96 and 0.97, R^2=0.998 and 0.998, respectively) adequately described the cell growth of *S. stipitis* ATCC 58784. Also they were accurately defined the cell growth of *S. stipitis* ATCC 58785 (slop=1.04 and 1.08, R^2=0.972 and 0.975; slop=1.01 and 1.01, R^2=0.995 and 0.995, respectively). Consequently, the specific growth equations-obtained for *S. stipitis* strains in glucose and xylose mediums can be used every time to determine the cell growth. In addition, the modified logistic and modified Richards equations proposed in this study indicated its generality to fit all cell growth curves.

REFERENCES

Flickinger M.C., 2013. Upstream industrial biotechnology, 2 volume Set. John Wiley & Sons.

Koch A.L., 2013. Microbial Growth Measurement. Encyclopedia of Industrial Biotechnology.

Lee J.-W., Zhu J., Scordia D., Jeffries T.W., 2011. Evaluation of ethanol production from corncob using *Scheffersomyces* (*Pichia*) *stipitis* CBS 6054 by volumetric scale-up. Applied biochemistry and biotechnology, 165(3-4): 814-822.

Madigan M.T., 2005. Brock Biology of Microorganisms, 11th edn, SciELO Espana.

Najafpour G., 2015. Biochemical engineering and biotechnology. Elsevier.

Pearl R., Reed L.J., 1920. On the rate of growth of the population of the United States since 1790 and its mathematical representation. Proceedings of the National Academy of Sciences, 6(6): 275-288.

Richards F., 1959. A flexible growth function for empirical use. Journal of experimental Botany, 10(2): 290-301.

Zhu J., Yang J., Zhu Y., Zhang L., Yong Q., Xu Y., Li X., Yu S., 2014. Cause analysis of the effects of acid-catalyzed steam-exploded corn stover prehydrolyzate on ethanol fermentation by *Pichia stipitis* CBS 5776. Bioprocess and biosystems engineering, 37(11): 2215-2222.

Zhu J., Yong Q., Xu Y., Yu S., 2011. Detoxification of corn stover prehydrolyzate by trialkylamine extraction to improve the ethanol production with *Pichia stipitis* CBS 5776. Bioresource technology, 102(2): 1663-1668.

Zwietering M., Jongenburger I., Rombouts F., Van't Riet K., 1990. Modeling of the bacterial growth curve. Applied and environmental microbiology, 56(6):1875-1881.

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FERMENTATION OF ACID-PRETREATED TEA PROCESSING WASTE FOR ETHANOL PRODUCTION USING *Saccharomyces cerevisiae*

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Abstract

Value-added products such as biofuels, enzymes, polysaccharides, pharmaceuticals, and organic acids can be produced from renewable resources such as carob pods, wheat straw, rice straw, rice husk, sugarcane bagasse, etc. by fermentation. Tea processing waste (TPW) is one of the renewable resources, which contains 13.60% cellulose, 32.16% hemicellulose, and 33.38% lignin. Due to its high carbohydrate content (total 45.76% wt), TPW can be used for production of value-added products. Therefore, the main objectives of this study were undertaken not only to determine the chemical composition of acid-pretreated tea processing waste hydrolysate (APTPWH) but also to investigate the effect of pH, inoculum size, agitation, and nitrogen sources on ethanol production from APTPWH without detoxification by using *Saccharomyces cerevisiae* in a stirred tank bioreactor. Results showed that it contains no HMF and lactic acid, 16.03 g/L fermentable sugar (FS), 0.43 g/L acetic acid, 2.61 g/L glucose, 4.14 g/L fructose+xylose, and 2.27 g/L phenolics. For batch ethanol fermentations, optimum conditions were found to be pH controlled at 5.5, 5% inoculum size (v/v), 150 rpm agitation speed, and yeast extract, which achieved as 25.55% yield ($Y_{P/S}$), 1.75 g/L ethanol production (P), and 0.38 g/L/h productivity (Q_P). Consequently, ethanol could be relatively produced from TPW without detoxification.

Key words: *tea processing waste, hydrolysis, chemical composition, ethanol fermentation.*

INTRODUCTION

Fossil fuels have been used to supply primarily the energy and also the organic chemicals requirements in the world for years. However, energy sector is confronted a problem such as reduction of petroleum fuel reserves. On the other hand, the world is face to face with some threats with respect to the large-scale utilization of petroleum fuels such as global warming, environmental pollution, and greenhouse effect. In 2050, the world population is expected to increase up to 10 billion, and thus this will increase the necessity to fuels of the world. Therefore, the attempts are enhanced for the production of biofuels from biomass due to increasing petroleum price and depletion of fossil fuel reserves. Accordingly, alternative sources need to be investigated in order to decrease the level of greenhouse gases released to environment and to supply the energy requirement of the world (Fatehi, 2013). Renewable resources are the most abundant and low-cost materials in nature, which generally exist in the form of pre- and post-harvest agricultural losses, agro-industrial wastes, wastes of food processing industries etc. (Galbe & Zacchi, 2012). TPW is also a kind of renewable resource released as a post-harvest waste of tea factory. Tea is a major commercial herbal crop in the tropical and subtropical regions. According to FAO data in 2013, the production amount of tea in the worldwide and Turkey are 5345523 and 212400 tons per year, respectively (FAOSTAT, 2015; Malkoc & Nuhoglu, 2007). Tea plant is grown in the Eastern Black Sea Region of Turkey, which is harvested three or four times a year in Turkey (Malkoc & Nuhoglu, 2007). In order to produce high quality tea, two and a half top leaves of the shoot on tea plant are harvested. But while tea producer cut the top leaves with special tea shears some overgrown woody shoots are mixed in the tea harvest. Therefore, these were untreated by tea factory

during the tea production process and thus formed into TPW, which is generated about 30-50 thousand tons per year in Turkey. Consequently, TPW is one of the most abundant renewable resources in tea growing countries such as China, Iran, Turkey, etc., which has not been evaluated for ethanol production (Malkoc & Nuhoglu, 2007).

Yeasts and bacteria have been used for ethanol production from pure carbon sources (glucose, sucrose, xylose, etc.), industrial plants (sugar cane, sugar beet, etc.) or by-products of food industry (whey, molasses, etc.) for many years. Among these, *S. cerevisiae* is the most used yeast from past to present as well as *Zymomonas mobilis* and *Pichia stipitis* (Atiyeh & Duvnjak, 2003). Therefore, the main objectives of this study are to determine the chemical composition of APTPWH and to examine the effect of pH, inoculation rate, agitation speed, and nitrogen sources on ethanol production from APTPWH by using *S. cerevisiae* in a stirred tank bioreactor.

MATERIALS AND METHODS

Raw material

TPW was provided from Çaykur Tea Company in Rize, a province of Turkey. It was milled to increase the hydrolysis efficiency by using a grinder (Bosch MKM6000, Ljubljana, Slovenia) and stored at room temperature until used. Besides, TPW composition analysis were studied before, which is consisted of 13.60% cellulose, 32.16% hemicellulose, 33.38% lignin, 20.86% extractives, and 0.10% moisture (Germec et al., 2016).

Dilute acid hydrolysis of TPW

Dilute acid hydrolysis of the milled TPW was performed using an autoclave (Hirayama HG-50, Saitama, Japan). Optimum hydrolysis conditions for TPW were studied by Germec et al. (2016). So the hydrolysis conditions of TPW were determined to be 120ºC, 12.5% solid loading (w/v), 1% dilute H_2SO_4 rate (w/v), and 15 min. After hydrolysis, the reaction mixture was cooled to room temperature and then filtered. The hydrolysate was stored at +4ºC until used for fermentation (Germec et al., 2016).

Microorganism and medium

S. cerevisiae ATCC 36858 was used for ethanol production from APTPWH, which was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). The stock culture was grown at 30ºC for 48 h in medium including 50 g of glucose, 6 g of yeast extract, 4 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 1 g of $MgSO_4.7H_2O$, and 0.3 g of $CaCI_2.2H_2O$ per liter of deionized water. The stock culture was stored at 4ºC and sub-cultured bimonthly in order to maintain viability. For a long-term storage, cultures were preserved at -80ºC in 20% glycerol (Turhan et al., 2010).

Ethanol fermentation medium

The reference and stock culture medium was composed of 50 g of glucose, 6 g of yeast extract, 4 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 1 g of $MgSO_4.7H_2O$, and 0.3 g of $CaCI_2.2H_2O$ per liter of deionized water. For fermentations, APTPWH was used as carbon source instead of glucose, but all other ingredients were added in the fermentation environment (Turhan et al., 2010).

Batch ethanol fermentation

Batch ethanol fermentations were carried out in a stirred tank bioreactor (Sartorius Biostad B Plus, Goettingen, Germany) with a 5-L vessel (working volume of 2.5 L). The reactor vessel was autoclaved at 121.1ºC for 15 min. After autoclaving and cooling down to room temperature, prepared inoculum at 30ºC for 24 h was used to inoculate the reactor and ethanol fermentations were performed for a period of 48 h. During ethanol fermentations, temperature was maintained at 30ºC and pH was controlled by using automatic addition of 4N NaOH. Samples were collected every 2 or 4 h for the first 12 h and every 6 or 12 h for the remainder of the fermentation and analyzed for residual sugar, ethanol production (P) as well as optical cell density for biomass concentration (X) in fermentation broth (Turhan et al., 2010).

Experimental design for ethanol fermentation

Five different ethanol production designs described as follows were evaluated in a stirred tank bioreactor.

- ***Effect of pH:*** Ethanol fermentation was carried out in enriched APTPWH with 3% inoculum at 30ºC, 150 rpm, and pH 5.5 or pH uncontrolled.
- ***Effect of inoculation rate:*** Three inoculation rates (1, 3, and 5%, v/v) were used to determine the effect of inoculation rate on the production of ethanol from APTPWH. Fermentations were performed at 30ºC, 150 rpm, and pH 5.5.
- ***Effect of agitation speed:*** In order to determine the effect of agitation speed on ethanol production from APTPWH, three different agitation speeds (100, 150, and 200 rpm) were used with the best inoculation rate.
- ***Effect of nitrogen source:*** Effect of three different nitrogen sources (yeast extract, beef extract, and ammonium nitrate) on the production of ethanol from APTPWH was examined with the best inoculation rate and agitation speed.
- ***Control:*** Non-enriched APTPWH was used for ethanol production with the best inoculation rate and agitation speed at pH 5.5 as control.

Analysis

Ethanol

Ethanol concentration was measured by using a bioanalyzer (Model YSI 2700, Yellow Springs, OH, USA) (Izmirlioglu & Demirci, 2012).

Sugars, organic acids, and total phenolics

Glucose, fructose, xylose, lactic acid, and acetic acid concentrations in APTPWH were determined by a HPLC system (LC-20 AD model, Shimadzu Kyoto, Japan) equipped with a refractive index detector. Separations were performed on a Transgenomics ORH-801 column (Apple Valley, MN, USA) at 65ºC using 0.0025 N H_2SO_4 as the mobile phase (20 µL injection volume, 0.6 ml/min). APTPWH was diluted with HPLC-grade water and filtered 0.45 µm membrane filters (Macharey-Nagel, Duren, Germany) prior to analysis (Kelebek et al., 2009).

The concentration of total FSs were analyzed by 3,5-dinitrosalicylic acid method (Miller, 1959). Absorbance values that measured at 575 nm were converted to FS concentration by using glucose standard curve. On the other hand, the analysis of total phenolic compounds were performed to Folin-Ciocalteu method (Singleton et al., 1999).

Hydroxymethyl furfural (HMF)

HMF concentration in the APTPWH was determined by a ThermoScientific HPLC system with a 20 µL sample loop and a Series 200 UV-Vis variable wavelength detector. Separation was performed on an Altima C18 column, 250 mm×4.6 mm, 5 µm particle size (Alltech, Sedriano, Italy) using 0.1 M H_2SO_4 solution in HPLC-grade water (resistance higher than 18 MΩ and methanol (Merc, Darmstadt, Germany) as the mobile phase (1.2 ml/min). The duration of HMF analysis was 23 min. The gradient mobile phase was used to be 90% of 0.1 M H_2SO_4 and 10% of methanol for 0-2 min, 70% of 0.1 M H_2SO_4 and 30% of methanol for 2-9 min, 70% of 0.1 M H_2SO_4 and 30% of methanol for 9-11 min, 40% of 0.1 M H_2SO_4 and 60% of methanol for 11-16 min, 40% of 0.1 M H_2SO_4 and 60% of methanol for 16-21 min, 90% of 0.1 M H_2SO_4 and 10% of methanol for 21-22 min, and 90% of 0.1 M H_2SO_4 and 10% of methanol for 22-23 min (Spano et al., 2009).

Biomass

The optical cell density was measured using a spectrophotometer (ThermoScientific 201 UV-Visible Evolution, Shanghai, China) at 620 nm. Uninoculated media was used as a blank. Absorbance values were converted to biomass concentrations by using a standard curve (Turhan et al., 2010).

Statistical analysis

The data were evaluated by using SAS statistical program (Version 9.00, SAS Institute INC., Cary, NC, USA). Duncan's multiple comparison test was used at significance level (P=0.05). All values were the average of two replicates and expressed in table as mean ± standard deviation.

RESULTS AND DISCUSSIONS

This study was undertaken not only to determine the chemical composition of APTPWH, but also to investigate separately the effect of fermentation parameters on ethanol

production from APTPWH without detoxification in stirred tank bioreactor.

Chemical composition of APTPWH

The inhibitors such as HMF, acetic acid, and phenolics are formed during the biomass pretreatment depending on the pretreatment severity, which are formed as a result of the degradation of cellulose, hemicellulose, and lignin in raw material (Uzuner & Cekmecelioglu, 2014). In this research, the acidic hydrolysis of TPW were performed at 120ºC, 12.5% solid loading (w/v), 1% (w/v) dilute H_2SO_4 rate, and 15 min (Germec et al., 2016). HMF and lactic acid were not detected, 16.03 g/L total FS, 0.43 g/L acetic acid, 2.61 g/L glucose, 4.14 g/L fructose+xylose, and 2.27 g/L total phenolic compounds were determined in APTPWH. The acid hydrolysis results showed that 16.03 g/L carbon source for ethanol fermentation was produced.

Similarly, Uzuner and Cekmecelioglu (2014) analyzed the level of chemical compounds in acid-pretreated hazelnut shell and reported that 2.59 g/L acetic acid, 0.0145 g/L HMF, 0.15 g/L phenolics, and no furfural was detected in hydrolysate. In conclusion, inhibitors such as HMF, acetic acid, furfural, and phenolics could be produced to be by-product during pretreatment of lignocellulosic materials.

Ethanol fermentation

Ethanol fermentation from APTPWH by using *S. cerevisiae* in a stirred tank bioreactor was evaluated in point of pH, inoculation rate, agitation speed, and nitrogen source.

Effect of pH on ethanol production

Effect of pH on ethanol production was initially evaluated at pH 5.5 and pH uncontrolled (Table 1, A and B). According to results, while $Y_{P/S}$, P, and Q_P values were calculated to be 20.07%, 1.61 g/L, and 0.31 g/L/h at pH 5.5, they were determined to be 10.17%, 0.89 g/L, and 0.13 g/L/h at pH uncontrolled, respectively. There was not significant difference between P values for pH controlled and uncontrolled fermentations but the $Y_{P/S}$ and Q_P values were significantly different ($p<0.05$). Therefore, the pH value for ethanol fermentation from APTPWH by *S. cerevisiae* was chosen to be 5.5.

The pH value of *S. cerevisiae* ethanol fermentations are generally ranged from 5 to 5.5 (Germec et al., 2015; Turhan et al., 2010; Yatmaz et al., 2013). The pH value of the fermentation medium is important. Ethanol production is completely inhibited if pH level drops to below 4 (Graves et al., 2006). Turhan et al. (2010), Yatmaz et al. (2013), and Germec et al. (2015) reported that the effect of pH on ethanol production from carob extract was statistically important and the optimal pH values were determined to be 5.5, 5.5, and 5.18, respectively.

Effect of inoculation rate on ethanol production

Three different inoculation rates (1, 3, and 5%, v/v) were tested to determine the effect on ethanol production from APTPWH. Agitation speed was fixed at 150 rpm while nitrogen source used in the media was yeast extract. According to inoculation rates fermentation results, the highest $Y_{P/S}$ (25.55%), P (1.75 g/L), and Q_P (0.38 g/L/h) was determined with 5% inoculation rate. In addition, lowest doubling time (t_d) was calculated for 5% inoculation rate. It means that conversion of sugar to ethanol is preformed faster and the microorganism with 5% inoculation rate was used sugar better than 1 and 3% inoculation rate (Table 1, D).

The effect of inoculation rate on ethanol production from carob extract by using immobilized *S. cerevisiae* cells in Ca-alginate in a stirred tank bioreactor was also studied. They reported that the best inoculation rate was found to be 5% v/v (Yatmaz et al., 2013). Results showed that inoculation rate had a significant effect on consumption rate (Q_S), growth rate (Q_X), specific growth rate (μ), and t_d values ($P<0.05$) while it had no significant effect on P, Q_P, and $Y_{P/S}$ values ($P>0.05$) (Table 1B-D). Besides P, Q_S, Q_P, Q_X, μ, and $Y_{P/S}$ values were generally enhanced with increasing of inoculation rate. Accordingly, the optimum inoculation rate for highest P, Q_P, and $Y_{P/S}$ was chosen to be 5% v/v, which was used for all following fermentations.

Effect of agitation speed on ethanol production

In order to determine the best agitation speed, three different agitation speeds (100, 150, and

200 rpm) were used for ethanol production from APTPWH. Nitrogen source was yeast extract. Results indicated that agitation speed had a significant effect on P, Q_S, μ, and t_d (P<0.05) (Table 1, D-F). The highest P, $Y_{P/S}$, Q_S, Q_P, Q_X, μ, and t_d values were obtained with 150 rpm agitation speed (Table 1, D). Yatmaz et al. (2013) studied the effect of agitation speed on P from carob extract by immobilized *S. cerevisiae* cells and reported the optimum agitation speed was 150 rpm. Consequently, kinetic parameter values were reduced at 100 and 200 rpm agitation speed, and thus the best agitation speed was determined to be 150 rpm, which was used for all following fermentations (Table 1, D-F).

Effect of nitrogen source on ethanol production

In order to investigate the effect of different nitrogen sources instead of yeast extract on ethanol production from APTPWH, beef extract and ammonium nitrate (6 g/L) were used individually and results were given in Table 1D, G, and H. The highest P and $Y_{P/S}$ were found to be 1.95 g/L and 28.72% by addition of ammonium nitrate in the media. However, there was no statistically an important effect between nitrogen sources in point of P and $Y_{P/S}$ (P>0.05). Besides, when yeast extract was used in the media; Q_X, μ, and t_d values were statistically significant according to the usage of ammonium nitrate in the media (P<0.05), but this was not valid for Q_X when beef extract was utilized in the media (P>0.05). On the other hand, Q_S and Q_P were not statistically important depending on the usage of yeast extract and ammonium nitrate in the media (P>0.05). Also, Q_S was decreased by addition of beef extract in the media compared to yeast extract and ammonium nitrate. Accordingly, nitrogen source used in the media had a statistically significant effect on P from APTPWH (P<0.05). Consequently, although higher P and $Y_{P/S}$ were obtained when ammonium nitrate was used in the media; higher Q_S, Q_P, Q_X, μ, and lower t_d were achieved by addition of yeast extract in the media. Therefore, the best results were obtained by using yeast extract in the fermentation environment. In conclusion, the best fermentation conditions were chosen to be pH 5.5, 5% inoculation rate (v/v), 150 rpm agitation speed, and yeast extract (Table 1, D).

Table 1. Summary of fermentation results.*

Kinetic parameters / Fermentation conditions	P (g/L)	$Y_{P/S}$ (%)	Q_S (g/L/h)	Q_P (g/L/h)	Q_X (g/L/h)	μ (h^{-1})	t_d (h)
A	$0.89^{abc} \pm 0.03$	$10.17^{b} \pm 1.21$	$0.35^{ab} \pm 0.10$	$0.13^{b} \pm 0.02$	$0.13^{bc} \pm 0.01$	$0.05^{de} \pm 0.01$	$13.77^{b} \pm 2.33$
B	$1.61^{abc} \pm 0.47$	$20.07^{ab} \pm 3.99$	$0.15^{b} \pm 0.02$	$0.31^{ab} \pm 0.06$	$0.11^{c} \pm 0.00$	$0.03^{e} \pm 0.00$	$25.26^{a} \pm 2.37$
C	$1.29^{abc} \pm 0.79$	$22.32^{ab} \pm 1.25$	$0.68^{a} \pm 0.09$	$0.21^{ab} \pm 0.10$	$0.19^{abc} \pm 0.05$	$0.07^{bcd} \pm 0.01$	$9.97^{c} \pm 1.4$
D	$1.75^{ab} \pm 0.05$	$25.55^{ab} \pm 0.88$	$0.68^{a} \pm 0.01$	$0.38^{a} \pm 0.03$	$0.24^{a} \pm 0.01$	$0.21^{a} \pm 0.02$	$3.35^{d} \pm 0.35$
E	$0.65^{c} \pm 0.02$	$10.66^{b} \pm 1.01$	$0.08^{b} \pm 0.00$	$0.21^{ab} \pm 0.12$	$0.15^{abc} \pm 0.04$	$0.07^{bcd} \pm 0.01$	$9.48^{bc} \pm 1.08$
F	$1.05^{abc} \pm 0.04$	$14.81^{ab} \pm 1.51$	$0.62^{a} \pm 0.15$	$0.30^{ab} \pm 0.06$	$0.21^{ab} \pm 0.03$	$0.11^{b} \pm 0.01$	$6.54^{cd} \pm 0.67$
G	$1.45^{abc} \pm 0.12$	$20.45^{ab} \pm 5.07$	$0.22^{b} \pm 0.00$	$0.27^{ab} \pm 0.03$	$0.16^{abc} \pm 0.00$	$0.10^{bc} \pm 0.00$	$7.04^{cd} \pm 0.27$
H	$1.95^{a} \pm 0.06$	$28.72^{a} \pm 2.65$	$0.66^{a} \pm 0.28$	$0.20^{ab} \pm 0.05$	$0.12^{bc} \pm 0.03$	$0.08^{bcd} \pm 0.01$	$9.33^{bc} \pm 1.71$
I	$0.81^{bc} \pm 0.03$	$9.51^{b} \pm 0.05$	$0.21^{b} \pm 0.04$	$0.20^{ab} \pm 0.01$	$0.14^{bc} \pm 0.01$	$0.06^{cde} \pm 0.00$	$11.04^{bc} \pm 0.07$

*All fermentation experiments were performed at 30ºC. **A:** 3% (v/v) inoculation rate, 150 rpm agitation speed, yeast extract, *pH uncontrolled*. **B:** 3% (v/v) inoculation rate, 150 rpm agitation speed, yeast extract, *pH 5.5*. **C:** *1% (v/v) inoculation rate*, 150 rpm agitation speed, yeast extract, pH 5.5. **D:** *5% (v/v) inoculation rate*, 150 rpm agitation speed, yeast extract, pH 5.5. **E:** 5% (v/v) inoculation rate, *100 rpm agitation speed*, yeast extract, pH 5.5. **F:** 5% (v/v) inoculation rate, *200 rpm agitation speed,* yeast extract, pH 5.5. **G:** 5% (v/v) inoculation rate, 150 rpm agitation speed, *beef extract*, pH 5.5. **H:** 5% (v/v) inoculation rate, 150 rpm agitation speed, *ammonium nitrate*, pH 5.5. **I:** 5% (v/v) inoculation rate, 150 rpm agitation speed, pH 5.5 (*non-enriched medium*). Values are given as the mean ± standard deviation of two replicates. Different letters in the same column indicate statistically significance between mean values (P<0.05).

Batch fermentation with using non-enriched APTPWH

To produce the ethanol from non-enriched APTPWH, fermentation conditions were set to be pH 5.5, 5% inoculation rate (v/v), and 150 rpm. The results were given in Table 1I, which were significantly low compared to the best fermentation conditions (Table 1, D). The P and $Y_{P/S}$ values for enriched medium at optimum conditions were 1.75 g/L and 25.55% while they were found to be 0.81 g/L and 9.51% for non-enriched medium, respectively (Table 1, D and I). The results for enriched and non-enriched medium demonstrated that using enriched medium for P from APTPWH was better than using non-enriched medium because of higher kinetic parameter values and ethanol concentration.

CONCLUSIONS

In this study, chemical composition of APTPWH was evaluated. According to results, no HMF and lactic acid, 16.03 g/L total FS, 0.43 g/L acetic acid, 2.61 g/L glucose, 4.14 g/L fructose+xylose, and 2.27 g/L phenolics were determined in APTPWH. *S. cerevisiae* was also used for *P* from APTPWH in a stirred tank bioreactor. The effect of pH, inoculation rate, agitation speed, nitrogen source as well as enrichment on *P* was investigated. The *P*, $Y_{P/S}$, Q_P, and Q_S were 1.75 g/L, 25.55%, 0.38 g/L/h, and 0.68 g/L/h respectively at optimized conditions for batch fermentations, respectively. Enrichment of medium had a statistically effect on ethanol production from APTPWH ($P<0.05$). In conclusion, TPW could be used as a potential substrate source for production of value-added products by fermentation.

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REFERENCES

Atiyeh H., Duvnjak Z., 2003. Production of fructose and ethanol from cane molasses using *Saccharomyces cerevisiae* ATCC 36858. Acta biotechnologica, 23(1): 37-48.

FAOSTAT., 2015. Food and Agriculture Organisation of the United Nations. Available from: http://faostat3.fao.org/download/Q/QC/E, Accessed 2015 November 21.

Fatehi P., 2013. Recent advancements in various steps of ethanol, butanol, and isobutanol productions from woody materials. Biotechnology progress, 29(2): 297-310.

Galbe M., Zacchi G., 2012. Pretreatment: the key to efficient utilization of lignocellulosic materials. Biomass and Bioenergy, 46: 70-78.

Germec M., Tarhan K., Yatmaz E., Tetik N., Karhan M., Demirci A., Turhan I., 2016. Ultrasound-assisted dilute acid hydrolysis of tea processing waste for production of fermentable sugar. Biotechnology progress. DOI: 10.1002/btpr.2225

Germec M., Turhan I., Karhan M., Demirci A., 2015. Ethanol production via repeated-batch fermentation from carob pod extract by using *Saccharomyces cerevisiae* in biofilm reactor. Fuel, 161: 304-311.

Graves T., Narendranath N.V., Dawson K., Power R., 2006. Effect of pH and lactic or acetic acid on ethanol productivity by *Saccharomyces cerevisiae* in corn mash. Journal of Industrial Microbiology and Biotechnology, 33(6): 469-474.

Izmirlioglu G., Demirci A., 2012. Ethanol production from waste potato mash by using *Saccharomyces cerevisiae*. Applied Sciences, 2(4): 738-753.

Kelebek H., Selli S., Canbas A., Cabaroglu T., 2009. HPLC determination of organic acids, sugars, phenolic compositions and antioxidant capacity of orange juice and orange wine made from a Turkish cv. Kozan. Microchemical Journal, 91(2): 187-192.

Malkoc E., Nuhoglu Y., 2007. Potential of tea factory waste for chromium (VI) removal from aqueous solutions: thermodynamic and kinetic studies. Separation and Purification Technology, 54(3): 291-298.

Miller G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry, 31(3): 426-428.

Singleton V.L., Orthofer R., Lamuela-Raventos R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in enzymology, 299: 152-178.

Spano N., Ciulu M., Floris I., Panzanelli A., Pilo M.I., Piu P.C., Salis S., Sanna G., 2009. A direct RP-HPLC method for the determination of furanic aldehydes and acids in honey. Talanta, 78(1): 310-314.

Turhan, I., Bialka, K.L., Demirci, A., Karhan, M. 2010. Ethanol production from carob extract by using *Saccharomyces cerevisiae*. Bioresource technology, 101(14), 5290-5296.

Uzuner, S., Cekmecelioglu, D. 2014. Hydrolysis of Hazelnut Shells as a Carbon Source for Bioprocessing Applications and Fermentation. International Journal of Food Engineering, 10(4), 799-808.

Yatmaz, E., Turhan, I., Karhan, M. 2013. Optimization of ethanol production from carob pod extract using immobilized *Saccharomyces cerevisiae* cells in a stirred tank bioreactor. Bioresource technology, 135, 365-371.

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SHORT TERM EXPERIMENTS ON SYNTHETIC WASTE WATER TREATMENT IN LABORATORY ACTIVATED SLUDGE SEQUENCING BATCH REACTOR

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Abstract

This paper presents the short term (5 hours) evolution of ammonium, nitrite, nitrate, total phosphorus and COD of a synthetic wastewater treated in a laboratory activated sludge sequencing batch reactor. In the experiment with 4.33 g/L activated sludge and the initial raport COD:N:P of 154:5.3:1 in about 4 hours 31% of COD and 83% of ammonium were removed, whereas nitrate shows an increase of 89%. In the experiment with 4.71 g/L activated sludge and the initial raport COD:N:P of 195:3.22:1 in about 4.5 hours 26% of COD and 92% of ammonium were removed, whereas nitrate shows an increase of 85%. In the experiment with 4.34 g/L activated sludge and the initial raport COD:N:P of 103:4.45:1 in about 4.5 hours 30% of COD and 48% of ammonium were removed, whereas nitrate shows an increase of 65%.

Key words*:synthetic waste water, activated sludge, sequencing batch reactor.*

INTRODUCTION

Wastewater treatment is essentially based on biological treatment using activated sludge, either in suspension or immobilized, containing prokaryotes (archaea and bacteria), eukaryote microorganisms as well some metazoa: nematodes, rotifers, copepods (Ardern and Lockett,1923; Grady and Lim, 1980 Vaicum, 1981;Negulescu, 1985; Arceivala, 1988; Bitton, 1999; Zarnea, 1994; Cheremisino, 2002; Burton and Stensel, 2003; Godeanu 2015). The activated sludge process is based on microorganisms which can use as nutrients organic and inorganic substances that are true contaminants in wastewater. The microorganisms thus growth and multiply, basically converting soluble chemicals (the pollutants) to particulate matter, microbial cells clumping together, thus increasing the biomass of activated sludge. These aggregates of microorganisms are in the form of flocs which can settle to the bottom of the tank, ideally, leaving an outlet water which should be relatively clear liquid free of organic material and suspended solids. .

The Sequencing Batch Reactor (SBR) is an activated sludge process designed to operate under non-steady state conditions. An SBR operates in a true batch mode with aeration and sludge settlement both occurring in the same tank. The major differences between SBR and conventional continuous-flow, activated sludge system is that the SBR tank carries out the functions of equalization aeration and sedimentation in a time sequence rather than in the conventional space sequence of continuous-flow systems (Norcross, 1992; Chambers, 1993; Larrea et al, 2007, Narcis et al., 2014). Furthermore, the SBR system can have the possibility to be functional to treat a wide range of influent volumes whereas the continuous system is based upon a fixed influent input. However, the appropriate function of a SBR deserves that the influent wastewater is admitted into the aeration in a controlled manner, an appropriately designed SBR process being a unique combination of equipment and software and skilled operators (Arora et al., 1985; Norcross, 1992; Chambers, 1993; Narcis et al., 2014). A balanced nutrient ratio is essential if the microorganism are to function at maximum efficiency during wastewater treatment. The most important of these nutrients are carbon, nitrogen and phosphorus. Microorganisms involved in the

removal of carbonaceous contaminants from wastewater require nitrogen and phosphorus for growth and multiplication. Microorganisms require nitrogen to form proteins, cell wall components and nucleic acids (Maier, 1999). It is usually stated that the ratio of COD:N:P in the wastewater to be treated should be aproximately 100:5:1 for aerobic treatment and 250:5:1 for anaerobic treatment (Metcalf and Eddy, 1991). Unfavourable nutrient ratio and high concentration of individual substances reduce the degradation efficiency of biological wastewater treatment proceses. Early recognition and continuous monitoring of critical parameters is therefore essential in order to enable plant operators to take rapid corective action when necessary. The aim of this paper is monitor the evolution of ammonium, nitrite, nitrate, total phosphorus and COD of the synthetic wastewater treated in a laboratory activated sludge in sequencing batch reactor.

MATERIALS AND METHODS

The sequencing batch reactor used in this study had an active volume of 2 L. The activated sludge comes from the wastewater treatment plant Constanta Nord. The activated sludge used has been taken from the discharge from the end of the aerobic bioreactor (Vazquez et al., 2003) in each experiment, 2 L of activated sludge was filtered through a filter paper (Filter Discs Grade 388 Munktell – Ahlstrom). The dry mass of activated sludge was measured according to ~~stass~~ SR EN 12880. For the experiment 1 the concentration of activated sludge, dry mass was of 4.33 g/L, for the experiment 2 concentration of activated sludge, dry mass was 4.71 g/L and for the experiment 3 the concentration was 4.34 g/L. In the sequencing batch reactor together with the activated sludge was introduced the synthetic sewage composed of: 1.24 g glucose, 64 mL standardised solution of ammonium 1000 mg/mL NH_4^+ (2,97g NH_4Cl / L H_2O) and 31.5 mL standardised solution of phosphorus 1000 mg/ml (KH_2PO_4 998g/l H_2O). The activated sludge together with the synthetic sewage were the subject of sequences of aeration and mixing both simultaneously and successively. The duration of both aeration and mixing was 60 minutes whereas the mixing time (in the absence of aeration) was 30 minutes. The duration of this experiment was 4.5 h, during which samples were taken as follows: initially – T_0 (after mixing the activated sludge with the synthetic sewage); after 1 hour of aeration and simultaneous mixing – T_1; after 1 hour aeration and mixing, and 30 minutes just mixing – T_2; after 2 hours aeration and mixing and 30 minutes just mixing – T_3; after 2 hours aeration and mixing and 60 minutes just mixing – T_4 ; after 3 hours aeration and mixing and 1 hour just mixing – T_5; after 3 hours aeration and mixing and 1.5 hour just mixing – T_6. For experiments 1 and 2 the samples were filtered through the filter of 0,45 mm type LCW 916, Hach Lange, and were made the following determinations: ammonium, nitrate, total phosphorus, chemical oxygen demand. For the experiment 3 samples were filtered twice - first through the filter type Filter Discs Grade 388 (Munktell - Ahlstrom) then through the filter of 0,45 mm filter type LCW 916, Hach Lange. The determinations were made on the Hach Lange kits: for nitrat was used LCK 339 1-60 mg/L NO_3, for nitrit was used LCK 541 0.005 – 0.10 mg/L NO_2 and LCK 342 2-60 mg/L NO_2, for ammonium was used LCK 303 2,5-60 mg/L NH_4, for total phosphorus LCK 349 0,15 – 4,50 mg/L PO_4 and LCK 350 6-60 mg/L PO_4 , for COD was used LCK 514 100-2000 mg/L O_2.

RESULTS AND DISCUSSIONS

In figure 1 there are presented the time evolution of ammonium, nitrite, nitrate and total phosphorus concentrantions of the synthetic wastewater during the first experiment. In the first experiment with an initial raport to the COD:N:P 154:5.3:1, with about 50% more COD than ideal ratio presented in literature, obtaining an efficiency of 31% for COD removal (figure 4) and 39% for nitrogen, showing the steps of nitrification/ denitrification (aeration/mixing) very well on the parameter to be analyzed - nitrate ($T_{1,3,5}$ times showing stages of aeration). It is observed a reduction of 21.95 mg/L for ammonium within 4 hours, the efficiency of removal of 83% compared with the nitrate which registers an increase of 37.79 mg/L in

the range of 4 hours, this fact is due to intense nitrification. In this experiment the concentration of activated sludge used was 4.33 g/L, in which the mineral part represented 33.4% and volatile part represented 66.6%, with a sedimentation of 367 mL, and an Mohlmann index of 85 mg/gf.

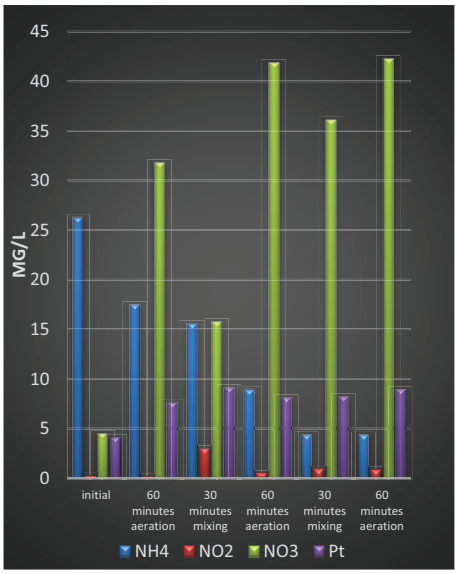

Figure 1 - Time evolution of ammonium, nitrate, nitrit and total phosphorus concentration during the first experiment

In figure 2 there are presented the time evolution of ammonium, nitrite, nitrate and total phosphorus concentrantions of the synthetic wastewater during the second experiment. In the second experiment with an initial raport to the COD:N:P of 195:3.22:1, with about 100% more COD than ideal ratio presented in literature, obtaining an efficiency of 26% for COD removal (figure 4) and 72% for nitrogen, showing the steps of nitrification/ denitrification (aeration/ mixing) very well on the parameter to be analyzed - nitrate ($T_{1,3,5}$ times showing stages of aeration). It is observed a reduction of 23.14 mg/L for ammonium within 4.5 hours, the efficiency of removal of 92% compared with the nitrate which registers an increase of 10.36 mg/L in the range of 4.5 hours, this fact is due to intense nitrification. In this experiment the concentration of activated sludge used was 4.71 g/L, in which the mineral part represented 32.3% and volatile part represented 67.7%, with a sedimentation of 400 mL, and an Mohlmann index of 85 mg / gf.

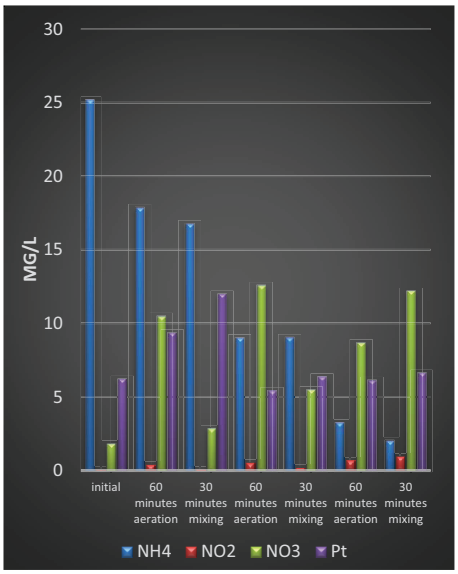

Figure 2 - Time evolution of ammonium, nitrate, nitrit and total phosphorus concentration during the second experiment

In figure 3 there are presented the time evolution of ammonium, nitrite, nitrate, total phosphorus of the synthetic wastewater during the third type of experiment.

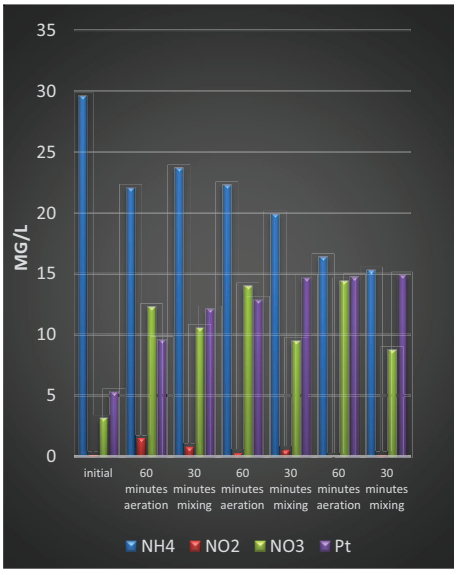

Figure 3 - Time evolution of ammonium, nitrate, nitrit and total phosphorus concentration during the third experiment

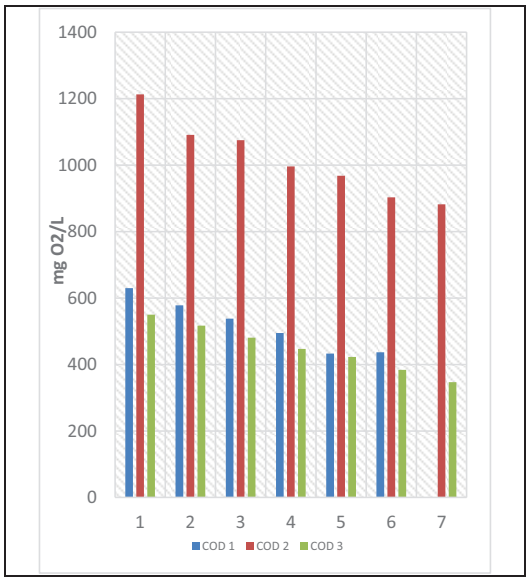

Figure 4 - The decrease in chemical oxygen demand in experiment 1 (COD1), experiment 2 (COD2) and experiment 3 (COD3)

In the third experiment with an initial raport to the COD:N:P of 103:4.45:1, aproximatly ideal ratio presented in literature, obtaining an efficiency of 30% for COD removal (figure 4) and 42% for nitrogen, showing the steps of nitrification/ denitrification (aeration/ mixing) very well on the parameter to be analyzed - nitrate ($T_{1,3,5}$ times showing stages of aeration). It is observed a reduction of 14.30 mg/L for ammonium within 4.5 hours, the efficiency of removal of 48% compared with the nitrate which registers an increase of 5.64 mg/L in the range of 4.5 hours, this fact is due to intense nitrification. In this experiment the concentration of activated sludge used was 4.34 g/L, in which the mineral part represented 31.8% and volatile part represented 68.2%, with a sedimentation of 410 mL, and an Mohlmann index of 95 mg / gf. In figure 4 there are presented the time evolution of COD of the synthetic wastewater during the three experiments.

CONCLUSIONS

a) In the experiment with 4.33 g/L activated sludge and the initial raport COD:N:P of 154:5.3:1 in about 4 hours 31% of COD and 83% of ammonium were removed, whereas nitrate which shows an increase of 89%.
b) In the experiment with 4.71 g/L activated sludge and the initial raport COD:N:P of 195:3.22:1 in about 4.5 hours 26% of COD and 92% of ammonium were removed, whereas nitrate which shows an increase of 85%.
c) In the experiment with 4.34 g/L activated sludge and the initial raport COD:N:P of 103:4.45:1 in about 4.5 hours 30% of COD and 48% of ammonium were removed, whereas nitrate shows an increase of 65%.

PERSPECTIVES

A) The use of immobilized activated sludge for easier physical separation between biological catalysts and (synthetic) waste water as well as for other advantages of immobilization;
B) Deeper caractrization of the activated sludge, mainly with respect to structural (flocs dimentions and structure) and functional traits (the rate of metabolic electron transport such as aerobic respiration, denitrification and resazurine reduction);
C) The selection and improvement of appropriate microbial populations to be used as starter cultures in futher experiments concerning (synthetic) waste water treatment in (laboratory) activated sludge sequencing batch reactor useful for the removal not only of wastes containing C and N but also of P.

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REFERENCES

Arceivala J.S., 1988. Wastewater treatment for pollution control, 2nd Ed. Tata McGraw-Hill Publishing Co., New Delhi
Ardern E., Lockett W.T., 1923. Activated Sludge Process. Withington Works. J. Soc. Chem. Ind. 225-230

Arora M.L., Edwin F. Barth and Margaret B., 1985. Umphres Journal (Water Pollution Control Federation), Vol. 57(8), pp. 867-875

Barsan Narcis, Joita Ion, Stanila Marius, Radu Cristian, Dascalu Mihaela, 2014. Modelling wastewater treatment process in a small plant using a sequencing batch reactor (SBR). Environmental Engineering & Management Journal (EEMJ), Vol. 13(7), pp. 1561-1566

Bitton G., 1999. Wastewater Microbiology. Wiley-Liss, New York.

Burton G., Stensel F.L., 2003. Engineering, Treatment, Disposal and Reuse. Tata McGraw-Hill Publishing Company, New Delhi, India

Chambers, B., 1993. Batch Operated Activated Sludge Plant for Production of High Effluent Quality at Small Works. Water Science and Technology, vol. 28(10)

Cheremisino N.P., 2002. Handbook of water and wastewater treatment technologies. Butterworth-Heinemann, Boston

Godeanu S. P., 2015. Biological wastewater treatment. ISBN:978-606-764-002-1, Bucharest

Grady C.P.L., Lim H.C., 1980. Biological Waste Treatment: Theory and Applications

Larrea L, Albizuri J, Irizar I, Hernández JM., 2007 Design and operation of SBR processes for small plants based on simulations.

Maier RM, 1999. Enviromental Microbiology Biochemical Cycling, Chapter 14. In: Maier RM, Pepper IL, Gerba CP (eds.). Academic Press, pp. 319-346

Metcalf & Eddy, Inc., 1991. Wastewater Engineering, Treatment, Disposal and Reuse, third edition, McGraw-Hill, Inc., New York

Negulescu M., 1985. Municipal waste water treatment. Ed.Elsevier, Amsterdam, Oxford, New-York, Tokio

Norcross K.L., 1992. Sequencing Batch Reactors-An Overview. Water Science and Technology, vol. 26(9-11)

Vaicum L.M., 1981. Waste Water Purification with Activated Sludge. Biochemical Bases Publishing House of the Academy of RS Romania (in Romanian)

Vazquez-Rodriguez G., Goma G. and J. L. Rols, 2003. Activated sludge as inoculum for ready biodegradability testing: Effect of source. Environmental Technology, 24(8):979-987

Zarnea G., 1994. Treatise of General microbiology. Romanian Academy Press, Vol. V, pp. 1002-1036 (in Romanian)

SR EN 12880 Determinarea reziduului uscat si a continutului din namoluri.

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LIFE CYCLE ASSESSMENT (LCA) ON EUROPEAN SKIMMED MILK POWDER PROCESSING PRODUCTION PLANT

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Abstract

The dairy industry consists one of the most energy intensive food industries, with milk powder production being the most energy consuming process. The aim of this work is to present the state of the art skimmed milk powder production processing chain in order to identify the processes with high environmental and energy impact. A life cycle assessment (LCA) has been performed to analyse the environmental footprint and energy balance derived from the skimmed milk powder (medium heat) production on the post-harvest chain. Therefore, a comparative gate to gate LCA was performed within the boundaries of the processing plant (i.e. standardization/separation, homogenization, pasteurization, evaporation, spray drying). In this study, two scenarios were evaluated on their environmental performance: a) the conventional production of skimmed milk powder (SMP) with the inclusion of Reverse Osmosis (Scenario 1) and b) the production of SMP exclusion of Reverse Osmosis (Scenario 2). The standard framework of LCA was followed according to the ISO 14044, which is also in line with the International Reference Life Cycle Data System (ILCD) Handbook. LCA study was performed on Gabi 6 software with databases from within the food industry. Inventory data were collected from the industry and completed using the literature and databases, impact categories were evaluated adopting a CML method with the energy analysis carried out based on the cumulative energy demand (CED).

Key words*: Dairy Processing, Energy Consumption, Life cycle Assessment (LCA), Skimmed Milk Powder, Spray Drying.*

INTRODUCTION

In recent years "green" economy aiming at a sustainable development without degrading the environment, avoiding the resources depletion and ensuring the prevention of human health and well-being. This trend has been the evident practice around the globe in all aspects of industry. As a consequence, governments, non-governmental organizations, companies and civil society are becoming interested in increasing the knowledge of how a product is processed and what is the environmental impact of its production. That implies taking into account the whole chain of a product's life cycle and all relevant external effects, in order to be able to make improvements that promote sustainability and environmental friendly production.

In accordance to this tendency, the dairy value chain has been actively working towards the reduction of greenhouse gas (GHG) emissions derived from the production, collection and processing of milk and delivery of dairy products, while satisfying the needs of the marketplace in the most sustainable manner. In compliance to that direction, various organizations such as International Dairy Federation (IDF, 2005 & 2010) and European Commission (EC) are supporting the evolution of efficient and sustainable businesses and technologies that contribute to a GHG emissions reduction.

The food industry is a major consumer of water and energy. It was positioned third in terms of water consumption and wastewater discharge, after the chemical and refinery industries. In the European Union about 31% of total GHG emissions are estimated to come from the food chain (EC, 2006). Dairy industry is considered to be the most energy and water consuming sector of the food industry (EC, 2008), with a consumption exceeding 8% of the total EU energy consumed.

The whole dairy value chain is divided into different segments (Figure 1, right), with each one of the segments having different impact on energy consumption (Figure 1, left). It is apparent that processing stage is the most energy intensive step of the dairy chain. This study is dealing with the post-harvest chain and the production of skimmed milk powder (SMP) and intends to evaluate the environmental state of the dairy processing plant.

Among the tools available to evaluate environmental performance, LCA has gained recognition as the most powerful tool for the comparison of environmental impacts of products, technologies or services with a view to their whole life cycle (cradle to grave) or to a targeted part of that life cycle (cradle to gate, gate to gate or gate to grave). The present study focuses on the application of LCA for the evaluation of SMP production by two different scenarios. In short, a comparative LCA study of the conventional dairy processing production of SMP in European area is performed. The inclusion or exclusion of reverse osmosis (RO) process in the dairy plant route forms the two different scenarios.

The study is based on the methodology for LCA, as specified in the standardized documents of ILCD, ISO and BSI:

- ILCD: International Reference Life Cycle Data System Handbook (2010)
- ISO: Environmental management–LCA-Requirements and guidelines (2006)
- PAS 2050: Specification for the assessment of the life cycle greenhouse gas emissions of goods and services (2008)

MATERIALS AND METHODS

Goal and Scope

The goal of this study was to evaluate the current technologies on skimmed milk production on their environmental performance. In this study two different production lines were examined, the conventional production of skimmed milk powder (SMP) with the inclusion of Reverse Osmosis (Scenario 1) and the production of SMP exclusion of Reverse Osmosis (Scenario 2). In the LCA the energy, water and environmental profile of the conventional dairy processing scenarios were evaluated within the boundaries of the dairy plant, while material and energy inputs and outputs on farming, packaging, distribution and retail were not taken into consideration in the assessment. The impact of the incorporated processes on human health, natural environment and natural resources caused by interventions between Techno-sphere and Ecosphere during operations was assessed against all relevant impact categories resulting from the analysis. Finally, the scope of the study was to provide data on the evaluation of the sustainability performances of the current state of skimmed milk production.

Function and Functional Unit

The functional unit selected is 1 kg of produced Skimmed Milk Powder unpacked, which was output related, while the function was the production of Skimmed Milk Powder in a dairy processing plant.

Description of Conventional Dairy Processing Plant

The system boundaries included all relevant life cycle stages and processes that were operated within the techno-sphere and related to the functional unit. The examined system was defined as everything involved in the production of skimmed milk powder in the limits of the dairy processing plant. It was a gate to gate LCA methodology and the impact derived from the transportation of the raw milk to the plant was included. Mass and energy balance flows was collected, as well as, data on the amount of waste and emissions to water and air. In addition, the mode and distance of all transportation within the system were taken into account. However, in the study buildings and machinery, and personnel and other capital goods were not taken into consideration.

The production line of the standard way to produce skimmed milk powder is depicted in Figure 2. In brief, milk from farm was transported to the dairy site with different batches (from different farms) being mixed and stored at the bulk storage/mixing unit. Raw milk enters the separator (cold or hot) and cream was separated from the skimmed milk. In separator, standardization of skimmed milk was performed with the addition of cream. The flow of cream that leaves the system was further processed, but since the current study aims for skimmed milk powder, the flow of cream was not further taken into account. The skimmed milk was pasteurized and stored in buffer silo after pasteurization. Although storage after each process step unit was not necessary, most factories apply storage as a safety margin. Pasteurization and preheating before evaporation was combined into one single step. A 3-stage evaporator was applied, while a combination of evaporation and reverse osmosis could also be combined. Although the potential for energy saving it was hardly implemented in milk powder plants.

This work examines both scenarios:

Scenario 1: Dairy Plant with RO

Scenario 2: Dairy plant without RO

A buffer tank for the storage of the concentrated milk coming out of the evaporator was applied. The drying part consisted of a 2 stage drying, a spray dryer followed by a fluidized bed dryer. The air exiting both dryers was filtered by a cyclone, preferably combined with a filter (depending on the exhaust air restrictions).

Life Cycle Inventory Analysis

The inventory analysis involves the compilation and qualitative/quantitative identification of inputs and outputs for a given product system throughout its life cycle or for a single process. The Life Cycle Inventory model has been implemented through GaBi 6. The data collection was related to the functional unit of 1 kg of produced SMP as defined in the goal and scope step. Primary data concerning the flows of the conventional dairy processing plant was collected in first stage through questionnaires and in second stage from similar operations and published data. It was based for a production rate of 200 kg SMP per hour, for a dairy operating 20 hours/day, while the remaining 4 hours/day were set for cleaning. In general, data collection and manipulation included:

- directly measured data, through completion of data sheet questionnaires;
- data from simulation tools (WU, 2015), which forms a credible model of industrial situation;
- specific data for milk industry from Food database of Gabi 6;
- literature data;

The inputs for all the operations were used in the calculation of mass balances linking all the subsystems in the system and estimating the outputs of each subsystem and of the overall system. Processes in the background system were inventoried on the basis of data taken from the dedicated database of the software GaBi 6 (Gabi and Ecoinvent databases). The LCI is fully described in the following paragraphs for both scenarios. Milk from farm is transported to the dairy site by a truck with a capacity of more than 32 t gross weight and 24.7 pt payload for 35 km with different batches (from different farms). Raw milk enters the plant and at first stage heads for bulk storage/mixing, which requires electrical energy of 0.6k Wh per ton per day. Raw milk is lead for separation and standardization to obtain skimmed milk. In this study, a cold separator is utilized, which requires 24.2 MJ/h Motor energy, 35 kg/h operating water and 69 kg/h cooling water. Skimmed milk after cold separation is pasteurized with requirements of 58 MJ/h thermal energy, brine solutions 4300 L/h (it is assumed that 10.000 L are recirculated in the system for 5 years utilization) and 58MJ/h cooling energy for the brine recirculation. In case of a plant implementing RO (Scenario 1) pasteurized skimmed milk is lead for reverse osmosis, a process that requires 12.9 MJ/h electricity and cooling water 0.3 m^3/1000 kg input milk. In case of a processing plant without RO (Scenario 2) this process is excluded. Buffer Silo is utilized for short storage between pasteurization or RO and evaporator and requires no energy due to solid insulation. Pre-heater is applied afterwards and requires 280MJ/h thermal energy and is set prior evaporation. In scenario 2, where no RO is performed the amount of required thermal energy is 509 MJ/h. Concentrated milk enters the 3-stages evaporator which requires 360 MJ/h thermal energy and 72 m^3/h cooling water in case of Scenario 1, where RO occurs. In Scenario 2, 541 MJ/h thermal Energy and 13 L/kg SMP produced cooling water are required. Prior Spray drying, short storage in a Buffer Tank is following and similar to the other buffer equipment requires no energy due to solid insulation. Spray drying is a 2-stage procedure with spray dryer being the first followed by fluidized bed dryer and cyclones. Spray dryer requires 758 MJ/h thermal energy and 90 MJ/h electricity. Fluidized bed dryer requires 53 MJ/h thermal energy and 115 MJ/h electricity, while Cyclone/Bag Filter requires 9.86 MJ/h electricity.
Cleaning in Place (CIP) is performed for the whole system and in total requires 0.889 MJ thermal Energy (Pasteurization, Separation, Evaporator, Dryer), 0.007 kg alkaline detergents, 0.003 kg acidic detergents and 20 kg water for cleaning per kg of SMP product. A transportation of 140 km by truck was assumed for both detergents, while a waste plan for solids with 40% landfilling, 40% incineration and 20% recycling is considered. Waste water treatment plan was considered for the whole system based on readily available plan of Gabi or the treatment of industrial waste water using the chemical reduction/oxidation process.

Life Cycle Impact Assessment (LCIA)

The LCIA identifies and evaluates the amount and significance in the potential environmental impacts arising from the examined scenarios and LCI. Inputs and outputs were assigned to impact categories and their potential impacts were quantified according to characterization factors and categorised in midpoint categories. The choice of the impact categories was based on the recommendations of the Product Environmental Footprint (PEF, 2012) and on the scope of the study. The corresponding impact categories considered include Global Warming Potential (GWP), Acidification Potential (AP), Water Depletion (WD) and Primary Energy Demand (PED), among others.

RESULTS AND DISCUSSIONS

The results of the LCIA are presented in Table 1 by reporting, for both scenarios, the total value of each impact category. The goal was to evaluate the environmental impact of the two scenarios

examined and identify the important differences between the two. From this study the main outcomes can be summarized in the following:

- The GWP (100 years) in Scenario 1 (1.271145183 kg CO2-eq) contributed 11.5% less than the GWP of Scenario 2 (1.417186565 kg CO2-eq.).
- PED in Scenario 1 (23.5607 MJ) contributed 11.2% less than Scenario 2 (26.2075785 MJ).
- POF in Scenario 1 (0.001654927 kg NMOVC eq.) contributed 9.3% less than Scenario 2 (0.001808785 kg NMOVC eq.).
- EP in Terrestrial in Scenario 1 (0.005989512 mol N eq.) contributed 8.7% less than Scenario 2 (0.006509838 mol N eq.)
- AP in Scenario 1 (0.000139725 kg PM2.5-eq.) contributed 4.7% less than Scenario 2 (0.000144701 kg PM2.5-eq.)
- PM/RI in Scenario 1 (0.000139725 kg PM2.5-eq.) contributed 3.6% less than Scenario 2 (0.000144701 kg PM2.5-eq.)
- WD in Scenario 1 (1.17682958 m3) contributed 1% less than Scenario 2 (1.188108965 m3)

The above calculated differences were mainly due to the smaller amounts of energy and water requirements on evaporator and pre-heater process.

The remaining examined impact categories (ARD, Ecotoxicity, Human toxicity cancer, Human toxicity non-cancer, Freshwater Eutrophication and Marine Eutrophication) proved to have identical results with minimal higher impact (less than 0.1%) in case of Scenario 2, while the only impact category that had minimal higher impact in Scenario 1 is ODP. In addition, Spray Drying (spray dryer, fluidized bed and cyclone/bag filter combined) had the highest contribution in GWP, PM/RI, AP, POF, Terrestrial Eutrophication, WD and PED with percentages more from 30% up to 45%. Moreover, the waste water treatment and CIP were the processes with the next higher impact for the above mentioned impact categories with percentages in the range of 14% to 24.2% and 11.7% to 18%, respectively, while the Evaporator came next for the same categories with contribution from 6.8% up to 17.5%.

In ODP for both scenarios the 78% was derived from the waste water treatment process due to iron chloride, and 21% was derived from pasteurization due to cooling energy. As far as ARD was concerned, for both scenarios the major contributors are pasteurization and waste water treatment processes with 49% and 34%, respectively. In case of pasteurization 98% of the impact is due to the cooling energy utilized, while in waste water treatment process 90% is due to iron chloride use. Finally, in Ecotoxicity, Human toxicity (cancer), Human toxicity (non-cancer), Eutrophication (Freshwater) and Eutrophication (Marine) for both scenarios the >98% is derived from the waste water treatment.

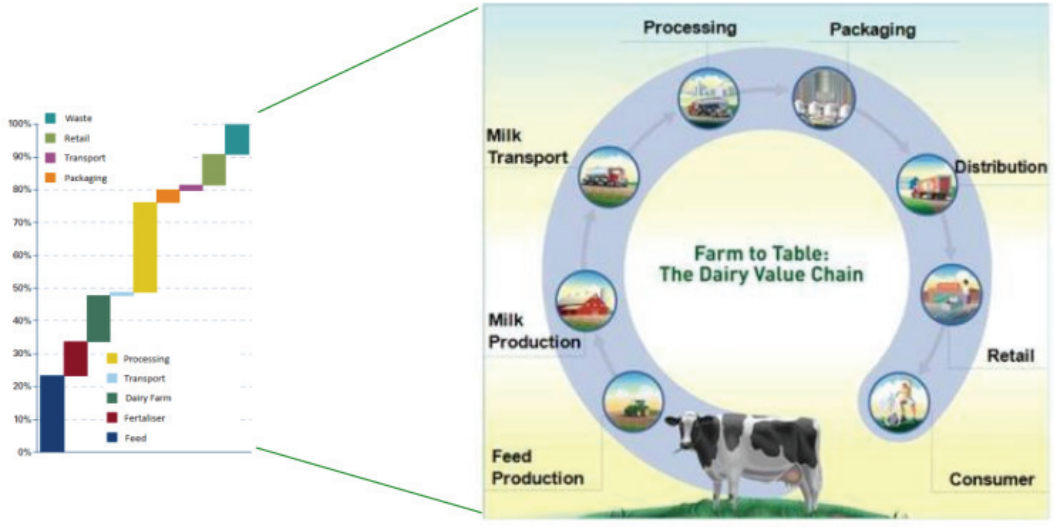

Figure 1. Dairy value chain (right) and energy consumption of segments (left).

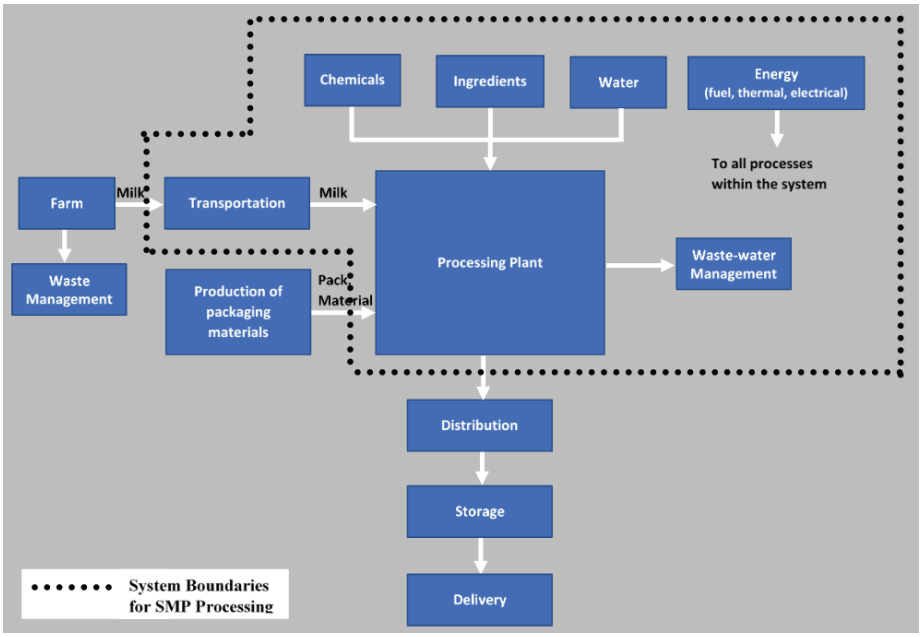

Figure 2. The boundaries of the system examined.

Table 1. Life Cycle Impact Assessment for the production of 1kg of Skimmed Milk Powder under the two Scenarios examined.

Impact Category*	Units	Scenario 1	Scenario 2	Impact of SC2 compared to SC 1
GWP	kg CO2-eq.	1.2711	1.4171	11.5% ↑
ODP	kg R11-eq.	2.28E-08	2.28E-08	≈0%
PM	kg PM2.5-eq.	0.000139725	0.0001447	3.6% ↑
AP	kg SO2-eq.	0.002161627	0.0022636	4.7% ↑
RD	kg Sb eq.	2.16E-06	2.20E-06	1.8% ↑
ECOTOXICITY	CTUe	25.11449628	25.116724	≈0%
HT (Carc.)	CTUh	5.30E-07	5.30E-07	≈0%
HT (non-carc.)	CTUh	4.39E-06	4.39E-06	≈0%
POF	kg NMOVC eq.	0.001654927	0.0018088	9.3% ↑
EP (FreshWater)	kg P eq.	0.000735751	0.0007362	≈0%
EP (Marine)	kg N eq.	0.006022652	0.0060257	≈0%
EP (Terestrial)	mol N eq.	0.005989512	0.0065098	8.7% ↑
WD	m3	1.176829588	1.188109	1% ↑
PED	MJ	23.56070008	26.207579	11.2% ↑

*GWP: Global Potential; ODP: Ozone Depletion Potential; PM: Particulate Matter Formation; AP: Acidification Potential; RD: Resource depletion, mineral and fossil; HT (carc.): Human Toxicity (carcinogenic); HT (non-carc.): Human Toxicity (non-carcinogenic); POF: Photochemical Oxidant Formation; EP: Eutrophication Potential; WD: Water Depletion; PED: Primary energy demand from renewable and non-renewable Resources

CONCLUSIONS

A comparative LCA was performed for two scenarios of the conventional production of SMP restricted to the dairy processing plant, based on the methodology for LCA, as specified in the standardized documents of ISO 14044 and ILCD Handbook. LCA study was performed on Gabi 6 software with databases from within the food industry. The function is the production of Skimmed Milk Powder in a dairy processing plant and the functional unit selected is 1 kg of produced Skimmed Milk Powder unpacked.

Overall, LCA analysis lead to the generic conclusion that inclusion of RO is beneficial in environmental terms. Although this is not a widely accepted practice in current dairy plants, it should be reconsidered. The Spray Drying proved to be the process causing the heaviest environmental burden, followed by CIP and waste water treatment processes. This is mainly due to the energy and water requirements.

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REFERENCES

EC, 2006. Environmental impact of products (EIPRO): analysis of the life cycle environmental impacts related to the total final consumption of the EU25, European Commission Technical Report, NO. EUR 22284 EN2006

Olmez H & Kretzschmar U, 2009. Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact, Food Science and Technology, Vol42, pp686–693

EC, 2008. Environmental improvements potentials of meat and dairy products, EUR 23491 EN

IDF, 2005. Guide on Life Cycle Assessment towards Sustainability in the Dairy Chain, Bulletin 398/2005

IDF, 2010. A common carbon footprint approach for dairy. The IDF guide to standard LCA assessment methodology for the dairy sector, Bulletin 445/2010.

ISO, 2006. International Standard, ISO 14044, Environmental management-LCA Requirements and guidelines

EC, 2010. The ILCD Handbook, Analysis of existing Environmental Impact Assessment methodologies for use in Life Cycle

Assessment. EUR 24708 EN, 2010

BSI, 2008. PAS 2050–Specification for the assessment of the life cycle greenhouse gas emissions of goods and services, 2008.

EC, 2012. Product Environmental Footprint (PEF) Guide

WU, 2015. Personal communication with Ass. Professor Ton van Boxtel from Wageningen University

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NITROGEN AND PHOSPHORUS REMOVAL FROM MUNICIPAL WASTEWATER USING CONSORTIA OF PHOTOSYNTHETIC MICROORGANISMS

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Abstract

In this paper there are presented experiments aiming to investigate the nutrient removal, either from the influent or from efluent of a wastewater treatment plant. The time of contact was 7 days. In order to determine the ratio volume of water – algal biomass we increased the volume of water and the retention time to 4 days. In the study photosynthetic micro-organisms were used, either free or immobilized, aiming to put them to work for consuming the nutrients. In relation mass / volume of 10 grams wet weight of free photosynthetic micro-organisms in 1,000 milliliters input water, removal efficiency of total nitrogen was 15% on the first day, 29% on the second day and 33% on day 4, while total phosphorus removal efficiency was 31% in the first day, 57% on second day and 80% on day 4. In relation mass / volume of 10 grams wet weight of free photosynthetic micro-organisms in 1,000 milliliters effluent water, removal efficiency of total nitrogen was 40% on the first day, 66% on day 2 and 79% on day 4, while the removal efficiency of total phosphorus was 22% on day 1,50% on day 2 and 67% on day 4. Experiments with photosynthetic micro-organisms immobilized in an artisanal cage showed greater efficiency of removing nutrients (nitrate decreased by 70% and phosphorus by 50%) compared to experiments with free photosynthetic micro-organisms (nitrate was removed by 64% and total phosphorus by 39%) subjected to the same experimental conditions.

Key words*: photosynthetic microorganisms, nitrogen, phosphorus, removal, wastewater.*

INTRODUCTION

Wastewaters are unique in their chemical profile and physical properties as compared with fresh and marine waters (Jin et al., 2014). The research of cultivation of algae on waste streams for wastewater treatment was conducted as early as the 1950s, and the symbiotic algal-bacterial relationship in waste stabilization pond was first proposed in which algae were used as tiny aeration devices to provide large amount of O_2 through photosynthesis for aerobic bacteria to oxidize and degrade the organic compounds in wastewaters while heterotrophic bacteria concomitantly release CO_2 and the nutrients needed by microalgae during photosynthesis (Oswald et al., 1957).

Organic matter from wastewater contains, in addition to significant amounts of carbon, also various compounds of N, S, P, H and O characteristics for each stage of the treatment cycle. As the amount of organic C and N decreases, these compounds are different depending on the treatment process used (aerobic or anaerobic). This phenomenon is best reflected in the nature of the resulting final compounds. In aerobic processes, predominate products are oxidized (nitrates, sulfates, etc.) and in anaerobic processes in particular ammonia, methane and sulfides. Resulting nitrogen compounds can be harmful to the quality of the receiving water effluent treatment plants. They may act in natural waters as fertilizer that stimulates the growth of algae and other aquatic plants, causing an accelerated eutrophication (Zarnea, 1994). This nutrient enrichment or eutrophication can profoundly alter the structure and function of aquatic ecosystems, potentially endangering human health, biodiversity and ecosystem sustainability. Therefore, both nitrogen and phosphorus in wastewater should be properly treated or reused thereby reducing their contaminant effects in aquatic ecosystems (An et al., 2003).

The N/P ratio and initial nutrient concentration are considered to be the

significant factors that affect algal growth and nutrient removal efficiency (Hee et al., 1991).
In the past, special attention has been focused on nitrogen and phosphorus removal from municipal wastewater using biological, physical and chemical methods (Blackall et al., 2002; Mallick, 2002). However, some harmful substances cannot be effectively eliminated because the conventional treatment technology used in wastewater treatment plants is insufficient for removing these specific compounds (Ternes, 1998; Saçan and Balcioglu, 2006). More often, the effluents from the wastewater treatment plant fail to meet with the national or local environmental standards. Recent studies have demonstrated that microalgae have a great potential for the removal of nitrogen and phosphorus from wastewater (An et al., 2003; Blackall et al., 2002; Mallick, 2002; Órpeza et al., 2009). Microalgae can be used for treatment of wastewater due to their capacity to assimilate nutrients including both nitrogen and phosphorus (Noüe et al., 1992; Shi et al., 2007).
The aim of this paper is to monitor in laboratory microcosms the nutrient removal ability of selected photosynthetic microorgansims, either free or immobilized, using either input waste water (after mechanichal purge) or effluent water (after traditional activated sludge treatment).

MATERIALS AND METHODS

Isolation of the photosynthetic microorganism consortia was made in BG_{11} medium using for the inoculation either purified water (effluent of the wastewater treatment plant) either waste water (input of the wastewater treatment plant); thus obtained consortia were further enriched by growing them together and mixed in the effluent water of the traditional activated sludge wastewater treatment plant and further in the waste water from sewage. The experiments were performed with either free or immobilized cells. Immobilization of the cells was made on solid substrate in a pyramid mesh made of plastic. Chemical analysis were performed according to ISO standards: spectrophotometric methods - ammonia SR ISO 7150-1/2001, nitrate SR ISO 7890-1:1998, nitrite SR ISO 6777:2002, total phosphorus SR EN ISO 6878:2005.
Laboratory equipment used consists of: Equipment for the determination of the dissolved oxygen - oxygen meter WTW type OXI730; Hach Lange DR 6000 Spectrophotometer - DR6000 spectrophotometer UV-VIS provides peak performance for both routine laboratory tasks and demanding photometric applications. This system is designed to work effectively in professional laboratories. Intelligent software assists the manager laboratory calibration routine quality assurance and custom application development; Hach Lange Thermostat LT 200 - for standard and special digestions; MultiLab mobile luxmeter Model: 545 light measurement with detachable sensor.
Microcosms with free photosynthetic microorganisms: The first experiment was done using a volume of 300 milliliters with effluent from wastewater treatment plant, held in contact with algal biomass; analyzed water was changed from three to seven days, determining the concentrations of nutrients in the initial phase and final phase.
The second experiment was done using a volume of 1500 mL and was kept in contact with microalgae biomass for 4 days to determine a suitable hydraulic retention time.
The third experiment was done in order to compare the efficiency of the microcosms with free photosynthetic microorganisms and microcosms with immobilized photosynthetic microorganisms using a volume of 1500 milliliters kept in contact with microalgae biomass for 2 days.
The fourth experiment lasted 4 days, using influent waste water (after mechanical treatment stage) and effluent (after traditional activated sludge treatment), volume of 1 liter left in contact with microalgae mass of 10 grams (wet weight), at an average temperature of 22 degrees Celsius, artificially illuminated by a fluorescent tube of 60 cm with a luminous intensity of 1150 lumens about 91.5 candelas for 12 hours, alternating with 12 hours of dark.
Microscopic observations: Photosynthetic microorganisms have been inspected at

optical microscope by using alkaline methylene blue as a metachromatic regent to test the presence (or absence) of intracellular deposits of phosphorus, in the form of polyphosphate, and Lugol to test the presence (or absence) of polysaccharides.

RESULTS AND DISCUSSIONS

The first experiment was done using a volume of 300 milliliters with effluent from wastewater treatment plant North Constanta, in an Erlenmeyer flask, held in contact with algal biomass; We found that samples of effluent loaded with nitrogen and phosphorus mixed with microorganisms microalgae and cyanobacteria for several days had a significant efficiency lowering concentrations of the nitrogen and phosphorus; multiple experiments were made in order to determine the best contact time, so contact time was of 3 days in some experiments, 5 days for others and longest contact time was of 7 days, determining the concentrations of nutrients in the initial day and final day.

In the case of samples left in contact for three days, efficiency of phosphorus removal was 99 % and the percentage of nitrogen removed was 73 %, and in the case of the samples left in contact for five days removal of nutrients in the medium was 96 % in the phosphorus and 99 % in the case of nitrate. In experiments with longer contact were found less favorable outcomes phosphorus removal of only 87 % and 100 % nitrogen.

In the second experiment we used the effluent of the wastewater treatment plant North Constanta, in a flask with 1,500 milliliters volume and was kept in contact with microalgae biomass for 4 days to determine a suitable hydraulic retention time. Initially the effluent was charged with 8.9 mg/L nitrate, 0.484 mg/L ammonium and 0.844 mg/L total phosphorus and after 2 days nitrate decreased by 94%, ammonium decreased by 93% and total phosphorus by 54%. After another 2 days (4 days after contact) decreased by 99.2% nitrate, ammonium decreased by 97% and total phosphorus decreased by only 61% difference between the concentration of day 2 and day 4 being very close.

The third experiment was made on 2 samples of water from the effluent of the treatment plant of 1500 milliliters left in contact with free photosynthetic microorganisms (Table 1), and with an immobilized cells in a pyramid mesh made of plastic (Table 2).

Table 1. – Nitrogen and phosphorus removal by free photosynthetic microorganisms (experiment 3)

	Day 0	Day 1	Day 2	Removal ratio %
NO_2 (mg/L)	0.027	0.155	0.01	100
NO_3 (mg/L)	16.2	2.62	2.61	83
NH_4 (mg/L)	0.108	0.007	0.001	99
P total (mg/L)	0.237	0.134	0.096	60

Table 2. - Nitrogen and phosphorus removal by photosynthetic microorganisms immobilized in pyramid mesh made of plastic (experiment 3)

	Day 0	Day 1	Day 2	Removal ratio %
NO_2 (mg/L)	0.027	0.753	0.022	100
NO_3 (mg/L)	16.2	5.26	2.55	84
NH_4 (mg/L)	0.108	0.001	0.001	99
Ptotal (mg/L)	0.237	0.047	0.037	85

The fourth experiment lasted 4 days, using influent wastewater (after mechanical treatment) and effluent (after traditional activated sludge treatment), volume of 1 liter left in contact with microalgae mass of 10 grams (wet weight), at an average temperature of 22 degrees Celsius, artificially illuminated by a fluorescent tube of 60 cm with a luminous intensity of 1150 lumens about 91.5 candelas for 12 hours, alternating with 12 hours of dark. The content of chemical elements in the water sample was big enough, especially the content of phosphorus and ammonia (directly influences the concentration of total nitrogen), so removal efficiency of ammonia in the sample was not large in terms of the experiment, probably would have needed a bigger amount of biomass a higher yield. The results are shown in Table 3 and 4, respectively.

Table 3. – Experiment 4 - Nitrogen and phosphorus removal by free photosynthetic microorganisms using influent water (after mechanical purge)

	Day 0	Day 1	Day 2	Day 4	Removal ratio %		
					Day 1	Day 2	Day 4
NH_4 (mg/L)	21,7	20,0	16,7	16,3	7,83	23,0	24,9
NO_2 (mg/L)	0,174	1,06	0,131	0,011	-	24,7	93,7
NO_3 (mg/L)	5,38	1,31	1,21	1,12	75,7	77,5	79,2
P total (mg/L)	5,12	3,56	2,21	1,05	30,5	56,8	79,5
COD (mg/LO_2)	90,6	68,5	63,2	51,6	24,4	30,2	43,0
N total (mg/L)	20,4	17,4	14,5	13,8	14,7	28,9	32,4

As seen in table 3, removal efficiency of total nitrogen was of 15% on the first day, 29% in the second day and 33 % on day 4, while the removal efficiency of phosphorus was 31% on day 1, 57% in day 2 and 80% on day 4.
The remaining of the nutrients have been removed successfully yield over 50%, taking into account our considerable percentage of high concentrations of nutrient from the input water (previously treated only mechanical).
It appeared that there were some inhibitory factors in the initial stage. This phenomenon was also found during the first days of growth of *Chlamydomonas reinhardtii* in wastewater (Kong et al., 2010). Wastewater often has high concentration of nutrients, much of the N in the form of NH4 -N which can inhibit algal growth at high concentration (Wrigley and Toerien, 1990). In addition, the presence of toxic heavy metals and organic compounds in wastewater, especially in industrial wastewater, is another critical inhibition factor for microalgal growth (Chinnasamy et al., 2010).Was observed a significant decrease in the chemical oxygen demand (COD), reaching 43 % in day 4.

In a study using *Chlorella* sp. (Changfu Wang et al.2013) showed higher removal ratios of total nitrogen in influent wastewater than effluent wastewater. The removal rate of NH4 -N was higher than 83% in influent wastewater. The removal rate of total phosphorus was of 90% in influent and 60% in effluent wastewaters.
Experiment have continued changing just the matrix to the effluent of the wastewater treatment plant North Constanta, to see the efficiency for a quantity of 10 grams algal biomass in a liter of effluent water (less loaded with nutrients). We used only free photosynthetic microorganisms in form of flakes, at an average temperature of 22 degrees Celsius, artificially illuminated with fluorescent light with a luminous intensity of 1150 lumens approximately 91.5 candelas, for 12 ore alternating with 12 hours dark. The concentrations of nutrients in this water ware much lower. The nitrate concentration is the only one that's higher because of nitrification stage, the water suffered in the conventional treatment process with activated sludge.

Table 4. – Experiment 4 - Nitrogen and phosphorus removal by free photosynthetic microorganisms using effluent water (after traditional activated sludge treatment)

	Day 0	Day 1	Day 2	Day 4	Removal ratio %		
					Day 1	Day 2	Day 4
NH_4 (mg/L)	1,70	0,540	0,466	0,280	68,2	72,6	83,5
NO_2 (mg/L)	0,077	0,340	0,046	0,002	-	40,3	97,4
NO_3 (mg/L)	13,1	8,82	4,34	3,96	32,7	66,9	69,8
P total (mg/L)	0,970	0,756	0,488	0,321	22,1	49,7	66,9
COD (mg/L O2)	20,20	4,98	3,85	3,04	75,3	80,9	85,0
N total (mg/L)	4,45	2,68	1,52	0,953	39,8	65,8	78,6

Nutrient removal efficiency was 79% for total nitrogen and 67% for total phosphorus; also chemical oxygen demand (COD) was reduced by 85%, as seen in Table 4. In previous research, *Chlorella vulgaris* was inoculated with final effluent of wastewater treatment

plant and 60% of nitrogen and phosphorus concentrations were removed from the system in 2 days (Rawiwan B. et al. 2012).

The production of biomass and growth rate of *Chlorella vulgaris* - reached a maximum cell density of 11.5x106 cells mL^{-1} and growth rate of 0.54 d^{-1} using KNO_3 as nitrogen source (Jeanfils J. et al 1993). Other studies, reported a lower density of 16 x106 cells mL^{-1} with similar inoculums size (1x 106 cells mL^{-1}) (Lau P.C et al 1995). Similarly, Lau et al. 1994 reported a growth rate for *C.vulgaris* of 0.364 d^{-1} , and Lau P.C. et al. 1997 reported a cell density of 26.5x106 cells mL^{-1} and growth rate of 0.362 d^{-1}, both studies used Bristol medium with 40 mg L^{-1} NO_3-N.

We used an oxygen meter to measure changes in the concentration of dissolved oxygen from water samples , both in light conditions (due to photosynthesis) and in the dark, by measuring the consumption of oxygen caused by the breath of the aerobic microbiota present in the samples.

We analyzed samples of effluent water, water that came from activated sludge bioreactors where oxygen is blown through a ventilation system so the water sample already have a fairly high content of dissolved oxygen. The water temperature during the tests was 19 degrees Celsius. As a result of the calculations carried out under light conditions we achieved an increase in oxygen concentration of approximately 0.02 mg/L O_2 per minute, reaching to be 2.20 mg/L O_2 in 10 minutes, starting at a concentration of 2 mg/L dissolved O_2; We alternated periods of darkness followed by observing the drop in the dissolved oxygen concentration of about 0.2 mg / L O_2 in 10 minutes. Oxygen concentration decreased more slowly in the dark than the speed with which occurred in light conditions. Once we changed the temperature above 22 degrees Celsius, analyzing the dissolved oxygen concentration we found slow growth in light conditions, increasing only 0.07 mg / L O_2 in 10 minutes.

Microscopic observations were, so far, focused on the presence (or absence) of polyphosphate inclusions and on the presence or absence of amidon/glycogen inclusions.

In Figure 1 (a and b) one can see the images of the photosynthetic consortium treated with alkaline methylene blue for (specific) labelling of polyphosphate granules (and other acidic structures).

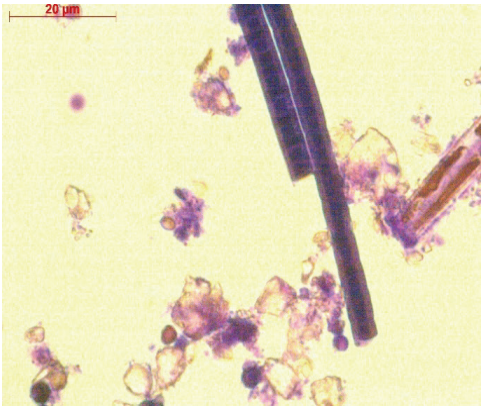

Figure 1a - Photosynthetic microorganisms grown in BG_{11}, labelled with alkaline methylene blue.

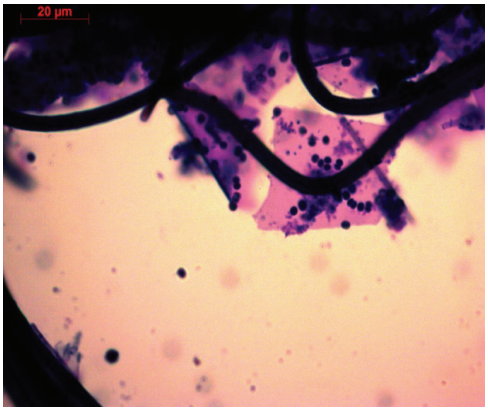

Figure 1b - Photosynthetic microorganisms grown in effluent water, labelled with alkaline methylene blue.

In both images one can see the absence of metachromatic (labeling of polyphosphate granules which suggest that the consortium do not accumulate polyphosphate in either of the growing condition (BG_{11} or effluent water). However, metachromatic label is visible in fig 5a, probably for acidic polysaccharides.

When it comes to polysaccharide inclusions, either glycogen or starch, one can see significant difference between the same consortium grown in BG_{11} or effluent water (Figure 2 a and 2 b).

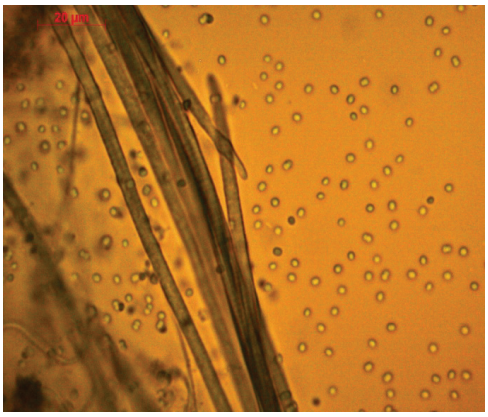

Figure 2a - Photosynthetic microorganisms grown in BG_{11}, labelled with Lugol solution, for polysaccharides inclusions.

One can see the absence of visible polysaccharides inclusions both in unicellular and filamentous photosynthetic microorganisms, when grown in BG_{11} medium

Figure 2b - Photosynthetic microorganisms grown in effluent water, labeled with Lugol solution for polysaccharides inclusions.

One can see the presence of visible polysaccharides inclusions filamentous photosynthetic microorganisms, when grown effluent water, suggesting that the intracellular accumulation of polysaccharides inclusions is sustained in these conditions of cultivation.

The signification of these results deserves further attention, including quantification of polyphosphate and of polysaccharides inclusions, in order to understand the mechanisms involved in N and P removal from effluent water by photosynthetic microorganisms.

CONCLUSIONS

Optimum contact time is of maximum 4 days, the most drastically decrease being recorded in the first two days; after two days, the changes in the concentrations of nutrients are rather low. A contact time of more than 4 days seems to influence the concentration of total phosphorus in the water, this starting to increase, the main cause of this could be the release of phosphorus by (injured/ dead) photosynthetic micro-organisms.

The efficient removal of the nutrients at the laboratory level using photosynthetic micro-organisms is both available on effluent (with low concentrations of nitrogen and phosphorus) but also on the influent if the concentration of biomass is higher enough. For 10 g WCW/1000mL influent water the removal efficiency of total nitrogen was 15% on the first day, 29% on the second day and 33% on day 4, while total phosphorus removal efficiency was 31% in the first day, 57% on second day and 80% on day 4.

For 10 g WCW/1000mL effluent, removal efficiency, calculated on initial concentration basis, of total nitrogen was 40% on the first day, 66% on day 2 and 79% on day 4, while the removal efficiency of total phosphorus was 22% on day 1, 50% on day 2 and 67% on day 4.

Experiments with photosynthetic micro-organisms immobilized in an artisanal cage showed greater efficiency of removing nutrients (nitrate decreased by 70% and phosphorus by 50%) compared to experiments with free photosynthetic micro-organisms (nitrate was removed by 64% and total phosphorus by 39%) subjected to the same experimental conditions.

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REFERENCES

An J.Y., S.J. Sim, J.S. Lee and B.W. Kim, 2003, Hydrocarbon production from secondarily treated piggery wastewater by green alga Botryococcus braunii. J. Appl. Phycol., 15, 185-191.

Blackall, L.L., G.R. Crocetti, A.M. Saunders and P.L. Bond, 2002, A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants. Antonie Leeuwenhoek, 81, 681-692.

Changfu W., Xiaoqing Y., Hong and Jun Yang, April 2013, Nitrogen and phosphorus removal from municipal wastewater by the green alga Chlorella sp., Journal of Environmental Biology, Vol 34, 421-425.

Chinnasamy, S., A. Bhatnagar, R.W. Hunt and K.C. Das, 2010, Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Biores. Technol., 101, 3097-3105.

Hee. M. Oh and Rhee G. Y., 1991, A comparative study of microalgae isolated from flooded rice paddies; Light limited growth, C fixation, growth efficiency and relative N and P requirement, J. Appl. Phycol., vol. 3, pp. 211-220.

Jeanfils J., Canisius M. F., and Burlion N., 1993, Effect of high nitrate concentration on growth and nitrate uptake by free – living and immobilized Chlorella vulgaris cells. Journal of applied phycology. vol. 5, pp. 369-374.

Jin L., Zheng S. and Henri G., 2014, Potential Applications of Microalgae in Wastewater Treatments.

Kong, Q.X., L. Li, B. Martinez, P. Chen and R. Ruan, 2010, Culture of microalgae Chlamydomonas reinhardtii in wastewater for biomass feedstock production. Appl. Biochem. Biotechnol., 160, 9-18.

Lau P.C., Tam N. F. Y., and Wong Y. S., 1995, Effect of algal density on nutrient removal from primary settled wastewater, Environmental Pollution, vol. 89, pp. 59-66.

Lau P. S., Tam N. F. Y., and Wong Y. S., 1994, Effect of organic-N sources on algal wastewater treatment system. Resour. Conser. Recyc. vol. 11, pp. 197-208.

Lau P. S., Tam. N. F. Y., and Wong. Y. S. 1997, Wastewater nutrients (N and P) removal by carrageenan and alginate immobilized Chlorella vulgaris. Environmental Technology. vol. 18, pp. 945-951.

Mallick, N., 2002, Biotechnological potential of immobilized algae for waste water N, P and metal removal: A review. Biometals, 15, 377-390.

Noüe, J., G. Laliberté and D. Proulx, 1992, Algae and waste water. J. Appl. Phycol., 4, 247-254.

Órpeza, R., M.E. Martínezb, G. Hodaifac, F.E. Yousfi, N. Jbarib and S. Sánchez: Growth of the microalga Botryococcus braunii in secondarily treated sewage. Desalination, 246, 625-630 (2009)

Oswald WJ, Gotaas HB, Golueke CG, Kellen WR, 1957, Algae in waste treatment. Sewage Ind Wastes 29: 437-455.

Rawiwan B., Gyu T. S., Da R. P., Chung Y. S., November 2012, Microalgae Photobioreactor for Nitrogen and Phosphorus Removal from Wastewater of Sewage Treatment Plant, International Journal of Bioscience, Biochemistry and Bioinformatics, Vol. 2, No. 6.

Saçan, M.T. and I.A. Balcioglu, 2006, A case study on algal response to raw and treated effluents from an aluminum plating plant and a pharmaceutical plant. Ecotox. Environ. Safe., 64, 234-243.

Shi, J., B. Podola and M. Melkonian, 2007, Removal of nitrogen and phosphorus from wastewater using microalgae immobilized on twin layers: An experimental study. J. Appl. Phycol., 19, 417- 423.

Ternes, T.A., 1998, Occurrence of drugs in German sewage treatment plants and rivers. Water Res., 32, 3245-3260 (1998).

Wrigley, T.J. and D.F. Toerien, 1990, Limnological aspects of small sewage ponds. Water Res., 24, 83–90.

Zarnea G., 1994, Tratat de microbiologie generală, Editura Academiei Republicii Socialiste România, volumul 5, pp 1039-1045.

SR ISO 7150-1/2001

SR ISO 7890-1:1998

SR ISO 6060/1989

SR EN ISO 6878:2005

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GASTROPROTECTIVE POTENTIAL OF FAGUS SYLVATICA LEAVES EXTRACTS ON STRESS-INDUCED ULCER MODEL ON RATS

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Abstract

Romanian folk medicine recommends Fagus sylvatica L. *bark and leaves derived products (infusions, decoctions and raw powders) for various skins, respiratory and digestive ailments. The presented work was based on this data, and it aimed to evaluate gastroprotective potential of one standardized product prepared from beech leaves collected in July, by testing it on stress-induced ulcer model on rats. Beech leaves derived product (FA) has been designed as a combination of two polar extracts: aqueous extract as source of polysaccharides compounds and ethanolic (defatted) extract as source of polyphenols compounds resulting in a final standardized product (powder) with exactly 2% (w/w) total flavones content expressed as rutin equivalents. The obtained results, the total length (mm) of superficial, medium and deep gastric lesions of exposed groups versus control group indicated that while the pre-treatment with chemical reference product Ranitidine, an inhibitor of histamine receptor (RH2), assured gastric protection percentages of 59%, 54% and 89%, the pre-treatment with beech leaves derived product (FA) demonstrated gastric protection percentages of 38%, 62% and 96% (n=6; p<0.05) on superficial, medium and deep lesions. Therefore, our results confirm gastroprotective potential of beech leaves derived products (precisely aqueous and ethanolic defatted extracts) suggesting potential use for the development of new phytomedicines targeted at the digestive system.*

Key words*: Fagus sylvatica L. folium, gastroprotective activity.*

INTRODUCTION

Romanian folk medicine (the information comes from the northern part of Romania) recommends the consumption of a few beech leaves (*Fagus sylvatica* L.) collected from spring to early summer, over 3 to 5 days, as an effective treatment of epigastric pain occurring in the spring season.

Japanese researchers (Tsutomu et al., Patent Jp. 05,139,972) have indeed proved the effectiveness of extracts from beech leaves against *Helicobacter pylori* gram-negative bacteria, the main cause for gastric lesions and subsequent chronic progression up to tumour process initiation as it is known (Konturek et al., 2006). Concerning the chemical nature of the active compounds, the same studies (Tsutomu et al., Patent Jp. 05,139,972) related anti-*Helicobacter* activity of beech leaves extracts with the presence of some epi(gallo)-catechin compounds similar to those found in green tea (*Camellia sinensis* L.) products.

On the other hand, very recent metabolomic studies (Cadahía et al., 2015) on leaves of *Fagus sylvatica* L. have revealed very complex chemical composition abounding in polyphenols compounds including numerous flavonoids derivates described with gastroproptective activity (de Lira Mota et al., 2009) such as naringenin, quercetin, cyanidol, apigenin, myricetin and luteolin derivates. Our previous studies (Pirvu et al., 2013) on beech leaves plant pieces indicated the dynamic of caffeoylquinic acid, kaempferol, apigenin, quercetin and catechin derivates along the vegetation time as well as flavonoids abundance in spring and early summer time; there were estimated total flavones content measuring from 12 to 9 mg (expressed as rutin equivalents) *per* 1 gram fresh material collected in May and, respectively, July period (±5%, w/w).

Concerning the pharmacological data, beech leaves extracts have been proved with antimicrobial (Pirvu et al., 2014) and anti-tumor (Frederich et al., 2009) properties.

Our previous studies (Pirvu et al., 2013) have also revealed high antioxidant potency of beech leaves polar extracts *versus* augmented pro-oxidant properties of non-polar extracts (dichloromethane fraction from whole ethanolic extract).

In view of these, this work was aimed to evaluate gastroprotective potential of one product prepared from beech leaves collected in July, by testing it on rats with stress-induced gastric lesions. The test product has been designed as a combination of two polar extracts: aqueous extract as source of polysaccharide compounds and ethanolic (defatted) extract as source of polyphenol compounds resulting in a final standardized product relating to total flavones content (as key compounds with gastroprotective potency).

The final interest is to better valorise Romanian folk medicine data and our previous results which indicated high antioxidant properties of beech leaves polar extracts.

MATERIALS AND METHODS

Plant material description: *Fagus sylvatica* L. *folium* (*Fagaceae* family) vegetal material was purchased from Romanian Carpathian Mountains, the Northern region called Bucovina. Taxonomic identification has been fulfilled by the team of botanists at the *National Institute of Chemical-Pharmaceutical Research and Development* (ICCF), Bucharest, Romania. Voucher specimens (FSPA20-25) are deposited in ICCF *Plant Material Storing Room*. Beech leaves vegetal material has been harvested early July, shade dried then minced as a fine plant powder.

Extracts' preparation: Technological studies started from the idea of a final product combining beech leaves polar extracts (our previous studies indicated the pro-oxidant potency of the non-polar compounds/fraction), polysaccharides and polyphenols compounds respectively, with potential citoprotective and antioxidant properties, thus assuring the chemical condition of a potential gastroprotective product.

This way, three charges of one hundred and fifty (150) grams beech leaves powder each were extracted with 1500 mL of distilled water at boiling temperature under continuous agitation system. The resulting aqueous extracts were (separately) concentrated at low pressure (*Büchi* Rotary Evaporator) and then atomized (BŰCHI Mini Spray Dryer B-290, *Switzerland*). Three brown powder products were obtained (codified F) further estimated with 50-55% total polysaccharides content (gravimetrical estimation), 0.91% total flavones content (rutin equivalents) and 1.18% total phenols content (gallic acid equivalents), (mean values, w/w).

The vegetable waste resulted after the first (hot water) extraction was further extracted with 900 mL ethanol (96%, v/v) and the resulting ethanolic extracts were concentrated at the residue. The three residues (corresponding to the three laboratory charges) were (separately) dissolved into 100 mL of distilled water then extracted with dichloromethane solvent (3x200 mL each operation, over night) in order to remove the non-polar compounds. The resulting aqueous fractions were also atomized. Three red-brown powder products have been obtained (codified A) further estimated with 5.10% total flavones content (rutin equivalents) and 12.40% total phenols content (gallic acid equivalents), (mean values, w/w).

F and A products were then combined in order to obtain the final standardized product (PA); practically, to a fixed amount of F product has been added the necessary amount of A product in order to obtain the exactly 2g% total flavones content (rutin equivalents, w/w).

Chemicals, reagents and references: Chemicals (aluminium chloride, sodium acetate, sodium carbonate), reagents (Folin-Ciocalteau, Natural Product and PEG4000 identification reagents - NP/PEG) and solvents (methanol, ethanol, dichloromethane, toluene, formic acid, acetic acid, ethyl acetate) as well as the *reference products* rutin (min. 95%), hyperoside (>97%), quercitin-3-O-xyloside (>97%), apigenin (>97%), cosmosiin (97%), vitexin (>96%), isovitexin (>98%), vitexin-2"O-rhamnoside (>98%), apiin (>99%), chlorogenic acid (>95%), caffeic acid (99%) were purchased of *Fluka* and *Sigma-Aldrich* Co (Bucharest, Romania).

An internal standard consisting in green tea (*Camellia sinensis* L. *folium*) 70% (v/v) ethanol extract has used too.

Qualitative analytical determination: Studies were performed according to *Plant Drug Analysis* (Wagner H. and Bladt S., 1996) and *High-Performance Thin - Layer Chromatography for the Analysis of Medicinal Plant* (Reich E. and Schibli A., 2008) methodologies, standard settings for polyphenols (system A) and catechins (system B) assessment: ethyl acetate - acetic acid - formic acid – water/100:12:12:26 (system A) and toluene – formic acid – acetone/9:9:2 (system B); plates (10x10) of Silica gel 60F254 – HPTLC (Merck, Darmstadt, Germany*);* reference compounds, *Sigma-Aldrich* polyphenols, were prepared as 10^{-3}M solutions in ethanol 70% (v/v).

Test products, beech leaves derived products F and A respectively, were prepared as 2g% (w/v) solutions in ethanol 70% (v/v) then filtered in vacuum system (filter paper blue). Volumes measuring from 1 to 5 µL test vegetal and test reference samples were loaded as 8 mm band length in the 10 x 10 cm Silica gel 60F HPTLC plate using Hamilton syringe and Linomat 5 instrument (CAMAG, Muttentz, Switzerland). The loaded plates were kept in TLC twin developing chamber at 18-19°C with respective mobile phase (system A and system B) up to 90 mm. The dried plates were exposed at 254 nm, then immersed into identification reagents (NP/PEG) and studied at 366 nm in order to asses the polyphenols content (yellow, orange, red, green, blue-green, or blue spots on black, non-fluorescent plate); catechins compounds were studied by the exposure at 254 nm since they appear as black spots on green fluorescent plate. Spots' assignments have been done by using reference compounds and literature data.

Estimation of Total Flavones content: Total flavones content was measured using *Romanian Pharmacopoeias* (1993) method. Briefly, 5.000 g of each, F, A and FA beech leaves derived products (powders) were twice (heat assisted) extracted with 50 mL of 50% (v/v) ethanol solvent and the resulting ethanolic extracts completed at 100 mL final volume with 50% (v/v) ethanol solution thus obtaining the *test solutions*. Three aliquots of 50 to 100 µL *test solution* (F, A and FA) were treated with 600 µL of 2.5% $AlCl_3$ and 1000 µL of 10% CH_3COONa then accurately finished at 5000 µL with (50%, v/v) ethanol. Mixtures were incubated at room temperature for 30 minutes and the absorbance of reactions at maximum absorption wavelength (386 nm) measured. Total flavones content was estimated by using rutin (ref.) standard calibration curve and the results were expressed as g total flavones (rutin equiv.) *per* 100 g vegetal product (r^2=0.9998).

Estimation of Total Phenols Content: Total phenols content was measured using *Folin-Ciocalteau* and *Romanian Pharmacopoeias* (1993) method. Briefly, three aliquots of 25 to 50 µL *solution test* (F, A and FA) were treated with 200 µL of *Folin-Ciocalteau* reagent and accurately finished at 5000 µL volumetric flasks with 5% (w/v) Na_2CO_3. Flasks were mixed and left in the dark place at room temperature for 5 min then the absorbance at 750 nm was measured. Total phenols content was estimated by using gallic acid (ref.) standard calibration curve (r^2=0.9997) and the results were expressed as g total phenols (gallic acid equivalents) *per* 100 g vegetal product.

Stress-induced rat ulcer model experiment description: Pharmacological *in vivo* studies were carried out on *Wistar* Albino rats, male, of 180 - 200 g purchased from the animal house of the Faculty of Natural and Agricultural Sciences, Constanta, Romania. The animals were maintained in a controlled environment at 22±2°C and 55±10% humidity with 12h light–dark cycle and fed with standard pellet food and water *ad libitum*. The stress-induced rat ulcer model experiment was developed as follows: animals were fasted over night; the next morning, control group animals were immobilized and immersed into cold water on dorsal position for four hours (preliminary studies indicated four hours as being the properly time necessary to achieve various type of gastric lesions and no mortality cases); in the case of treated groups, one hour before the stress experiment the animals received *per oral* (*p.o.*) the respective doses of test products (beech leaves derived product PA and chemical reference Ranitidine). At the end, control animals and treated animals were anesthetised and euthanized, the stomachs excised and washed, then the total length of each type of gastric lesions (superficial, medium and deep gastric lesions) were measured; the obtained values (mm) were compared, thus resulting the final gastric protection results expressed in percentages (GP%).

Statistical analysis: The total length (mm) of each type of superficial, medium and deep gastric lesions, were calculated as mean ± SD, n=6; differences were significantly different (Student's *t* test) if $p<0.05$; results were expressed as percentages (GP%).

RESULTS AND DISCUSSIONS

Analytical screening results

Figure 1 shows qualitative (HP-TLC) aspects referring to polyphenols content (System A setting study) of the two test products, aqueous extract enriched in polysaccharides compounds (F) and ethanolic defatted extract enriched in polyphenols compounds (A), obtained through processing *Fagus sylvatica* L. *folium* raw material collected early July by comparing with several reference products mixtures (ref.), polyphenols compounds.

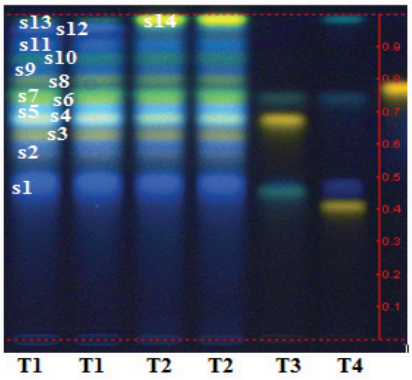

Figure 1. (HP) TLC aspects of beech leaves polar extracts comparatively to reference compounds (ref.). Tracks 1, beech leaves aqueous extract (F product, duplicate sample); Tracks 2, beech leaves ethanol (defatted) extract (A product, duplicate sample); Track 3, vitexin-2"O-rhamnoside, hyperoside, vitexin and caffeic acid mixture (ref.); Track 4, rutin, chlorogenic acid, cosmosiin and kaempferol mixture (ref.)

As shown in Figure 1, the two beech leaves polar extracts, aqueous extract (Tracks 1, duplicate sample) and ethanolic defatted extract (Tracks 2, duplicate sample) indicated similar polyphenol content with the mention that, differing on the aqueous extract, ethanolic extract favoured the extraction of important amounts of quercetin aglycone (the yellow fluorescent spot, s14, at the FRONT region). Also, numerous caffeoylquinic acids were revealed: caffeic (s12), chlorogenic (s1), neochlorohenic (s2) and isochlorogenic (s5, s9, s11) acids respectively. Quantities of apigenin (s7, s8, s10), quercetin (s3, s6) and kampferol (s4) glycosides along with apigenin (s13) and quercetin (s14) aglycones have also been revealed.

Aimed to observe the catechins presence, system B setting study (Figure 2**)** and comparison with the internal standard consisting in green tea (*Camellia sinensis* L. *folium*) 70% (v/v) ethanolic extract indicated the occurrence of at least two catechin derivates, epicatechin (*Rf*~0.68) and epigallocatechin (*Rf*~0.52), in both polar extracts (F product and A product).

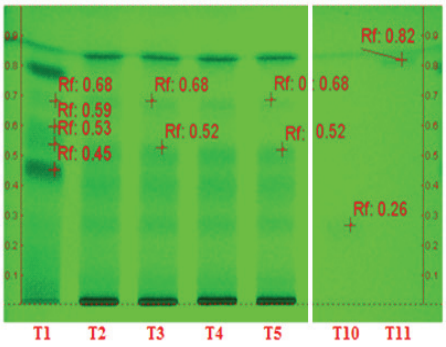

Figure 2. (HP)TLC aspects of beech leaves aqueous extract (F product) and beech leaves ethanolic extract (A product) face to green tea 70% (v/v) ethanolic extract and two caffeoylquinic acids (reference products). Track 1, green tea ethanolic extract (the internal standard); Tracks 2 - 3, beech leaves aqueous extract (F product); Tracks 4 - 5, beech leaves ethanolic extract (A product); Track 10, chlorogenic acid (ref.); Track 11, caffeic acid (ref.)

Also, green tea ethanolic extract confirmed the occurrence of the four catechin derivates, as the literature data reports [8]: epicatechin (*Rf*~0.68), epigallocatechin (*Rf*~0.53), epicatechin gallate (*Rf*~0.59) and epigaleocatechin gallate (*Rf*~0.45).

Therefore, chemical qualitative analyses indicated two similar, but not identical, beech leaves polar extracts, differing as of quercetin aglycone, found in ethanolic extract (A) only.

These extracts were further quantitatively analysed thus resulting the algorithm of the final standardized product (FA) with exactly 2g% (w/w) total flavones content expressed as rutin equivalents (see *Extract's preparation*).

***In vivo* pharmacological results**

The main purpose of pharmacological studies was the evaluation of gastro-protective potential of beech leaves derived product (FA) on gastric lesions obtained *via* stress-induced ulcer model on rats.

Concerning the stress-induced rat ulcer model experiment *in vivo*, it is well known that immobilization on dorsal position associated with low temperature both lead to the decrease of microcirculation in gastric tissue.

Exposing the vulnerable gastric mucosa to augmented quantities of gastric acid (induced by stress state) results in various gastric lesions, from superficial to medium and deep lesions, explaining stress-induced rat ulcer model on rats.

The three animals' groups were as follows:

- *Group 1* (control group, C) - fasted animals were stressed through immobilization and immersion into cold water on dorsal position, then euthanized and the total length (mm) of each gastric lesion (superficial, medium and deep lesion) measured;
- *Group 2* (group treated with chemical reference Ranitidine, R) - one hour before the stress experiment, fasted animals received (*p.o.*) the human corresponding dose of Ranitidine (27 mg/kg body), then they underwent the stress experiment;
- *Group 3* (group treated with beech leaves derived product, FA) - similarly, one hour before the stress experiment, fasted animals received (*p.o.*) the vegetal test product (FA) in dose of 500 mg/kg body (the respective dose was selected based on previous exploratory studies (Pirvu et al., 2015) and literature data as well (Eswaran et al., 2010; El-Shenawy, 2009) then they underwent the stress experiment.

At the end, the treated animals were anesthetized and euthanized, and the total length (mm) of each type of gastric lesion was measured.

Thus, by comparing the total length (mm) of each, superficial (mucosal irritations), medium (haemorrhagic) and deep (necrotic) lesions of the treated groups (R and FA) with the total length (mm) of superficial, medium and deep lesions of the control group (C) gastro-protective activity (GP%) has been estimated.

Results are summarized in Figure 3.

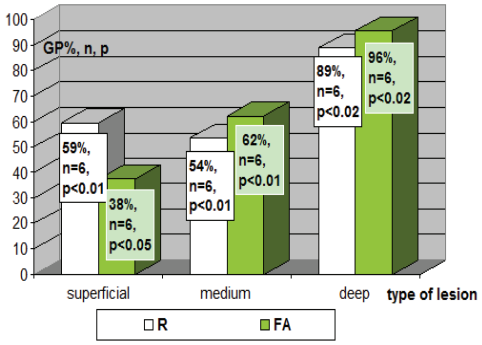

Figure 3. Gastroprotective activity of *Fagus sylvatica* L. *folium* derived product (FA) compared to chemical reference Ranitidine (R)

As shows in Figure 3, the pre-treatment with chemical reference product/Ranitidine (R) assured gastric protection percentages (GP%) of 59% ($p<0.01$), 54% ($p<0.01$) and 89% ($p<0.02$) on superficial, medium and deep gastric lesions ($n=6$); the pre-treatment with beech leaves derived product (FA) indicated gastric protection percentages of 38% ($p<0.05$), 62% ($p<0.01$) and 96% ($p<0.02$) on the same type of mucosal lesions ($n=6$). Therefore, the comparison with chemical reference, Ranitidine (a well known inhibitor of histamine receptor/RH2 and subsequently gastric acid synthesis controller) suggests gastric mucosa protective properties of polar extracts isolated from beech leaves plant part. Besides, the presence of a fine *veil* of vegetal product crossing the entire gastric mucosa has been noticed, the most probable due by tanning properties of catechin derivates; these observations could explain the short time of treatment recommended by Romanian folk medicine practice.

Together, gastroprotective potential of beech leaves derived products is sustained by present data (stress-induced ulcer model on rats) suggesting gastric mucosa protection against acid secretion but also through previous studies demonstrating high antioxidant potency of beech leaves polar extracts (Pirvu et al., 2013) as well as by means of Japanese researchers' experiments demonstrating their efficacy in controlling *H. pylori* infection (Tsutomu et al., Patent Jp. 05,139,972).

Other tree leaves extracts demonstrated with gastroprotective activity are those from *Cinnamomum tamala* T. Nees & Eberm species (*Lauraceae* family) (Eswaran et al., 2010),

traditionally used in Indian System of Medicine in order to improve numerous digestive system ailments; it was proved that leaves extract of *C. tamala* in doses of 50, 100 and 200 mg/kg body weight, *p.o.*, administered as preventive treatment (twice a day over a period of 5 days) in rats with gastric lesions induced *via* ethanol, cold-restraint and pylorus-ligation models results in a significant gastric lesion index reduction compared to ulcerated rats, in all studied models. It was concluded that the free radical scavenging activity of this tree leaves mainly sustained gastroprotective activity.

Similarly, studies upon the tropical tree *Eugenia jambolana* Lam. (*Myrtaceae* family) (El-Shenawy, 2009), aiming to evaluate potential benefits on indomethacin-induced (25 mg/kg, *p.o*) ulcer on rats, indicated that the acute gastric mucosal lesions were significantly reduced when ethanol extract of seeds, pericarp and leaves (250 and 500 mg/kg, *p.o.*) were administered.

Studies (Fernandes et al., 2010) on the ethanolic extracts of leaves from *Parkia platycephala* Benth. (*Leguminosae - Mimosoideae* family) found in Brazil indicated a protective effect (on rodents) in absolute ethanol, ethanol-HCl, ischemia-reperfusion lesion models (66%, 48% and 52% gastroprotective percentage) but not in indomethacin-induced ulcer. The results also suggested antioxidant activity as the most probable mechanism for gastric protection.

Also, studies (Speroni et al., 2011) on *Laurus nobilis* L. plant species (*Lauraceae* family), a tree found in the Mediterranean area and Europe, indicated that leaves extracts (obtained with different solvents and methods) significantly reduced animal gastric damages, chloroform and methanol crude extracts providing the most important gastroprotective effects. Similarly, the results obtained were in good agreement with antioxidant capacity also suggesting a relationship between biological effects of leaves extracts from *L. nobilis* and their scavenging activity.

CONCLUSIONS

Despite the numerous potential therapeutic benefits (based on its valuable chemical content), the main commercial applications of *Fagus sylvatica* L. leaves plant part are several external products (cosmetics) assumed with high antioxidant activity; beech leaves derived products for internal use (food supplements, dietary supplements or traditionally medicines) are quite missing.

Based on Romanian folk medicine data, our studies have revealed gastroprotective potential of beech leaves derived products, precisely the capacity of the beech leaves polar extracts to offer rat gastric mucosa protection against acid secretion stimulated *via* stress-induced ulcer model, suggesting their compliance for the development of some internal use natural medicines targeted at the digestive system.

These results should be evaluated in the context of our former results (Pirvu et al., 2013) which indicated high antioxidant potency of beech leaves polar extracts and Japanese researchers' experiments (Tsutomu et al., Patent Jp. 05,139,972) demonstrating their efficacy in controlling *Helicobacter pylori* infection but also in the context of literature data indicating potential toxic effect of different beech tree products (Husgafvel et al., 2014) and augmented pro-oxidant effects of the non-polar compounds found in dichloromethane fraction from 70% ethanol extracts (Pirvu et al., 2013).

Therefore, extensive studies on beech tree plant parts and subsequent beech extracts should to be performed to develop novel and safe phyto-medicines targeted at the digestive system.

REFERENCES

Cadahia E., Fernandez de Simon B., Aranda I., Sanz M., Sanchez-Gomez D., Pinto E., 2015. Non-targeted metabolomic profile of Fagus sylvatica L. leaves using liquid chromatography with mass spectrometry and gas chromatography with mass spectrometry. Phytochem Anal, 26(2):171-182.

El-Shenawy S. M., 2009. Evaluation of some pharmacological activities of ethanol extracts of seeds, pericarp and leaves of Eugenia Jambolana in rats. Inflammopharmacology, 17(2):85-92.

Eswaran M. B., Surendran S., Vijayakumar M., Ojha S.K., Rawat A.K., Rao Ch. V., 2010. Gastroprotective activity of Cinnamomum tamala leaves on experimental gastric ulcers in rats. J Ethnopharmacol, 128(2):537-540.

Fernandes H. B., Silva F.V., Passos F. F., Bezerra R. D., Chaves M. H., Oliveira F. A., Oliveira R. C., 2010. Gastroprotective effect of the ethanolic extract of Parkia platycephala Benth. leaves against acute gastric lesion models in rodents. Biol. Res., 43(4):451-457.

Frederich M., Marcowycz A., Cieckiewicz E., Megalizzi V., Angenot L., Kiss R., 2009. In vitro anticancer potential of tree extracts from the Walloon Region forest. Planta Med, 5(15):1634-1637.

Husgafvel Pursiainen K., 2014. Occupational Cancers, Sisko Anttila and Paolo Boffetta Publishing House, Springer, 158.

Konturek P.C., Konturek S.J., Brzozowski T., 2006. Gastric cancer and Helicobacter pylori infection. J Physiol Pharmacol, 57(3):51-65.

de Lira Mota K. S., Nunes Dias G. E., Ferreira Pinto M. E., Luiz-Ferreira A., Souza-Brito A. R. M., Hiruma-Lima C.A., Barbosa-Filho J. M., Maria Batista L., 2009. Flavonoids with Gastroprotective Activity. Molecules, 14, 979-1012.

Pirvu L., Grigore A., Bubueanu C., Draghici E., 2013. Comparative analytical and antioxidant activity studies on a series of *Fagus sylvatica* L. leaves extracts. JPC. Journal of Planar Chromatography, 26(3):237-242.

Pirvu L., Hlevca C., Nicu I., Bubueanu C., 2014. Comparative Analytical, Antioxidant and Antimicrobial Activity Studies on a Series of Vegetal Extracts Prepared from Eight Plant Species Growing in Romania. JPC. Journal of Planar Chromatography, 27(5):346-356.

Pirvu L., Panteli M., Bubueanu C., Coprean D., 2015. *Centaurea cyanus* L. Polysaccharides and Polyphenols Cooperation in Achieving Strong Rat Gastric Ulcer Protection. Open Chemistry, 13(1): 910-921.

Reich E., Schibli A., 2008. HPTLC for the Analysis of Medicinal Plants, Thieme Publishing House, N.Y.-Stuttgart.

Romanian Pharmacopoeia, 1993. Xth Edition, Medicala Publishing House, Bucharest, 334-335, 1063-1064.

Speroni E., Cervellati R., Dall'Acqua S., Guerra M. C., Greco E., Govoni P., Innocenti G., 2011. Gastroprotective effect and antioxidant properties of different Laurus nobilis L. leaf extracts. J Med Food, 14(5):499-504.

Tsutomu O., Hidehisa T., Masaru F. Patent no. Jp. 05,139,972.

Wagner H., Bladt S., 1996. Plant Drug Analysis, Second Ed., Springer Publishing House.

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OPTIMIZATION OF HYDROLASE ENZYME DOSAGE IN THE PROCESS OF CELLULOSE HYDROLYSIS OF REJECT PULP AND BIOETHANOL FERMENTATION BY MICROBIAL CONSORTIUM

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Abstract
The purpose of this study was to obtain effective dose of hydrolase enzyme (α-amylase, hemicellulase, cellulase and amyloglucosidase) to produce highest levels of reducing sugars and dextrose equivalent (DE), and also to obtain the best microbial consortium in the fermentation of cellulose from pulp waste to produce bioethanol. This research used descriptive and experimental methods. The descriptive method was used in the optimization of hydrolase enzyme dosage, while the experimental method was used in cellulose of reject pulp fermentation. Parameters used in this study consist in ethanol content, reducing sugar content, microbial population and DE. Our results showed that the highest levels of reducing sugars in the optimization process of α-amylase enzyme was obtained at a dose of 0,52 µL g-1whereas hemicellulase enzyme was at a dose of 2/3 or 0.00067 g g-1 and cellulase as well as amyloglucosidase was at a dose of 1 + 1 or 0.83 µL g-1 and 0,56 µL g-1, respectively. Additionally, the most effective and optimum fermentation was obtained by a consortium of Kluyveromyces marxianus and Zymomonas mobilis (K2), with ethanol content and fermentation efficiency as much as 6.27 and 59.48%, respectively. High levels of ethanol produced was also supported by the lowest reducing sugar content of 1.32% with the average of DE 1.69. Moreover, ethanol yield (Yp/s), maximum specific ethanol productivity (qp) and cell yield (Yx/s) were also obtained at 21, 76 and 26%, respectively. We observed that consortium K. marxianus and Z. mobilis (K2) reached the highest logarithmic phase at 36th hour, with a population of 11,80x1010 cfu mL-1.

Key words*: Reject Pulp, Hyrolase Enzyme, Microbial Consortium, dextrose equivalent (DE).*

Abbreviations: DE = dextrose equivalent; ADH = alcohol dehydrogenase ; YEPDA = yeast extract peptone dextrose; YEPDB = yeast extract peptone dextrose broth; SFS = Simultaneous Saccharification and Fermentation

INTRODUCTION

The use of fossil fuels continues to increase along with a wide range of human needs, such as industrial activities, usage for power generation and as fuel for transportation.
The elevated usage of fossil fuels has given unfavourable impact on the environment.
One of the solutions to overcome this problem is to use alternative fuels that are environmental friendly.
Currently, ethanol has been recognized as one source of energy that can replace fossil fuels.
Ethanol is usually made chemically, but this method is less environmental friendly. Therefore, ethanol needs to be produced using microorganisms through fermentation.

Ethanol produced by microorganisms, is recognized as bioethanol. This substance is a liquid that is produced through the fermentation of sugars from the decomposition of carbohydrate sources with the help of microorganisms (Retno and Nuri, 2011). Basic ingredients for creating bioethanol can be obtained from biomass waste, which contains a lot of carbohydrates or lignocellulose, such as agricultural waste (banana stem and palm oil), industrial waste (paper and pulp) and others. Organic components of lignocellulose is abundant in nature and consists of three types of polymers, namely cellulose, hemicellulose and lignin (Maulana et al 2013).

Reject pulp is a lignocellulosic biomass derived from wood pieces that are not perfectly processed in the digester of pulp and paper industry. From preliminary research, it was known that the lignocellulose in the reject pulp contains cellulose (57.89%), hemicellulose (11%), starch (9.03%), lignin (16.41%), extractive (1.16%) and ash (1.09%). High content of cellulose and hemicellulose, make the reject pulp as one of the potential source to be used as raw material for bioethanol. The production of bioethanol from lignocellulosic containing industrial waste such as reject pulp can be made through the conversion of carbohydrates into sugar or glucose by several methods, for instance, by acid and enzymatic hydrolysis. Enzymatic hydrolysis has been used more frequent because it is considerably more environmental friendly than the acid catalyst. An enzyme catalyst has been used more often in the process of hydrolysis enzymatic. For instance, the α-amylase enzyme, hemicellulase, cellulase and amyloglucosidase were used in different dosage: 1, 2/3 and 1/3 of the recommended dose of commercial enzymes. Glucose obtained from the hydrolysis is then processed through fermentation by adding yeast and fermentative bacteria to obtain bioethanol. The combination of yeast and bacteria in a microbial consortium is able to optimize the fermentation process from lignocellulosic waste into bioethanol. This is due to the combined action of the alcohol dehydrogenase enzyme (ADH) that is more active in yeast, such as the yeast *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* as well as the enzyme pyruvate decarboxylase more active in fermentative bacteria such as the bacterium *Zymomonas mobilis*. Thus, in the present study, the reject pulp fermentation is processed by using microbial consortium with a combination of bacteria and yeasts, with variations in microbial consortium, the consortium of *S. cerevisiae* and *Z. moblis* (K1), a consortium of *K. marxianus* and *Z. mobilis* (K2), and a consortium of *S. cerevisiae-K. marxianus-Z. mobilis* (K3).

MATERIALS AND METHODS

The materials required in this research were the reject pulp that has been was obtained from the Laboratory of Chemical and Energy Management of Forest Products, Research and Development Center of Forest, Bogor. They consisted of distilled water, alcohol (75%), [NH4]2SO4 (4%, b/v), 1 N HCl, H2SO4, commercial enzyme of cellulase, α-amylase, hemicellulase and amyloglucosidase, isolates of *S. cerevisiae* isolates *of Z. mobilis,* isolates of K. marxianus, potassium dichromate, 1 N NaOH, peptone, DNS reagent, yeast extract, YEPDA (yeast extract peptone Dextrose), and YEPDB (yeast extract peptone dextrose broth).

Methods

In general, this study was devided into two phases: 1) optimization of enzyme dosage of α-amylase, hemicellulase, cellulase, amyloglucosidase (Produces by SIGMA) for enzymatic hydrolysis and 2) fermentation of reject pulp into ethanol using three types of consortium: consortium of *S. cerevisiae* and *Z. mobilis* (K1); consortium of *K. marxianus* and *Z. mobilis* (K2); consortium of *S. cerevisiae* + *K. marxianus* + *Z. mobilis* (K3). The fermentation stage was carried out by using the Simultaneous Saccharification and Fermentation (SFS).

Procedures

Optimization of Hydrolase Enzyme Dosage: Dose optimization consisted of three stages enzyme addition.

The first stage was the addition of α-amylase enzyme, which was incubated for 60 minutes at 104^{o}C with agitation 500 rpm.

The second stage was the addition of hemicellulase enzymes, which was incubated for 360 minutes at 55°C with agitation 500 rpm.

The third stage was the addition of cellulase enzymes and amyloglucosidase, which was incubated at 60^{o}-62^{o}C for 48 h with agitation 500 rpm.

The four types of enzymes are added into the hydrolyzate with doses of 0, 1/3, 2/3, and 1 of the recommended dose commercially. The parameters measured in this process were a reducing sugar content (by DNS method) and dextrose equivalent (DE) value.

Fermentation Process: SFS hydrolyzate produced by enzymatic hydrolysis was then fermented by using consortium of *S. cerevisiae* (5%, v/v) and *Z. mobilis* (5%, v/v) (K1); *K. marxianus* (5%, v/v) and *Z. mobilis* (5%, v/v)

(K2); *S. cerevisiae* (3.3%, v/v) + *K. marxianus* (3.3%, v/v) + *Z. mobilis* (3.3%, v/v) (K3) to produce bioethanol. The fermentation process was conducted for 3 days (72 h) and parameters were measured every 12 h.

Data analysis

The parameters measured in fermentation process were ethanol content (dichromate oxidation method), reducing sugar content (DNS method), microbial population (TPC method), and the DE value. All data were statistically analyzed using analysis of variance (ANOVA) and when the difference is significant, it was then followed by Duncan's Multiple Range Test with significance level of 5%.

RESULTS AND DISCUSSIONS

Optimization of Hydrolase Enzyme Dosage.

The aim of optimization of hydrolase enzyme dosage was to obtain an effective dose of an enzyme that enables to produce the optimum value of reducing sugars. The enzyme concentration was proportional to the rate of reaction, thus the higher enzyme concentration, the faster the rate of reaction.

Optimization of α-Amylase Dosage

The purpose of liquefaction of starch gel melting process was to obtain a lower viscosity by hydrolyzing starch into simpler molecules such as oligosaccharides or dextrins through the help of α-amylase enzyme (Robi'a and Sutrisno 2015).

In this study, α-amylase enzyme was used by *SIGMA* production, according to the dosage recommended by the product of the enzyme. Liquefaction process was lasted for 60 minutes, at 104°C and pH 6 using three variations dosage of α-amylase enzyme: dose 1 (0.52 µL g^{-1}), dose 2/3 (0.346 µL g^{-1}), and dose 1/3 (0.173 µL g^{-1}).

Reducing sugar levels and DE obtained as a result of optimization of amylase dosage was shown in Figure 1.

Highest reducing sugar levels and DE values were observed in the reject pulp as a result of the addition of α-amylase enzyme at a dose 1 (Fig. 1). At this dose, levels of reducing sugars and DE produced was 6.89% and 8.836, respectively. These results is consistent with the effective dose of the α-amylase enzyme to hydrolyze 11.27% starch content in the newsprint waste, which was equivalent to a dose of 1 or 0.52 µL g^{-1} (Pangaribuan, 2014). Reducing sugar levels and high DE value is influenced by the increasing number of enzymes used. In addition, α-amylase will be more easily dispersed completely into a solution that has a high viscosity (Havier, 2007).

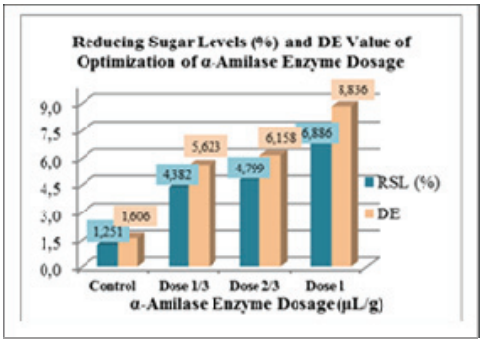

Figure 1. Reducing Sugars Level (RSL) and Dextrose Equivalent (DE, %) Value of Optimization of α-Amylase Enzyme Dosage.

Optimization of Hemicelullase Dosage

Hemicellulose is one of heteropolimer that is composed by polysaccharides, and many are composed by 1,4-β-xylose. In addition, these molecules are found in three groups, namely: xylan, mannan and galactans (Taherzadeh, and Karimi, 2007). Hemicellulase enzyme is known to break monomers in hemicellulose xylan into xylose (Samsuri et al., 2007). Hemicellulase enzyme used in this study is provided by SIGMA, the hydrolysis process lasted for 360 minutes, at 55°C with pH 6. Variation in enzyme dosage used was based on the recommended dosage of the enzyme product. Three variations of the enzyme dose were 1 (0.001 g g^{-1}), 2/3 (0.00067 g g^{-1}) and 1/3 (0.00033 g g^{-1}). Results obtained from hemicellulose hydrolysis step were shown in Figure 2.

Figure 2 showed that the highest levels of reducing sugar and DE values in the waste substrate reject pulp, were obtained by the addition a hemicellulase dose equivalent to 2/3 or 0.00067 g g^{-1}. At this dose, the production levels of reducing sugar were 12.52% and followed by a DE value of 16.067.

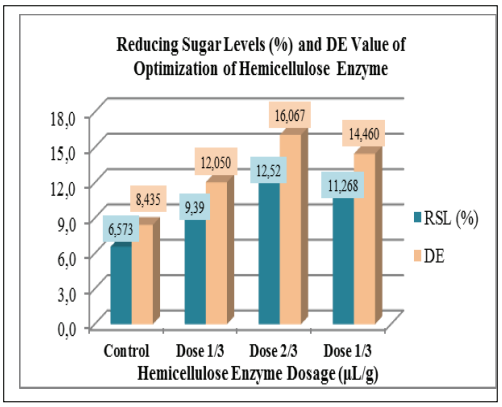

Figure 2. Reducing Sugars Level (RSL) and Dextrose Equivalent (DE, %) Value of Optimization of Hemicellulase Enzyme Dosage

This result was consistent with this dose of hemicellulase enzyme. At similar dose, the most effective in hydrolyzing hemicellulose was also obtained for banana pseudostem waste with levels of 16.02%, when a dose of 2/3 of recommended dosage for this product was used (Lubiana, 2014). In addition, the hemicellulase enzyme dosage by 2/3 of dose was recognized effective in hydrolyzing hemicellulose in stem pith of sago starch substrate, and it was able to produce a reducing sugar content by 26.6% (Fadia, 2008).

Optimization of Cellulase and Amyloglucosidase Dosage

Optimization of cellulase and amyloglucosidase enzymes dosage (saccharification process), was the final stage of the optimization of hydrolase enzyme dosage. At this stage, dextrin produced in the liquefaction process was broken down into glucose by amyloglucosidase. In addition, the cellulose molecules still being contained in the reject pulp were able to be broken down by the cellulase. Cellulase and Amyloglucosidase (AMG) enzymes used were provided by SIGMA. These two enzymes have worked synergistically and reached an optimum value at pH 4.8 at 60°-62°C for ± 48 h. Both of these enzymes were incorporated into the reject pulp waste substrate simultaneously in different dosage: dose of 1 (equivalent to 0.83 µL g^{-1} and 0.56 µL g^{-1}), 2/3 dose (equivalent to 0.553 µL g^{-1} and 0.373 µL g^{-1}), and 1/3 dose (equivalent to 0.277 µL g^{-1} and 0.187 µL g^{-1}). Results obtained in the optimization of enzyme dosage of cellulase and amyloglucosidase can be seen in Figure 3.

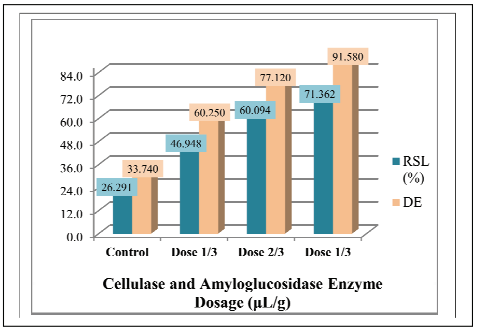

Figure 3. Reducing Sugar Levels (RSL) and Dextrose Equivalent Value (%) of Optimization of Cellulase and Amyloglucosidase Enzymes Dosage.

In Figure was shown that the the dose of the enzyme that producing the highest reducing sugar levels and DE values was obtained at dose 1+1 of cellulase and amyloglucosidase, or the equivalent to 0.83 and 0.56 µL g-1, respectively. The levels of reducing sugars produced was 71.362% with DE 91.580. In accordance with the study (Pangaribuan, 2014), the cellulose content of 43.17%, was also contained in the newspaper waste and it was effectively hydrolyzed by using a dose of 1 + 1 cellulase and amyloglucosidase enzymes with a reducing sugar levels and the resulting final DE value: respectively by 70.42 and 86.28%. In addition, the process of saccharification produced reducing sugar levels and the highest DE values with a dose of 0.5 µL g/1, or about the dose of 1 (Musanif, 2014).

Based on the data listed in Table 1 it was shown that the consortium *Kluyveromyces marxianus* and *Zymomonas mobilis* (K2) was the consortium that produced the highest levels of ethanol during bioethanol fermentation of reject pulp, reaching the amount of 6.274%. The increased levels of ethanol during bioethanol fermentation of reject pulp is presented in Figure 4.

Table 1. Parameter of Analysis of Bioethanol Fermentation of *Reject Pulp* using Microbial Consortium

Parameter	Consorsium *S. cerevisiae* & *Z. mobilis* (K1)	Consortium *K. marxianus* & *Z. mobilis* (K2)	Consortium *S. cerevisiae*-*K. marxianus*-*Z. mobilis* (K3)
Reducing Sugar Levels (0h, %)	22.410	22.347	22.535
Reducing Sugar Levels (72h, %)	1.565	1.315	1.440
Dextrose Equivalent Value (72h)	2.01	1.69	1.85
Ethanol Content (72h, %)	5.819	6.274	6.083
Efficiency of Fermentation (%)	55.01	59.48	56.99
Microbial Population in Logarithmic Phase (x 10^{10} CFU mL^{-1})	10.94 x 10^{10}	11.80 X 10^{10}	13.19 X 10^{10}
***Yp/s* (ethanol yield, %)**	20	21	19
q_p (maximum ethanol productivity, %)	62	76	72
***Yx/s* (cell yield, %)**	21	26	23

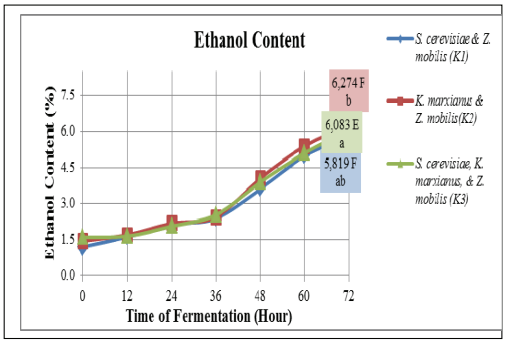

Figure 4. Graphic of Ethanol Content (%) During Bioethanol Fermentation from *Reject Pulp* Process.

Consortium of *K. marxianus* and *Z. mobilis* (K2) produced the highest ethanol content (6.274%), whereas consortium of *S. cerevisiae*-*K.marxianus*- *Z. mobilis* (K3) produced lower ethanol content (6.083%). Additionally, the consortium of *S. cerevisiae* and *Z. mobilis* (K1) produced the lowest ethanol during fermentation (5.819%). Study conducted by Gunasekaran and Kamini (1991), observed that the use of the consortium *K. fragilis* (synonymous with *K. marxianus*) (Rosa et al., 1986) and *Z. mobilis* was able to produce 64.4 g L^{-1} ethanol in 200 g L^{-1} of lactose medium. The use of consortium of *K. marxianus* and *Z. mobilis* in the ethanol production from Jarusalem artichoke tuber origin showed a reduction in sugar yield (168 g kg^{-1}) with ethanol production by 9.9% (v/v) after the distillation process was made (Szambelan, Nowak, and Jelen, 2005). Additionally, efficiency of fermentation (EF) of different microbial consortium during bioethanol fermentation process on the reject pulp was calculated. Our findings showed that the highest EF was obtained in K2 (59.48%).

Whereas K1 and K3 had lower value of EF (55.01 and 56.99%). These results indicated that the consortium K2 were the best in the fermentation process from the reject pulp, which is confirmed by the highest production of ethanol and EF. The microbial consortium was capable to produce a combined activity of various enzymes of microorganisms so that the ethanol production run more optimally (Howard, Masoko and Abotsi, 2003). *K. marxianus* yeasts was able to utilize glucose, xylose, mannose and galactose, and turned it into higher amount of ethanol compared to the one produced by the yeast *Saccharomyces cerevisae* (Rouhollah et al., 2007, Gao et al., 2015). The activity of yeast *K. marixanus* that was used to produce ethanol was also supported by the ability of fermentative bacteria *Z.*

Mobilis, which enable to convert sucrose, fructose and glucose to ethanol via the Entner-Duodoroff (Abate et al., 1996).

Reducing Sugar Levels

Consortium of *K. marxianus* and *Z. mobilis* (K2) became a consortium that has reached the highest level RSL during bioethanol fermentation process of the reject pulp (Table 1.) RSL observed at 0 h was 22.347% and decreased to 1.315% at 72 h. The decreased in RSL indicated the conversion of glucose into pyruvic acid via the Embden-Meyerhof-Parnas lane. This pyruvic acid enables to transform decarboxylated into acetaldehyde, then it underwent dehydrogenation into ethanol. In general, this process was catalyzed by enzymes produced by microbes. Decrease in RSL during fermentation was shown in Figure 5.

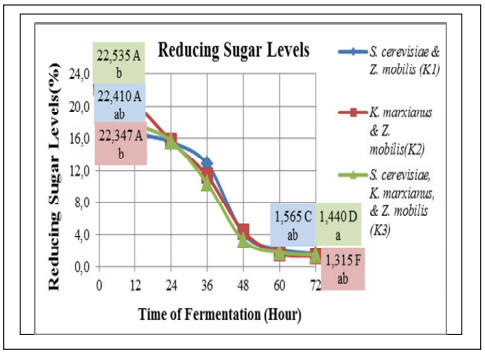

Figure 5. Graphic of Reducing Sugar Levels (%) During Bioethanol Fermentation from Reject Pulp.

Consortium *K. marxianus* and *Z. mobilis* (K2) produced the lowest RSL, from 22.347% (0 h) to 1.315% (72 h) (Fig.5) Decrease in RSL observed in consortium K2 was significantly lower compared to the consortium of K1 and K3. In addition, the total RSL used by consortium K2 was approximately 94.11%. This result was similar to the study from (Szambelan, Nowak and Jelen (2005), that the consortium of *K. fragilis* and *Z. mobilis* used 99% of reducing sugar in ethanol production from Jarusalem artichoke tuber origin, where ethanol production was quite high (9.9%) after distillation process. Lower RSL produced by microbial fermentation indicates that the microbes perform optimum in fermentation process (Pandey, 2009). Therefore, the consortium K2 is considered as the best in the reject pulp fermentation process due to its ability in using 94% sugar substrates for its growth as well as generating the highest levels of ethanol compared to the two other microbial consortium.

Microbial Populations

Table 1 showed that consortium K3 at 48 h was the consortium with the largest population of microbes during fermentation bioethanol of reject pulp (13.196 x 1010 CFU mL-1). However, this large number of microbial populations in K3 was not accompanied by higher ethanol production compared to consortium K2 (Figure 4). A consortium of microbial population growth during bioethanol fermentation process was presented in Figure 6.

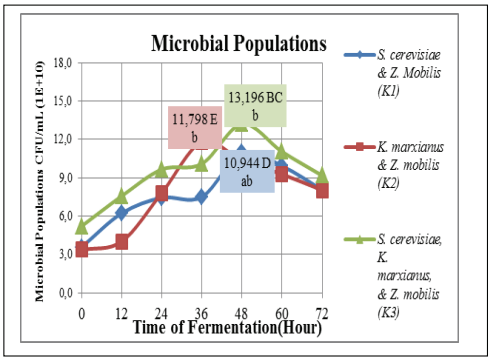

Figure 6. Graphic of Microbial Consortium Populations (x1010 CFU/mL) During Bioethanol Fermentation from Reject Pulp

At the beginning of the fermentation process, the addition in cell mass has been caused by adaptation or adjustment to the new environment. Then, the three consortiums enter in logarithmic phase at different times. Consortium K1 and K3 enter the logarithmic phase at the same time (48 h). However, the microbial population produced by K3 was larger than K1 (13.196 x 10^{10} and 10.944 x 10^{10} CFU mL^{-1}, respectively). Moreover, consortium K2 entered the logarithmic phase at 36 h, with a population of 11.798 x 10^{10} CFU mL^{-1}. Number of microbial population produced by consortium K3, was caused by the composition of the consortium itself, which consisted of three microbes. It was previously confirmed that microbial populations of mixed cultures in fermentation process is higher than

in single culture. However, microbial population was not proportional to ethanol production (Figure 4). Thus, consortium K3 were less effective in bioethanol fermentation from reject pulp. Furthermore, consortium K2 produced relatively less microbial populations compared to the two other consortium variations. Nevertheless, this consortium was able to produce higher ethanol content (Figure 4). Therefore, consortium K2 was considered as the best consortium in bioethanol fermentation due to their ability to take advantage of reducing sugar and to produce a high ethanol content despite their low microbial population.

Relation between Reducing Sugar Level, Ethanol Content, and Microbial Populations

To identify the relationship between RSL, ethanol content and microbial population, the ethanol yield (*Yp/s*), maximum productivity of ethanol-specific (q_p) and the yield of microbial biomass (*Yx/s*) were calculated. *Yp/s* or ethanol yield represents the concentration of ethanol in comparison to the concentration of glucose consumed in the substrate. While *Yx/s* value or microbial yield indicates microbial biomass formed per mass of substrate consumed. Additionally, q_p value or the maximum ethanol productivity represents the concentration of ethanol produced per unit of time, where higher concentration of ethanol resulting higher productivity of ethanol (Mulyanto et al., 2009). Consortium K2 gained the highest value of *Yp/s*, q_p and *Yx/s* compared to other consortium (i.e. 21%, 76% and 26%, respectively). However, several studies on bioethanol fermentation using consortium of *K. marxianus* and *Z. mobilis* showed that it was able to produce ethanol yield by 42-48% (g ethanol/g substrate) (Gunasekaran and Kamini, 1991, Szambelan, Nowak and Jelen, 2005). Theoretically, from 1 g of glucose, ethanol produced was only half of the consumption of glucose, with clean ethanol coefficient (yield) for about 51% of ethanol. When the net was above 51%, there are number of substrates that are not measurable. But if the net coefficient below 51%, allegedly in the fermentation process, in addition to producing ethanol, also produced byproduct compounds, such as acids, volatile organic such as lactic acid, acetic acid, acetaldehyde (Fadia, 2008). The low yield of ethanol produced in this study could be caused by the formation of by product compounds from ethanol fermentation process.

However, consortium K2 was the best consortium in bioethanol fermentation from pulp waste because it can produce the highest ethanol yield compared to the two others microbial consortium. Highest ethanol yield was supported by the growth of microbial biomass produced.

***Dextrose Equivalent* (DE) Value**

Table 1 showed that the consortium K2 produce the lowest DE (1.690) during the bioethanol fermentation from reject pulp. This was due to consortium K2 were able to consume glucose available on the substrate of reject pulp as source of nutrients. DE value during bioethanol fermentation process from reject pulp was presented in Figure 7.

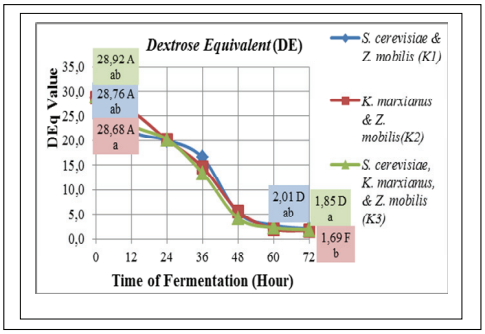

Figure 7. Graphic of *Dextrose Equivalent* (DE) Value During Bioethanol Fermentation from *Reject Pulp* Process.

DE or the degree of conversion indicates the ability of each microbial fermentative in conversion of sugar into ethanol during the biethanol production from the reject pulp, from the total sugar content of substrate (77.92%). DE generated by each consortium of the decreased microbes (Figure 7). This decline was proportional to the decreased of (RSL) that occured during bioethanol fermentation. DE in consortium K2 decreased to the lowest point from 28.68 (0 h) to 1.69 (72 h). Consortium K1 and K3 were also able to decrease DE, for instance, from 28.76 (0 h) to 2.01 (72 h) and from 28.92 (0 h) to 1.85 (72 h) for K1 and K3, respectively.

Based on the impairment of DE during the bioethanol fermentation, consortium of *K. marxianus* and *Z. mobilis* was considered as the best and the most effective consortium, due to their lower DE during fermentation process. Lower DE value was achieved by better and more optimum of microbes used for fermentation process (Pandey, 2009).

CONCLUSIONS

Based on this study, the effective dose in the optimization of α-amylase enzyme dosage was dose of 1 with RSL 6.88% and a DE value 8.84; hemicellulase enzyme dosage was 2/3 dose with RSL 12.52% and DE value 16.07; and cellulase and amyloglucosidase enzymes dosage was dose of 1 with RSL 71.36% and DE value 91.58. Consortium *Kluyveromyces marxianus* and *Zymomonas mobilis* (K2) was the best consortium in the fermentation process of reject pulp for bioethanol since it was able to generate the highest levels of ethanol (6.274%) with 59.48% fermentation efficiency.

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REFERENCES

Abate, C., D. Callieri, E. Rodrigues, O. Garro. 1996. Ethanol Production by Mixed Culture of flocculent strains of *Zymomonas mobilis* and *Saccharomyces sp*. *Appl Microbiol Bioethanol*. 45:580-583.

Aryani, Dian., Tjahjadi Purwoko, Ratna Setyaningsih. 2004. Ethanol fermentation of sweet potato (Ipomoea batatas) by Mixed Culture of *Rhizopus oryzae* and *Saccharomyces cerevisiae*.*Jurnal Bioteknologi*. 1(1): 13-18, ISSN: 0216-6887.

Fadia, H.N. 2008. Hydrolysis Sago pith Trunk powder (*Metroxylon sago Rottb*.) By Enzymatic and its hydrolyzate fermentation into ethanol by Saccharomyces cerevisiae DI / P3GI. [Essay]. Jatinangor, Department of Biology, State University of Padjadjaran.

Gao, Jiaoqi., Wenjie Yuan, Yimin Li, Ruijuan Xiang, Shengbo Hou, Shijun Zhong, & Fengwu Bai. 2015. Transcriptional Analysis of *Kluyveromyces marxianus* for Ethanol Production From Inulin Using Consolidated Bioprocessing Technology. *Biotechnology for Biofuels*. 8:115

Gunasekaran, P., dan N.R. Kamini. 1991. High Ethanol Productivity from lactose by immobilized Cells of *Kluyveromyces fragilis* and *Zymomonas mobilis*. *World Journal of Microbiology and Biotechnology*. Vol (7).

Havier, M.R. 2007. Sago Starch Hydrolysis (*Metroxylon sago Rottb*.) And its fermentation by *Saccharomyces cervisiae* FNCC 3012 and stren Bacteria isolated from Tape. [Essay]. Department of Biology, State University of Padjadjaran. Jatinangor.

Howard R.L., P. Masoko and E. Abotsi. 2003. Enzyme Activity of Phanerochaete chrysosporium Cellobiohydrolase (CBHI.1) Expressed as a Heterologous Protein From *Escherichia coli*. *African J. Biotechnology*, 2(9):296-300.

Lubiana, Kamila. 2014. Simultaneous saccharification and Separated Waste pseudostem of Pisang Ambon (*Musa x paradisiaca var. Sapientum L*.) And its hydrolyzate Fermentation for Bioethanol Production. [Essay]. Jatinangor: Department of Biological Science Unpad

Maulana, Arbi., Chairul, Muhammad Iwan Fermi. 2013. Effect of pH in fermentation Bioethanol from reject pulp using a method of co-fermentation Simultaneous Saccharification (SKFS). Chemical Engineering Department, University of Riau.

Mulyanto., Tri Widjadja, Abdul Hakim M., Eko Frastiawan. 2009. Productivity Ethanol from molasses by Continuous Fermentation Process Using *Zymomonas mobilis* by Cell Immobilization Technique K-carrageenan in Packed-Bed Bioreactor. Proceedings of the National Seminar XIV-FTI-ITS. ISBN: 979-545-043-3.

Musanif, J. 2014. *Bioetanol*. Kementrian Pertanian Indonesia.

Nugroho, A. 2007. Population Dynamics Consortium hydrocarbonoclastic bacteria In Case Study Biodegradation of Petroleum Hydrocarbons Laboratory Scale. Journal of Basic Science. 8 (1): 13-23.

Pandey, A. (ed). 2009. *Handbook of Plant-Based Biofuels*. CRC Press. USA.

Pangaribuan, Franky Gamaliel. 2014. Optimization of Enzyme dose and concentration of sulfuric acid (H2SO4) Hydrolysis Against Waste Paper, and its hydrolyzate fermentation. [Essay]. Jatinangor: Department of Biology, State University of Padjadjaran

Retno, D.T. & W. Nuri. 2011. Bioethanol manufacture of Banana peel. Proceedings of the National Seminar on Chemical Engineering "Kejuangan": Development of Chemical Technology for Processing of Natural Resources for Indonesia.

Robi'a., Aji Sutrisno. 2015. Characteristics of Wheat Glucose Syrup Sweet Purple (Study of liquefaction temperature and concentrations of Alpha-Amylase): Reader Review. Journal of Food and Agro-Industry, Vol 3 (4). p: 1531-1537.

Rosa, M. F., A. M. Vieira, dan M. L. Bartolomeu. 1986. Production of High Concentration of Ethanol from Mash, Juice, and Pulp of Jerusalem Artichoke tubers by *Kluyveromyces fragilis*. *Enzyme Microb. Technol*, Vol(8).

Rouhollah, H.; N. Iraj; E. Giti; dan A. Sorah. 2007. Mixed Sugar Fermentation by *Pichia stipitis, Saccharomyces cerevisiae*, and an Isolated Xylose Fermenting *Kluyveromyces marxianus* and Tehir Cocultures. *African Journal of Biotechnology* Vol. 6 (9), pp. 1110-1114.

Samsuri, M.; M. Gozan; R. Mardias; M. Baiquni; H. Hermansyah; A. Wijanarko; B. Prasetya; dan M. Nasikin. 2007. Utilization of cellulose Bagas For Ethanol Production Through Simultaneous Saccharification And Fermentation With xylanase enzyme. Makara, *Teknologi*, Vol. 11, No. 1, April 2007: 17-24.

Szambelan, K., J. Nowak, dan H. Jelen. 2005. The Composition of Jerusalem Artichoke *(Helianthus tuberosus* L.) Spirits Obtained from Fermentation with Bacteria and Yeast. Eng. *Life Science.*Vol(5). No(1).

Taherzadeh, M.J. dan K. Karimi. 2007. *Enzyme-Based Hydrolysis Processes for Ethanol from Lignocellulosic Material*.a Review. [Online]. Tersedia: http://www.ncsu.edu /bioresources/BioRes_02/BioRes (Diakses pada: 25/01/2015).

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LEVAN - A MINI REVIEW

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Abstract

This review aimed to present a short summary of the biosynthesis, properties and industrial applications of levan, as a multiuse biopolymer. During the past years, a great number of bacterial polysaccharides have been discovered and nowadays, many studies about their molecular structure, biosynthesis and industrial development, or their functional properties establish correlations emphasizing their significant industrial value, especially as biomaterials. Levan and inulin are the main representative molecules, in the fructans group (as non-structural carbohydrates - fructose polymers). Levan is an extracellular polysaccharide (EPS), a biologically active polymer. It is a naturally occurring homopolymer of fructose, which can be found in plants and many microbial strains. Its main plant sources are: Agropyron cristatum, Dactylis glomerata, Poa secunda, Triticum aestivum, Cocksfoot and Pachysandra terminalis. As an EPS, levan is also produced, usually from sucrose-based substrates, by a variety of microorganisms: the most known microbial levan producers belong to the genera Zymomonas, Bacillus, Acetobacter, Aerobacter, Pseudomonas, Erwinia, Gluconobacter, Streptococcus and Corynebacterium. Many research works attribute levan a variety of potential applications in various fields, like: medical, chemical, pharmaceutical, cosmetics and food industries. General properties like film-forming ability, flexibility, renewability, biocompatibility, biodegradability and ecofriendliness, along with a number of remarkable physical, chemical and biomedical properties, made levan a superior biopolymer in many commercial sectors.

Key words*: biopolymers, levan, biosynthesis, properties, applications.*

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INTRODUCTION

During recent years, **polysaccharides** – natural polymers, offered a considerable promise as sustainable materials that embrace two highly important properties, biodegradability and biocompatibility (Poli et al, 2009; Chen et al, 2014).

Their natural origin is characterized by a wide variety of sources: bacterial, fungal, algal and plant (Donot et al, 2012).

This mini review proposes to present some general aspects regarding biosynthesis properties, and applications of levan polymers.

Exopolysaccharides (EPS) – polysaccharides exuded into the extracellular environment (e.g. homopolysaccharides like dextran or levan and heteropolysaccharides like xanthans or gellans) have received considerable research attention, due to both their environmental and human compatibility (Donot et al, 2012; Ates, 2015).

These polysaccharides (monomers of monosaccharides) as natural materials (highly stable and safe) derived from microbial sources have an outstanding potential for various industrial and medical applications, due largely to their susceptibility to biodegradation (comparative with synthetic polymers) (Liu et al, 2008; Rehm, 2010; Liang and Wang, 2015).

Microorganisms can synthesize a large number of polysaccharides that play important roles in biological functions such as adhesion, infection, immune response etc. Therefore, the carbon sources available in culture media are converted into a various range of polymers as materials with different properties, in many cases more advantageous than other gums (Ernandes and Cruz, 2011; Rehm, 2010; Öner, 2013).

During the last years, a great number of bacterial polysaccharides have been discovered and nowadays, many studies about their molecular structure, biosynthesis and industrial

development, or their functional properties, establish correlations emphasizing their significant industrial value, especially as biomaterials. These microbial polysaccharides, categorized as environmentally friendly materials, are called "the sleeping giant of biotechnology" (Freitaset al, 2011; Esawyet al, 2012).

In order to improve their properties (e.g. biocompatibility) or to enhance the productivity yield, new approaches that target the biological processes of EPS synthesis. Such studies involve many aspects (for example – related to fermentation process optimization by using renewable resources as cheaper substrates), the most important being the reduction of production costs (van Dyk et al, 2012).

Besides the EPS roles in nature, microbial polysaccharides have got several important industrial and medical applications. Due to their complex structures, biopolymers – superior to petrochemical – derived polymers, have various valuable properties in many sectors such as: pharmacology and medicine (drug delivery, anti-tumor, anti-mutant, anti-coagulant, antioxidant, immunostimulatory, antiviral and anti-inflammatory activities), cosmetology, textiles, adhesives, detergents, depollution – wastewater treatment (biofloculants, heavy metal removal agents, bioabsorbents), brewing and food production (additives – gelling, thickeners, emulsifying and stabilizing agents) (Poli et al, 2009; Rehm, 2010;Donot et al, 2012; van Dyk et al, 2012; Sarilmiser and Öner, 2014).

FRUCTANS

An important group of polysaccharides as non-structural carbohydrates (fructose polymers) can naturally occur in various microbial (bacteria, fungi) and plant species (monocots, dicots). Depending on their origin, fructans are characterized by β-(2,6) linkages (levan type) and β-(2,1) bonds (inulin type) (Kim et al, 2005; Banguela and Hernandez, 2006; Franken et al, 2013).

Levan and inulin are the main molecules, very representative for the fructans group, but also a third type of fructose polymers exists, namely fructo-oligosaccharides, FOS (short-chain sugar molecules) (Banguela and Hernandez, 2006; Linde et al, 2012).

A broad range of microorganisms, including Gram positive and Gram negative bacteria (*Pseudomonas, Bacillus subtilis, Xanthomonas, Streptococcus mutants, S. salivarius, Azotobacter chroococcum, Lactobacillus reuteri, Leuconostoc citreum, Zymomonas mobilis, Arthrobacter ureafaciens, Rothiadento cariosa*) and different fungal genera (*Fusarium, Aspergillus, Trichoderma, Aureobasidium, Penicillium, Phytophora*, *Pestaloptiosis, Myrotecium*) are currently known to synthesize fructans (Banguela and Hernandez, 2006; Gupta et al, 2011).

These homopolymers of fructose have various biological functions/roles in microorganisms such as: physical barrier forming, enhanced resistance against abiotic and biotic stress, improved nutrient assimilation, role in pathogenesis (Franken et al, 2013).

Fructosyltransferases, enzymes capable to catalyse trans-glycosylation and sucrose hydrolysis reactions, play a very important role in fructans biosynthesis. In addition, bacterial levansucrases (EC 2.4.1.10) and inulosucrases (EC 2.4.1.9) contribute to the sucrose conversion into high polymerization degrees (DP) fructans (e.g. levan polymers have a DP > 100, whereas inulin polymers range between 20 and 10000). Fungal fructans, fructo-oligosaccharides, predominantly have a DP of 3 to 10 (Banguela and Hernandez, 2006; Franken et al, 2013).

Fructose polymers are well known for their various applications in nutraceuticals, food and non-food industries (e.g. FOS are typical representatives of prebiotics with bifidogenic effect; inulin and levan are used in fructose syrups production; levan is used as an emulsifying or encapsulating agent, blood plasma volume extender etc.) (Abdel-Fatah et al, 2005; Banguela and Hernandez, 2006; Linde et al, 2012; Wang, 2015).

Due to their properties and beneficial roles for human and animals health, these molecules have been extensively used in different industry sectors and medicine.

LEVAN

Levan is an extracellular polysaccharide, a biologically active fructan polymer. It is a naturally occurring homopolymer of fructose, which can be found in plants and many microbial strains (Melo et al, 2007; Shih et al, 2010; Silbir et al, 2014; Sarilmiser et al, 2015).

It was firstly reported by Lipmann, in 1881 as a type of microbial fructan (similar with bacterial dextran) under the name of "levulan" (Gupta et al, 2011; Liang and Wang, 2015).

Levan, commonly reffered as polyfructose is made of repeating fructose sub-units which form a main chain with β-(2→6) fructofuranosidic bonds and occasionally with β-(2→1) branching. This backbone make levan a unique biopolymer, being at the same time one of the few natural polymers in which carbohydrate is found in the furanose form (Szwengiel et al, 2004; Gupta et al, 2011; Divya and Sugumaran, 2015; Liang and Wang, 2015; Srikanth et al, 2015).

STRUCTURE AND PROPERTIES

Over the years, the chemical structure and physical properties of levan were well studied and characterized.

Empirical formula: $(C_6O_{10}H_5)_n$(Han, 1989)

Structural formula: Fig. 1 (Han and Clarke, 1996).

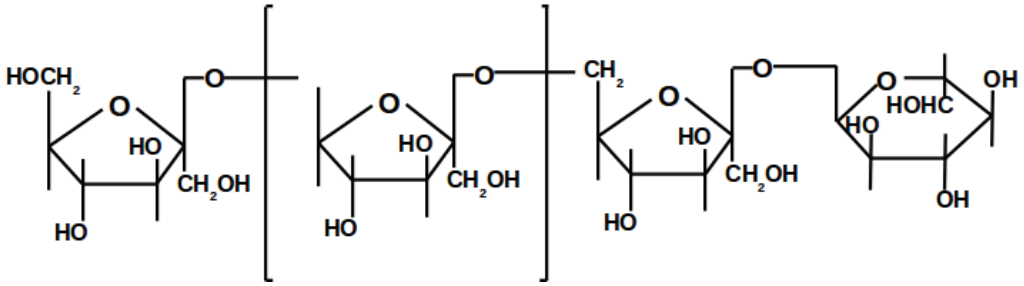

Fig. 1 Structural formula of levan (Han and Clarke, 1996)

The molecular weight of levan, its degree of polymerization and the branching of the repeating unit depend on the source. Levans from the plants are much smaller than those produced by microorganisms, usually with molecular weights between 2000 and 33000 Daltons (Da) (Esawy, 2012; Srikanth et al, 2015). On the other side, microbial levans generally have molecular weights up to several millions Da (10^5 to 10^8 Da; e.g. high molecular weight levans from *Bacillus polymyxa* – $2x10^7$ Da and *Z. mobilis* – 10^7 Da) (Chung et al, 1997; Freitas et al, 2011; Esawy et al, 2012; Report - CIR, 2012; Chen et al, 2014; Srikanth et al, 2015).

The main properties of levans are very similar to those of dextrans (Gupta et al, 2011). It is known that levan has some distinguished and interesting biochemical and biomedical properties and, in this context, they are shortly reviewed in Table 1 (Zhurina, 2009; Sarilmiser et al, 2015).

General properties like film-forming ability, flexibility, renewability, biocompatibility, biodegradability and ecofriendliness, along with a number of remarkable physical, chemical and biomedical properties, made levan a superior biopolymer in many commercial sectors and also, determined its classification in the most valuable and versatile polymers of the future (Srikanth et al, 2015; Adamberg et al, 2016).

BIOSYNTHESIS

Levan history begun in the years 1870-1881 with its discovery by Lipmann, followed in 1902 by Greig-Smith and Steel who reported levan produced by microorganisms isolated from a secretion of *Eucalyptus stuarina*. In the immediate following period (1870-1940), the research work in the field was focused on the biosynthesis, production and collection of levan, especially in Germany, England and France. Starting from 1930s, insights on the microbial levan production were brought to the researchers' attention and opened a new horizon for polymers applications, while in the USA a commercial market for polysaccharides started to develop (Gupta et al, 2011; Zhang et al, 2014; Srikanth et al, 2015).

Table 1. The main properties of levan

PHYSICO-CHEMICAL PROPERTIES		BIOMEDICAL PROPERTIES
Solubility (Manandhar et al, 2009; Zhurina, 2009; Belghith et al, 2011; Freitas et al, 2011; Gupta et al, 2011; Report - CIR, 2012; Sarilmiser and Öner, 2014;Silbir et al, 2014; Sarilmiser et al, 2015; Srikanth et al, 2015)	Water and oil soluble, due to its β-(2→6) linkage (high soluble in water at room temperature)	Not hydrolyzed by human digestive enzymes → bifidogenic effect (Belghithet al, 2011)
	Non-gelling in water	
	Insoluble in organic solvents like methanol, acetone, ethanol, n-propanol, methylethylketone, isopropanol, ethyl lactate, toluene (exception: dimethyl sulfoxide – DMSO); also, resistant to jet fuel and d-limonene	
Viscosity (Esawy et al, 2012; Report - CIR, 2012; Sarilmiser and Öner, 2014;Srikanth et al, 2015)	Low intrinsic viscosity (0,07 to 0,18 dL/g for levan with molecular weight between 16 to 24 million Da)	Antioxidant (Srikanth et al, 2015)
Particle size (Report - CIR, 2012)	Partially forms nanoparticle in water (224,3 nm) and in ethanol (251,8 nm)	Anti-inflammatory (Freitas et al, 2011; Srikanth et al, 2015)
Amorphous or microcrystalline (Gupta et al, 2011)		Anticarcinogenic (Abdel-Fatah et al, 2005; Freitas et al, 2011; Gupta et al, 2011; Srikanth et al, 2015)
Amphiphilic (Sezer et al, 2011)		Anti-AIDS (Srikanth et al, 2015)
Non-ionic (Report - CIR, 2012)		Antihyperlipidemic (Yussef et al, 2014), hypocholesterolemic (Abdel-Fatah et al, 2005)
Levorotatory (Gupta et al, 2011)		Antiviral effects (Esawy et al, 2011)
Self-assembling (Sarilmiser and Öner, 2014)		Non-toxic and ocular non-iritant (Sarilmiser and Öner, 2014; Srikanth et al, 2015)
Heat stable: melting point, 225°C (Manandhar et al, 2009; Zhurina, 2009)		Hyperglycaemic inhibitor (Srikanth et al, 2015)
Stability to heat acid and alkali media (Sarilmiser and Öner, 2014; Silbir et al, 2014)		
Glass transition temperature: 141°C (Manandhar et al, 2009)		
Tensile strength: "green" and strongly adhesive (easily removed with water) (Freitas et al, 2011; Sezer et al, 2011; Sarilmiser et al, 2015; Srikanth et al, 2015)		
Compatibility with salts and surfactants (Silbir et al, 2014)		

Levan is diversily distributed in plants and microorganisms.

Its main plant sources are: *Agropyron cristatum, Dactylis glomerata, Poa secunda* (levan is usually found in their stems and leaf sheats), *Triticum aestivum, Cocksfoot* and *Pachysandra terminalis* (Gupta et al, 2011; Silbir et al, 2014; Srikanth et al, 2015).

Levan is also produced as an exopolysaccharide, usually from sucrose-based substrates by a variety of microorganisms. There are some reports on microbial levan for which fructose, sugar cane syrup, glucose, molasses, glycerol or raffinose substrates were used (Jathore et al, 2012; Sarilmiser et al, 2015; Srikanth et al, 2015).

The main reaction involved in its biosynthesis is the transfructosylation and is carried out by an extracellular enzyme namely levan sucrase (sucrose 6-fructosyltransferase, EC 2.4.1.10) (Shih et al, 2010; Zhurina, 2009; Srikanth et al, 2015). Therefore, levansucrase (LSC) is considered to be key point of microbial levan production (Donot et al, 2012). The fructosyl-transferases were grouped into glycoside hydrolases 68 family (GH68) by CAZY (Carbohydrate-active enzymes) database(Alamäeet al, 2012) and besides transfructosylation (LSC catabolizes the sucrose and converts the fructose into levan) they are known to catalyse two more distinct reactions such as hydrolysis (of sucrose, when water is used as an acceptor) and polymerization (of fructose) (Inthanavong et al, 2013; Youssef et al, 2014; Goncalves et al, 2015). Studies on LSCs activities showed that they are involved in

phytopathogenesis (*Erwinia* and *Pseudomonas* strains), symbiosis (*Paenibacillus polymyxa*) and also in the survival of bacteria in soil (*Bacillus subtilis*) (Esawy et al, 2012). LSCs were also isolated from various strains like *Streptococcus* sp., *S. mutants, S. salivarius, Leuconostoc mesenteroides, Lactobacillus* sp., *L. johnsonii, L. gaserii, Acetobacter diazotrophicus, Gluconobacter oxydans, Bacillus amyloliquefaciens, B. natto, Pediococcus acidilactici, Aerobacter levanicum, Z. mobilis* (Zhurina, 2009; Youssef et al, 2014; Srikanth et al, 2015).

Many microorganisms, including Gram negative and Gram positive bacteria, yeasts and fungi are capable to produce levan. The main levan producers are: *Aspergillus sidawi* and *Aspergillus versicolor* (fungi) and *Zymomonas mobilis, Acetobacter xylinum, Gluconacetobacter diazotrophicus, Microbacterium laevaniformans, Bacillus subtilis, B.amyloliquefaciens, B. polymyxa, B.circulans, B.lentus, B.licheniformis, B.methylotrophicus, B.megaterium, Geobacillus stearothermophilus, Paenibacillus polymyxa, Pseudomonas syringae, Ps.syringae* pv. *glycinea, Ps.syringae* pv. *phaseolicola, Ps.fluorescens, Ps.prunicola, Lactobacillus sanfranciscensis, L.reuteri, Leuconostoc mesenteroides, Rahnella aquatilis, Erwinia amylovora, E.herbicola, Streptococcus salivarius, Seratia* sp., *S.laevanicum, Arthrobacter ureafaciens, A.acetigenum, Halomonas sp., H.smyrnensis, Aerobacter levanicum, A.aerogenes, Phytobacterium vitrosum, Xanthomona* ssp., *X.pruni, Actinomyces viscosus, Azotobacter* sp., *Mycobacterium* sp. (bacteria) (Han, 1989; Han and Clarke, 1996; Szwengiel et al, 2004; de Oliveira et al, 2007; Szwengiel et al, 2007; Bae et al, 2008; Zhurina, 2009; Ernandes et al, 2011; Gupta et al, 2011; Alamäe et al 2012;Donot et al, 2012; Jathore et al, 2012; Liu et al, 2012; Report CIR - 2012; Molinari and Boiardi, 2013; Sarilmiser andÖner, 2014; Silbir et al, 2014; Youssef et al, 2014; Zhang et al, 2014; Abou-Taleb et al, 2015; Divya and Sugumaran, 2015; Sarilmiser et al, 2015; Srikanth et al, 2015).

Therefore, the most known microbial levan producers belong to the genera: *Zymomonas, Bacillus, Acetobacter, Aerobacter, Pseudomonas, Erwinia, Gluconobacter, Streptococcus, Corynebacterium*.

APPLICATIONS

It is recognized that levan is a homopolysaccharide with multi-functional features and a wide range of potential industrial applications. In recent years, commercial interest for the levan production received considerable attention (Abdel-Fattah et al, 2005; Gupta et al, 2014; Ates, 2015; Sarilmiser et al, 2015).

Some recent studies proposed that this novel biopolymer together with pullulan, xanthan and curdlan will have a promising future in the polysaccharides industrial market. Many research works attribute levan a variety of potential applications in diverse fields like: medical, chemical, pharmaceutical, cosmetics and food industries (Adamberg et al, 2016).

In this context, some of the most relevant applications of levan will be overviewed below.

In **medical and pharmaceutical sectors**, levan finds many applications due to its biodegradability, biocompatibility and film-forming ability, especially. It can be used as a plasma substitute (e.g. levan with a molecular weight between 3000 and 100000 Da) (Barone and Medynets, 2007; Rairakhwada et al, 2007; Shih et al, 2010; Esawy et al, 2011; Sezer et al, 2011; van Dyk, 2012; Liu et al, 2012; Abou-Taleb et al, 2014; Santos et al, 2014; Sarilmiser and Öner, 2014; Silbir et al, 2014; Youssef et al, 2014; Zhang et al, 2014; Srikanth et al, 2015), prolongator of drug activity (Sarilmiser and Öner, 2014; Silbir et al, 2014; Zhang et al, 2014; Youssef et al, 2014; Sarilmiser et al, 2015), radioprotector, coating material in drug delivery systems (Sezer et al, 2011; Abou-Taleb et al, 2014), tablet binder (Abou-Taleb et a, 2014; Wang, 2015) or drug carrier of nano-scale size range for peptides and proteins (e.g. levan obtained from *Halomoas smyrnensis* AAD6^T) (Sezer et al, 2011; Ates, 2015), biofloculating agent (Sarilmiser and Öner, 2014; Sarilmiser et al, 2015), colour and flavour enhancer in the manufacturing of tablets and capsules (Srikanth et al, 2015), dietary supplement to common carp, *Cyprinuscarpio* juveniles (levan increases the total erythrocyte and haemoglobin content with immunostimulant and immunomodulatory effects on *C.*

carpio and *Labeorohita*; it offers protection against *Aeromonashydrophila* infection) (Rairakhwada et al, 2007; Gupta et al, 2014; Srikanth et al, 2015). Many studies reported levan's multiple beneficial effects on human and animal health. Therefore, levan is best known to have anti-tumour, antioxidant and anti-inflammatory effects.

As an anti-tumour agent, there are studies on levan's effects on the immunologic system, tumour suppression and enhancement of leukocyte anti-tumour activity (Abdel-Fatah et al, 2005), its ability to modify tumour cells permeability (Abou-Taleb et al, 2014) (increase cell permeability to cytotoxic agents) (Srikanth et al, 2015). Levans from *Z. mobilis* and *M. laevaniformans* are considered to be anti-tumour immune modulators in humans (Rairakhwada et al, 2007) and showing immune-modulatory effects on macrophage, B and T-cells (Srikanth et al, 2015); levan from *Z. mobilis* was studied against Sarcoma-180 cell and it was proven that its anti-tumour activity depends on its molecular weight (Moosavi-Nasab et al, 2010). Also, some studies on levan from *Aerobacter* sp. and *Microbacterium* sp. against stomach cancer demonstrated that it exhibits higher anti-proliferative activity against human gastric cancer cells (Srikanth et al, 2015). Levan's anti-tumour effect, obtained from *Z. mobilis, Rahnella aquatilis* and *Microbacterium laevaniformans*) has been shown against 8 different tumour cell lines (Esawy et al, 2011; Fattah et al, 2012).

Its role in alleviating oxidative stress and free radicals demonstrate levan's antioxidant potential (e.g. in high glucose condition in the pancreatic beta cells) (Dahech et al, 2011; Dahech et al, 2013; Sarilmiser and Öner, 2014; Srikanth et al, 2015).

Besides hypocholesterolemic (anti- hyperli-pidemic agent) (Zhang et al, 2014; Youssef et al, 2014; Sarilmiser et al, 2015; Srikanth et al, 2015); levan prevents hypercholesterolemic atherosclerosis (Abdel-Fattah et al, 2005; Shih et al, 2010; Sezer et al, 2011; BelghithBelghith, 2012; Esawy et al, 2012; Fattah et al, 2012; Liu et al, 2012; van Dyk et al, 2012; Dahech et al, 2013; Abou-Taleb, 2014; Santos et al, 2014; Sarilmiser and Öner, 2014; Silbir et al, 2014; Youssef et al, 2014), shows hypoglycaemic (Dahech et al, 2013; Srikanth et al, 2015) (antidiabetic agent) (Sarilmiser and Öner, 2014; Youssef et al, 2014) and anti-inflammatory effects (Sarilmiser and Öner, 2014, 2014; Srikanth et al, 2015). Levan is recognized for its bifidogenic effect. It can be used as a human or animal prebiotic, which can significantly modulate the colonic microbiota by stimulating the growth of lactic acid bacteria like *Bifido bacteria* and at the same time protecting the colon from carcinogens (Szwengiel et al, 2007; Gupta et al, 2011; Belghith et al, 2012; dos Santos et al, 2012; Esawy et al, 2012; van Dyk et al, 2012; Santos et al, 2014; Srikanth et al, 2015).

In other studies the levan's effects were demonstrated in the protection of liver and kidneys, pancreas and heart tissue from the damage in alloxan-induced diabetic rats and in enhancing enzymatic defensis (Dahech et al, 2011; Srikanth et al, 2015).

Furthermore, levan distinguished properties contribute in reducing risk factors for coronary artery disease (Belghith et al, 2012), prevention of infections and necrosis or in dermatological wounds healing (Srikanth et al, 2015). Also, it has a therapeutic role in dental caries (e.g. subcutaneous filling) (Fattah et al, 2012; Srikanth et al, 2015) and exhibits anti-clotting factor during the surgery of heart patients and also in the treatment of restenosis after angioplasty (Srikanth et al, 2015).

Esawy and colab.have mentioned the probable suitability of levan as a cheap and natural product in antiviral treatments (antiviral activity of levan was studied against respiratory virus HPA1, H5N1 and enteric virus, adenovirus type 40) (Esawy et al, 2011).

Levan sulphates, phosphates and acetates as levan derivatives can be used in the treatment of AIDS or as inhibitors for muscle proliferation (Barone, 2007; Abou-Talebet al, 2014; Divya and Sugumaran, 2015; Srikanth et al, 2015).

In **food industry**, levans can be used as industrial gums (e.g. substitute for gum Arabic) (Chung, 1997; Shih et al, 2010; Abou-Taleb et al, 2014), sweeteners (e.g. as a fructose source) (Baroneand Medynets, 2007; Shih et al, 2010; van Dyk et al, 2012; Abou-Taleb et al, 2014; Silbir et al, 2014; Dvya and Sugumaran, 2015), fat substitues (Santos et al, 2014), fillers (bulking agents) (Abou-Taleb et al, 2014),

emulsifiers and texture forming compounds (Barone and Medynets, 2007; Bae et al , 2008; Shih et al, 2010; Esawy et al, 2011; Liu et al, 2012; Jathore et al, 2012; van Dyk et al, 2012; Abou-Taleb et al, 2014; Santos et al, 2014; Youssef et al, 2014; Zhang et al, 2014; Silbir et al, 2014; Divya and Sugumaran, 2015; Sarilmiser et al, 2015), encapsulating agents and carriers for flavours (aromatic enhancers) (Shih et al, 2010; Esawy et al, 2011; Jathore et al, 2012; Liu, 2012; van Dyk et al, 2012; Abou-Taleb et al, 2014; Santos et al, 2014; Zhang et al, 2014; Divya and Sugumaran, 2015; Sarilmiser et al, 2015), food coating materials (e.g. bio-based plastics for packaging applications) (Barone and Medynets, 2007; Bae et al, 2008; Chen et al, 2014) and stabilizers or thickeners (Barone and Medynets, 2007; Bae et al, 2008; Shih et al, 2010; Esawy, 2011; Zhang, 2014; Jathore, 2012; Liu et al, 2012; van Dyk, 2012; Abou-Taleb, 2014; Santos, 2014; Youssef, 2014; Divya and Sugumaran, 2015; Sarilmiser, 2015).

As a formulation aid in **cosmetic** products (blending component), levan has been shown to exert excellent cell proliferation, skin-irritation-alleviating and skin moisturizing effects. Also, in hair care products, levan derivatives can be used (Shih et al, 2010; Gupta et al, 2011; Sezer et al, 2011; Fattah et al, 2012; Divya and Sugumaran, 2015; Srikanth et al, 2015).

Levan has also proven to be a promising biopolymer in **other industrial applications**. It can be used as a surface-finishing agent (Shih et al, 2010; van Dyk et al, 2012; Zhang et al, 2014), "green" adhesive (e.g. Montana Biotech SE Inc. produces 2 levan adhesives for indoor and external use) (Srikanth et al, 2015), surfactant for household use (Gupta et al, 2011; Esawy et al, 2012), or plugging agent (e.g. to plug pores of high permeability soils) (Ghaly et al, 2007). Another special uses of levan are in laser direct writing technologies (LDW) in order to obtain novel bioactive surfaces (Sarilmiser et al, 2015) and in the purification of biological materials through a PEG/levan two phase liquid system (Srikanth et al, 2015).

CONCLUSIONS

Due to an increased interest in discovering and developing biopolymers with innovative chemical structures, resulted from a continuous demand for natural products, biocompatible and biodegradable, this review proposed a summary analysis of levan biosynthesis, properties and potential applications in various industrial sectors.

FUTURE PROSPECTS

The increasing need for environmentally friendly products, in biomedical and pharmaceutical sectors or food and feed (e.g. aquaculture) has opened a new commercial market for bioproducts, among which levan made its way, succesfully. So far, the available information about levan's effects used as different bioactive agents in human and animal health is not complete. There is a need to extensively exploit its beneficial properties in biomedical sectors, especially in nanotechnology field (e.g. developing prophylactic medicines or preparation of novel nanocomposites – biopolymeric nanoscale drug carrier) or in aquaculture (as an immune stimulatory agent) (Gupta et al, 2011; Sezer et al, 2011; Srikanth et al, 2015).

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REFERENCES

Abdel-Fattah A. F., Mahmoud D. A. R., Esawy M. A. T., 2005.Production of levansucrasefrum B. subtilis NRC 33a and enzymatic synthesis of levan and fructo-oligosaccharides, Current microbiology, 51, 402-407.

Abou-Taleb K. A., Abdel-Monem M. O., Yassin M. H., Draz A. A., 2014. Nutritional factors affecting levan production by *Bacillus* sp. V8 strain isolated from rhizosphere bean (Viceafaba) plant, Journal of Agricultural Technology, 10(4), 899-914.

Abou-Taleb K. A., Abdel-Monem M. O., Yassin M. H., Draz A. A., 2015. Production, purification and characterization of levan polymer from *Bacillus lentus* strain, British Microbiology Research Journal, 5(1), 22-32.

Adamberg K., Tomson K., Talve T., pudova K., Puurand M., Visnapuu T., Alamäe T., Adamberg S., 2016. Levan enhances associated growth of bacteroides, *Escherichia*, *Streptococcus* and *Faecali bacterium* in Fecal Microbiota, PLOS ONE, 10(12), e0144042, doi: 10.1371/journal.pone.0144042.

Alamäe T., Visnapuu T., Mardo K., Mäe A., Zamfir A. D., 2012. Levan sucrases from *Pseudomonas* bacteria: novel approaches for protein expression, assay of enzymes, fructo oligosaccharides and heterooligofructans, Carbohydr. Chem., 38, 176-191.

Ates O., 2015. Systems biology of microbial exopolysaccharides production, Frontiers in Bioengineering and Biotechnology, 3 (article 200), doi: 10.3389/fbioe.2015.00200.

Bae I. Y, Oh I-K., Lee S., Yoo S-H., Lee H. G., 2008. Rheological characterization of levan polysaccharides from *Microbacteriumlaevaniformans*, International Journal of Biological Macromolecules, 42, 10-13.

Banguela A., Hernandez L., 2006.,Fructans: from natural sources to transgenic plants, BiotecnologiaAplicada, 23(3), 202-210.

Barone J. R., Medynets M., 2007. Thermally processed levanploymers, Carbohydrate polymers, 69, 554-561.

Bathena Z. P., Sahu N. P., Jha A., Mukherjee S. C., 2007. Dietary microbial levan enhances cellular non-specific immunity and survival of common carp (*Cyprinuscarpio*) juveniles, Fish &Sellfish Immunology, 22, 477-486.

Belghith K. S., Dahech I., Hamden K., Feki A., Mejdoub H., Belghith H., 2012.Hypolipidimic effect of diet supplementation with bacterial levan in cholesterol-fed rats, International Journal of Biological Macromolecules, 50, 1070-1074.

Chen X., Gao H., Ploehn H. J., 2014. Montmorillonite-levannanocomposites with improved thermal and mechanical properties, Carbohydrate Polymers, 101, 565-573.

Chung B. H., Kim W. K., Song K. B., Kim C. H., Rhee S. K., 1997. Novel polyethylene glycol/levan aqueous two-phase system for protein partitioning, Biotechnology Techniques, 11(5), 327-329.

Combie J., 2006. Properties of levan and potential medical uses. Chapter: 13, Polysaccharides for Drug Delivery and Pharmaceutical Applications, 263-269.

Dahech I., Belghith K. S., Hamden K., Feki A., Belghith H., Mejdoub H., 2011.Antidiabetic activity of levan polysaccharide alloxan-induced diabetic rats, International Journal of Biological Macromolecules, 49, 742-746.

Dahech I., Harrabi B., Hamden K., Feki A., Mejdoub H., BelghtihH., Belghith K. S., 2013. International Journal of Biological Macromolecules, 58, 281-286.

De Oliveira M. R., de Silva R. S. S. F., Buzato J. B., Celligoi M. A. P. C., 2007.Study of levan production by Z. mobilis using regional low-cost carbohydrate sources, Biochemical Engineering Journal, 37, 177-183.

Divya J. M., Sugumaran K. R., 2015. Fermentation parameters and condition affecting levan production and its applications, Journal of Chemical and Pharmaceutical Research, 7(2), 861-865.

Donot F., Fontana A., Baccou J. C., Galindo S. S., 2012. Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction, Carbohydrate Polymers, 87, 951-962.

Dos Santos F. L., Pineda E. A. G., de Melo F. C. B. C.., Celligoi M. A. P. C., Cavalcanti O. A., 2012. Levan in the developing of new colon-specific polymer material: evaluation of the permeability moisture and thermal analysis in free films of Eudragit FS30D, ActaScientiarum. Health Sciences, 34(2), 185-191.

Ernandes F. M. P. G., Garcia-Cruz, 2011. Nutritional requirements of *Zymomonasmobilis* CCT 4494 for levan production, Braz. Arch. Biol. Technol., 54(3), 589-600.

Esawy M. A., Ahmed E. F., Helmy W. A., Mansour N. M., El-Senausy W. M., El-Safty M. M., 2012. Antiviral levans from *Bacillus* spp. Isolated from honey, http://dx.doi.org/10.5772/52483.

Esawy M. A., Ahmed E. F., Helmy W. A., Mansour N. M., El-Senousy W. M., El-Safty M. M., 2011. Production of levansucrase from novel honey *Bacillus subtilis* isolates capable of producing antiviral levans, Carbohydrate Polymers, 86, 823-830.

Fattah A. M. A., Eldeen A. M. G., Helmy W. A., Esawy M. A., 2012. Carbohydrate Polymers, 89, 314-322.

Franken J., Brandt B. A., Tai S. L., Bauer F. F., 2013. Biosynthesis of levan, a bacterial extracellular polysaccharide, in the yeast *Sacharomycescerevisiae*, PLOS ONE, 8(10), 1-14, e77499.doi: 10.1371/journal.pone.0077499.

Freitas F., Alves V. D., Reis M. A. M., 2011. Advances in bacterial EPS from production to biotechnological applications, Trends in Biotechnology, 29(8), 388-398.

Freitas F., Alves V. D., Reis M. A. M., 2011. Advances in bacterial exopolysaccharides: from production to biotechnological applications, Trends in Biotechnology, 29(8), 388-398.

Ghaly A. E., Arab F., Mahmoud N. S., Higgins J., 2007. Production of levan by B. licheniformis for use as a soil sealant in Earthen Manure Structures, American Journal of Biotechnology and Biochemistry, 3(2), 47-54.

Goncalves B. C. M., Baldo C., Celligoi M. A. P. C., 2015. Levan and levansucrase – A mini review, International Journal of Scientific & Technology Research, 4(5), 100-104.

Gupta S. K., Das P., Singh S. K., Akhtar M. S., Meena D. K., Mandal S. C., 2011. Microbial levan, an ideal prebiotic and immunonutrient in aquaculture, World Aquaculture, 61-66, DOI: 10.1142/9789814360807-0004.

Gupta S. K., Pal A. K., Sahu N. P., Saharan N., Mandal S. C., Prakash C., Akhtar M. S., Prusty A. K., 2014. Dietary microbial levan ameliorates stress and augments immunity in Ciprinuscario fry (Linnaeus, 1758) exposed to sublethal toxicity fipronil, 45, 893-906.

Han Y. W., 1989. Levan production by Bacillus polymyxa, Journal of Industrial Microbiology, 4, 447-452.

Han Y. W., Clarke M. A., 1996.Production of fructan (levan) polyfructose polymers using Bacillus polymyxa, US Patent no. 5547863/20 august 1996.

Inthanovong L., Tian F., Khodadadi, Karboune S., 2013. Properties of Geobacillus stearothermophilus levansucrase as potential biocatalyst for the synthesis of levan and fructooligosaccharides, Biotechnol. Prog., 29(6), 1405-1415.

Jathore N. R., Bule M. V., Tilay A. V., Annapure U. S., 2012. Microbial levan from Ps. Fluorescens: characterization on medium optimization for enhanced production, Food Sci. Biotechnol., 21 (4), 1045-1053.

Jathore N. R., Bule M. V., Tilay A. V., Annapure U. S., 2012. Microbial levan from Pseudomonas fluorescens; characterization and medium optimization for enhanced production, Food Sci. Biotechnol., 21(4), 1045-1053.

Kim K. H., Chung C. B., Kim Y. H., Kim K. S., Han C. S., Kim C. H., 2005. Cosmeceutical properties of levan produced by Zymomonasmobilis. J. Cosmet. Sci. 56, 395-406.

Linde D., Rodriguez-Colinas B., Estevez M., Poveda A., Plou F. J., Lobato M. F., 2012. Analysis of neofructooligosaccharides production mediated by the extracellular β-fructofuranosidase from *Xanthophyllomycesdendrorhous*, Bioresource Technology, 109, 123-130.

Liu J., Luo J., Ye H., Zeng X., 2012. Preparation, antioxidant and antitumor activities in vitro of different derivatives of levan from endophytic bacterium *Paenibacilluspolymyxa* EJS-3, Food and Chemical Toxicology, 50, 767-772.

Liu Z., Jiao Y., Wang Y., Zhou C., Zhang Z., 2008.Polysaccharides-based nanoparticle as drug delivery systems, Advanced Drug Delivery Reviews, 60, 1650-1662.

Manandhar S., D'Souza N., Vidhate S., 2009. Water soluble levan polysaccharide biopolymer electrospum fibers, Carbohydrate polymers, 78, 794-798.

Mathur V., Mathur N. K., 2006. Microbial polysaccharides based food hydrocolloid additives, Science Tech Entrepreneur, 1-10.

Melo I. R., Pimentel M. F., Lopes C. E., Calazans G. M. T., 2007. Application of fractional factorial design to levan production by Z. mobilis, Brazilian Journal of Microbiology, 38, 45-51.

Molinari M. L., Boiardi J. L., 2013. Levan production by Gluconacetobacterdiazotrophicus, Electronic Journal of Biotechnology, DOI: 10.2225/vol16-issue 3-fulltext-9.

Moosavi-Nasab M., Layegh B., Aminlary L., Hashemi M. B., 2010.Microbial production of levan using date syrup and investigation of its properties, International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering, 4(8), 603-609.

Őner E. T., 2013.Microbial production of extracellular polysaccharides from biomass. Chapter 2, DOI: 10.1007/978-3-642-32735-32.

Poli A., Kazak H., Gürleyndağ B., Tommonaro G., Pieretti G., Őner E. T., Nicolaus B., 2009. High level synthesis of levan by a novel *Halomonas* species growing on defined media, Carbohydrate Polymers, 78, 651-657.

Rehm B. H. A., 2010. Bacterial polymers: biosynthesis, modifications and applications, Nature Reviews Microbiology, doi: 10.1038/nrmicro2354.

Report - Safety assessment of microbial polysaccharides gums as used in cosmetics, 2012. Cosmetic Ingredient Review (CIR).

Rhee S., Song K., Kim C., 2002. Levan. Chapter: 14, Biopolymers, 351-377.

Santos V. A. Q., del Bianchi V. L., Garcia-Cruz C. H., 2014. Effect of initial pH in levanproductionby Z. mobilis immobilized in sodium alginate, 36(2), 349-354.

Sarilmiser H. K., Őner E. T, 2014. Investigation of anti-cancer activity of linear and aldehyde-activated levan from *Halomonassmyrnensis* AAD6^T, Biochemical Engineering Journal, 92, 28-34.

Sarilmiser K., Ates O., Ozdemir G., Arga K. Y., Őner E. T., 2015. Effective stimulating factors for microbial levan production by *Halomonassmyrnensis* AAD6^T, Journal of Bioscience and Bioengineering, 119(4), 455-463.

Sezer A. D., Kazak H., Öner E. T., Akbuğa J., 2011. Levan-based nanocarrier system for peptide and protein drug delivery: Optimization and influence of experimental parameters on the nanoparticle characteristics, Carbohydrate Polymers, 84, 358-363.

Shih Ing-Lung, Chen Li-Dar, Wu Jane-Yii, 2010.Levan production using B. subtilisnatto cells immobilized on alginate, Carbohydrate Polymers, 82, 111-117.

Silbir S., Dagbagli S., Yegin S., Baysal T., Goksungur Y., 2014. Levan production by *Zymomonasmobilis* in batch and continuous fermentation systems, Carbohydrate Polymers, 99, 454-461.

Srikanth R., Reddy C. H. S. S., Siddartha G., Ramaiah M. J., Uppuluri K. B., 2015. Review on production, characterization and applications of levan, Carbohydrate Polymers, 120, 102-114.

Szweigel A., Czarnecka M., Czarneki Z., 2007. Levan synthesis during associated action of levansucrase and *Candida cacaoi* DSM 2226 yeast, Polish Journal of Food and nutrition sciences, 57(4), 433-440.

Szwengiel A., Czarnecka M., Roszyk H., Czarnecki Z., 2004. Levan production by *Bacillussubtilis*DSM 347 strain, Electronic Journal of Polish Agricultural Universities, 7(2), 1-7.

Van Dyk S. J., Ah Kee N. L., Frost C. L., Pletschke B. I., 2012. Extracellular polysaccharides production in *Bacillus licheniformis* SVD1 and its immunomodulatory effect, BioResources, 7(4), 4976-4993.

Van Hijum A. F. T., Szalowska E., van Maarel M. J. E. C., Dijkhuizen L., 2004. Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*, Microbiology, 150, 621-630.

Wang T. H., 2015. Synthesis of neofructo-oligosaccharides, Organic chemistry insights, 5, doi: 10.4137/OCI.S13222.

Youssef G. A., Youssef A. S., Talha S., El-Aassar S. A., 2014. Increased fructosyltransferase (levansucrase) production by optimizing culture condition from *Pediococcusacidilactici* strain in shaking batch cultures, Life Science Journal, 11(7), 33-47.

Zhang T., Li R., Qian H., Mu W., Miao M., Jiang B., 2014. Biosynthesis of levan by levansucrase from *Bacillus methylotrophicus* SK 21.002, Carbohydrate Polymers, 101, 975-981.

Zhurina D., 2009. PhD Thesis - Identification and potential characterization of transcriptional regulators involved in temperature-dependent expression of levansucrase in *Pseudomonassyringae*, https://www.marmic.mpg.de/marmic/uploads_edit/Daria_Zhurina.pdf

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VALIDATION OF RT-QPCR TECHNIQUE FOR DETECTION OF BRUCELLA GENOME IN MILK SHEEP AND GOAT IN WEST BANK PART OF PALESTINE

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Abstract

Brucella melitensis is a severe pathogen for human and animals, even at low concentrations. The milk of sheep and goat and the fresh dairy products, including white cheese, are the main source of consumers' contamination. Early detection, using reliable validated diagnostic tools, is crucial for the control and eradication of the disease. The aim of this study was to develop fast molecular in-house techniques, such as RT-qPCR, to detect Brucella genome in milk. The validation of the method was carried out according to the specifications of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organization for Animal Health OIE in chapter 1.1.5: Validation and quality control of polymerase chain reaction methods used for diagnostic of infection disease and with the requests of the ISO/IEC 17025:2005: General requirements for the competence of testing and calibration laboratories. Tests for the RT-qPCR validation have proved his ability to detect 3.4 copies of Brucella's genome, into 5µl amplification product. Furthermore, DNA from non-Brucella microorganisms was not detected by developed method. While the identification of Brucella melitensis by traditional methods is time-consuming and may impair the outbreaks control, the RT-qPCR proposed can be used as a complementary, rapid and sensitive diagnostic tool for Brucella spp *in Palestine, contributing to properly implement the control policy of authorities.*

Key words*: Brucella genome, real-time PCR optimization, validation, protocol in-house*

INTRODUCTION

Brucellosis is a zoonotic disease and an important public health problem worldwide, especially in Mediterranean countries [Doganay et al., 2003; Gul et al., 2007].
The brucellosis is produced in different animal species by different *Brucella* species. The most significant and important, as zoonotic pathogen, in this genus is *Brucella melitensis* [Scholz et al., 2013; Mayer-Scholl et al., 2010].
Brucella infections are causing tremendous economic losses due to the decrease of the productivity as a result of abortion weakness of offspring and reduced milk production and may be associated with the loss of trade opportunities [FAO, 2010].
The human infections usually occur due to the ingestion of the contaminated dairy products or following the close contact with infected small ruminants [Saleem et al., 2010; Kaoud et al., 2010; Doganay et al., 2003; Zvizdic et al., 2006].
Milk of animals is the foremost source of humans' infection with *Brucella* and its bacteriological isolation has low sensitivity (Ning et al., 2013), depending on the viability and number of *Brucella* in the sample, as well as the nature of the sample that is usually cross-contaminated with various bacteria species. Thus, culture methods are not always successful, moreover, they are time-consuming and their handling could be hazardous [Hinic, 2009; Refai et al., 2002].

Serologic methods are rapid but inconclusive, because not all infected animals produce detectable levels of antibodies, and the cross-reactivity against other antigens can give false-positive results [Gwida et al., 2011]. Early detection of *Brucella* genome, by using valid diagnostic tools, is crucial for the control and eradication of this disease [Al-Garadi et al., 2011; Bricker et al., 2002] The molecular diagnostic techniques represent an important breakthrough in the diagnostic practice. The most of the authors confirmed that real-time PCR is a highly sensitive method for the *Brucella* detection from various samples [Doosti et al., 2011; Safarpoor Denkordi et al. 2014; Newby et al., 2003; Al-Garadi et al., 2011; Mirnejad et al., 2012; Foster, 2008].

Laboratory tests for any infectious agent, by molecular diagnostic techniques, are requesting standardization, optimization and quality assurance [Sloan, 2007]. This is the request of the international quality standard for veterinary laboratories in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organization for Animal Health (OIE) chapter 1.1.5: Validation and quality control of polymerase chain reaction methods used for diagnostic of infection disease and the guide ISO/IEC 17025:2005: General requirements for the competence of testing and calibration laboratory which demands for verification and validation procedures for each in-house assay (OIE, 2008; ISO/IEC 17025, 2005).

The real time quantification is based on the relationship between initial template amount and Ct value, obtained during amplification, an optimal qPCR assay absolutely essential for accurate and reproducible quantification of samples.

The hallmarks of an optimized qPCR assay are: linear standard curve (R^2> 0,980 or r > 1-0.9901), high amplification efficiency 90-105% and consistency across replicate reactions [Bio Rad laboratories, 2006; Applied Biosystem, 2003].

The main objective of this study was to develop and validate an in-house Real-Time qPCR protocol, in order to provide a sensitive diagnostic tool for rapid detection of *Brucella* genome in sheep and goat milk. The most important epidemiological target was to implement a rapid preventive tool against brucellosis in Palestine.

MATERIALS AND METHODS

Samples preparation

Control strains

The reference control strains were: different non-*Brucella* bacteria used for optimization and validation RT-qPCR, were retrieved as loops from Oxoid Company; vaccine strain *Brucella melitensis* Rev 1 from Ovejero Company (Spain) and *Brucella melitensis* pure bacteria derived from our laboratory.

In this study were also used wild *Brucella melitensis* strains and positive and negative milk samples (Table 2). All milk samples were obtained from animals during their lactation period: 10 ml of each milk sample was used for detection of *Brucella* genome by Real-Time PCR assay.

Preparation of control reference strains

Each reference strain of bacteria was inoculated onto specific nutrient agar. Plates were then incubated overnight at 37ºC under different specific condition, up to the requirements of each bacterium. After 24 hours each bacterium strain was confirmed by specific biochemical test; then few colonies were harvested from nutrient agar and immersed in 200 µl of phosphate buffer saline. From this, *Brucella melitensis* was inoculated on specific agar and harvested 48 hours later.

DNA extraction

All reference strains of non-*Brucella* and *Brucella* bacteria, *B. melitensis* Rev 1 vaccine strain, *B. mellitensis* wild strain, positive and negative milk samples, were extracted using a commercial kit QIAamp RNA Mini Kit (Qiagen, 52906) according to the manufacturer's instructions. Before extraction milk samples were centrifuged at 8000 rpm for 15 min to settle out the bacteria [Khan et al., 2011; Romero et al., 1999]. The fatty top layer and supernatant were discarded and 200µl pellet were used for the extraction procedure.

Determination of DNA concentration of Brucella melitensis Rev 1 vaccine

In this study, for validation procedure and calculation was used *Brucella melitensis* Rev 1 vaccines strain (Ovejero Company, Spain), as reference material. The DNA concentration was evaluated by using NanoDrop® ND-1000 Spectrophotometer Genomic (Thermo Scientific, USA). The concentration was given in ng/µl and then converted into gene copies per µl by using URI Genomics & Sequencing Center - dedicated software, created by Andrew Staroscik (2004).

Validation of RT-qPCR

Specificityand sensitivityof RT-qPCR

Brucella spp. was identified using the primers and probe targeting the bcsp31 gene (GenBank accession number M20404) [Probert et al., 2004]. The specificity of the primers and of the probes used in this study (Table 1) were analysed by using Standard Nucleotide BLAST (Basic Local Alignment Search Tool) administered by the National Centre for Biotechnology Information (NCBI).The sensitivity and specificity of each qPCR assay were studied using different dilutions of DNA the *Brucella melitensis* Rev 1 and DNA the different non-*Brucella* bacteria, positive and negative *Brucella* milk samples and negative control (Table 2).

Table 1. Specific real-time PCR oligonucleotides primers and probe for *Brucella* group (Probert et al., 2004)

PCR identification	Primer sequence	5'Fluorophore/3' quencher
***Brucella* spp F**	5 –GCTCGGTTGCCAATATCAATGC-3	-
***Brucella* spp R**	5- GGGTAAAGCGTCGCCAGAAG-3	-
***Brucella* spp Probe**	5-AAATCTTCCACCTTGCCCTTGCCATCA-3	6-FAM/BHQ1

Limit of detection (LOD) and standard curve preparation

The evaluation of LOD was performed by standard suspension of genomic DNA of *Brucella melitensis* Rev 1 vaccine strain, as initial stock, and 11 four-fold dilutions with three PCR replicates. Dilutions were chosen within the linear dynamic range of the assay and expected concentrations of DNA within possible specimens. Ct values were determined by running the RT-qPCR using 5µl of each dilution together with known standard PCR in the same run. The log-linear regression analysis, standard deviation and correlation coefficient of the Cq-values of each concentration was performed using Microsoft Excel 2007 software.

Repeatability, reproducibility and efficiency of the test

The efficiency of the RT-qPCR assays was evaluated in one run with 11 serial dilutions. The repeatability was evaluated by testing all dilutions in three replicates, in three PCR runs, and the assay was repeated in three different days. Tests of reproducibility were performed through running RT-qPCR protocol by another technician. The efficiency of the PCR was calculated from the slope of the logarithmic regression of Ct values plotted against DNA concentrations by $E = e(-1/slope)-1$. The efficiency of the assay was then given by equation: $E (100\%) = (10\text{-}1/k – 1) \times 100$, where k is the slope of the standard curve obtained by linear regression with calculation y-intercept which corresponds C_t value for a single copy of the target molecule and coeficientul of determination (R^2) such that $0 \leq r^2 \leq 1$, which denotes the strength of the linear association between *x* and *y* and represents the percent of the data that is the closest to the line of best fit and is a measure of how well the regression line represents the data [Eurogentec, 2013, Life technologies, 2012, Bio-Rad laboratories, 2006].

Robustness and gel electrophoresis

Robustness was evaluated by running all dilutions of *Brucella melitensis* Rev 1 strain on three different real time instruments: two instruments Real-Time PCR of SmartCycler® - Cepheid and LightCycler® - Roche. PCR products were analysed by 2% agarose (Promega, UK)gel electrophoresis.

RESULTS AND DISCUSSIONS

Concentration of DNA

The concentration of genome stock *Brucella melitensis* Rev 1 vaccine was 2.5 ng/µl and equal to 3.52×10^6 copies of genomic DNA, considering the approximate size of *Brucella* genome 3290000000 bp [DelVecchio, 2002] and represents only one copy of the bcsp31 gene on the *Brucella* genome.

Evaluation specificity and sensitivity of RT-qPCR

The BLAST search showed that the primers and the probes did not have identity with other organisms. The primers sequences were

identical 100% to their respective target. In order to perform the validation of RT-qPCR detection of the gene bcsp31 of *Brucella*spp. were used: different dilutions of the vaccine strain *Brucella melitensis* Rev 1 as positive control and *Brucella* wild strains, and, as negative controls, negative milk samples and non-*Brucella* bacteria. Real time PCR was performed with a set of primers and probe showed in Table 1. DNA amplification mixture was composed of 7.12µl of nuclease free water, 5 µl Qiagen 1 step RT PCR buffer x5 (Qiagen, Cat. No: 210212), 0.8µl dNTPs [200µM] (Qiagen, Ct. No: 210112), 0.5 µl forward and reverse primer [20µM/µl], 0.5 µl [5µM/µl] probe (Syntheza, Israel), 1µl Taq polymerase enzyme (Sigma, USA, Cat. No D4545) and 5µl of DNA product. Amplification was performed in SmartCycler, Cepheid. Reaction was initiated with denaturation at 95°C for 3 minutes followed by 50 cycles: 95°C for 15 sec, annealing and extension at 60°C for 35 minutes. No amplification products were observed in real-time PCR of negative controls, whatever is the targets the non-*Brucella* micro-organisms tested, *Brucella melitensis* negative milk or water samples [Table 2].

Limit of detection (LOD), preparation of standard curve and calculation of efficiency

The stock suspension and 11 four-fold dilutions of template DNA *Brucella melitensis* Rev 1 vaccine strain, ranging from $3.52x10^6$ to 0.8 gene copies per reaction indicate that 3.4 copies of bacterial genomes in 5µl of DNA the sample detected by developed RT-qPCR assay protocol. At these concentrations all *Brucella* positive bacteria and *Brucella melitensis* positive milk samples were positive. In this assay was calculated standard deviation and coefficient of variation [Table 3].

Table 2. Control and reference strains of bacteria used in validation of RT-qPCR and specificity evaluation result

Strain	Reference and origin	Type of samples	No of samples	Results
Brucella melitensis Rev 1	Elberg strain of vaccine, Ovejero	Vaccine	2	Positive
Brucella melitensis	Field strain, our laboratory	Pure bacteria	2	Positive
Brucella melitensis positive milk samples	Field strain, our laboratory	Milk	10	Positive
Bacillus cereus	ATCC 11778, Oxoid	Pure bacteria	2	Negative
Campylobacter jejuni	ATCC 29428, Oxoid	Pure bacteria	2	Negative
E.coli	ATCC 12229, Oxoid	Pure bacteria	2	Negative
Salmonella enerica subsp *Enteritidis*	ATCC 13076, Oxoid	Pure bacteria	2	Negative
Listeria monocytogenes	ATCC 7644, Oxoid	Pure bacteria	2	Negative
Staphylococcus aureus	ATCC 33862, Oxoid	Pure bacteria	2	Negative
Yersinia enterocolitica	ATCC 23715, Oxoid	Pure bacteria	2	Negative
Brucella melitensis negative milk samples	Our laboratory	Milk	10	Negative
Negative control		Water	2	Negative

Table 3. DNA concentrations and Ct values, standard deviation and coefficient of correlation obtained from experiment

No of dilution	Concentration (ng/µl)	No of copies in 1 µl of RT-qPCR product	No of genes copies in 5µl	Ct Mean ± SD	CV (%)
Stock	2.5	704000	$3.52x10^6$	19.04 ± 0.07	0.003
Dilution 1	0.625	176000	8.8×10^5	22.20 ±0.28	0.013
Dilution 2	0.156	43900	2.2×10^5	24.37 ± 0.46	0.019
Dilution 3	0.039	11000	$5.5x10^4$	26.72 ± 0.23	0.009
Dilution 4	0.00975	2750	13750	29.52 ± 0.41	0.014
Dilution 5	0.00244	687	3940	31.15 ± 0.26	0.008
Dilution 6	0.000609	171	860	32.91 ± 0.23	0.007
Dilution 7	0.000152	42.8	214	34.85 ± 0.68	0.019
Dilution 8	0.0000380	10.7	53.5	36.21 ± 0.75	0.021
Dilution 9	0.00000952	2.68	13.4	38.13 ± 0.09	0.002
Dilution 10	0.00000238	0.67	3.4	39.04 ± 0.07	0.002
Dilution 11	0.000000595	0.17	0.8	Not detect	-

The LOD for each assay was the lowest concentration consistently detected in all three PCR runs. The first ten dilutions were detectable and produced standard curve with correlation coefficient 0.996881. Analysis of the stock suspension and of the different dilutions of standard DNA revealed that the efficiency of the assay is 96.5%, with y-intercept 18.7 and R^2 value 0.09984 (Figure 1).

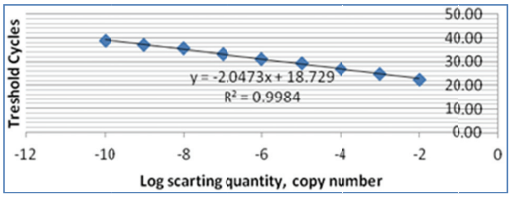

Figure 1.The linearity and linear regression of RT-qPCR standard curve the bscp31 gene of *Brucell*a spp.

Repeatability, Reproducibility, Robustness, and gel electrophoresis

Results were found almost similarly for all three replicates of the dilution and also for the replicates evaluated in three different days and on three different instruments. Given results by following criteria were established in order to validate the analysis: samples were considered positive for *Brucella* spp when their amplification curves were similar to the positive control curve and exceeded the threshold with Ct values lower than 40. The electrophoresis of amplified product revealed a single band, corresponding at the expected size and the decrease of the DNA concentrations of amplicons is showed by the decrease in fluorescence [Figure 2].

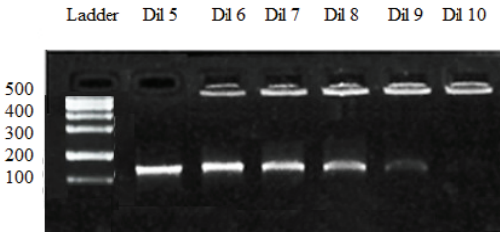

Figure 2. Different concentration of RT-qPCR products are show equal different visibility of bands in gel electrophoresis

DISCUSSION

Despite the intensive ocular vaccination program, brucellosis in Palestine remains a significant problem. Our previously study revealed that during the years 2013 and 2015 several new isolates of *Brucella* spp. were detected in small ruminants from the northern districts of the West Bank [Awwad et al., 2015].

The chosen specific target of *Brucella* - bcsp31 is a conserved, single-copy gene, coding a 31-kDa outer membrane protein and is found in all strains of *Brucella*. This gene is commonly used as target for detection of *Brucella* by PCR, due to the small size of the amplicon (151 bp) that facilitates its use in real-time PCR. [Bounaadja, 2009; Da Costa, 1996; Debeaumont, 2005].

A genus-specific target was chosen to establish in-house RT-qPCR protocols that detect all species of *Brucella* in the milk of the infected animals, in order to increase the possibility of the detection and to improve the control of brucellosis in Palestine. The developed assay performed well in the analytical sense and was able to detect very low concentrations (3.4 copies) of *Brucella* genomic DNA. The test was performed in accordance with requirements OIE and to the requirements of ISO 17025 for optimization laboratory methods. The efficiency of this assay was 96.5%, with y-intercepts 18.7 and R^2 value 0.09984. Although standard curve parameters of genomic DNA *Brucella melitensis* Rev 1 vaccine strain was subtly altered, there was no reduction in percentage amplification at very low bacterial DNA concentrations. A few possible reasons have been taken into consideration for the relatively inconsistent performance of *Brucella* detection by different laboratories. The stage of infection may influence the number and location of bacteria [Alton et al., 1988]; the sample type used for diagnostic purposes may affect the results [O'Leary et al., 2006]; the presence of large amounts of host genomic DNA may inhibit the PCR reaction [Navarro et al., 2002]; the DNA extraction method used may be crucial in determining the ability of the PCR assay to detect the bacterium [Romero et al., 1999and Lopez-Goni et al.,1999]. Factors that may

compromise DNA recovery from milk include difficulties in disrupting bacterial cell walls, loss of DNA template through extraction procedures, or the presence of potential polymerase inhibitors. In addition, the amount of milk used for PCR is much smaller than that required for bacteriological methods, and the number of organisms contained in a sample may thus not reach PCR detection limits [Yousef-Beingi, 2005]. For this purpose the milk samples were centrifuged and only concentrated bacteria (pellet) was used for extraction.

Above of all these reasons, the quality of a laboratorial result is linked to the use of procedures such as validated methods, quality internal controls, participation in inter-laboratorial comparison programs, the proper use of certified reference materials, and the compliance with requirements of standards. Some of the parameters used in validation such as the specificity and sensitivity of the method, the detection limit, linearity, the repeatability, the reproducibility, and the robustness, are crucial to produce reliable in-house method.

In summary, we evaluated the feasibility of molecular assays as improved and very sensitive diagnostic tools for detection of *Brucella* spp. in fresh milk, especially during outbreaks. The advantages of this technique are that it can be performed very quickly, it allows the direct identification of the organism and it decrease the number of false-positive result [Soherbi et al., 2011; Redkar et al., 2001; Yousef-Beingi et al., 2009]. In addition, along with molecular assay, serology must always be performed and, in accord with the goal of the investigation, will be confirmed by bacterial isolation.

CONCLUSIONS

The RT-qPCR for the detection of *Brucella* spp. in fresh milk, above described, proved to be a sensitive and specific tool for the detection of *Brucella* genome.

The efficiency of this assay was 96.5%, with y-intercepts 18.7 and R^2 value 0.09984.

The proposed protocol is fast performed, it allows the direct identification of the organism and it increases the specificity of diagnostic.

The critical step for our RT-qPCR is the quantity of the *Brucella* DNA in the milk samples: to reduce its impact, the milk samples have been centrifuged and the DNA extraction has been carried out only on the sediment bacteria (pellet).

The novel molecular technique such as RT-qPCR in-house is cost-efficiency affordable and is useful as a reliable screening method for the rapid detection of the infectious agent: the use of this technique could be a huge step in order to rapidly implement the measures for the outbreak control, to prevent spread of the disease and to avoid the human infections. This method is designed to be performed using raw milk.

REFERENCES

Al-Garadi A., Khairani-Bejo S., Zunita Z., Omar A.R., 2011. Detection of *Brucella melitensis* in blood samples collected from goats. Journal of Animal and Veterinary Advances, 10:1437-1444.

Alton G., Jones L., Angus R., Verger J., 1988. Techniques for the *Brucellosis* Laboratory. Institut National de la RechercheAgronomique Publications, Paris, France.

Applied Biosystems, 2003. Creating Standard Curve with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR. 4371090 Revision A. Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd, http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042486.pdf.

Awwad E., Adwan K., Farraj M., Essawi T., Rumi I., Manasra A., Baraitareanu S., Gurau R.M., Danes D., 2015. Cell Envelope Virulence Genes among Field Strains of *Brucella melitensis* Isolated in West Bank Part of Palestine. Agriculture and Agricultural Science Procedia, 6:281-6.

Bio Rad Laboratories, 2006. Real-time PCR application guide, http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf.

Bounaadja L., Albert D., Chenois B., 2009. Real-time PCR for identification of *Brucella* spp.: a comparative study of IS711, bcsp31 and per target genes. Vet Microbiol, 137:156-64.

Bricker B., 2002. PCR as a diagnostic tool for brucellosis. Veterinary Microbiology, 90:435-46.

Da Costa M., Guillou J.P., Garin-Bastuji B., Thiebaud M., Dubray G., 1996. Specificity of six gene sequences for the detection of the genus *Brucella* by DNA amplification. J ApplBacteriol, 81:267–275.

Debeaumont C., Falconnet P., Maurin M., 2005. Real-time PCR for detection of *Brucella* spp. DNA in human serum samples. Eur J ClinMicrobiol Infect Dis, 24:842–845.

DelVecchio V., Kapatral V., Redkar R., Patra G., Mujer C., Los T., Ivanova N., Anderson I., Bhattacharyya

A., Lykidis A., Reznik G., Jablonski L., Larsen N., D'Souza M., Bernal A., Mazur M., Goltsman E., Selkov E., Elzer P., Hagius S., O'Callaghan D., Letesson J., Haselkorn R., Kyrpides N., Overbeek R., 2002. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. ProcNatlAcadSci USA, 99(1):443-8.

Doganay M., Aygen B., 2003. Human brucellosis: an overview. Int J Infect Dis, 7:173-182.

Doosti A., GhasemiDencodri P., 2011. Application of real time PCR for identification and differentiation of *Brucella abortus* and *Brucella melitensis* in cattle. Bulgarian J of Vet Med, 14(2):109-5.

FAO, 2010. *Brucella melitensis* in Eurasia and the Middle East. FAO Animal Production and Health Proceedings. No. 10. Rome.http://www.fao.org/ docrep/ 012/i1402e/i1402e00.pdf.

Foster T., Okinaka R., Svensson R., Shaw K., De B., Robison R., Probert W., Kenefic L., Brown W., Keim P., 2008. Real-time PCR assays of single- nucleotide polymorphisms defining the major *Brucella* clades. Journal of Clinical Microbiology, 46:296-301.

Gul T., Khan A., 2007. Epidemiology and epizootology of brucellosis: A review. Pakistan Veterinary Journal, 27:145-51.

Gwida M., El-Gohary H., Melzer F., Tomaso H., Rösler U., Wernery U., Wernery R., Elschner C., Khan I., Eickhoff M., Schöner D., Neubauer H., 2011. Comparison of diagnostic tests for the detection of *Brucella* spp. in camel sera. BMC Research Notes, 4:525.

Hinic V., Brodard I., Thomann A., Holub M., Miserez R., Abril C., 2009. IS711-based real-time PCR assay as a tool for detection of *Brucella* spp. in wild boars and comparison with bacterial isolation and serology. BMC Vet Res., 5:22.

ISO/IEC 17025:2005 General requirements for the competence of testing and calibration laboratories, 2005. https://www.iso.org/obp/ui/#iso:std:iso-iec: 17025:ed-2:v1:en

Kaoud A., Zaki M., El-Dahshan A., Nasr S., 2010. Epidemiology of brucellosis among farm animals. Nature and Science, 8:190-7.

Khan M., Weary D, and Keyserlingk A. 2011. Invited review: Effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. J. Dairy Sci. 94 :1071–1081

LópezGoñi I, García Y., Marín C., De Miguel M., Muñoz P., Blasco J., Jacques I., Grayon M., Cloeckaert A., Ferreira A., Cardoso R., SáMi C., Walravens K., Albert D., Garin-Bastuji B., 2009. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. J ClinMicrobiol, 46(10):3484-7.

Mayer-Scholl A., Draeger A., Gollner C., Scholz C., Nockler K., 2010. Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species. J. microbiol. Meth., 80(1):112-4.

Mirnejad R., Doust H., Kachuei R., Mortazavi M., Khoobdel M., Ahamadi A., 2012. Simultaneous detection and differentiates of *Brucella abortus* and *Brucella melitensis* by combinatorial PCR. Asian Pacific Journal of Tropical Medicine 5: 24-8.

Navarro E., Escribano J., Fernandez J., Solera J., 2002. Comparison of three different PCR methods for detection of *Brucella* spp. in human blood samples. FEMS Immunol. Med. Microbiol. 34:147-51.

Newby T., Hadfield T., Roberto F., 2003. Real-time PCR detection of *Brucella abortus*: A comparative study of SYBRgreen I, 5′-exonuclease, and hybridization probe assays. Applied and Environmental Microbiology, 69:4753-9.

Ning P., Guo M., Guo K., Xu L., Ren M., Cheng Y., Zhang Y., 2013. Identification and Effect Decomposition of Risk Factors for *Brucella* Contamination of Raw Whole Milk in China. PLoS ONE 8(7): e68230.

OIE, World Organisation for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE, Paris, France, 5th edition, 2008, http://www.oie.int/eng/normes/manual/Asummry.htm

O'Leary S., Sheahan M., Sweeney T., 2006. *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Res. Vet. Sci. 81:170-6.

Probert W., Schrader K., Khuong N., Bystrom S., Graves M., 2004. Real-Time Multiplex PCR Assay for Detection of *Brucella*spp, *B. abortus*, *B. melitensis*, Journal of Clinical Microbiology, 42:1290-3.

Redkar R., Rose S., Bricker B., DelVecchio V., 2001. Real-time detection of *Brucella abortus*, *Brucella melitensis* and *Brucella suis*. Molecular and Cellular Probes, 15:43-52.

Refai M., 2002. Incidence and control of brucellosis in the Near East region. Veterinary Microbiology, 90:81−110.

Romero C., Lopez-Goni I., 1999. Improved Method for Purification of Bacterial DNA from Bovine Milk for Detection of *Brucella* spp. by PCR. Applied and environmental microbiology, 65(8):3735-7.

SafarpoorDenkordi F, Kahamesipour F, Momeni M., 2014. *Brucella abortus* and *Brucella melitensis* in Iranian Bovine and Buffalo Semen Samples: The First Clinical Trial on Seasonal, Senile and Geographical Distribution Using Culture, Conventional and Real-time Polymerase Chain Reaction Assays. KafkasUniv Vet FakDerg, 20(6):821-8.

Saleem N., Boyle M., Sriranganathan N., 2010. Brucellosis: a reemerging zoonosis. Veterinary Microbiology, 140(3-4):392-8.

Scholz H., Vergnaud G., 2013. Molecular characterisation of *Brucella* species. Rev. sci. tech. Off. int. Epiz., 32(1):149-62.

Sloan L.M., 2007. Real-time PCR in clinical microbiology: Verification, validation, and contamination control. ClinMicrob Newsletter, 29:87-95.

Sohrabi M., MohabatiMobarez A., Behmanesh M., Khoramabadi N., Doust R., 2011. Evaluation of a new set of Real-Time PCR for *Brucella* detection within human and animal samples. JPHS, 1(2):13-7.

WHO (World Health Organization), 1999. Human and animal brucellosis: Epidemiological Surveillance in the MZCP Countries. Report of a WHO/MZCP

Workshop, Damascus, Syrian Arab Requblic, 4-5 May 1998. Athens 1999.

Yousef-Beingi G., Rad M., Hosseine M., Jafari A., 2005. Effect of centrifugation of milk samples on bacteriological culture. Iranian Journal of Veterinary Research, 6(1):59-61.

Zvizdic S., Cengic D., Bratic M., Mehanic S., Pinjo F., Hamzic S., 2006. *Brucella melitensis* review of the human infection case. Bosnian Journal of Basic Medical Sciences, 6:15-8.

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STUDIES CONCERNING THE OPTIMISATION OF REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION TECHNIQUE OF PAN-SIMBU VIRUS GROUP

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Abstract

Several molecular methods have been developed for diagnostic or surveillance of those agents of emerging infectious diseases, including for the Schmallenberg-Simbu group viruses. Serological surveillance of the Schmallenberg-Simbu group viruses in Romania revealed the presence of positive ruminants and it rise up the question about the presence of virus into the environment. In this frame, the paper has described preliminary studies concerning the optimisation of classical RT-PCR of pan-Simbu virus group. We used the OneStep RT-PCR Kit and made minor changes as follows. For one reaction were used 5 µl 5x OneStep RT-PCR Buffer, 1.5 µl dNTP 10 mM, 1.5 µl OneStep RT-PCR Enzyme Mix, 4 µl primer panOBV-L-2959 F 10 µM, 4 µl primer panOBV-L-3274R 10 µM and 9 µl RNase-free water. Into reaction tubes were transferred 25 µL master mix + 10 µL sample. Thermal cycling program consisted of one cycle of 50°C - 30 min and one cycle of 95°C - 15 min, followed by 42 cycles of 95°C - 30 s, 55°C - 30 s, 72°C - 30 s and 72°C - 10 min. All results obtained by real time RT-PCR (virotype SBV RT-PCR Kit) and classical RT-PCR were correlated with the quantity of estimated RNA by fluorometry. The sensitivity of classical RT-PCR was lower than sensitivity of real time RT-PCR, the positive result being acquired at a minimum of 3.91 ng/µl RNA per sample. The specificity of methods was the same, without non-specific electrophoretic bands detection. Therefore, our classical RT-PCR protocol can be a useful tool in evaluation of virus circulation in countries with or without history of associated Simbu disease in livestock, or with reported seroconversion.

Key words*: PCR, diagnostic, real-time RT-PCR, Orthobunyavirus, Schmallenberg virus.*

INTRODUCTION

Simbu serogroup viruses are arthropod-borne bunyaviruses antigenically related (Horne and Vanlandingham, 2014), some of them involved in livestock's pathological disorders (Coverdale et al., 1978; Jagoe et al., 1993; Hoffmann et al., 2012).

Several molecular methods have been developed for diagnostic or surveillance of those agents of emerging infectious diseases (Baraitareanu and Danes, 2014), including for the Schmallenberg-Simbu group viruses (Hoffmann et al., 2012).

Since October 2011, when a novel orthobunyavirus of the Simbu serogroup, subsequently named Schmallenberg virus (SBV) was first identified in German cows, several European groups of researchers have developed molecular diagnostic tools able to identify SBV or Simbu serogroup (PSV) (Fischer et al., 2013; Hoffmann et al., 2012; Afonso et al., 2014; Balenghien et al., 2014; Schulz et al., 2015). Also, the retrospective studies or meta-analyses concerning molecular tools used in the diagnostic of orthobunyavirus, are already available (Afonso et al., 2014; Balenghien et al., 2014; Manescu et al., 2015; Schulz et al., 2015).

The PCR optimization strategies aim to correct one or more parameters, in order to enhance specificity and sensitivity at an optimal confidence level (Roux, 2009).

The confidence of diagnostic method can be quantified by interlaboratory comparison of results. For this reason, Schulz et al. (2015) conducted the European interlaboratory comparison of real-time RT-PCR for Schmallenberg virus (SBV) detection on experimental and field samples. In this study, they identified that the confidence of the results can affected by the method of extraction of

RNA-SBV from semen samples (Schulz et al. 2015).

Fischer et al. (2013) developed a pan-Simbu real-time reverse transcriptase PCR able to detect several viruses of Simbu serogroup (Aino virus, Akabane virus, Douglas virus, Oropouche virus, Peaton virus, Sabo virus, Sango virus, Sathuperi virus, Schmallenberg virus, Shamonda virus, Shuni virus, Simbu virus, Thimiri virus, Tinaroo virus), Bunyamwera serogroup (Batai virus, Bunyamwera virus, Ngari virus) and, probably, California serogroup (Tahyna virus, Chatanga virus, La Crosse virus, Jamestown Canyon virus, Snowshoe hare virus, Inkoo virus). This broad molecular tool for screening allows the identification of targeted viruses both in mammalian samples and in the samples of the vector insect (Fischer et al., 2013), which recommends it as an excellent method of epidemiological surveillance.

Serological surveillance of the Schmallenberg-Simbu group viruses in Romania has revealed the presence of positive ruminants (Danes et al., 2014) and it rises up the question about the presence of virus into the environment. In light of these circumstances, the paper described the preliminary studies concerning the optimisation of reverse transcription polymerase chain reaction technique of pan-Simbu virus group.

MATERIALS AND METHODS

In order to optimise the RT-PCR technique for the detection of pan-Simbu Virus Group (PSV) RNA, the following materials were used:
(1) Five dilution series of positive control RNA of the Schmallenberg virus (Friedrich-Loeffler-Institut, Greifswald–Insel, Riems, Germany);
(2) Qubit RNA HS Assay Kit and Qubit 3.0 Fluorometer (Invitrogen, Canada);
(3) *virotype* SBV RT-PCR Kit (Qiagen, Germany);
(4) SmartCycler and Life Science 2d SmarCycler software (Cepheid, USA);
(5) OneStep RT-PCR Kit (Qiagen, Germany);
(6) Agarose + TAE/TBE (1x) + Ethidium bromide (10 mg/ml).
Forward and revers primers for amplification of L-Segment are presented in table 1.
RNA quantification of the dilution series of SBV-RNA were in accord with the recommendations of the manufacturer (Invitrogen, Canada).

Table 1. Sequence of forward and revers primers used for amplification of L-Segment and size of expected PCR product (Fischer et al., 2013)

Primer name	Sequence	Product size
panOBV-L-2959 F	5'-TTGGAGARTATGARGCTAARATGTG-3'	279 bp
panOBV-L-3274R	5'-TGAGCACTCCATTTNGACATRTC-3'	

Detection of RNA from Schmallenberg virus by real-time RT-PCR was performed in accord with the protocol described in *virotype* SBV RT-PCR Kit Handbook (Qiagen, Germany). Briefly, preparation of reaction mix was performed in 25 µL/sample (20 µL master mix + 5 µL sample), and the real-time RT-PCR protocol consisted in one cycle of 45°C - 10 min and one cycle of 95°C - 10 min, followed by 40 cycles of 95°C - 15 s, 56°C - 30 s, 72°C - 30 s.
Classical RT-PCR for RNA-SBV detection was performed by adapting a previously described RT-PCR protocol developed for the detection of pan-Simbu Viruses (Fischer et al., 2013). We used the OneStep RT-PCR Kit (Qiagen, Germany) and made minor changes as follows.
For one reaction were used 5 µl 5x OneStep RT-PCR Buffer (Qiagen, Germany), 1.5 µldNTP 10 mM (Qiagen, Germany), 1.5 µl OneStep RT-PCR Enzyme Mix (Qiagen, Germany), 4 µl primer panOBV-L-2959 F 10 µM (Fischer et al., 2013), 4 µl primer panOBV-L-3274R 10 µM (Fischer et al., 2013) and 9 µlRNase-free water. Into reaction tubes were transferred 25 µL master mix + 10 µL sample. Thermal cycling program consisted of one cycle of 50°C - 30 min and one cycle of 95°C - 15 min, followed by 42 cycles of 95°C - 30 s, 55°C - 30 s, 72°C - 30 s and 72°C - 10 min.
Agarose electrophoresis was performed to visualize the PCR products (0.9 g agarose, 60 ml TAE/TBE 1x, 5 µl ethidium bromide; 10 mg/ml; 100V; 1,5A; 35 min).

RESULTS AND DISCUSSIONS

RNA quantification of the dilution series of SBV-RNA (provided by Friedrich-Loeffler-Institut) have been done with Qubit RNA HS Assay Kit in Qubit 3.0 Fluorometer. The quantities of total RNA in each dilution are presented in table 2.

Table 2. Dilution series of positive control RNA of the Schmallenberg virus

Dilution	RNA quantity (ng/µl)*
10^{-2}	4.92
10^{-3}	3.91
10^{-4}	2.46
10^{-5}	1.36
10^{-6}	0.84

* RNA quantification with Qubit RNA HS Assay Kit in Qubit 3.0 Fluorometer

Real-time RT-PCR technique provided quantification cycle (Cq±SD) 17.19±0.141 for dilution 10^{-2}, 21.55±0.140 for dilution 10^{-3}, 24.16±0.164 for dilution 10^{-4}, 28.61±0.139 for dilution 10^{-5}, and 30.93±0.113 for dilution 10^{-6} (figure 1).

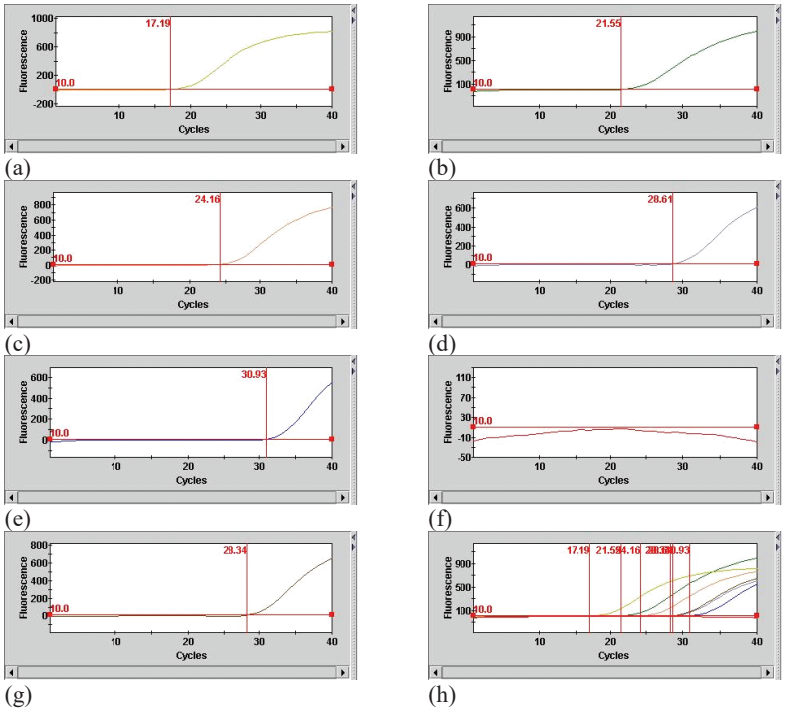
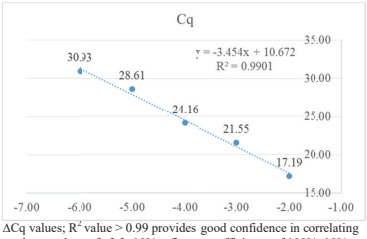


Figure 1. Quantification cycle (Cq) values obtained by Real-time RT-PCR for five dilutions of positive control RNA of the Schmallenberg virus (Friederich-LoefflerInstitut, Germany), positive and negative controls of *virotipe* SBV RT-PCR Kit (Qiagen, Germany). (a) Cq value of dilution 10^{-2}; (b) Cq value of dilution 10^{-3}; (c) Cq value of dilution 10^{-4}; (d) Cq value of dilution 10^{-5}; (e) Cq value of dilution 10^{-6}; (f) Cq value of negative control supplied by *virotipe* SBV RT-PCR Kit; (g) Cq value of positive control supplied by *virotipe* SBV RT-PCR Kit; (h) Cq values of all amplification curves (analysis with Smart Cycler Life science software 2.0d)

Also, Cq value of positive control supplied by *virotipe* SBV RT-PCR Kit validated the reaction (Qiagen, Germany). All dilutions were analysed in triplicate, in three runs. The efficiency of real-time RT-PCR technique was evaluated by slope (-3.454) and R^2 value (0.9901) and the precision by standard deviation (<0.164) of all Cq values obtained in each dilution (figure 2).

The results of classical RT-PCR for RNA-SBV protocol are synthesized in table 3.

All results obtained by real time RT-PCR and classical RT-PCR were correlated with the quantity of estimated RNA by fluorometry.



ΔCq values; R^2 value > 0.99 provides good confidence in correlating values; a slope of ~3.3±10% reflects an efficiency of 100%±10%.

Figure 2. Linear regression, R^2 value and slope analysis of results for five serial decimal dilutions

The sensitivity of classical RT-PCR was lower than real time RT-PCR, the positive result being acquired only for dilutions 10^{-2} and 10^{-3}.

Table 3. Result of classical RT-PCR for RNA-SBV

Dilution	Results*
10^{-2}	++
10^{-3}	+
10^{-4}	±
10^{-5}	-
10^{-6}	-

*++ obviously band, + clear band, ± weak band, - no band

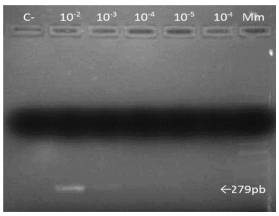


Figure 3. PCR electrophoresis results in classical PCR

Based on the recorded data the sensitivity of classical RT-PCR was estimated at a minimum of 3.91 ng/µl RNA per sample. The specificity of methods was identical, without the detection of nonspecific electrophoretic bands (figure 3). The results were similar with those of Fischer et al. (2013).

CONCLUSIONS

A classical RT-PCR assay was optimised to provide a rapid and sensitive molecular method for the surveillance of orthobunyaviruses' Simbu serogroup.

The assay can be a useful tool in evaluation of virus circulation in countries with or without history of associated Simbu disease in livestock, or with reported seroconversion.

However, to obtain reliable results using our classical RT-PCR protocol, the sample should contain minimum amount of 3.91 ng/µl of RNA.

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REFERENCES

Afonso A., Abrahantes J.C., Conraths F., Veldhuis A., Elbers A., Roberts H., Van der Stede Y., Méroc E., Gache K., Richardson J., 2014. The Schmallenberg virus epidemic in Europe-2011-2013, Prev. Vet. Med., 116(4):391-403.

Balenghien T., Pages N., Goffredo M., Carpenter S., Augot D., Jacquier E., Talavera S., Monaco F., Depaquit J.,Grillet C., Pujols J., Satta G., Kasbari M., Setier-Rio M.L., Izzo F., Alkan C., Delécolle JC, Quaglia M, Charrel R, Polci A, Bréard E., Federici V., Cetre-Sossah C., Garros C., 2014. The emergence of Schmallenberg virus across Culicoides communities and ecosystems in Europe, Prev. Vet. Med., 116(4):360-369.

Baraitareanu S., Danes D., 2014. Pathology related with "novel" emerging infectious agents in livestock, Scientific Works. Series C. Vet. Med., LX(1):41-46.

Coverdale O.R., Cybinski D.H., St George T.D. 1978. Congenital abnormalities in calves associated with Akabane virus and Aino virus. AustVetJ.54(3):151-2.

Danes D., Baraitareanu S., Gurau M.R., Dan M., Bartoiu I.A., Moldovan H., Danes M., 2014. Preliminary Results of Schmallenberg virus Serosurveillance in Romania. Advances in Environmental Technology and Biotechnology. Energy, Environmental and Structural Engineering Series 26:112-116.

Fischer M., Schirrmeier H., Wernike K., Wegelt A., Beer M., Hoffmann B., 2013. Development of a pan-Simbu real-time reverse transcriptase PCR for the detection of Simbu serogroup viruses and comparison with SBV diagnostic PCR systems, Virol. J., 10:327.

Hoffmann B., Scheuch M., Höper D., Jungblut R., Holsteg M., Schirrmeier H., Eschbaumer M., Goller K.V., Wernike K., Fischer M., Breithaupt A., Mettenleiter T.C., Beer M., 2012. Novel Orthobunyavirus in Cattle, Europe, 2011, Emerging Infectious Diseases. 18:469-472.

Horne K.M., Vanlandingham D.L., 2014. Bunyavirus-vector interactions. Viruses. 6(11):4373-97.

Jagoe S., Kirkland P.D., Harper P.A., 1993. An outbreak of Akabane virus-induced abnormalities in calves after agistment in an endemic region. Aust Vet J.70(2):56–58.

Manescu M.A., Baraitareanu S., Gurau M.R., Danes D., 2015. Molecular tools available for the detection of Schmallenberg-Simbu group viruses. 4th ISAA. Proc. Rom. Acad., Series B, Supplement 1, 120-123.

Roux K.H., 2009. Optimization and troubleshooting in PCR. Cold Spring HarbProtoc. 2009(4):pdb.ip66. http://cshprotocols.cshlp.org/content/2009/4/pdb.ip66.long

Schulz C., van der Poel W.H.,Ponsart C., Cay A.B., Steinbach F.,Zientara S., Beer M., Hoffmann B., 2015. European interlaboratory comparison of Schmallenberg virus (SBV) real-time RT-PCR detection in experimental and field samples: The method of extraction is critical for SBV RNA detection in semen. J.Vet.Diagn.Invest.27(4):422-30.

MISCELLANEOUS

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MICROBIAL XYLANASE: A REVIEW

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Abstract
Xylan is the major constituent of hemicellulose, the second most abundant natural polymer on earth. There are four main categories of xylans: arabinoxylans, glucuronoxylans, glucurono arabinoxylans and galacto glucurono arabinoxylans. The side chains of each xylan are being responsible for the solubility, physical conformation and reactivity of the xylan molecule with other components of the hemicellulose and therefore, influencing the mode and extent of enzymatic cleavage. Xylanases, as glycoside hydrolase members, are able to catalyse the hydrolysis of xylan, by breaking the β-1,4-glycoside linkages, in order to produce simpler compounds such as xylose. Because of the heterogeneity and complex chemical nature of xylan, the complete breakdown requires the action of several hydrolytic enzymes that are different considering their structure, the substrate specificities, their mode of action or biochemical properties. Many degrading microorganisms produce xylanases such as fungi (Aspergillus spp., Trichoderma spp.), bacteria (Bacillus spp., Streptomyces spp.), yeast (Cryptococcus spp.), marine algae etc. Depending on the source, microbial xylanases have different characteristics, that makes them useful for an application or another. Worldwide, the market of xylanases has expanded rapidly because of its potential in industrial use, especially in the biotechnological applications. In this review, are presented the significant aspects concerning the complete hydrolysis of xylan, and therefore of hemicellulose.

Key words*: xylan, xylanases, bacteria, fungi, applications.*

INTRODUCTION

Lignocellulose is the most abundant renewable biomass on earth. The main constituents of lignocellulosic materials are cellulose, hemicellulose and lignin, along with smaller amounts of pectin, protein etc. The variation of the proportions of these components depends on: plant species, age, stage of growth or other conditions (Kumar et al., 2009)

The term "hemicellulose" was introduced by Schulze in 1891, describing the fractions isolated or extracted from plant materials with dilute alkali (Beg et al., 2001). Hemicellulose is not a well-defined compound (Polizeli et al., 2005), but a class of polymers, found in plant cell wall, based on pentose and hexose sugars, with xylan (the polymer of xylose) as the most abundant constituent (Uday et al., 2016). The names of the classes of the hemicellulose match the main sugar unit. Therefore, when a polymer is hydrolysed and produce xylose, it is a xylan (Polizeli et al., 2005).

Xylans or the hemicelluloses are located in plants between the lignin and the cellulose fibers underneath, being interspersed and covalently linked at various points with lignin, while covering cellulose, via hydrogen bonding (Beg et al., 2001). The term xylans is used to describe a group of non-cellulose polysaccharides, based on monosaccharides units such as D-xylose, D-mannose, D-glucose, L-arabinose, D-galactose, D-glucuronic acid and D-galacturonic acid (Polizeli et al., 2005; Shallom and Shoham, 2003). The xylose residues are linked by β-1,4-glycosidic bonds. Depending on their source and extraction method, these polysaccharides have different structure and composition. (Harmsen et al., 2010).

Xylanases represents a class of enzymes, which are responsible for the complete hydrolysis of the linear polysaccharide β-1,4-xylan into simpler compounds which are mainly consisted of xylose, thus breaking down hemicellulose.

XYLAN STRUCTURE

Based on the nature of its substituents, four main categories of xylans (Motta et al., 2013) can be considered:

- Arabinoxylans, comprising only side chains of single terminal units of α-L-arabinofuranosyl;

- Glucuronoxylans, based only on α-D-glucuronic acid and its 4-O-methyl ether derivative;
- Glucurono arabinoxylans, in which α-D-glucuronic (and 4-O-methyl- α-D-glucuronic) acid and α-L-arabinose are both present;
- Galacto glucurono arabinoxylans, characterized by the presence of terminal β-D-galactopyranosyl residues on complex oligosaccharide side chains of xylans.

cells. It is found in large quantities in hardwoods (15-30% of the cell wall content) and softwoods (7-10%), as well in annual plants (<30%). In hardwoods, xylan exists as O-acetyl-4-O-methylglucuronoxylan and in softwoods, as arabino-4-O-methylglucuronoxylan, while in grasses and annual plants, it is as arabinoxylans (Beg et al., 2001; Kulkarni et al., 1999). Linear unsubstituted xylan has also been reported in esparto grass, tobacco and certain marine algae, with the latter containing xylopyranosyl residues linked by both β-1,3 and β-1,4 linkages (Motta et al., 2013).

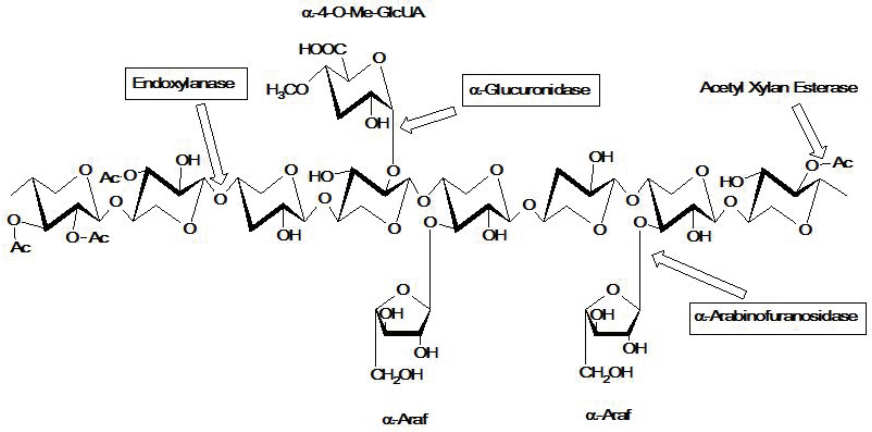

Figure 1. Structure of xylan and the xylanolytic enzymes involved in its degradation.
Ac: Acetyl group; α-Araf: α-arabinofuranose; α-4-O-Me-GlcA: α-4-O-methylglucuronic acid.
Source: Sunna and Antranikian, 1997

In the Figure 1 are presented the enzymes involved in the hydrolysis of xylan.
Additionaly there is another category, named homoxylans, consisting exclusively of xylosyl residues, but this type of xylans are not widespread in nature, being isolated from limited sources (tobacco stalks, guar seed husks) (Sunna and Antranikian, 1997).
Among these categories, the complexity increases from linear to highly substituted xylans. The side chains are responsible for the solubility, physical conformation and reactivity of the xylan molecule with other hemicellulosic components and therefore, influence the mode and extent of enzymatic cleavage (Kulkarni et al., 1999).
Xylan is present in a variety of plant species, being distributed in several types of tissues and

ENZYMATIC HYDROLYSIS OF XYLAN

The role of enzymes in the breakdown of xylan was observed by Hopper-Seyler over 100 years ago (Bastawde, 1992). Xylan is a complex chemical compound with a heterogeneous nature. Therefore, its complete breakdown requires the action of several hydrolytic enzymes with diverse modes of action and specificities.
The xylanolytic enzyme system includes β-1,4-endoxylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetyl xylan esterase (Motta et al., 2013) and phenolic acid (ferulic and p-coumaric acid) esterase (Beg et al., 2001; Dhiman et al., 2008). The synergic action of all these enzymes convert xylan into its constituent sugars. Among all of xylanases, endoxylanases

and β-xylosidases are the most important in depolymerizing xylan molecule into monomeric pentose units. Endoxylanases are involved in cleaving the glycosidic bonds and in liberating short xylooligosaccharides, while β-xylosidase releases xylose residues from the nonreducing ends of xylooligosaccharides (Motta et al., 2013). Acetyl esterase, ferulic esterase, glucuronidase , and arabinosidase are required for the release of different side chains from the xylan backbone (Dhiman et al., 2008).

Endo-1,4-β-xylanases (E.C.3.2.1.8) are reported to be produced mainly by microorganisms such as bacteria (*Bacillus sp.*, *Streptomyces sp.*) and fungi (*Aspergillus sp.*, *Trichoderma sp.*). However, there are reports that indicate several other sources, such as: Japanese pear fruit during the over-ripening period, the flour of European wheat (*Triticum aestivum*) , or fresh water mollusc (Subramaniyan and Prema, 2002).

Exo-β-1,4-D-xylosidase (E.C.3.2.1.37) removes succesive D-xylose residues from the non-reducing end, by catalysing the hydrolysis of β-1,4-D-xylo-oligosaccharides. β-xylosidase can easily hydrolise xylobiose that isn't affected by the endoxylanases that release xylose during the hydrolysis of xylan. Among the producing microorganisms, there are reports that include *Bacillus* sp. and different fungi (Subramaniyan and Prema, 2002).

α-L-arabinofuranosidases (E.C.3.2.1.55) hydrolyse the terminal, non-reducing α-L-arabinofuranosyl groups of arabinans, arabinoxylans and arabinogalactans. This type of enzyme is produced by fungi, actinomycetes and other bacteria (*Bacillus polymyxa*, *Rhodothermus marinus*).

α-D-glucuronidases (E.C.3.2.1.1) catalyse the hydrolysis of the α-1,2-glycosidic linkages between xylose and D-glucuronic acid or its 4-O-methyl ether linkage.

For the complete hydrolysis of natural glucuronoxylans is necessary to use esterases to remove the bound acetic and phenolic acids. Acetyl xylan esterase (E.C. 3.1.1.6) breaks the bonds of xylose to acetic acid, feruloyl esterase (E.C. 3.1.1.73) the arabinose side chain residues to ferulic acid and p-coumaroyl esterase the arabinose side chain residue to p-coumaric acid.

Usually, xylanases are inducible enzymes secreted in media containing pure xylan or xylan-rich residues (Balakrishnan, 1997). The immobilization of xylanases is necessary for practical purposes. Therefore, either the microorganism or the enzymes are immobilized on solid material, technique that offers several advantages such as repetead usage on enzyme, ease of product separation and improvement of enzyme stability (Beg et al., 2001).

SOURCES OF XYLANASE

Many studies have reported the production of xylanase from fungi, bacteria, yeast, marine algae (Mandal, 2015), seeds, crustaceans, snails (Polizeli et al., 2005) but the main sources for these enzymes are fungi and bacteria. According to the source, xylanases have different characteristics which makes them useful for an application or another.

Bacterial xylanases

Xylanases produced by bacteria and actinomycetes (*Bacillus* sp*., Pseudomonas* sp., *Streptomyces* sp*.)* are effective in a broader pH range of 5-9, with the optimum temperature for xylanase activity between 35ºC to 60ºC (Beg et al., 2001; Mandal, 2015; Motta et al., 2013).

Bacterial strains studied for their xylanase activity are shown in the Table 1 (Mandal, 2015; Amore et al., 2014; Dhiman et. al, 2008; Maheshwari and Chandra, 2000).

Table 1. Xylanase producing bacteria

Microorganism
Bacillus pumilus
Bacillus subtilis
Bacillus amyloliquefaciens
Bacillus cereus
Bacillus circulans
Bacillus megatorium
Bacillus licheniformis
Bacillus stearothermophilus
Streptomyces sp.
Streptomyces roseiscleroticus
Streptomyces cuspidosporus
Streptomyces actuosus
Pseudonomas sp.
Clostridium absonum
Thermoactinomyces thalophilus

Studies on *Bacillus* spp. showed higher xylanase activity at alkaline pH and high temperature. Therefore, bacterial xylanases are used in industrial application due to their alkali tolerance and thermostability (Mandal, 2015).

Fungal xylanases

Fungi (*Aspergillus* spp., *Fusarium* spp., *Penicillium* spp.) are important producers of xylanase due to high yields and extracellular release of the enzymes (Nair and Shashidhar, 2008). Also, fungal xylanases have higher activity, compared with bacteria or yeast. However, xylanases derived from fungal sources have some characteristics that makes them unavailable for some industrial applications (Mandal, 2015). Most of these xylanases are efficient at temperature below 50ºC and a pH range of 4-6 (Beg et al., 2000). For example, fungal xylanases can't be used in the pulp and paper industry, that needs an alkaline pH and temperature more than 60ºC (Mandal, 2015). Another problem with fungal xylanases is the presence of a cellulase, few studies reporting fungal xylanase without cellulase activity (Subramaniyan and Prema, 2002).

Fungal strains studied for their xylanase activity are shown in the Table 2 (Mandal, 2015; Huitron et al., 2008; Ja'afaru, 2013; Taneja et al., 2002; Haltrich et al., 1993; Ghanen et.al, 2000; Haltrich et al., 1996).

Table 2. Xylanase producing fungi

Microorganism
Aspergillus niger
A. foetidus
A. brasiliensis
A. flavus
A. nidulans
A. terreus
Penicilium sp.
Trichoderma reesei
T. longibrachiatum
T. harzianum
T. viride
T. atroviride
Fusarium oxysporum
Thermomyces lanuginosus
Alternaria sp.
Talaromyces emersonii
Schizophyllum commune
Piromyces sp.

Xylanases can be produced either in solid state fermentation (SSF) or in submerged fermentation (SmF), the enzyme productivity in SSF being much higher than in SmF (Nair and Shashidhar, 2008).

Additionaly, large scale production of fungal xylanase is difficult due to the slow generation time and coproduction of highly viscous polymer that lowers the oxygen transfer (Mandal, 2015).

APPLICATIONS OF MICROBIAL XYLANASE

The market of xylanase has increased significantly worldwide, over the past few years (Techapun et al., 2003; Haki and Rakshit, 2003; Dhiman et al., 2008). Microbial xylanases have attracted a great deal of attention, because of their biotechnological potential in various industrial processes such as food, feed and pulp and paper industry.

Also, they have shown an immense potential for increasing the production of several useful products in a most economical way. The main possibilities are the production of SCPs, enzymes, liquid or gaseous fuels and solvents and sugar syrups, which can be used as such or as feed stock for other microbiological processes (Kuhad and Singh, 1993). Therefore, xylanases are considered as "one of the more industrially important enzymes" (Dhiman et al., 2008)

Pulp and paper industry

Chemical bleaching in the pulp and paper industry is used to increase the paper brightness. Unfortunately, this causes serious damages of the cellulose components and reduce the yield and viscosity of the pulp. The level of viscosity is related to the degree of cellulose polymerization and to the paper strength (Cheng et al., 2013), so decreasing viscosity is not desirable.

The role of xylanase in the biobleaching of pulp was first reported by Viikari et al. in 1986. Since then, many studies were conducted, toward newer microbial isolates (Sunna and Antranikian, 1997; Beg et al., 2000; Gilbert and Hazlewood, 1993; Liu et al., 1998), as well as bleaching experiments (Cheng et al., 2013;

Manimaran et al., 2009; Garg et al., 2011; Khandeparkar and Bhosle, 2007; Li et al., 2005). The researchers, focused on using xylanase mainly for reducing the chemical consumption, few studies being centered on the effects of this enzyme on the yield and viscosity of the pulp (Cheng et al., 2013)

With this technique, the pulp is usually treated with xylanase before chemical bleaching (Martin-Sampedro et al., 2012). The reprecipitated xylan is hydrolyzed, in the presence of xylanase, this facilitating pulp bleaching and lowering the chemical consumption. By this means, this technique reduces the toxic compounds discharged into the environment (Cheng et al., 2013).

Animal feed

Including xylanases into a rye-based diet of broiler chickens results in reduced intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency (Bedford and Classen, 1992)

Xylanases, used as pretreatment of forage crops, improve the nutritional properties of agricultural silage and grain feed (Subramaniyan and Prema, 2002; Kuhad and Singh, 1993; Bedford and Classen, 1992), thus improving the digestibility of ruminant feeds and facilitating composting (Gilbert and Hazlewood, 1993).

However, the complete removal of xylan is not wanted, because hemicelluloses are important components of diet and their removal may increase bowel diseases (Mandal, 2015)

Food industry

Xylanases improve the quality of bread, by increasing the specific bread volume. This is further enhanced when amylase is used in combination with xylanase (Maat et al., 1992). Also, they are applied in rye baking, where the addition of xylanase makes the doughs soft and slack (Subramaniyan and Prema, 2002). During the bread-baking process, they delay crumb formation, allowing the dough to grow (Mandal, 2015).

Another use of xylanases is as dough strengthners, because they provide excellent tolerance to the dough towards variations in processing parameters and in flour quality (Subramaniyan and Prema, 2002). Also, a larger amount of arabinoxylo-oligosaccharides in bread would be beneficial to health (Mandal, 2015). In biscuit-making, xylanase is recommended for making cream crackers lighter and improving the texture and tastiness (Mandal, 2015).

Along with cellulase and pectinase, xylanase can be used for the preparation of dextrans, used as food thickeners (Mandal, 2015).

Hemicellulosic wastes

Xylan is present in large amounts in hemicellulosic wastes. There is a massive accumulation of agricultural, forestry and municipal solid waste residues, therefore the development of an efficient process of enzymatic hydrolysis offers new prospects for treating wastes (Subramaniyan and Prema, 2002; Rani and Nand, 1996).

Biofuels

Production of biofuels is gaining great importance as the energy resources are shrinking. The combined action of xylanase with several enzymes such as mannanase, ligninase, xylosidase, glucanase, glucosidase etc. can be applied for the generation of biofuels (ethanol and xylitol), from lignocellulosic biomass (Dominguez, 1998). The production of bioethanol requires the delignification of lignocellulose to liberate cellulose and hemicellulose. The next steps include the depolymerization of the carbohydrate polymers to produce free sugars and the fermentation of mixed pentose and hexose to produce bioethanol (Lee, 1997).

Fabric bio-processing

Xylanase treatment can significantly remove hemicellulosic impurities, thus increasing the water absorbing properties of fiber, without affecting the fibre strength during the spinning process. In the end, fiber becomes more soft and smooth after desizing (Dhiman et al., 2008).

Treatment of plant cells

Treatment of tobacco suspension cells (*Nicotiana tabacum* CV.KY 14) with a purified endoxylanase from *Trichoderma viride* increased the levels of acylated sterol glycosides and induces the synthesis of

phytoalexins (Moreau et al., 1994). Additionaly, a truncated bacterial xylanase gene from *Clostridium thermocellum* has been demonstrated in rhizosecretion in transgenic tobacco plants. (Borisjuk et al., 1999). Some xylanases improve cell wall maceration for the production of plant protoplasts (Beg et al., 2001).

Beverage and juices industry

Xylanases used in combination with cellulase and pectinase helps clarifying must and juices, liquefying fruits and vegetables (Beg et al., 2001), stabilization of the fruit pulp, reduction of viscosity, hydrolysing the substances that hinder the physical or chemical clearing of the juice,or that may cause cloudiness in the concentrate (Polizeli et al., 2005; Mandal, 2015).

Particularly, α-L-arabinofuranosidase and β-D-glucopyranosidase are used for aromatizing musts, wines and fruit juices (Spagna et al., 1998). Also, the xylanolytics enzymes are employed for extracting coffee, plant oils and starches (Subramaniyan and Prema, 2002).

In the fermentation industries, such as beer brewing, xylanases, used as a pre-treatment of the arabinoxylans containing substrates (barley, wheat) reduce the viscosity, thus increasing process efficiency (Subramaniyan and Prema, 2002).

Surfactants

Alkyl glycosides are surfactants widely used in industrial applications, being produced commercially from monomeric sugars. Using polysaccharide is more feasible for their industrial production, because several steps in the process can be omitted (Matsumara et al., 1999). Therefore, xylanase presents a challenging opportunity.

Other application of xylanase is in the detergent industry, as it improves the cleaning ability of detergents that are more efficient in cleaning fruit, vegetable, soils and grass stains (Kumar et al, 2004; Dhiman et al., 2008).

Retting of Flax fibers

A combined xylanase-pectinase system is used in the debarking process, which is the first step in wood processing, the addition of xylanases enhancing the retting process. Other applications of this combined system are used in the degumming of bast fibers such as flax, hemp, jute and ramie or the fiber liberation from plant instead of retting (Beg et al., 2001).

CONCLUSIONS

For the breakdown of xylan, the main component of hemicellulose, is requierd the combined action of xylanolytic enzymes such as: β-1,4-endoxylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetyl xylan esterase and phenolic acid esterase.

Depending on the source, mainly bacteria or fungi, xylanases have different characteristics which makes them useful for an application or another.

Xylanases present immense potential in various industrial areas or research fields such as: pulp and paper, animal feed, food industry, hemicellulosic wastes, biofuels, fabric bio-processing, treatment of plant cells, surfactants, retting of flax fibers, beverages and food industry.

REFERENCES

Amore A., Parameswaran B., Kumar R., Birolo L., Vinciguerra R., Marcolongo L., Ionata E., La Cara F., Pandey A., Faraco V., 2014. Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR 44A in brewer's spent grain saccharification. Journal of Chemical Technology and Biotechnology 90(3): 573-581.

Balakrishnan H., Srinivasan M.C., Rele M.V., 1997. Extracellular protease activities in relation to xylanase secretion in an alkalophilic Bacillus sp. Biotechnology Letters, 18:599-601

Bastawde K.B., 1992. Xylan structure, microbial xylanases, and their mode of action. World Journal of Microbiology and Biotechnology, 8:353-368

Bedford M.R., Classen H.L., 1992. The influence of dietary xylanase on intestinal viscosity and molecular weight distribution of carbohydrates in rye-fed broiler chick. In: Visser J., Beldman G., vanSomeren M.A.K., Voragen A.G.J. (Eds) Xylans and xylanases. Elsevier, Amsterdam, 361-370

Beg Q.K., Bhushan B., Kapoor M., Hoondal G.S., 2000. Production and characterization of thermostable xylanase and pectinase from a Streptomyces sp. QG-11-3. Journal of Industrial Microbiology and Biotechnology, 24:396-402

Beg Q.K., Kapoor M., Mahajan L., Hoondal G.S., 2001. Microbial xylanases and their industrial applications: a review. Applied Microbiology and Biotechnology, 56:326-338

Borisjuk N.V., Borisjuk L.G., Logendra S., Petersen F., Gleba Y., Raskin I., 1999. Production of recombinant proteins in plant root exudates. Nature Biotechnology, 17:466-469

Cheng X., Chen G., Huang S., Liang Z., 2013. Biobleaching effects of crude xylanase from Streptomyces griseorubens LH-3 on Eucalyptus kraft pulp. BioResources, 8(4):6424-6433

Dhiman S.S., Sharma J., Battan B., 2008. Industrial applications and future prospects of microbial xylanases: a review. BioResources, 3(4):1377-1402

Dominguez J.M., 1998. Xylitol production by free and immobilized Debaryomyces hansenii. Biotechnology Letters, 20:53-56

Garg G., Dhiman S.S., Mahajan R., Kaur A., Sharma J., 2011. Bleach-boosting effect of crude xylanase from Bacillus stearothermophilus SDX on wheat straw pulp. New Biotechnology, 28(1):58-64

Ghanen N.B., Yusef H.H., Mahrouse H.K., 2000. Production of *Aspergillus terreus* xylanase in solid-state cultures: application of the Plackett-Burman experimental design to evaluate nutritional requirements. Bioresource Technology, 73: 113-121

Gilbert H.J., Hazlewood G.P., 1993. Bacterial cellulases and xylanases. Journal of General Microbiology, 139:187-194

Haki G.D., Rakshit S.K., 2003. Developments in industrially important thermostable enzymes: a review. Bioresource Technology, 89:17-34

Haltrich D., Preiss M., Steiner W., 1993. Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune*. Enzyme Microbial Technology, 15: 137-161

Haltrich D., Nidetzky B., Kulbe K.D., 1996. Production of fungal xylanases. Bioresource Technology, 15: 137-161

Harmsen P., Huijgen W., Bermudez L., Bakker R., 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic. Biomass, 1-49

Huitron C., Perez R., Sanchez A.E., Lappe P., Rocha-Zavaleta L., 2007. Agricultural waste from the tequila industry as substrate for the production of commercially important enzymes. Journal of Environmental Biology 29(1): 37-41

Ja'afaru M.I., 2013. Screening of fungi isolated from environmental samples for xylanase and cellulase production. ISRN microbiology 2013: 1-7

Khandeparkar R., Bhosle N.B., 2007. Application of thermoalkalophilic xylanase from Arthrobacter sp. MTCC 5214 in biobleaching of kraft pulp. Bioresource Technology, 98(4):897-903

Kuhad R.C., Singh A., 1993. Lignocellulosic biotechnology: current and future prospects. Critical Reviews in Biotechnology, 13:151-172

Kulkarni N., Shendye A., Rao M., 1999. Molecular and biotechnological aspects of xylanases. FEMS Microbiology Reviews, 23:411-456

Kumar B.K., Balakrishnan H., Rele M.V., 2004. Compatibility of alkaline xylanases from an alkaliphilic Bacillus NCL (87-6-10) with commercial detergents and proteases. Journal of Industrial Microbiology and Biotechnology, 31:83-87

Kumar P., Barrett D.M., Delwiche M.J., Stroeve P., 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial & Engineering Chemistry Reasearch, 48(8):3713-3729

Lee J., 1997. Biological conversion of lignocellulosic biomass to ethanol. Journal of Biotechnology, 56:1-24

Li X.T., Jiang Z.Q., Li L.T., Yang S.Q., Feng W.Y., Fan J.Y., Kusakabe I., 2005. Characterization of a cellulase-free, neutral xylanase from Thermomyces lanuginosus CBS 288.54 and its biobleaching effect on wheat straw pulp. Bioresource Technology, 96(12):1370-1379

Liu W., Zhu W., Lu Y., Kong Y., Ma G., 1998. Production, partial purification and characterization of xylanase from Trichosporon cutaneum SL409. Process Biochemistry, 33:331-326

Maat J., Roza M., Verbakel J., Stam H., daSilra M.J.S., Egmond M.R., Hagermans M.L.D., vanGarcom R.F.M., Hessing J.G.M., vanDerhondel C.A.M.J.J., vanRotterdam C., 1992. Xylanases and their application in bakery. In: Visser J., Beldman G., vanSomeren M.A.K., Voragen A.G.J. (Eds) Xylans and xylanases. Elsevier, Amsterdam, 349-360

Maheswari U., Chandra T.S., 2000. Production and potential applications of a xylanase from a new strain of *Streptomyces cuspidosporus*. World Journal of Microbiology and Biotechnology, 16: 257-263

Mandal A., 2015. Review on microbial xylanases and their applications. International Journal of Life Sciences, 4(3):178-187

Manimaran A., Kumar K.S., Permaul K., Singh S., 2009. Hyper production of cellulase-free xylanase by Thermomyces lanuginosus SSBP on bagasse pulp and its application in biobleaching. Applied Microbiology and Biotechnology, 81(5):887-893

Martin-Sampedro R., Rodriguez A., Ferrer A., Garcia-Fuentevilla L.L., Eugenio M.E., 2012. Biobleaching of pulp from oil palm empty fruit bunches with laccase and xylanase. Bioresource Technology, 110:371-378

Matsumara S., Sakiyama K., Toshima K., 1999. Preparation of octyl-β-D-xylobioside and xyloside by xylanase catalyzed direct transglycosylation reaction of xylan and octanol. Biotechnology Letters, 21:17-22

Motta F.L., Andrade C.C.P., Santana M.H.A., 2013. A review of xylanase production by the fermentation of xylan: classification, characterization and applications. In: Chandel A.K., da Silva S.S. (Eds) Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization, InTech, Croatia, 251-266

Moreau R.A., Powell M.J., Whitaker B.D., Bailey B.A., Anderson J.D., 1994. Xylanase treatment of plant cells induces glycosylation and fatty acylation of phytosterols. Physiologia Plantarum, 91:575-580

Nair S.G., Shashidhar S., 2008. Fungal xylanase production under solid state and submerged fermentation conditions. African Journal of Microbiology Research, 2(4):82-86

Polizeli M.L.T.M., Rizzatti A.C.S., Monti R., Terezni H.F., Jorge J.A.,Amorim D.S., 2005. Xylanases from fungi: properties and industrial applications. Applied Microbiology and Biotechnology, 67:577-591

Rani S., Nand K., 1996. Development of cellulase-free xylanase producing anaerobic consortia for the use of lignocellulosic wastes. Enzyme and Microbial Technology, 18:23-28

Shallom D., Shoham Y., 2003. Microbial hemicellulases. Current opinion in microbiology, 6(3):219-228

Spagna G., Ramagnoli D., Angela M., Biochi G., Pifferi P.G., 1998. A simple method for purifying glycosidase: α-L-arabinofuranosidase and β-D-glucopyranosidase from A. niger to increase the aroma of wine. Enzyme and Microbial Technology, 22:298-304

Subramaniyan S., Prema P., 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Critical Reviews in Biotechnology, 22(1):33-64

Sunna A., Antranikian G., 1997. Xylanolytic enzymes from fungi and bacteria. Critical Reviews in Biotechnology, 17:39-67

Taneja K., Saurabh G., Kuhad R.C., 2002. Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. Bioresource Technology, 85: 39-42

Techapun C., Poosaran N., Watanabe M., Sasaki K., 2003. Thermostable and alkaline-tolerant microbial cellulase-free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocess: a review. Process Biochemistry, 38:1327-1340

Uday U.S.P., Choudhury P., Bandopadhyay T.K., Bhunia B., 2016. Classification, mode of action and production strategy of xylanase and its application for biofuel production from water hyacinth. International Journal of Biological Macromolecules, 82:1041-1054

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NEW BIOACTIVE COMPOSITES BASED ON BACTERIAL CELLULOSE AND NATURAL PRODUCTS

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Abstract

Bacterial cellulose (BC) has a variety of applications in biomedical fields. However, the native BC lacks certain properties, which limits its applications in various fields. The trend is nowadays towards the development of organic polymers using natural materials. In this sense, the possibility of obtaining new composite biomaterials with improved properties will prove as an interesting solution for achieving green composites BC, satisfying the need to explore minimal cost, biodegradable and renewable materials. Following these principles, this paper presents some of our studies carried out in order to obtain new eco-friendly composite biomaterials based on BC and valuable natural products such as sericin, propolis and royal jelly with appropriate biomedical applications. The main objective consisted in the biosynthesis and characterization of bacterial cellulose-based composites (BC) combined with natural products with antimicrobial properties.

Key words*: eco-friendly composites, bacterial cellulose, antimicrobial activity.*

INTRODUCTION

Bacterial cellulose (BC) has received a substantial interest owing to its unique structural features and impressive physical-mechanical properties. BC has a variety of applications in biomedical fields, including use as biomaterial for artificial skin, artificial blood vessels, vascular grafts, scaffolds for tissue engineering and wound dressing. However, the native BC lacks certain properties, which limits its applications in various fields. Therefore, synthesis of BC composites will be conducted to removing these limitations.

As like other polymers, BC has limitations which restrict the application to a certain extent. For example, BC is an excellent material for dressing as it provides a moist environment that allows rapid healing of the wound, however, it has not any antimicrobial properties to prevent infection (Maneerung et al., 2007; Maria et al., 2010; Saibuatong et al., 2010; Zhang et al., 2011).

Therefore, it cannot be used directly in treating infections but should be treated with bioactive ingredients in order to form polymer composites.

The trend is nowadays towards the development of organic polymers using natural materials. Thus, the possibility of replacing synthetic polymer composites, with ecological composite materials obtained from natural polymers, harmless environment and human health, represents an important objective for the current period. Following these principles, this paper presents some of our studies carried out in order to obtain new eco-friendly composite biomaterials based on BC and valuable natural products such as sericin, propolis and royal jelly with appropriate biomedical applications. The main objective consisted in the synthesis and characterization of bacterial cellulose - based composites (BC) combined with natural products, functionalized for biocompatibility, antimicrobial properties and healing chronic wounds.

In this sense, the possibility of obtaining new composite biomaterials with improved properties will prove as an interesting solution for achieving green composites BC,

satisfying the need to explore minimal cost, biodegradable and renewable materials.

MATERIALS AND METHODS

Bacterial Cellulose composite materials

In the present study, we have explored a novel biomaterial, and we prepared different bacterial cellulose composites (BC); 1) Pure BC, 2) BC with sericin, 3) BC with propolis and 4) BC with royal jelly.

Bacterial cellulose nanocomposites were obtained by immersion of sericin, propolis, royal jelly into culture media (in situ).

Bacterial cellulose (BC) has been obtained as pellicle in our laboratory, from *Acetobacter xylinum* DSMZ-2004. The culture medium used for the fermentation of *A. xylinum* DSMZ-2004 (German Collection of Microorganisms and Cell Cultures) contained an extract obtained from inadequate quality apples, 7.5% glucose, 2% glycerol, 0.2% ammonium sulfate, 0.5% citric acid, and various amounts of sericin, propolis and royal jelly, with the pH being adjusted to 5.5 by acetic acid.

The culture media prepared in 500 mL Erlenmeyer flasks was sterilized by autoclaving at 121°C, for 15 min and then it was inoculated with 10% (v/v) *A. xylinum* DSMZ-2004 inoculum. A single *A. xylinum* colony grown on agar culture medium was transferred to a Petri dish filled with liquid glucose medium and incubated for two days to create a cell suspension. Then, the cell suspension was introduced into the sericin, propolis, royal jelly-dispersed culture medium at 30°C and incubated for 14 days. That sericin, propolis, royal jelly-incorporated BC membrane which was biosynthesized in the medium (in situ) was purified by 1N sodium hydroxide for 2 days at 30°C, in order to remove the cells included in the pellicles. The pellicles were then immersed in water solution of NaN_3 (0.02%) to reduce microbial contamination, neutralized with 1% acetic acid and washed repeatedly with distilled water until its pH was 7.0 and finally, stored at 4°C.

BC/sericin, BC/propolis, BC/royal jelly membranes were obtained with 1%, 1%, and respectivelly 3% sericin, propolis and royal jelly content.

Microbiological studies

Test organisms

The tests were carried out on three microorganisms: a Gram-negative bacterium (*Pseudomonas aeruginosa* ATCC 9027) and two Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228).

Inoculum preparation

The three test-strains were grown on Casein soya broth agar medium (CaSoA). Before each experiment, the strain was activated by passaging the cells on CaSoA and incubated for 18-24 hours at 30-35ºC. When the bacterial culture was optimal, with a sterile loop, it was added in sterile purified water in order to obtain a bacterial suspension with a concentration of 108-109 colony forming units (CFU) /mL.

Antimicrobial assay

The tests were performed in sterile Petri dishes, each of them containing 15-20 ml of culture medium previously inoculated with 104-105 CFU/ml. On each dish, 3 or 4 samples (cut with a pair of sterile scissors) of approximately 20-25 mm diameter were placed on the solidified surface of the medium. The Petri dishes were incubated 18-24 hours at 30-35°C. After the incubation period, the growth inhibition zones were measured and the microbial growth was assayed in the contact zone between the sample (BC composite) and the agar media.

The microbial growth was assayed in the following manner:

- no growth - „none”;
- some colonies, but less that the control sample - „weak”;
- same as the control sample - „important”.

RESULTS AND DISCUSSIONS

The analysis of the experimental results presented in the Table 1 shows the antibacterial effect of our BC- samples on all those three strains tested, the lowest effect being of the Sample 4 (over the two strains of *Staphylococcus*) because these samples are missing the inhibition zone, the antibacterial effect being limited to the contact area between the material and the environment culture. However, we can see a certain antibacterial

effect of all the samples over *Pseudomonas aeruginosa*, especially of samples BC-1, 3 and 4, with the inhibition zone measuring between 4,0- 4,5 mm (Figures 1-4).

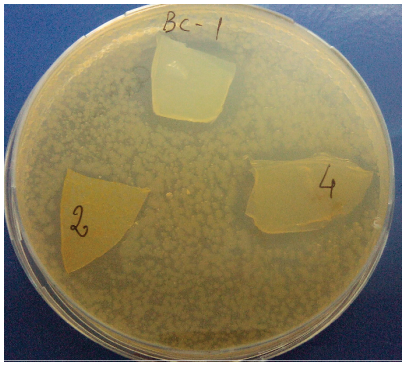

Figure 1. Antimicrobial activity of BC films

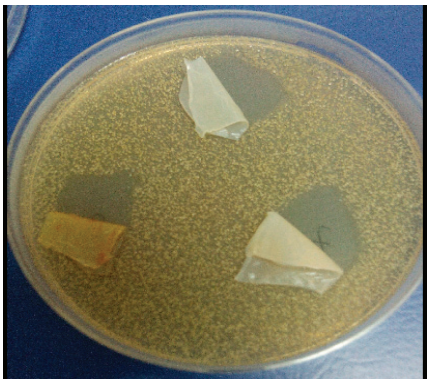

Figure 2. Antimicrobial activity of composite films BC-sericin 1%

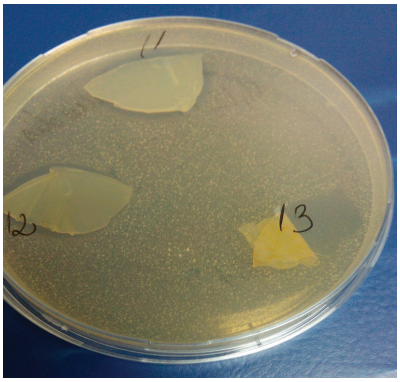

Figure 3. Antimicrobial activity of composite films BC-propolis 1%

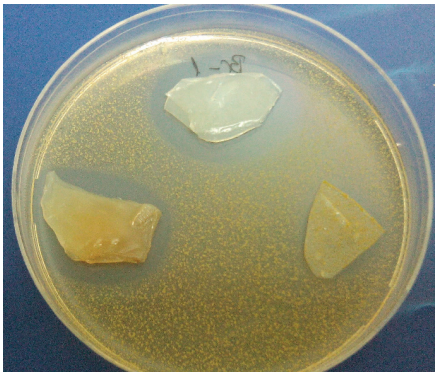

Figure 4. Antimicrobial activity of composite films BC-royal jelly 3%

Table 1. The antimicrobial activity of bacterial cellulose composites on *S. epidermidis, S. aureus* and *P. aeruginosa*

Sample	**Test - Organism Bacterial strain**	**Inhibition Zone (mm)**	**Breeding microbial (contact area)**
1) Pure BC BC-1	*S. epidermidis* ATCC 1228	4	None
	S. aureus ATCC 6538	6	None
	P. aeruginosa ATCC 9027	4	None
2) BC with sericin 1%	*S. epidermidis* ATCC 1228	2.5	None
	S. aureus ATCC 6538	2	None
	P. aeruginosa ATCC 9027	3	None
3) BC with propolis 1%	*S. epidermidis* ATCC 1228	3	None
	S. aureus ATCC 6538	4.5	None
	P. aeruginosa ATCC 9027	4.5	None
4) BC with royal jelly 3%	*S. epidermidis* ATCC 1228	0	Weak
	S. aureus ATCC 6538	0	None
	P. aeruginosa ATCC 9027	4	None

CONCLUSIONS

Three types of eco-friendly BC composite materials were presented in this study. The matrix of composites is the Bacterial Cellulose (BC), while the reinforcements of BC composites are represented by sericin (BC/sericin), propolis (BC/propolis) and royal jelly (BC/royal jelly).

All composites were obtained by static culture method, using valuable ingredients added directly into the biocellulose culture medium (in situ modification).

All of these three presented BC composite materials had an obviously antibacterial effect on all three strains tested.

ACKNOWLEDGEMENTS

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REFERENCES

Maneerung, T., Tokura, S., & Rujiravanit, R. 2007, CarbohydratePolymers, 72(1), 43–51.

Maria, L. C. S., Santos, A. L. C., Oliveira, P. C., & Valle, A. S. S., 2010, Polímeros: Ciência e Tecnologia, 20, 72–77.

Saibuatong, O., & Philsalaphong, M., 2010, Carbohydrate Polymers, 79, 455–460.

Zhang, S., & Luo, J., 2011, Journal of Engineered Fibers and Fabrics, 6(3), 69–72.

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SELECTIVE FRACTIONS WITH ANTIOXIDANT ACTIVITY FROM ROMANIAN CULTIVATED *CYNARA SCOLYMUS* L.

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Abstract
It is known that oxidative stress and inflammation play an important role in the onset of arterial disorders, very common in the elderly. Cynara scolymus L. *is one of the best recommended species for prevention and control of diseases associated with aging processes, mostly due to its high polyphenol content - luteolin, luteolin-7-glucoside, caffeic acid, chlorogenic acid.*
The aim of the study was to obtain some selective fractions from Cynara scolymus L. *leaves with various contents of caffeic, chlorogenic and rosmarinic acids, cynarin, luteolin-7-glucoside, apigenin-7-glucoside and rutin determined by HPLC and to establish the relationship between concentration and antioxidant activity.*
Eight selective fractions obtained by two distinct methods containing 0-0122% caffeic acid, 0-0.443% rosmarinic acid, 0.007-1.504% chlorogenic acid, 0-0.097% cynarin, 0.054-1.6662% luteolin-7-glucoside, 0.009-1.366% apigenin-7-glucoside and 0-0.396% rutin exhibited antioxidant activity at 0.001, 0.01, 0.1 and 1% dilution, varying from 0.27 to 87.77%. More precisely, selective fraction C_6 containing 16.662% luteolin-7-glucoside and C_8 selective fraction containing 5.568% luteolin-7-glucoside and 1.504% chlorogenic acid exhibited 87.77%, respectively 84.44% antioxidant activity at 1% dilution and 85.27%, respectively 69.44%. antioxidant activity at 0,1% dilution. The reference substance luteolin-7-glucoside showed 87.16% antioxidant activity at 1% concentration and 85.56% at 0.1% concentration. All selective fractions exhibited antioxidant activity and the action was correlated with their active substances concentration.

Key words*: selective fractions, Cynara scolymus, aging, antioxidant activity.*

INTRODUCTION

Cynara scolymus L. (Asteraceae) is one of the best recommended species for prevention and control of diseases associated with aging processes.
It is known that oxidative stress and inflammation play an important role in the onset of arterial disorders. It was demonstrated that extracts from the leaves of *Cynara scolymus* show antioxidant effect against oxidative stress-inducing factors and exhibit a cytoprotective effect both *in vitro* on rat hepatocytes (Gebhardt, 1997, 1998; Miccadei et al., 2008) and erythrocytes (Jimenez-Escrig et al., 2003), on human cells: neutrophil leukocytes (Perez-Garcia et al., 2000), endothelial cells and monocytes (Miccadei et al., 2008; Zapolska-Downar et al., 2002; Wang et al., 2003) but also *in vivo* (Jimenez-Escrig et al., 2003). The antioxidant effect is due to the polyphenolic content of this species including luteolin, luteolin-7-glucoside, caffeic acid, chlorogenic acid. (Gebhardt, 1997, 1998; Perez-Garcia et al., 2000; Wang et al., 2003).
Hypercholesterolemia is associated with an increased risk of coronary heart disorders and other sequelae of atherosclerosis. Extracts or some vegetal active substances such as luteolin, luteolin-7-glucoside from the *Cynara scolymus* L. leaves show hypocholesterolemic properties on rat hepatocytes cultures (Gebhardt, 1997) or on human hepatocytes cultures (Gebhardt, 2002).
Moreover, intraperitoneally administration of these extracts on rats decreases cholesterol and triglycerides levels (Saenz Rodriguez et al., 2002).
Other positive effects of extracts from *Cynara scolymus* extracts consist in: increasing the bile secretion demonstrated both *in vitro* on heaptocytes cultures (Gebhardt and Fausel, 1997)

and *in vivo*; hepatoprotective effects demonstrated *in vitro* on rat hepatocytes due to caffeic acid and less to cynarin (Gebhardt, 2002) and also *in vivo* by oral administration in rats (Adzet et al., 1987); spasmolytic effect demonstrated on guinea-pig ileum (Emendorfer et al., 2005).
The therapeutic properties of total extracts from the leaves of *Cynara scolymus* have been demonstrated also by clinical testing. These extracts are capable to decrease cholesterol and triglycerides levels (Petrowicz et al., 1997; Wider et al., 2007), to improve LDL / HDL ratio (Schmiedel, 2002; Fintelmann and Petrowicz, 1998), to exhibit choleretic (Kirchhof et al., 1994) and antidispeptic effects by improving symptoms like vomiting, abdominal pain, nausea, flatulence (Fintelmann and Petrowicz, 1998) and generally improving quality of life (Bundy et al., 2004) and also to have benefficial effects in the treatment of irritable colon syndrome (Holtmann et al., 2003).
The aim of this work was to study the antioxidant activity of fractions obtained by processing *Cynara scolymus* containing caffeic, chlorogenic and rosmarinic acids, cynarin, luteolin-7-glucoside apigenin-7-glucoside and rutin, secondary plant metabolites selectively extracted and distributed in fractions.

MATERIALS AND METHODS

The vegetal material consisted of *Cynara scolymus* L. leaves (Cynarae folium) obtained from cultivated crops, dried and ground as powder with a IV sieve, containing 1.38 polyphenolcarboxylic acids expressed as chlorogenic acid and 1.75 total flavones expressed as luteolin-7-glucoside .
Chemicals: 2,2-diphenyl-1-picrylhydrazyl (DPPH), cynarin, chlorogenic, rosmarinic and caffeic acids, luteolin-7-glucoside apigenin-7-glucoside and rutin were purchased from Sigma Aldrich-Fluka. All other chemicals were analytical reagent grade.

Extraction of selective fractions

Method I consisted in repeated extraction - two times of the active substances from 100g Cynarae folium with methylic alcohol (selective fraction C_1), ethylic alcohol (selective fraction C_2) 50% ethylic alcohol v/v (selective fraction C_3) (vegetal material / solvent ratio = 1/10 m/v for the first extraction and 1/5 m/v for the second extraction), at boiling temperature of the mixture for 1 hour per extraction, cooling and filtering, gathering all extractive solutions, solvent removal at reduced pressure (72-74 mmHg) and drying of remaining residue at 40^0C (Figure 1).

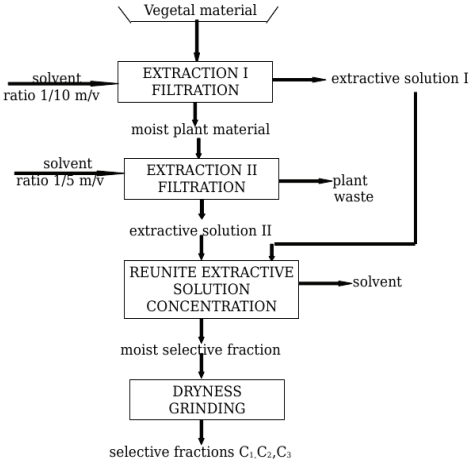

Figure 1 - Extraction scheme, method I

Method II consisted in repeated extraction of the active substances from 100g plant material with 50% ethanol (vegetal material / solvent ratio = 1/10 m/v for the first extraction and 1/5 m/v for the second extraction) at boiling temperature of the mixture for 1 hour per extraction, cooling and filtering, gathering all extractive solutions, hydroalcoholic solution concentration at reduced pressure (72-74 mmHg) to a volume of 1/1 V/m from plant material. The aqueous solution obtained was centrifuged at 4000 rot/min, the insoluble substances were dried at a temperature of 40^0C and grinding as fine powder with a IV sieve to obtain selective fraction C_4. The selective fractions C_5, C_6, C_7 from clear aqueous solution were obtained by successive liquid-liquid extraction three times with methylene chloride, four times with ethyl acetate and six times with n-butyl alcohol, followed by solvent removal, drying and grinding.. In remaining aqueous solution, a small quantity of acetone was added to obtain a precipitate which was further filtrated, dried and grinded resulting in selective fraction C_8 (Figure 2).

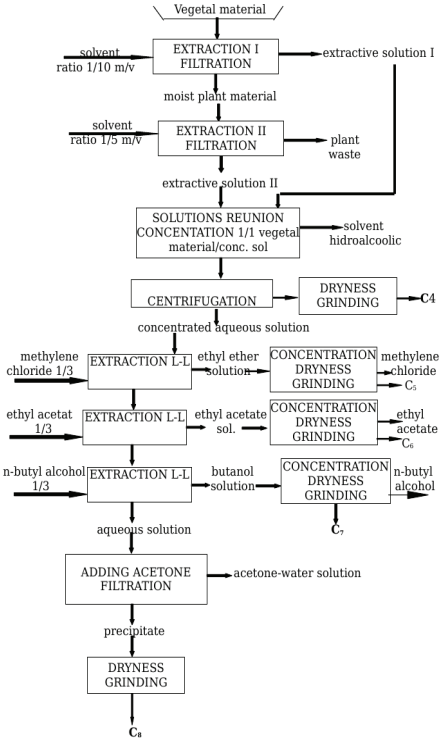

Figure 2 - Extraction scheme, method 2

Selective fractions analysis by HPLC

Chromatographic separation was achieved on a Kromasil ODS column (250 x 4.6 mm, 5µm) at 40ºC, using a gradient elution. The mobile phase was a binary gradient: water with ortho-phosphoric acid (pH = 2.0) and acetonitrile. The first step, the linear gradient started at 10% to 25% acetonitrile in 25 minutes, followed by isocratic elution with 25% acetonitrile over 8 minutes. The second step, the gradient elution was from 25% to 90% acetonitrile in 7 minutes, followed by isocratic elution with 90% acetonitrile for 5 minutes. The eluent absorbance was monitored at 330 nm.

Determination of antioxidant activity

DPPH assay: In each reaction tube 100 µL vegetal extract of different concentrations was mixed with 3900µL of 0.0025g/L DPPH at room temperature for 30 min. 50% methanol solution was used as control. The reduction of the DPPH free radical was measured by reading the absorbance at 515 nm. Luteolin-7-glucoside (PHYTOPLAN Diehm & Neuberger GmbH) was used as positive control. Inhibition ratio (percent) was calculated from the following equation:

% inhibition = [(absorbance of control – absorbance of sample)/ absorbance of control] x 100

DPPH radicals react with suitable reducing agents losing color stoichometrically with the number of electrons consumed which is measured spectrophotometrically at 515 nm (Sanchez Moreno, 1998).

RESULTS AND DISCUSSIONS

Using the experimental methods mentioned above, eight selective fractions from *Cynarae folium* were obtained and further analyzed by HPLC for polyphenolcarboxylic acids and flavones content in order to establish the relationship between the content in active substances and antioxidant activity.

The chosen methods allowed an excellent separation of reference substances (Figure 3) and also of the specific phytochemical compounds (Figure 4).

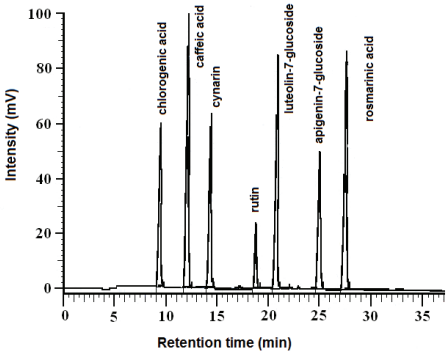

Figure 3 Standard solution chromatogram obtained under the selected chromatographic conditions (HPLC)

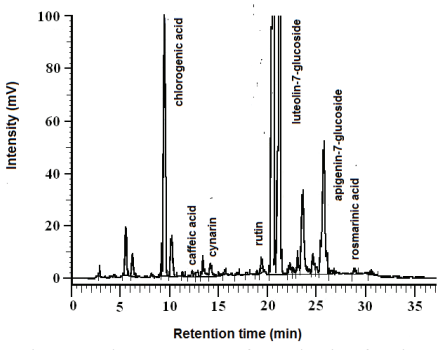

Figure 4 Chromatogram of C_8 selective fraction obtained from *Cynara scolymus*

Table 1. The content of polyphenolcarboxylic acids of selective fraction

Active substance/ Selective fraction	Quantity (g)	Polyphenolcarboxylic acids			
		Chlorogenic %	Rosmarinic %	Caffeic %	Cynarin %
1	12.08	0.112	0.014	0	0.018
2	8.02	0.052	0.008	0	0.013
3	18.773	0.365	0.011	0.018	0.088
4	8.330	0.120	0.356	0.004	0.012
5	1.670	0.306	0.443	0.014	0.013
6	0.534	0.113	0.240	0.122	0
7	5.770	0.007	0	0	0.004
8	2.370	1.504	0.022	0.019	0.097

The results regarding the amount of selective fraction and their content of active substance are presented in Tables 1 and 2.

Table 2. The content of flavones of selective fraction

Active substance/ Selective fraction	Quantity (g)	Flavones		
		Luteolin-7-glucoside %	Apigenin-7-glucoside %	Rutin %
1	12.08	1.672	0.110	0.056
2	8.02	1.909	0.111	0
3	18.773	1.846	0.254	0.218
4	8.330	1.625	0.106	0.043
5	1.670	1.599	0.043	0.117
6	0.534	16.662	1.366	0.034
7	5.770	0.054	0.009	0.009
8	2.370	5.576	1.253	0.396

The antioxidant activity of the pure active substances (reference substances) is presented in Table 3.

Table 3. Antioxidant activity of reference substances (%)

Dilution/ active substances	1%	0.1%	0.01%	0.001%
cynarin	89.44%	88.61%	27.22%	9.50%
chlorogenic acid	90.83%	87.77%	51.38%	7.50%
rosmarinic acid	90.27%	90.55%	61.66%	7.77%
caffeic acid	90.27%	90.55%	85.27%	17.50%
luteolin-7-glucoside	87.16%	85.56%	43.31%	3.74%
rutin	88.23%	86.55%	29.97%	4.76%

The experiments performed confirmed that all polyphenolcarboxylic acids, their derivatives and flavonosides, in pure form or as constituents of *Cynarae folium* selective fractions, exhibit a significant antioxidant activity as determined by DPPH method.

The antioxidant activity of the selective fractions are presented in Table 4.

The results obtained confirm the findings presented in the literature (Gebhardt, 1997, 1998; Perez-Garcia et al., 2000; Wang et al., 2003) concerning the antioxidant activity of extracts obtained from *Cynara scolymus* L. leaves and also the fact that the antioxidant effect is due to the polyphenols content (luteolin-7-glucoside, caffeic acid and chlorogenic acid).

The correlation between the chemical composition of flavonosides and polyphenolcarboxylic acids content and the antioxidant activity of each fraction is showed in Tables 1, 2 and 4.

Table 4. Antioxidant activity of selective fractions (%)

Dilution/ selective fraction	1%	0.1%	0.01%	0.001%
C1	78.05%	21.38%	3.88%	0.27%
C2	71.38%	20.55%	2.77%	2.50%
C3	70.83%	40.83%	8.05%	4.44%
C4	62.22%	33.33%	7.22%	4.72%
C5	60.83%	28,61%	8.05%	4.16%
C6	87.77%	85.27%	17.50%	5.27%
C7	37.22%	11.94%	4.72%	3.33%
C8	84.44%	69.44%	11.66%	4.65%

Comparing the antioxidant activity of selective fractions with the activity of reference substances, it can be said that antioxidant activity depends on the active substances concentration, namely it increase with the increase of concentration.

Selective fraction C_6 especially, which is rich in luteolin-7-glucoside (16.662%) exhibits an antioxidant activity higher than other fractions (87.77% at 1% dilution and 85.27% at 0,1% dilution), similar to the reference substance luteolin-7-glucoside (87.16% at 1% dilution and 85.56% at 0.1% dilution) which shows a significant antioxidant activity.

A dose-effect correlation is obvious, certifying that both flavones and polyphenolcarboxylic acids are responsible for antioxidant activity. In this respect, we showed that C_8 selective fraction containing only 5.576% luteolin-7-glucoside but a higher amount of chlorogenic acid (1.504%) than the other selective fractions

exhibits a good antioxidant activity (84.44% at 1% dilution and 69.44% at 0.1% dilution), close to selective fraction C_6 .
Still, some selective fractions exhibit antioxidant activity even though they have low concentrations in active substances (for example C_7) which might show that these compounds act synergistically.

CONCLUSIONS

Eight selective fractions from *Cynara scolymus* leaves were obtained; their concentration of active substances varies with the chosen extraction method from 0-0.122% caffeic acid, 0.007-1.504% chlorogenic acid, 0-0.443% rosmarinic acid, 0-0.097% cynarin, 0.054-16.662 % luteolin-7-glucoside, 0.009 - 1.253% apigenin-7-glucoside and 0-0.396% rutin.
All selective fractions exhibited antioxidant activity and the action is correlated with their active substances concentration.

REFERENCES

Adzet T., Camarasa J., Laguna J., 1987. Hepatoprotective activity of polyphenolic compounds from Cynara scolymus against CCl4 toxicity in isolated rat hepatocytes. J Nat Prod, 50(4), 612-617

Bundy R., Walker A., Middleton R., Marakis G., Booth J., 2004. Artichoke leaf extract reduces symptoms of irritable bowel syndrome and improves quality of life in otherwise healthy volunteers suffering from concomitant dyspepsia: a subset analysis. J Altern Complement Med, 10(4), 667-669

Emendorfer F., Bellato F., Noldin V., Cechinel-Filho V., Yunes R., Delle Monache F., Cardozo A., 2005. Antispasmodic activity of fractions and cynaropicrin from Cynara scolymus on guinea-pig ileum. Biol Pharm Bull; 28(5), 902-904

Fantini N., Colombo G., Giori A., Riva A., Morazzoni P., Bombardelli E., Carai M., 2011. Evidence of glycemia-lowering effect by a Cynara scolymus L. extract in normal and obese rats. Phytother Res., 25(3), 463-5

Fintelmann V., Petrowicz O., 1998. Long term adminostration of artichoke extracts for dyspepsia symptoms. Results of an observation study. Natura Med 13, 17-26

Gebhardt R., 1997. Antioxidative and protective properties of extracts from leaves of the artichoke (Cynara scolymus), against hydroperoxide induced oxidative stress in cultured rat hepatocytes. Toxicol Appl Pharmacol, 144, 279-286

Gebhardt R., Fausel M., 1997. Antioxidant hepatoprotective effects of artichoke extracts and constituents in cultured rat hepatocytes. Toxicol in Vitro, 11, 669-672

Gebhardt R., 1998. Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (Cynara scolymus L.) extracts. J Pharmacol Exp Ther., 286(3), 1122-8

Gebhardt R., 2002. Prevention of taurolithate-induced hepatic bile canalicular distortions by HPLC-characterized extracts of artichoke (Cynara scolymus) leaves. Planta Med, 68(9), 776–779

Gebhardt R., 2002 Inhibition of cholesterol biosynthesis in HepG2 cells by artichoke extracts is reinforced by glucosidase pretreatment. Phytother Res 16(4), 368-372

Holtmann G., Adam B., Haag S., Collet W., Grunewald E., Windeck T., 2003. Efficacy of artichoke leaf extract in the treatmentn of patients with functional dyspepsia: a six-week placebo-controlled, double-blind, multicentre trial. Aliment Pharmacol Ther, 18, 1099-1105

Jimenez-Escrig A., Dragsted L., Daneshvar B., Pulido R., Saura-Calixto F., 2003. In vitro antioxidant activities of edible artichoke (Cynara Scolymus L.) and effect on biomarkers of antioxidant in rats. J Agric Food Chem. 51(18), 5540-5

Kirchhoff R., et al. 1994. Increase in choleresis by means of artichoke extract. Phytomedicine, 1, 107-115

Miccadei S., Di Venere D., Cardinali A., Romano F., Durazzo A., Foddai M., Fraioli R., Mobarhan S., Maiani G., 2008. Antioxidative and apoptotic properties of polyphenolic extracts from edible part of artichoke (Cynara scolymus L.) on cultured rat hepatocytes and on human hepatoma cells. Nutr Cancer. 60(2), 276-83

Pérez-García F. et al. 2000, Activity of artichoke leaf extract on reactive oxygen in human leukocytes. Free Rad Res. 33(5), 661-65

Petrowicz O., Gebhardt R., Donner M., Schwandt P., Kraft K., 1997. Effects of artichoke leaf extract (ALE) on lipoprotein metabolism in vitro and in vivo. Atherosclerosis, 129, 147

Saenz Rodriguez T., Garcia Gimenez D., de la Puerta Vazquez R., 2002. Choleretic activity and biliary elimination of lipids and bile acids induced by an artichoke leaf extract in rats. Phytomedicine; 9(8), 687-693

SánchezMoreno C., Larrauri J., Saura-Calixto F., 1998. A procedure to measure the antiradical efficiency of polyphenols. J. Sci. Food Agric., 76, 270-276.

Schmiedel V., 2002. Senkung des Cholesterinspiegels durch Artischocke und Ballaststoffe. Erfahrungsheilkunde, 51, 405-414

Wang M. et al., 2003. Analysis of antioxidative phenolic compounds in artichoke (Cynara scolymus L.). J Agric Food Chem. 51(3). 601-608

Wider B., Pittler M., Thompson-Coon J., Ernst E., 2007. Artichoke leaf extract for treating Hypercholesterolaemia, Cochrane Database Syst rev. 4, CD003335

Zapolska-Downar D., Zapolski-Downar A., Naruszewicz M., Siennicka A., Krasnodebska B., Koldziej B., 2002. Protective properties of artichoke (Cynara Scolymus L.) against oxidative stress induced in cultured endothelial cells and monocytes. Life Sci., 71(24), 2897-08

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THE DYNAMICS OF LIVE AND DEAD CELLS, AND COLONY FORMING UNITS OF *E. COLI* DH5 α STRAIN KEPT FOR FIVE DAYS AT 18°C IN MICROCOSMS CONTAINING FILTERED AND UNFILTERED SEA WATER

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Abstract

The aim of this study is to determine the time-evolution of live and dead cells as well as the number of colony forming units of E. coli strain DH5α after the passage from LB medium to seawater indoor microcosms. In order to determine the dynamics and survivability capacity with respect to the absence or presence of bacterivores microorganisms E. coli sub-samples were housed in three different microcosms containing filtered (0.22 or 0.45 µm pores) and unfiltered sea water. The microcosms were kept for five days in a sterile environment at a constant temperature of 18 °C in the dark. The samples collected were examined by fluorescent microscopy (SYBER green and Ethidium homodimer) for the determination of cell density (total /dead) and colony forming units quantification (LB and Levine media) in order to establish C.F.U. growth. The results presented in this paper show that the shocks associated with the passage from LB medium to sea water indoor microcosms (hypothermic, hypo-osmotic, including low levels of nutrients, and the presence of bacterivores microorganisms) play a key role in the survival of E. coli cells in this five days period.

Key words: *E. coli, live cells, colony forming units, protozoa predation, microcosm.*

INTRODUCTION

During the last decades the Black Sea waters have suffered multiple changes due to pollutant discharge. Human interventions and hydraulic regime of the rivers played an important role in the changes of the phytoplankton, zooplankton and zoobenthos (Bologa et al., 1995; Bakan and Buyukgungor, 2000).

Similar unsteadiness in sea waters worldwide has driven researchers to study the harmful contingent risks of multiplication of potential pathogenic bacteria such as *E. coli* (Greenberg, 1956; Lessard et al., 1983; Davies et al., 1995). Numerous studies show that changes in sea water like high temperatures due to global warming, salinity, nutrient availability, light radiation and protozoa predation influences the survival of potential pathogen bacteria (Carlucci et al., 1961; Gameson and Gould, 1975; Fujioka et al., 1981; Anderson et al., 1983; Ingraham and Marr, 1996; Trousseller, 1998).

Taking all into consideration, we felt that a study that features the current stage of the Black Sea waters particularities regarding potential pathogenic cell multiplication is necessary and important in order to monitor the evolution of bacteria such as *E. coli* that had accidentally reached sea water by shedding and polluted sewage.

The aim of this paper is to monitor the dynamics of *Escherichia coli* cells (live, dead) and their ability to grow and multiply after being kept in three different microcosms containing filtered (0.22 or 0.45 µm pores) and unfiltered sea water at 18 °C for five days.

MATERIALS AND METHODS

Strain and Sampling

We used a nonpathogenic strain of *E. coli* (DH5α) that was previously grown in LB 37 °C on an orbital shaker (150 rpm). The cells were collected after 18 hours of growth and were aseptically washed two times in sterile phosphate buffer saline to eliminate organic substrate.

The sea water was collected in the autumn from the Black Sea territorial waters (Constanta) at 1 m depth. The water was filtrated into sterile bottles using either 0.22 µm Millipore or 0.45 µm Millipore filters.

Microcosms

Three microcosms were housed in sterile 1 L bottles. The first one contains sea water filtered by 0.22 µm in order to assure a sterile environment by eliminating all other cells, including different types of bacterivores pre-existing in the water. The second microcosm contains 0.45 µm filtered water to avoid the inclusion of bacterivores microorganism, but allowing the presence of other small microorganisms; the third one was with unfiltered water, a configuration more similar to the natural conditions. As many studies show that other concurrent bacteria and protozoa predation play an important role in the survivability of *E. coli* population, (Enzinger and Cooper, 1976; Barcina et al., 1992; Gonzales et al., 1992; Sherr and Sherr, 2002) the choice of having three different microcosms with the particularities described above is fundamentally important, in order to establish the ability of *E. coli* cells to remain intact and to keep the capacity to grow and multiply after reaching the sea water. In each of the three microcosms 0.5 mL *E. coli* DH5α sub-culture previously adjusted to an OD =1.0 was added. All were kept for five days in the dark at a constant temperature of 18 °C. The samples were collected immediately after inoculation (time zero), and also at 1 hour, 3 hours, 22 hours, 46 hours, 3 days, 4 days and 5 days.

Enumeration of live and dead *E. coli* cells

The samples were analyzed in order to assess the viability of the *E. coli* populations and also their capacity of multiplication under the environmental stress of each microcosm. Fluorescence microscopy was used to determine the cell densities in order to ascertain the number of remaining live *E. coli* cells in each of the three microcosms. The samples were stained with SYBER green for labelling the DNA of all the *E. coli* cells, the living cells, with intact and functional plasma membrane (live cells), and also cells with altered plasma membrane (dead cells) (Figure 1a). We also used Ethidium homodimer as a membrane impermeable dye to stain the same samples in order to highlight dead cells) (Figure 1b), cells with plasma membranes that have an unphysiological permeability, allowing the passage of large, negatively charged molecules (Manini and Danovaro, 2006).

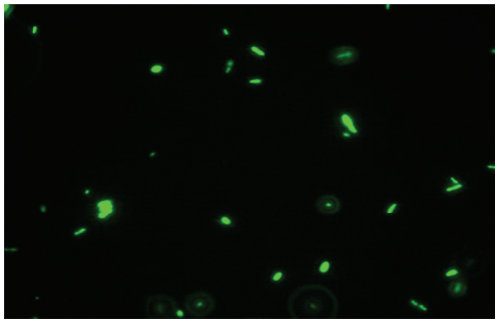

Figure 1a. *E. coli* cells stained with SYBER-green

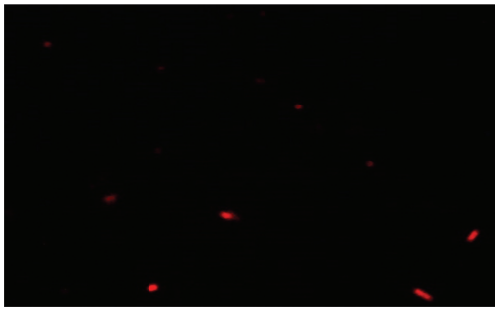

Figure 1b. *E .coli* cells stained with Ethidium homodimer (the same microscopic field as in Figure 1a)

For the fluorescence microscopy the samples were filtered through polycarbonate Nucleopore filters with Millipore funnel attempting an uniform distribution of bacterial cells over the filtration surface. The quantification was realized for a media of 15 microscopic fields for each probe and these readings were realized with Ob.100x, oil immersion. The cell density for each probe was determined with the following formula (Manini and Danovaro, 2007):

Cell density/ml = { [3,14 x (75000x75000)]:(46 x 98) }x number of counted cell/

First the number of total cells (SYBER-green labeled) was calculated and then in parallel the number of dead cells (Ethidium homodimer labeled). By deducting the number of dead cells from the total cell number for each microscopic field the number of live cell was obtained; these numbers were further used to calculate the density of live cells per volume (mL).

Quantification of colony forming units

In order to establish the ability of the *E. coli* cells to grow and multiply after being kept in these three microcosms, the number of colony forming units was determined. Towards avoiding any interference from the marine bacteria present in the 0.45 µm filtered microcosms and the unfiltered one, the samples were inoculated on two different culture media: LB and Levin (Figure 2a and Figure 2b). Although we have used before in our similar experiment Luria-Bertani culture medium as a specific media for coliform bacteria, adding a highly specific medium such as Levin in order to highlight the colonies of *E. coli* was mandatory (Cristina and Ardelean, 2015).

From each probe 10 µl were inoculated by using the droplet method (Neblett, 1976; Hoben and Somasegaran, 1982). The plates were then incubated at 37 °C for 24 hours.

RESULTS AND DISCUSSIONS

By maintaining all three microcosms in the same constant environmental parameters (18°C, darkness) we assured that the differrences between the evolutions of the *E. coli* populations relies only on the dissimilarities of the filtration of the sea water, namely the absence or the presence of bacterivores microorganisms.

Regarding the evolution of live cells density in these five days of study we argue that the nutritional competition and protozoa predation plays an important role in the survival of *E. coli*. As it is notable in the following graphic (Figure 3) the number of live cells decreases considerable in the unfiltered sea water microcosm with respect to the first two microcosm systems (the one with sea water filtered through 0.22 µm Millipore filters and the one filtrered through 0.45 µm Millipore filters). Like many studies show, protistis such as some dinoflagellates, including those from the Black Sea may act as predators for bacteria (Kofoid and Swezy., 1921; Barker, 1935; Schnepf and Elbrächter., 1992). In our five days experiment the number of total cell count from the unfiltered seawater microcosm decreases drastically, as does the number of live cells (Figure 4).

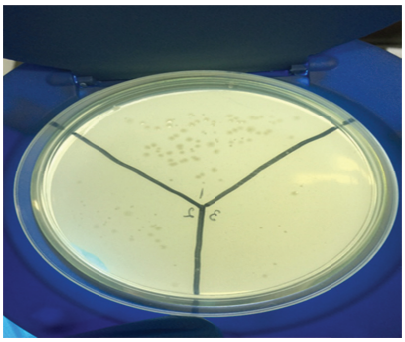

Figure 2a – *E. coli* C.F.U. on LB culture media

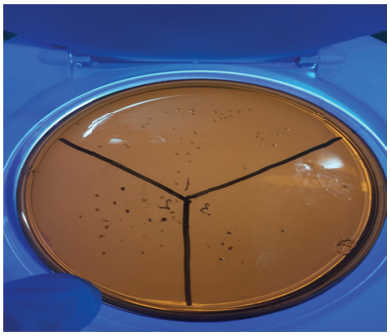

Figure 2b – *E. coli* blue-metalic C.F.U. on Levine culture medium

However, the number of total cell count (SYBER-Green labelled cells) from the other two microcosms, with filtered sea water, is maintained relatively constant, suggesting, as it has been previously demonstrated, that for short periods of time cell destruction does not occur (Cristina and Ardelean, 2015).

The loss of noteworthy cells due to the contingent formation of biofilms was not taken into consideration as *E. coli* DH5α strain used in this experiment expresses poor mobility and cell attachment, which are essential for biofilm formation (Wood et.al. 2006).

The number of dead cells increases sharply within the five days period in each of the three microcosms (Figure 5). Loss of cell integrity when submerged for different periods of time in sea water microcosms systems was described for *E. coli* strains in other studies as well. As it has been previously described, the osmotic stress suffered by *E. coli* population cells when submerged by direct inoculation from the culture in sea water microcosm determines a high mortality rate from the first

hour (Cristina and Ardelean, 2015; Omrane et al., 2011).

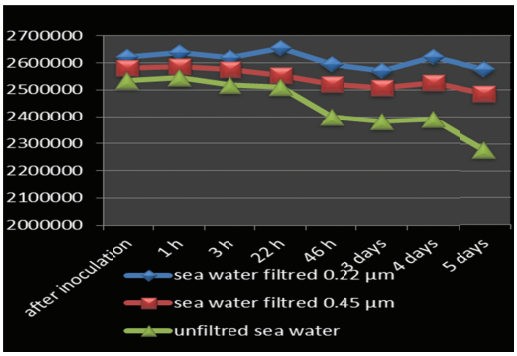

Figure 3. The evolution of total cells count during the five days experiment (SYBER Green *E. coli* labelled cells) in each of the three microcosms systems at 18 °C

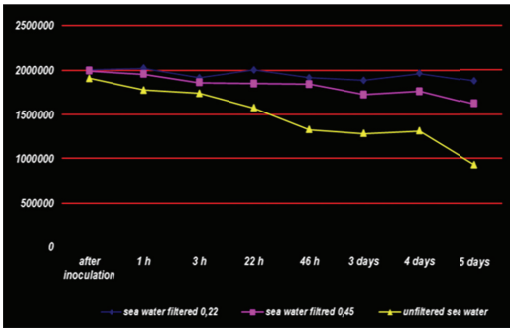

Figure 4 – The evolution of living cells during the five days experiment (SYBER Green *E. coli* labeled cells minus Ethidium homodimer – labeled cells) in each of the three microcosms systems at 18 °C

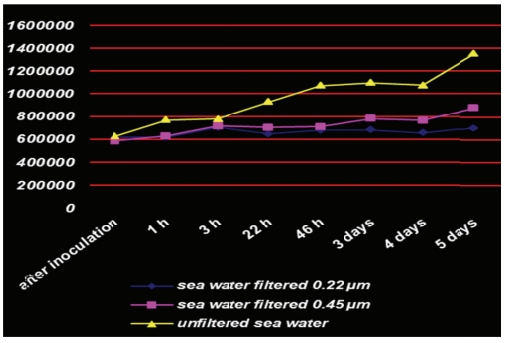

Figure 5 - The evolution of dead cells during the five days experiment (Ethidium homodimer – *E. coli* labelled cells) in each of the three microcosms systems at 18 °C

On the other hand, the stress factors of marine environment induce a loss of the colony forming ability although the cells maintain their viability (Figure 6a and Figure 6b). We can assume that this fact is due to their capacity to converge in a viable but nonculturable state. The nonculturable state, as explained in different studies is a state in which cells, though viable, are unable to divide on nutritional specific media (Roszakt and Colwell, 1987; Pommepuy 1996). The multiplication capacity does not vary substantially between the microcosm with sea water filtered 0.22 µm and the microcosm with sea water filtered 0.45 µm on the five days period of our study. By difference the culturable state of the *E. coli* cells from the microcosm with unfiltered sea water suffer a notable decrease from the 22 hours point on. After four days there was not any C.F.U. growth among the probes from the unfiltered water microcosm on culture medium, LB, or Levin. We repeated these probes in order to present an accurate result. Taking this into consideration and also due to the fact that there was a sharp decrease of C.F.U. in both media, LB and Levine, after five days in the unfiltered sea water microcosm, we could argue that the nutritional competition of the sea water microorganisms but mostly the activity of bacterivores is crucial to the survivability of *E. coli*.

Though not as dramatically, the number of *E. coli* C.F.U. from the microcosm with filtered sea water (0.22 µm) decreased linearly through the whole time period of the experiment. As proven in other experiments as well, the reduction of *E. coli* cells cultivability when submerged in seawater is substantially even in a five days period (Omrane et al., 2011). The cells from the microcosm with sea water filtered through 0.45 µm undertake a similar pattern.

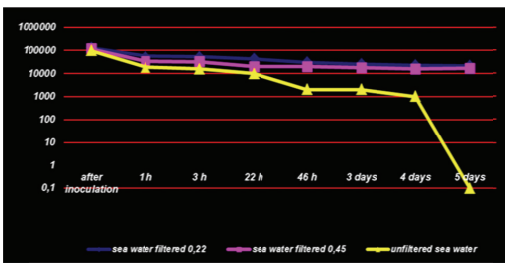

Figure 6a – The evolution of colony forming units of *E. coli* cells in each of the three microcosms systems at 18 °C, after inoculation in LB culture medium, during the five days experiment

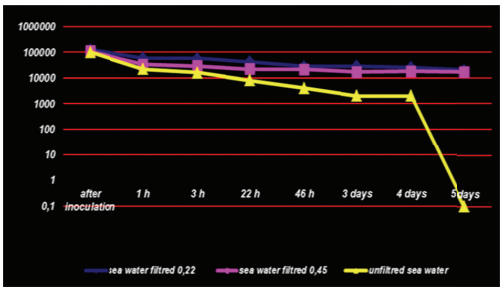

Figure 6b –The evolution of colony forming units of *E. coli* cells in each of the three microcosms systems at 18 °C, after inoculation in Levine culture medium, during the five days experiment

The slightly higher number of *E. coli* C.F.U. obtained on the Levin medium by comparison with LB culture medium could be probably explained by the superior nutritive composition of the Levin medium as compared with LB.

CONCLUSIONS

1. Within the five days period of this study there was a steadfast decrease of the density of live cells from each of the three microcosms. The decline suffered by the cell population was notable higher in the unfiltered sea water microcosm as compared with the results obtained in both microcosms with filtered sea water (0.22 µm or 0.45 µm).
2. Total cell count (SYBER- Green labelled cells) in the sterile microcosm (0.22 µm filtered sea water) was maintained constant during this study whereas in non-sterile microcosms (unfiltered or 0.45 µm filtered sea water) total cell count decreased. This suggest that n the sterile microcosm (0.22 µm- filtered sea water) physical disaggregation of cells does not occur, while for the other two microcosms nutritional competition with other microorganisms and microbial predation seems to play a (key) role in the reduction of total cell density of *E. coli* cells.
3. The density of colony forming units seems to be parallel both in sterile and 0.45 µm - filtered sea water microcosms but after 22 hours the decrease in C.F.U. is much higher in non-filtered microcosm, reaching extinction after 5 days both in LB and Levin media, suggesting the dramatic effect of predators on *E. coli*.

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REFERENCES

Anderson I.C., Rhodes M.W., Kator H.I., 1983. Seasonal variation in survival of Escherichia coli exposed in situ in membrane diffusion chambers containing filtered and nonfiltered estuarine water. ,Appl Environ Microbiol 45(6):1877–1883.

Barker H.A., 1935.The culture and physiology of the marine dinoflagellates, Arch. Mikrobiol. 6 (1–5): 157–181.Bakan, G. & H. Buyukgungor, 2000. *The Black Sea*. Marine Pollution Bulletin 41: 24–43.

Barcina I., Gonzalez J.M., Iriberri, J., Egea, L., 1992. Role of protozoa in the regulation of enteric bacteria populations in seawater. Marine Microb. Food Webs 5, 179-188.

Bologa A. S., N. Bodeanu, A. Petranu, V. Tiganus, Zaitsev, Y.P., 1995. Major modifications of the *Black Sea* benthic andplanktonic biota in the last three decades. Bulletin d'Institute Oceanographique de Monaco n. sp. 15: 85–110.

Carlucci A.F., Scarpino P.V., Pramer D., 1961. Evaluation of Factors Affecting Survival of *Escherichia coli* in Sea Water, V. Studies with Heat- and Filter-sterilized Sea Water .Appl Microbiol. 9(5): 400–404.

Cristina R.G. and Ardelean I., 2015. The viability of *E.coli* in sea water at different temperatures. Land Reclamation, Earth Observation & Surveying, Environmental Engineering. Vol. IV, 79-83.

Davies, C.M., Long, J.A., Donald, M., Ashbolt, N.J., 1995. Survival of fecal microorganisms in marine and freshwater sediments. Appl. Environ. Microbiol. 61: 1888-1896

Enzinger, R.M. and Cooper, R.C., 1976. Role of bacteria and protozoa in the removal of *E. coli* from estuarine waters. Appl. Environ. Microbiol. 31:758-76

Fujioka, R.S., Hashimoto, H.H., Siwak, E.B., Young, R.H., 1981. Effect of sunlight on survival of indicator bacteria in seawater. Appl. Environ. Microbiol. 41: 690-696.

Gameson, A.L.H. and Gould, D.J. 1975 Effects of solar radiation on the mortality of some terrestrial bacteria in sea water. Proceedings of the International Symposium on Discharge of Sewage from Sea Outfalls, 209-219.

Greenberg, A.E., 1956. Survival of enteric organisms in sea water. Public Health Rep. 71 :77-86.

Gonzalez, J.M., Iriberri, J., Egea, L., Barcina, I., 1992. Characterization of culturability, protistan grazing, and death of entericbacteria in aquatic ecosystems. Appl. Environ. Microbiol. 58: 998-1004.

Hoben H. J. and Somasegaran P., 1982. Comparison of the Pour, Spread, and Drop Plate Methods

forEnumeration of *Rhizobium* spp. in Inoculants Made from Presterilized Peat. Appl. Environ. Microbiol 44(5): 1246-1247

Ingraham, J.L. and Marr, A.G., 1996. Efect of temperature, pressure, pH, and osmotic stress on growth. In: *Escherichia coli* and Salmonella: Cellular and Molecular Biology. Vol. 2. ASM Press, Washington, DC.

Kofoid C.A., Swezy O.,1921. The free-living unarmoured dinoflagellata, Mere. Univ. Calif.

Lessard, E.J. and Sieburth, J.M., 1983. Survival of natural sewage populations of enteric bacteria in difusion and batch chambers in the marine environment. Appl. Environ. Microbiol. 45: 950-959.

Manini E., and Danovaro R., 2006. Synoptic determination of living/dead and active/dormant bacterial fractions in marine sediments, FEMS Microbiol. Ecol., Vol. 55: 416-423.

Neblett T.R., 1976. Use of droplet plating method and cystine-lactose-lactng electrolyte-deficient medium in routine quantitative urine culturing procedure. J. Clin. Microbiol. 4(3):296

Omrane B.B., El Bour M., Mejri S., Mraounai R, Got P., Troussellier M., Boudabous A., 2011. Survival study of enterotoxigenic *Escherichia coli* strain in seawater and wastewater microcosms. Archives de L'institut Pasteur de Tunis. ·

Pommepuy, M., Butin, M., Derrien, A., Gourmelon, M., Colwell, R.R., Cormier, M., 1996. Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight. Appl. Environ. Microbiol. 62: 4621-4626.

Roszakt D.B. and Colwell R.R., 1987; Metabolic Activity of Bacterial Cells Enumerated by Direct Viable Count. Appl. Environ. Microbiol.53 (12) :2889-2983.

Schnepf E., Elbrächter M., 1992. Nutritional strategies in dinoflagellates: A review with emphasis on cell biological aspects, Eur. J. Protistol. 28 (1): 3–24.

Sherr E., Sherr B., 2002. Significance of predation by protists in aquatic microbial food webs, Ant van Leeuwen, Vol. 81, 293–308.

Troussellier, M.,1998. Responses of enteric bacteria to environmental stresses in seawater. Oceanol. Acta 21: 965-981.

Wood T. K., González Barrios A.E., Herzberg M., Lee J., 2006. Motility influences biofilm architecture in *E. coli*. Appl Microbiol Biotechnol 72: 361-367.

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SATISFACTION LEVEL OF STUDYING THROUGH E-LEARNING SYSTEM AT THE STUDENTS ENROLLED IN THE BIOTECHNOLOGY EDUCATION

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Abstract

A lot of studies about online education are meant to answer about how efficient is this new way of a learning system, or how interested are students to learn using online platform, choosing themselves when or where to study without a program which requires their simultaneous presence in the same place and at the same time. In 1999, American Teachers Federation and National Association for Education initiate a research about distance learning efficiency. From that year till now, many researchers or teachers have been interested in how good is the e-learning system, the feedback from students being anytime important.

Key words: *biotechnology, education, e-learning, MOODLE.*

INTRODUCTION

We would say that somebody's dream can become reality someday. This is what happened to George Robert Stibitz, the American researcher who projected, in 1940, the first computer named CNC (Complex Number Calculator). The first modem, developing digital phone lines, first online communities, SAGE air defense system, SABRE, Carterfone and so on were steps in technology development. And when technology was created why not to use it for online learning?

The distance learning is a 19th Century concept that came from Isaac Pitman. In the first years of 90's, some schools were prepared for online courses, putting people together from the distance and educate them. Fifteen years ago, companies begin to use the e-learning system to train their employees. In the present days, there are many universities who adapted their knowledge in packages of courses to deliver the content for students all over the world, even for free.

Richey (2008) defined educational technology as "the study and ethical practice of facilitating learning and improving performance by creating, using and managing appropriate technological processes and resources".

Educational technology is not restricted to high technology, also called e-learning, and includes numerous types of media that deliver text, audio, images, animation, and streaming video, and includes technology applications and processes such as audio or video tape, CD-ROM, satellite TV, and computer-based learning.

Accordingly, virtual education refers to a form of distance learning, in which course content is delivered by various methods like a management applications course.

The learning management system (LMS) is software used for delivering, tracking and managing training and education. For example, an LMS tracks attendance, time on task, and student progress. Teachers can post announcements, grade assignments, check on course activity, and participate in class discussions. Students can submit their work, read and respond to discussion questions, and take quizzes.

Around the world, online college course enrollment has seen a 29% increase in enrollment with nearly one third of all college students, or an estimated 6.7 million students are currently enrolled in online classes.

But how are students reacting to e-learning system? We consider the e-learning system implemented through the Moodle platform was welcomed by students enrolled in the biotechnological education.

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MATERIALS AND METHODS

So, at the end of a semester, we conducted a study on the level of satisfaction between the beneficiaries of e-learning PC - Operating and Programming course from the first year of study here at the Faculty of Biotechnology. At this study, we obtained answers from 89 students. Our quantitative questionnaire contains a set of 14 simple questions targeting on our e-learning course through Moodle platform. The results of this study were processed through the Moodle platform and synthetically presented below.

RESULTS AND DISCUSSIONS

From the beginning, we conducted our quantitative questionnaire through the same Moodle platform used for the e-learning course. Every student log in her or his account where they can find easy questions described below and answer under the anonymous status. Our target was focused on:

- evaluation of students' expectations of e-learning,
- their evaluations of Moodle platform experiences,
- assessments of course outcomes (learning achievements, course satisfaction).

The first question is about the level of difficulty to create an account on the Moodle platform which is accessible at http://moodle.biotehnologii.usamv.ro

From all the tasted subjects none chose the respose "very hard", one chose "hard", 21 (23.60%) went to" easy" and 67 (over 75%) to "very easy" (Figure 1).

To the second question about how do the students classify the profile editing in their account (changing the password, picture modifying etc.), the answers repartition goes between (figure 2):

- easy for 32.58%
- very easy for 67,42%.

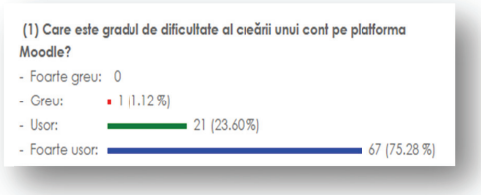

Figure 1. Repartition of the difficulty level in creating an account on the Moodle platform

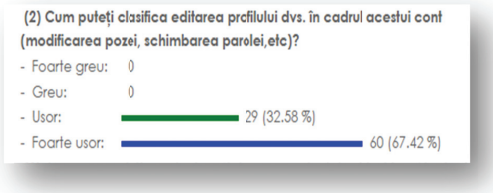

Figure 2. Repartition of the difficulty level in profile editing into an account on the Moodle platform

For the mobility in platform accessibility, with multiples accepted answers (Figure 3):

- over 97% enter from a laptop or a desktop,
- over 56% from a smartphone
- only over 17% from a tablet.

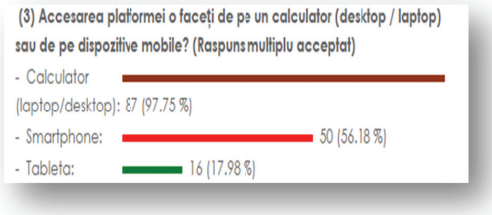

Figure 3. Accessibility of the Moodle platform from a PC desktop or laptop or mobile devices

Also, in the spirit of mobility, with multiple accepted answers, the login to the platform from a mobile device is doing (Figure 4):

- over 77% from home,
- over 50% from a classroom,
- over 58% in traffic (bus, subway, train) or another location different from home, laboratory (such as lunch time, library etc.).

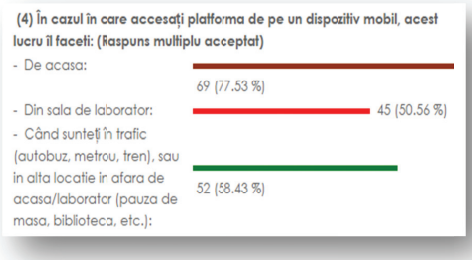

Figure 4. Repartition of the login to the Moodle platform on mobile devices from different locations

In unanimity, 100% of students responded that:

- they consider useful, for the individual study, to access the course material and learn also outside the traditional space (home or school),
- course material is easy to understand,
- the video packaging inserted in the platform for exemplifications are relevant,
- the online system of testing the knowledge achieved at PC-Operating and Programming course through the Moodle platform allows improvement of results,
- studying through the Moodle platform is a progress in education,
- studying through the Moodle platform is a modern element useful in education at the universities level,
- it is recommendable to introduce more disciplines which allowed online teaching and testing in the e-learning system.

Over 87% (Figure 5) of the students never used LMS (learning management systems) such as Moodle, Dokeos or Blackboard or else. The other students studied in high school chemistry, informatics or participated at the coursera.org courses.

To improve the PC - Operating and Programming course in Moodle system the single proposal received was to improve the mobile application and it comes from a single subject.

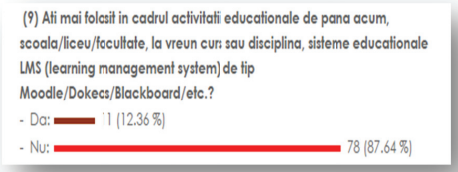

Figure 5. The repartition of the answers if our students are beneficiaries of the learning management system in the past

CONCLUSIONS

The e-learning system developed on the Moodle platform at the OP – Operating and Programming course was very well welcomed by the students from the Faculty of Biotechnologies.

The data gathered in our study are reflecting the following observations:

- to create an account on the Moodle platform is very easy,
- it is not imperative to be an expert on the navigation through an account to set up a password, or insert a picture, or describe in which class or group study is that student or modify each of it,
- the e-learning system through the Moodle platform is flexible as time and space, and allows for everybody mobility, free choice for the best focusing time, liberty to participate again and again at the practical stuff using the examples inserted as video materials,
- the platform has been overwhelming accessed from the PC desktop or laptop and from the smartphones,
- this new era of learning gave a new way to pass over the boring moments in the traffic and make something useful,
- the most part of our subjects never faced before with a learning management system. Their impression was very enthusiastic and came very interested at our meetings,

- learning outside the traditional space (home or school) was considered useful for the individual studies,
- the online system of testing the knowledge achieved at PC-Operating and Programming course through the Moodle platform allows improvement of results,
- studying through the Moodle platform is a progress in education, a modern element useful in education at the university level,
- it is recommendable to introduce more disciplines which allow online teaching and testing in the e-learning system.

The type of the platform, course material structure and examples inserted in the practical part are provided by professional work of the main teacher from this course. It is obvious the teacher assures a professional expertise when we analyze the answers about ideas of the course ~~i~~improvement and we do not receive comments about the structure and the coherence of the course package. Also, the results of the testing system revealed that the students were stimulated in learning motivation and they are beneficiaries of a collaborative learning.

REFERENCES

Merisotis, J.P., Phipps, R.A., Ed.D., 1999. What's the Difference? A Review of Contemporary Research on the Effectiveness of Distance Learning in Higher Education, The Institute for Higher Education Policy

Paechter, M.,Maier, B., Macher, D., 2009. Students' expectations of, and experiences in e-learning: Their relation to learning achievements and course satisfaction, Elsevier

Pomohaci C., Toma R.C., 2005. Spre un învăţământ realizat de computer? Agricultorul Român, anul VII, nr. 6(78)

Richey, R.C. (2008). "Reflections on the 2008 AECT Definitions of the Field".*TechTrends* **52** (1): 24–25. doi:10.1007/s11528-008-0108-2

Toma R.C., 2013. *Operarea şi programarea calculatoarelor*" Curs si aplicatii practice, AMC – USAMV Bucureşti

Vogel, M., 2015. A good peer review experience with moodle Workshop

http://www.ziare.com/internet-si-tehnologie/calculator/istoria-computerului-din-1939-pana-in-zilele-noastre-1076329

http://www.click.ro/news/lume/cum-ii-transforma-tehnologia-pe-tinerii-de-azi-sunt-niste-mutanti-spun-cercetatorii

http://www.talentlms.com/elearning/benefits-and-drawbacks-of-online-learning

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ANTIMICROBIAL ACTIVITY OF TEXTILES TREATED WITH ROSEMARY AND ORANGE ESSENTIAL OILS AGAINST A SELECTION OF PATHOGENIC FUNGI

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Abstract

The aim of the research was the evaluation of antimicrobial activity of a textile fabric treated with essential oils extracted from Rosmarinus officinalis (rosemary) and Citrus sinensis (orange), against pathogenic strains of Aspergillus niger (IMI 45551), Candida albicans (ATCC 90028), Trichoderma viride (isolated from agricultural soil), Aspergillus flavus (isolated from agricultural soil) and Epidermophyton floccosum (CCM 8339). Plant extracts were obtained by steam distillation from rosemary vegetal mater and orange peel. GC-MS analysis, carried out in hexane and diethyl-ether, allowed identification of main compounds, with high quantities of eucalyptol, camphor and α – pinene, in rosemary oil, respectively limonene, limonene oxide, α – pinene and β-phellandrene in orange oil. A textile substrate (56% cotton/44% polyester) was treated with concentrations of 1%, 3% and 5% of each oil and antimicrobial activity was assessed against each strain. Bioassays registered various percentage reduction rates, depending on oil concentration and tested strain: on Aspergillus niger, rosemary treatment registered a maximum of 22.12%, whilst orange treatment reached 51.45%; on Candida albicans, both treatment yielded 100% reduction rates for all tested concentrations; on Trichoderma viride, textiles treated with rosemary oil reached a maximum of 76.48% reduction rates, and 100% on orange treatment; on Aspergillus flavus, maximum efficiency on rosemary treatment was of 18.3% and 60.57% on orange treated materials; on Epidermophyton floccosum dermatophyte, maximum reduction rate on rosemary treatment was of 56.99% whilst on orange treatment it registered a maximum of 92.48%. The obtained results promote textiles functionalized with rosemary and orange essential oils as efficient active antimicrobial barriers.

Key words*: plant extracts, fungi, antimicrobial, textiles.*

INTRODUCTION

Fungi are ubiquitous microorganisms, with representative species that pose highly pathogenic potential to human hosts, as some of them are significant infectious agents to immunocompromised individuals but also to immunocompetent ones. Pathogenicity represents the ability of a microorganism to damage a living host (Casadevall et al., 1999) by affecting the target homeostasis, triggering an immune response or mechanical action at tisular level (Arturo, 2007).

Essential oils derived from plants pose great potential as antimicrobial agents, against a wide range of pathogens (Friedman et al., 2002; Mimica-Dukić et al., 2004). The biocidal effect of plant extracts is caused by its constituent types, such as alcohols, ethers, phenols, aldehydes, ketones, which renders them highly efficient against a wide range of microbial strains (Kalemba et Kunicka, 2003). Public awareness of pathogenic effects caused by microorganisms lead to a continuously increasing demand for antimicrobial solutions. Textiles are constantly exposed to microorganisms, thus functionalization of these materials can prove to be an efficient method of obtaining antimicrobial active barriers. Actual global market promotes synthetic chemicals (metallic salts, triclosan, quaternary ammonium compounds, photocatalitic agents etc.)

(Ghoranneviss et Shahidi, 2013) as finishing agents for inducing antimicrobial properties, whilst plant derived chemicals can prove viable alternatives with similar efficiency and smaller footprint on the environment (Kalemba et Kunicka, 2003).

Most modern antimicrobial finished textiles are based on synthetic products, and current consumer demands must be correlated with obtaining environmental friendly final products. Conventional antimicrobial finishing includes treatment with quaternary ammonium compounds, triclosan, N-halamines, polybiguanides, nanoparticles of noble metals (nanosilver treatment) and metal oxides (Thilagavathi et Kannaian, 2010) but also treatment with titanium oxide doped with various elements for photocatalytic and antimicrobial induced properties. New trend in antimicrobial finishing promotes plant based dyes (Dumitrescu et al., 2012) over synthetic ones that can also act as antimicrobial agents (Lee et al., 2009).

Plant extracts can be used as finishing agents during textile processes or can be encapsulated for inducing controlled release properties (acacia based capsule wall filled with herbal extracts (Lazko et al., 2004). Current researches regarding use of various plants extracts for the treatment of antimicrobial finished fabrics include functionalization of 100% cotton bed linen fabric with neem (*Azadirachta indica*) and Mexican daisy extracts (Tilagavathi et Bala, 2007), fabrics treated with turmeric rhizomes extract (*Curcuma longa*) pomegranate fruit rinds extract (*Punica granatum*), aloe vera extract (Jothi, 2009), tea oil, eucalyptus oil, tulsi leaves extract, with high antimicrobial efficiency (Joshi et al., 2009) against a series of fungi and Gram positive and Gram negative bacteria (Arsene et al., 2015). Beside extracts treatment, bioactive functionalisation of textile fibers include compounds such as phenolic and polyphenols, alkaloids, lectins, poypeptide, polyacetylene, terpenoids etc (Cown, 1999).

Beside antimicrobial properties, various plants extracts can also be used for inducing UV protection properties, such as annatto, ratanjot, manjistha, babool, grapefruit extract, honeysuckle extract etc (Latarzyna et Prezewozna, 2009).

Although the antimicrobial properties of various plants extracts has been thoroughly researched, the antimicrobial active functionalization of textile materials using plants extracts still require intensive documentation.

MATERIALS AND METHODS

Plant extracts. Essential oils were obtained by steam distillation from rosemary vegetal mater and orange peel. Steam distillation was preferred to direct extraction by heating, in order to avoid loss and denaturation of constituent chemicals. Textile materials composed of a mix of cotton (56%) and polyester (44%) (mass: 156 g/m^2; thickness: 0.392mm; density/10cm: 350 per warp and 290 per weft) were treated by impregnation with essential oils of rosemary and orange, diluted by 1%, 3% and 5%, in Cosmol mineral oil.

GC-MS analysis. Main constituents of selected oils were assessed by Gas Chromatography-Mass Spectrometry (GC-MS) on a Agilent 6890N Gas Chromatograph system, 5973N MS detector (70 eV), Agilent ChemStation data system, HP-5ms (5% phenyl-methylpolysiloxane), 0.25 μmx 30 m x 0.25 mm column. The GC operating conditions were as follows: 70 to 290°C at a heating rate of 5°C/min and then isothermally held for 10 min, injector temperature of 270°C, injected volume was of 1 μL of the oil solutions in diethyl ether and hexane (1:100), pulsed split mode, with flow rate -1.5 mL/min for 0.5 min and then 1.0 mL/min, split ratio 40:1 and He gas used as carrier gas, at 1mL/min. MS conditions were set to the following parameters: ionization voltage: 70 eV; ion source temperature: 280°C; mass range: 35-500 and scan time 0.32s. The identification of each oil constituents was made by comparison with their mass spectra from Wiley 6, NIST02, Mass Finder 2.3 software.

Antimicrobial activity. Antimicrobial activity of the functionalized textile materials was tested according to ISO 20743:2007, modified absorption method, which is an evaluation method where the microbial suspension is inoculated directly onto the treated samples. The standard is used in order to test the efficiency of antimicrobial finished textiles products, including nonwovens. Fresh cultures were obtained for each microbial strain, preceding the tests and serial dilutions of 10^{-4}

were made for filamentous strains and 10^{-3} for *Candida albicans.* Treated textile samples (surface area of ~ 1cm^2) were inoculated in, sterilized flasks, with 50mL of last dilution of each strain, and incubated (24h) at 28^{o}C for filamentous strains and 37^{o}C for *Candida albicans*. After incubation period, each sample was vortexed for approx. 20 seconds in 1mL of sterile deionized water, and plated on Petri dishes, with Czapek-Dox media used for filamentous strains and Sabouraud-Agar with chloramphenicol media for *Candida albicans*. Colony plate count method was used as quantification method for treatment efficiency and enumeration of CFUs, following incubation period. Untreated textile fabric was used as control for validation of growth condition of tested strains.

RESULTS AND DISCUSSIONS

Composition and quantity of constituent components in a volatile oil can determine its efficiency against certain microbial strains. Assessment of chromatographic profiles of selected oils allowed identification of the main compounds, carried out in two solvents, namely hexane and diethyl ether (table 1-4).

Table 1. Main components of rosemary oil extracted in hexane

Rosemary oil compounds	Percentage
Eucalyptol	42.05 %
α - Pinene	14.41 %
β - Pinene	5.59 %
Camphor	14.92 %
Camphene	7.96 %
Linalool	0.79 %
Limonene	4.70 %
Terpineol	2.45 %
2 - Thujene	0.66 %
Borneol	1.98 %
Bornyl acetate	1.20 %
Isoborneol	1.23 %
2 - Bornene	0.66 %
Caryophyllene	1.39 %

According to results from table 1, eucalyptol is the main component in rosemary oil, with 42.05%, followed by camphor (14.92%) and α–pinene (14.41%). The rest of the constituents are in small quantities, not more than 8%. Extraction of compounds was also carried out in diethyl ether solvent (table 2).
Results obtained on diethyl ether are similar with those obtained on hexane, for rosemary oil, with eucalyptol having the highest prevalence (37.92%), followed by camphor, with 15.76% and α-pinene, with 14.42%. The other compounds are in high quantity, with camphene having the highest concentration of under 10%. Moving forward, extraction of main compounds of orange oil was carried out in hexane solvent (table 3) and diethyl ether (table 4).

Table 2. Main components of rosemary oil extracted in diethyl ether

Rosemary oil compounds	Percentage
Eucalyptol	37.92 %
α - Pinene	14.42 %
β - Pinene	6.64 %
Camphor	15.76 %
Camphene	8.56 %
Linalool	0.88 %
Limonene	4.86 %
α - Terpineol	3.7 %
Terpinene	0.12 %
Borneol	1.28 %
Bornyl acetate	1.25 %
Verbenone	0.20 %
Caryophyllene oxide	0.54 %
Caryophyllene	1.60 %
α - Caryophyllene	0.20 %

Table 3. Main components of orange oil extracted in hexane

Orange oil compounds	Percentage
Limonene	97.75 %
Limonene oxide	0.48 %
β - Pinene	0.51 %
β - Phellandrene	1.26 %

Extraction of orange main components in hexane solvent allowed quantification of 4 compounds, with limonene having the highest prevalence (97.75%) followed by small traces of compounds in quantities of under 2%.

Table 4. Main components of orange oil extracted in diethyl ether

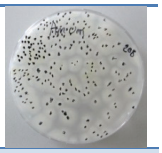
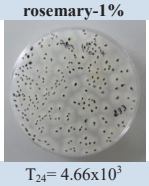
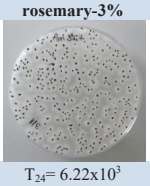
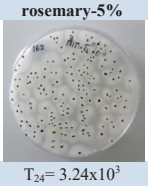
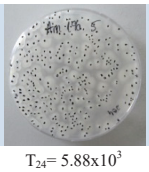
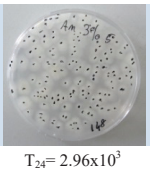
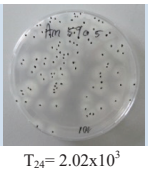
Orange oil compounds	Percentage
Limonene	94.07 %
Limonene oxide	0.63 %
α - Pinene	0.74 %
β-Phellandrene	0.34 %
2 - Thujene	0.27 %
Linalool	0.52 %
Carveol	0.21 %
B - Myrcene	1.59 %
Decanal	0.16 %
cis - Carveol	0.19 %
p - Mentha-6,8-dien-2-one	0.27 %

Similar to the extraction in hexane, chromatographic profile carried out in diethyl ether allowed identification of the first 4 compounds as being limonene, limonene oxide, α–pinene and β-phellandrene. Extraction in diethyl ether

also allowed identification and quantification of 7 more constituents, totaling approx. 3.21% of total compounds in orange oil.

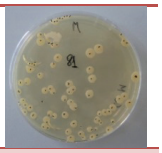
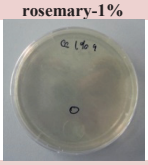
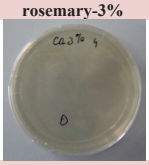
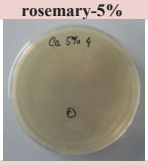
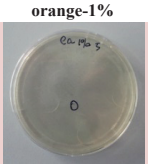
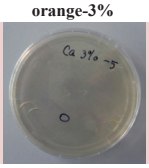
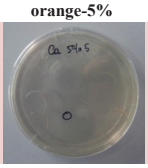
As can be noticed, for both oils matrixes, each constituent compound has different extraction percentage degree, depending on the solvent used. Antimicrobial assays (table 5-9) allowed screening of efficiency of selected plants, in concentrations of 1%, 3% and 5% when tested against four strains of filamentous fungi and one yeast strain.

Table 5. Antimicrobial activity of selected plants against *Aspergillus niger*

Control $4.16x10^3$ CFU/mL		
rosemary-1%	**rosemary-3%**	**rosemary-5%**
		
T_{24}= $4.66x10^3$ CFU/mL % red.=-11%	T_{24}= $6.22x10^3$ CFU/mL % red.=-33.2%	T_{24}= $3.24x10^3$ CFU/mL % red.=22.12%
orange-1%	**orange-3%**	**orange-5%**
		
T_{24}= $5.88x10^3$ CFU/mL % red.=-22.26%	T_{24}= $2.96x10^3$ CFU/mL % red.=28.85%	T_{24}= $2.02x10^3$ CFU/mL % red.=51.45%

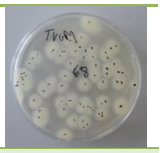
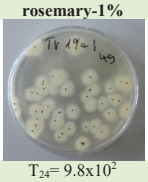
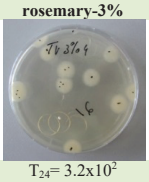
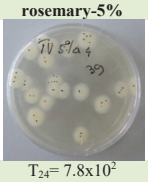
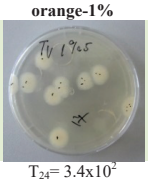
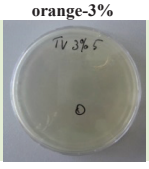
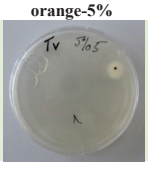
Results of efficiency of treated textiles when tested against *Aspergillus niger* revealed very mixed reduction rates, as only a few of the samples were able to percentually reduce the microbial population inoculated on the fabrics, with maximum of 51.45% for textiles treated with orange oil in 5% concentration, whilst for the materials treated with rosemary oil, the maximum was of only 22.12%, in 5% concentration. For three of the treated materials (two with rosemary oil and one with orange oil) the treatment not only that didn't reduce the strain concentration, but allowed cell proliferation (expressed here as negative percentage growth), underlining that the used concentrations were too low in order to properly induce efficient antimicrobial properties on the treated textiles. In terms of antimicrobial efficiency, orange oil proved to be more efficient against strain of *Aspergillus niger*.

Table 6. Antimicrobial activity of selected plants against *Candida albicans*

Control $1.62x10^3$ CFU/mL		
rosemary-1%	**rosemary-3%**	**rosemary-5%**
		
T_{24}= 0 CFU/mL % red.= 100%	T_{24}= 0 CFU/mL % red.= 100%	T_{24}= 0 CFU/mL % red.= 100%
orange-1%	**orange-3%**	**orange-5%**
		
T_{24}= 0 CFU/mL % red.= 100%	T_{24}= 0 CFU/mL % red.= 100%	T_{24}= 0 CFU/mL % red.= 100%

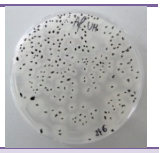
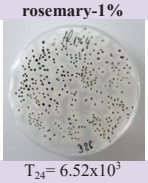
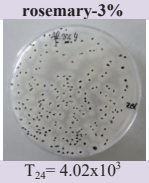
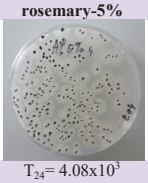
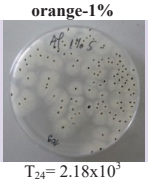
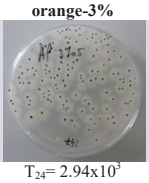
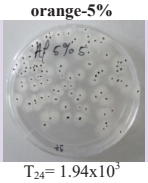
As can be seen on table 6, all textile materials treated with rosemary and orange oil reduced 100% of the strain inoculated on them, regardless of the oil and concentration used.

Table 7. Antimicrobial activity of selected plants against *Trichoderma viride*

Control $1.36x10^3$ CFU/mL		
rosemary-1%	**rosemary-3%**	**rosemary-5%**
		
T_{24}= $9.8x10^2$ CFU/mL % red.= 27.95%	T_{24}= $3.2x10^2$ CFU/mL % red.= 76.48%	T_{24}= $7.8x10^2$ CFU/mL % red.= 42.65%
orange-1%	**orange-3%**	**orange-5%**
		
T_{24}= $3.4x10^2$ CFU/mL % red.= 75%	T_{24}= 0 CFU/mL % red.= 100%	T_{24}= $2x10^1$ CFU/mL % red.= 98.53%

Antimicrobial efficiency assessment of textiles treated with rosemary and orange oil, tested against *Trichoderma viride* revealed better reduction rates of materials treated with orange oil when compared with the ones treated with rosemary oil, with average rate of 91.17% for orange oil and average rate of 49.02% for treatment with rosemary oil.

Table 8. Antimicrobial activity of selected plants against *Aspergillus flavus*

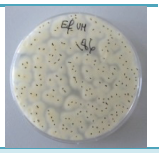
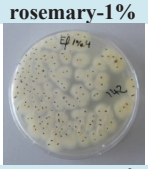
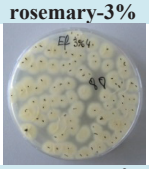
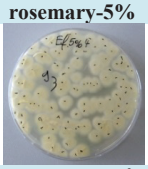
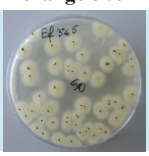
Control $4.92x10^3$ CFU/mL 		
rosemary-1%	**rosemary-3%**	**rosemary-5%**
		
T_{24}= $6.52x10^3$ CFU/mL % red.= -24.54%	T_{24}= $4.02x10^3$ CFU/mL % red.= 18.3%	T_{24}= $4.08x10^3$ CFU/mL % red.= 17.08%
orange-1%	**orange-3%**	**orange-5%**
		
T_{24}= $2.18x10^3$ CFU/mL % red.= 55.7%	T_{24}= $2.94x10^3$ CFU/mL % red.= 40.25%	T_{24}= $1.94x10^3$ CFU/mL % red.= 60.57%

Results of tests against *Aspergillus flavus* showed poor efficiency against the strain on textiles treated with rosemary oil in concentration of 1%, allowing strain proliferation, whilst the other treatments didn't achieve reduction rates higher than 18.3%. On the other side, orange treatment yielded satisfactorily efficiency against the strain, with maximum percentage reduction rate of 60.57%, on the material treated with 5% orange oil concentration. However, when the results were compared with the ones obtained on testing against the other *Aspergillus* strain, the reduction rates were better, only the material treated with orange oil in concentration of 5% yielding similar reduction rates with the ones tested against *Aspergillus flavus* (of 51.45%).

Antimicrobial efficiency testing of treated fabrics against *Epidermophyton floccosum* dermatophyte strain revealed satisfactorily reduction rates on textiles treated with rosemary oil, with minimum of 23.66%, for 1% oil concentration, and maximum of 56.99%, for 3% oil concentration, while for materials treated with orange oil, the minimum was of 37.64%, for 1% oil concentration, whilst maximum of 92.48% was achieved for the fabric treated with 5% orange oil concentration. As a general pattern, materials treated with orange oil extract presented higher percentage reduction rates, with total average of 66.08%, when compared to the ones treated with rosemary oil extract, with total average reduction rate of 37.76, thus promoting orange extract as better antimicrobial finishing agent.

Furthermore, comparative analysis focused on efficiency of each treatment, taking into consideration the oil concentration used and the tested strain (figure 1-2).

Table 9. Antimicrobial activity of selected plants against *Epidermophyton floccosum*

Control $3.72x10^3$ CFU/mL 		
rosemary-1%	**rosemary-3%**	**rosemary-5%**
		
T_{24}= $2.84x10^3$ CFU/mL % red.= 23.66%	T_{24}= $1.6x10^3$ CFU/mL % red.= 56.99%	T_{24}= $1.86x10^3$ CFU/mL % red.= 50%
orange-1%	**orange-3%**	**orange-5%**
		
T_{24}= $2.32x10^3$ CFU/mL % red.= 37.64%	T_{24}= $1x10^3$ CFU/mL % red.= 73.12	T_{24}= $2.8x10^2$ CFU/mL % red.= 92.48%

Results show high resistance of *Aspergillus niger* strain to rosemary treatment in all three concentrations, while textiles treated with orange oil yielded higher reduction rates, with significant reduction rates for concentrations of 3% and 5%. The textiles treated with 1% rosemary concentration did not present any reduction rate, allowing the cells to proliferate, when compared to control.

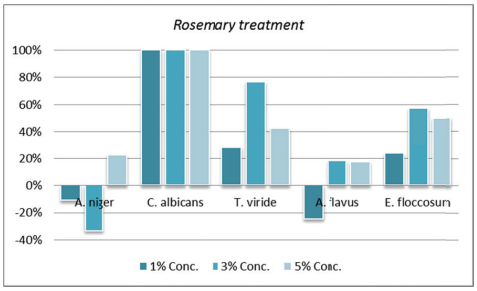


Figure 1. Rosemary treated textiles antimicrobial efficiency at different concentrations

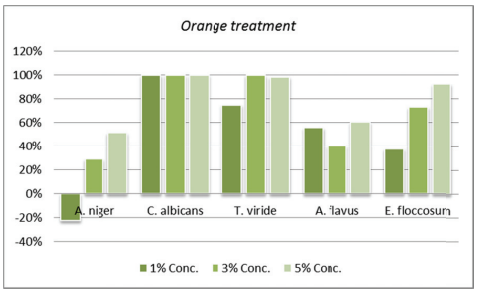


Figure 2. Orange treated textiles antimicrobial efficiency at different concentrations

When tested against *Candida albicans*, treated materials yielded maximum reduction rates for all concentrations and type of treatment, thus eliminating the need for concentration higher than 1%.

Antimicrobial assays carried out against *Trichoderma viride* strain indicated poor antimicrobial activity of fabrics treated with rosemary oil for concentrations 1% and 5%, and good percentage reduction rate for materials treated with 3% oil concentration. On the other side, the orange treatment proved to be highly efficient against this strain, with good reduction rate for 1% oil concentration and above 98% for concentrations of 3% and 5%. Following *Candida albicans* results, the bioassays carried out against *Trichoderma viride* yielded the second bets set of results, in terms of antimicrobial treatment efficiency.

Similar to results of *Aspergillus niger* testing, the antimicrobial activity of rosemary treated textiles was very poor when tested against *Aspergillus flavus*, with additional growth on material treated with 1%, when compared to control, and poor reduction rates of 3% and 5% oil concentration treatments (not exceeding 20% in reduction rates). Orange treatment proved to be far more efficient against this strain, with reduction rates above 40%, reaching its maximum on materials treated with 5% concentration, of 60.75%.

Bioassays carried out against dermatophyte strain of *Epidermophyton floccosum* indicate poor efficiency against textiles treated with 1% rosemary oil concentration, and medium reduction rates for the other two treatment concentration, with maximum of 56.99%. When tested against the same strain, textiles treated with orange oil showed improved efficiency with increasing of concentration, with minimum of 37.64%, for 1% concentration, to 73.12%, for 3% concentration and 92.48%, for 5% orange oil concentration treated fabrics.

Even though there are numerous studies regarding antimicrobial efficiency of plants extracts against several fungal and bacterial strains (Liolious et al., 2007) (Pereira, 2007) (Rodriguez et al., 2007), very few studies treat the antimicrobial efficiency of plants extracts functionalized fabrics against fungi strains.

Eucalyptus oil exhibits significant antimicrobial activity against both fungi (*Aspergillus niger, Candida albicans* etc.) and bacteria strains (*Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli, Staphylococcus epidermidis, Proteus vulgaris, Shigella dysenteriae, Salmonella paratyphi* etc.) (Safaei-Ghomi et Abbasi Ahd, 2010). Cotton and wool fabrics were treated with *Eucalyptus odorata* and *Eucalyptus cinerea* extracts for antimicrobial effectiveness against *Staphylococcus aureus* and *Escherichia coli* bacterial strains. Antibacterial efficiency in terms of bacterial reduction percentage for directly applied neem extract on fabric samples (scoured and bleached 100% cotton bed linen fabric) against *Staphyloccocus aureus* (100% reduction rate) and against *Escherichia coli* (78.44% reduction rate) (Thilagavathi et Kannaian, 2010). Quantitative tests carried out on 100% cotton fabric treated with similar concentrations used in the present study (3% and 5%) of turmeric, pomegranate and neem extracts against strains of *Bacillus cereus* and *Escherichia coli* revealed the following percentage effectiveness of plants extracts coated fabrics: for pomegranate treated fabrics, the yielded antimicrobial efficiency was of

62.83% for 3% extract and 82.42% for 5% extract against *Escherichia coli* and 36.39% for 3% extract and 46.049% for 5% extract against *Bacillus cereus*; for neem treated fabrics, the yielded antimicrobial efficiency was of 30.66% for 3% extract and 39.77% for 5% extract against *Escherichia coli* and 31.55% for 3% extract and 41.89% for 5% extract against *Bacillus cereus*; for turmeric treated fabrics, the yielded antimicrobial efficiency was of 37.48% for 3% extract and 46.65% for 5% extract against *Escherichia coli* and 22.89% for 3% extract and 28.76% for 5% extract against *Bacillus cereus* (Mahesh et al., 2011).

CONCLUSIONS

The results show great potential of rosemary and orange oils in functionalization of textile substrates for obtaining highly efficient antimicrobial textiles. Efficiency of treatment is highly dependent on type of oil, concentration and strain type. General pattern dictates higher concentrations to be used in order to induce maximum of efficiency, nevertheless, presented data shows good antimicrobial efficiency of rosemary and especially orange treated fabrics, at relatively small concentrations, thus promoting them as efficient bioactive barriers.

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REFERENCES

Arsene A.L., Rodino S., Butu A., Petrache P., Iordache O., Butu M., 2015. Study On Antimicrobial And Antioxidant Activity And Phenolic Content Of Ethanolic Extract Of Humulus Lupulus. FARMACIA, Vol. 63, 6;

Casadevall A., 2007. Determinants of virulence in the pathogenic fungi. Fungal Biol Rev., 21(4): 130–132;

Casadevall A., Pirofski L., 1999. Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infection and Immunity, 67:3703–3713;

Cown M.M., 1999. Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev., 12,564-582;

Iuliana Dumitrescu, Padma S. Vankar, Jyoti Srivastava, Ana Maria Mocioiu, Ovidiu Iordache, 2012. Dyeing of cotton, silk and wool with Bixa orellana in the presence of enzymes. Industria textilă 63(6):327-333;

Friedman M., Henika R.P., Mandrell E.R., 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Protect, 65, 1545-1560;

Ghoranneviss M., Shahidi S., 2013. Effect of Various Metallic Salts on Antibacterial Activity and Physical Properties of Cotton Fabrics. Journal of Industrial Textiles, 2013 vol. 42 no. 3 193-203;

Joshi M., Wazed Ali S., Purwar R., 2009. Ecofriendly antimicrobial finishing of textiles using bioactive agents based on natural products. Int J. Fibre Text. Res. 34:295-304;

Jothi D., 2009. Experimental study on antimicrobial activity of cotton fabric treated with aloe gel extract from Aloe vera plant for controlling the Staphylococcus aureus (bacterium). African Journal of Microbiology Research Vol. 3(5) pp. 228-232;

Kalemba D., Kunicka A., 2003. Antibacterial and antifungal properties of essential oils. Curr. Med. Chem., 10, 813-829;

Latarzyna P., Prezewozna S., 2009. Natural dyeing plants as a source of compounds protecting against UV irradiation. Herba Policia 55:56-59;

Lazko J., Popineanu Y., Legrand J., 2004. Soy glycinin microcapsules by simple coacervation method. Colloids Surfaces B: Bio Interfaces, 37-128;

Lee L.H., Hwang E.H., Kim, 2009. Colorimetric assay and antimicrobial activity of cotton, silk and fabrics dyed with Peony, Clove, Coptis chinensis and Gallnut. Materials. 2, 10- 21;

Liolious C., Laouer C.H., Boulaacheb N.O., Gortzi C., Ioanna, 2007. Chemical composition and antimicrobial activity of the essential oil of Algerian Phlomins Bover DeNoe subp.bovei. Molecules. 12, 772- 781;

Mahesh S., Manjunatha Reddy A.H., Vijaya Kumar G., 2011. Studies on Antimicrobial Textile Finish Using Certain Plant Natural Products. International Conference on Advances in Biotechnology and Pharmaceutical Sciences (ICABPS'2011) Bangkok Dec.;

Mimica-Dukić N., Bozin B, Soković M., Simin N., 2004. Antimicrobial and antioxidant activities of *Melissa officinalis* L. (Lamiaceae) essential oil. J. Agric. Food Chem., 52, 2485-2489.

Pereira A.P., 2007. Phenolic compounds and antimicrobial activity of olive (Olea europaea L. Cv. Cobrancosa) leaves. Molecules, 12, 1153-1162;

Rodríguez D. Jasso, Hernández-Castillo D., Angulo-Sánchez J.L., Rodríguez-García R., Villarreal Quintanilla J.A., Lira-Saldivar R.H., 2007. Antifungal activity in vitro of flourensia sp. extracts

on Alternaria sp. Rhizoctonia solani and Fusarium oxysporum. Indian Crops Products. 25, 111-116;
Safaei-Ghomi J., Abbasi Ahd A., 2010. Antimicrobial and antifungal properties of the essential oil and methanol extracts of Eucalyptus largiflorens and Eucalyptus intertexta. Pharmacogn Mag. Jul-Sep; 6(23): 172–175;
Thilagavathi G., Kannaian T., 2010. Combined antimicrobial and aroma finishing treatment for cotton, using microencapsulated geranium (Pelargonium graveolens L.) leaves extract. Indian Jounal of Natural Products and Resources. 1(3), 348-352;
Tilagavathi G., Krishna Bala S., 2007. Microencapsulation of herbal extracts for microbial resistance in healthcare textiles. Indian Journal of Fibre & Textile Research, Vol. 32, September 2007, pp. 351-354.

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BENEFITS OF THE E-LEARNING PLATFORMS AND CLOUD COMPUTING IN THE BIOTECHNOLOGY EDUCATION

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Abstract

Online education represents a new approach of learning process, in which substantive elements remain the same, only the means of exchange of knowledge and learning is different.
Few benefits of the e-learning platforms and cloud computing in educational area are: a reduced infrastructure and IT costs, an increased accessibility, a better collaboration, and allow organizations more flexibility. But cloud computing is having other effects as well, which have the potential to greatly change how education works, both in online and offline (traditional classrooms) courses like: no more expensive textbooks, no more outdated learning materials, no expensive hardware and software required, reaching more diverse students.

Key words: *biotechnology, education, e-learning, internet, MOODLE.*

INTRODUCTION

The future is about internet access, online learning and collaboration, both locally and globally. Schools of the future could have a traditional cohort of students, as well as online students.
In the field of education, in recent years, there has been notable progress in terms of teaching and learning techniques. Using the Internet and modern technology in education has resulted in changes of substance.
But sometimes technology can be a barrier to teaching and learning. Preparing teachers to use ICT (information and communications technology) in teaching activity effectively occurs such as desideratum in the coordination of the educational process. In this context, the educational „***E-learning*** type" platforms represent reliable tools for so-called computer assisted learning.
E-learning solutions are the result of evolution of technologies that support these features. Thus, due to large storage capacities, classic distance education using printed materials sent by mail has been replaced by electronic books. Subsequently, interactive communication technologies have become elements of new models of education using the electronic support.
Computer and electronic (digital)/ multimedia materials are used as support in teaching, learning, assessment, or as a means of communication.
There are three generally accepted patterns in the world of online education, each occupying a roughly equal share of this market:
Online independent education represents the model whereby individual user takes the course over the Internet or on CD, studying it by himself. This model represents the advantage of a very large amount of information that can be accessed in a short time, as well as extensive multimedia facilities, but being very rigid in terms of instructor-student communication.
Online asynchronous education allows transmission of information at any given time, but to only one partner in instructor-student relationship. The instructor can provide information to students but they can't interact while receiving. The major advantage, in this case, is that the student keeps the facility to work at his own pace, and also getting answers to his requests in an acceptable time frame.
Online synchronous education allows interactive information transfer with any other user at any time. For example, the instructor and students are transferring information during the class or seminar, usually in real time. This model is the most advanced in terms of facilitation of communication, audio as well as video integrated methods creating the concept of the *virtual classroom.*

Unlike traditional educational system, e-learning can enumerate as **advantages**: geographical mobility; accessibility of on-line; individualization of learning process; various pedagogical methods; low cost of distribution; reduced time for study; synchronous and asynchronous interactions and it is based on a dynamic technology.
Among the **disadvantages** of eLearning learning system are: high rate of drop-out students; this system requires experience in the use of computers and a high cost for design and maintenance.

MATERIALS AND METHODS

Faculty of Biotechnology (U.A.S.M.V. of Bucharest) has developed its own ICT infrastructure since its founding in the mid-90s through:

- Purchase of computers (desktops, notebooks, servers, projectors, multi-function printers, etc.) and specialized software (operating systems Windows suite of applications: Office, etc.);
- Aided calculator editing textbooks, laboratory notebooks and university courses;
- Connection to INTERNET to all faculty buildings and offering free access for all teachers but also students through wireless connections, both in lecture halls, laboratories, seminar rooms, and also outside them.

All these were followed by a natural shift from the classical manner of course presentations to the computer and projector aided courses, through PowerPoint and multimedia presentations.
Together with the faculty leadership, we have developed some strategies to ensure the concept of eLearning resulting in a **MOODLE type platform** dedicated to faculty staff and graduate students.

MOODLE**,** an acronym for *Modular Object - Oriented Dynamic Learning Environment*, **(**http://www.moodle.org) is a free and open-source software learning management system written in PHP and distributed under the GNU General Public License that lets developers build an education solution for institution needs.
MOODLE is used for blended learning, flipped classroom and other e-learning projects in schools, universities and other sectors.
With customizable management features, MOODLE allows for extending and tailoring learning environments using community sourced plugins.

RESULTS AND DISCUSSIONS

The online educational platform is accessible at http://moodle.biotehnologii.usamv.ro
By using this platform student have the following benefits:

- Technically the platform allows the simultaneous access of a large number of users;
- The platform provides access to all registered users, regardless of where the access is requested;
- The platform allows the simultaneous access of users who use different types of connection;
- The platform allows the simultaneous access of users who use different types of equipment (desktop, laptop, tablet, smartphone, etc.);
- The platform allows the simultaneous access of users who use different types of operating systems (Windows, Linux, MacOS, Android, iOS, etc.).

Some suggestive screenshots of the platform of the faculty are given below. The screen shots are taken from the course *Operation and computer programming* (Figure 1- 6).

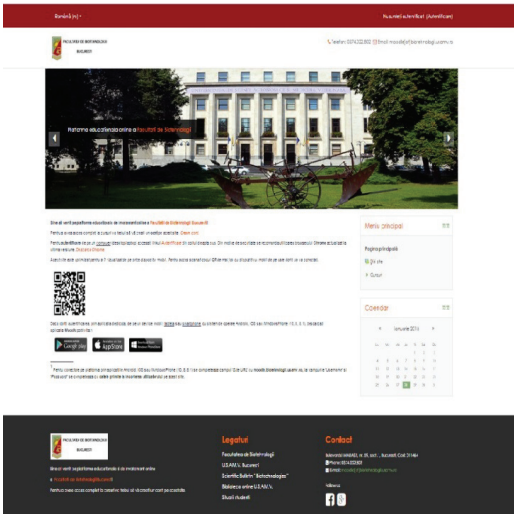

Figure 1 Homepage of the e-learning platform

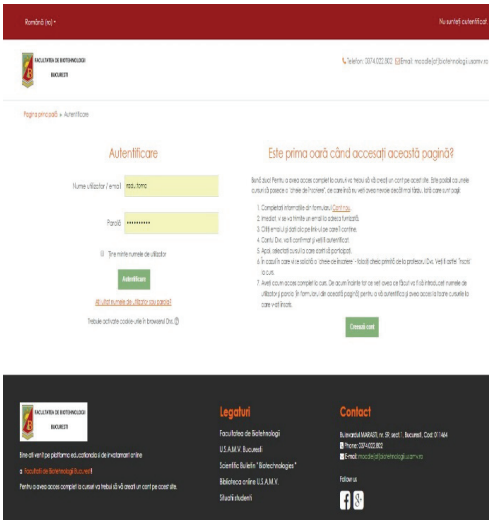

Figure 2 The login area

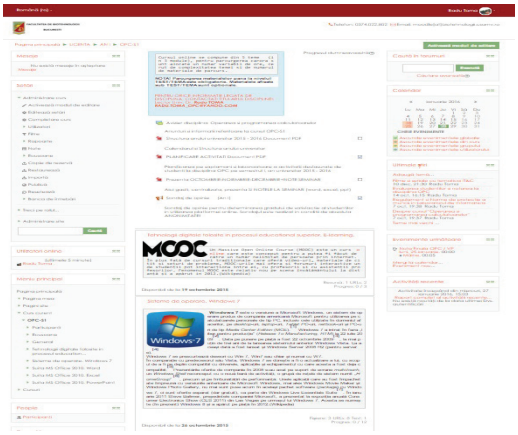

Figure 3 Presentation of the course resources

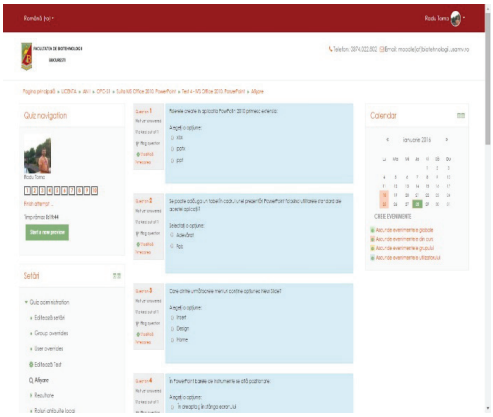

Figure 4 The online examination. Quiz questions

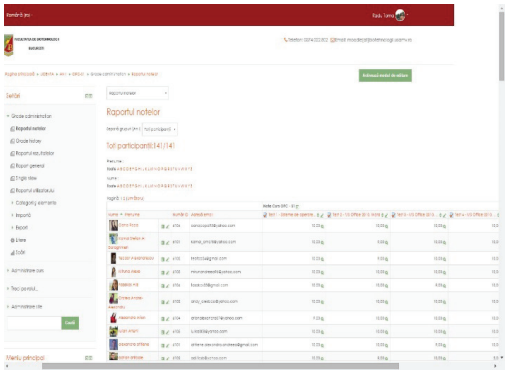

Figure 5 The grades situation of students enrolled in the course

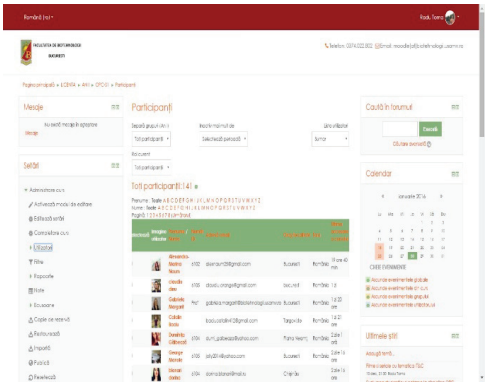

Figure 6 The online presence of students enrolled in the course

Some considerations about the using of the online platform:

- It is necessary to accumulate a certain number of hours logged on the platform in order to be considered and noted the presence in a classic format;
- The student is required to complete a minimum number of resources in each chapter to be regarded as he has passed the course material / seminar;
- The grading is done in a blended format both online examination through online quiz questions and/or essays plus other forms of online examinations as well as in the classic format through direct examination, face to face teacher and students, for two main reasons:
    - Faculty structure contains no "at distance" programs, having only "with frequency" specializations;
    - The Romanian online education system is at the beginning and is not fully developed in all cases and the students don't have a culture of assuming the grades exclusively through the prism of their own performances so fraud attempts are possible in order to obtain bigger grades.

CONCLUSIONS

Online education represents a new approach of the learning process, in which substantive elements remain the same, only the means of exchange of knowledge and learning is different.

Few benefits of the e-learning platforms and cloud computing in educational area are:

- A reduced infrastructure and IT costs;
- An increased accessibility;
- A better collaboration, and allow organizations more flexibility.

But these systems are also having other effects as well, which have the potential to greatly change the way education works, both in online and offline (traditional classrooms) courses like:

- No more expensive textbooks. It's no secret that university-level textbooks are expensive. Cloud-based textbooks can solve this problem as digital content, that is significantly less expensive than printed content;
- No more outdated learning materials. Cloud-based applications can be run on Internet browsers, but most are compatible with mobile devices as well. This means that schools and students do not need to own expensive computers. Students also don't need to purchase external storage devices as there are companies, like Google, that offer free cloud - based storage (http://drive.google.com);
- No expensive hardware and software required, reaching more diverse students. Many software programs are now available either free or on a low-cost subscription basis, which substantially lowers the cost of essential applications for students. For example, instead of purchasing a single Microsoft Office student license, students and their families can purchase a cloud-based subscription for five computers and five mobile devices for a modest fee monthly. Even better, they can use Google Docs for free or other suites like OpenOffice, Kingsoft Office, etc.

REFERENCES

Toma R.C., 2016. Informatică aplicată în biotehnologii. Ed. Sf. Ierarh Nicolae, Iași, ISBN: 978-606-30-0579-4.

Pomohaci C., Toma R.C., 2005. Spre un învăţământ realizat de computer? Agricultorul Român, anul VII, nr. 6(78)

Toma R.C., 2013. Operarea şi programarea calculatoarelor. Curs si aplicatii practice, AMC – USAMV Bucureşti

Toma R.C., Pomohaci C.M., 2005. Linux şi SST o soluţie fiabilă pentru infrastructura sistemului de educaţie informatizată Lucrări Ştiinţifice, USAMV Bucureşti, Seria F, Vol. IX.

Toma R.C., Vamanu E., Vamanu A., Stokovic I., 2005. Analiza si modelarea unui sistem informatic, Lucrari stiintifice USAMV Bucuresti, Seria F, vol. X, 141 - 147, ISSN 1224-7774

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CULTIVATION OF FLORIDA OYSTER MUSHROOM ON VARIOUS TYPES OF SUBSTRATE

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Abstract

Valorisation of agricultural wastes is one of the main objectives for activity optimization in agriculture industry. One method for waste utilization implies their use as substrate for obtaining edible mushrooms, which are a raw material of interest in current food industry. The aim of the paper was the cultivation of Pleurotus ostreatus var. florida species, on diverse common plant wastes. The species had good fructification, resulting numerous pins on the surface of the substrate, the cap diameter reaching a maximum of 4 to 5 cm. The color of the basidium was brighter, because the cultivation temperatures were above 20°C most of the time. The fructification time decreased with the increase of inoculation rate. Although major differences haven't been determined for morphological characteristics of the fructification body, the supplementation of the substrate formula with other components determined approximately 10% increase in productivity. The study proved that applying supplements to the substrate formula lead to the optimization of valorisation of plant wastes taken into consideration. Also, the use of supplements did not stimulate infection rate of the substrate.

Key words*: mycelium, Pleurotus, pins, productivity, substrate.*

INTRODUCTION

Pleurotus ostreatus is a mushroom industrially cultivated for over 50 years. Due to the composition rich in biologically active substances it is commercialized in food markets. In Romania, it is often used to replace meat, because the texture, after cooking, is relatively similar (Yang et al., 2013). Usually only the cap is consumed. The stipe of the fungus is slightly difficult to digest and it is not consumed directly.

Currently, agricultural research is being carried out in order to exploit diverse plant wastes (Zervakis et al., 2013). Also, different species are tested, for the implementation in industrial mushroom farms: *P. ostreatus* var. *florida*, *P. djamor* or *P.citrinopileatus*. In Romania, partial experiments were realized so far in order to adapt *P. ostreatus* var. *florida* (*Florida Oyster Mushroom*), also known as *Hiratake* (Alananbeh et al., 2014). In our country, there are cultivated (excepting summer) common species, known as Winter Oyster Mushroom (Rahi & Malik, 2016). In both cases productivity is directly dependent on temperature (Vamanu, 2012). The purpose of the paper was the adaptation of the species *P. ostreatus* var. *florida* M 2125 for valorization of household plant wastes (straw from different types of cereals), in the farm of Denisa Stănescu student's family. There were used wheat, oats, sorghum straw, clover leaves and chopped corn stalks.

MATERIALS AND METHODS

Biological material: *P. ostreatus* var. *florida* M 2125 was obtained from Mycelia BVBA, Belgium.

The mycelium was stored on wheat grains, at -20˚C in glycerol. The revitalization was achieved by cultivation on PDA medium. Wheat grains were previously sterilized at 121˚C and complete colonization was achieved in approximately 10 days at 25˚C in a LabTech thermostat (Dinu & Vamanu, 2015).

Obtaining the substrate: The raw material was obtained from Teleorman County, Romania. The substrate formulas (Exp. 1) were supplemented with broken rice (Exp.2) and grain mixture (Exp.3) (**Table 1**). The substrates were sterilized with hot water, 60 - 80˚C. The experiments were carried out in plastic bottles of at least 5 liter. Inoculation was 2 - 3%. Colonization of the substrate was performed at

20 - 24˚C in the dark. Fructification phase took place after 10 to 14 days, humidity 50-60%, 800-1000 lumens (Zervakis et al., 2013). Humidity was maintained by regular spraying with a water (Konan et al., 2014, Dinu & Vamanu, 2015).

Determining productivity. The following parameters were calculated:

Productivity = total amount of harvested mushrooms into a wave,

Biological efficiency (%) = (amount of harvested mushrooms / substrate weight) × 100 (Yang et al., 2013; Dinu & Vamanu, 2015).

Table 1. Substrate formulas

No.	Formula
Experiment 1	Control: 100% wheat straw
	100% clover
	100% dried leaves stalks
	100% dried oat
	100% dried sorghum
Experiment 2	Formula Experiment 1 supplemented with 10% broken rice
Experiment 3	Formula Experiment 1 supplemented with 10% grain mixture

Statistical analysis. All experiments were assessed in triplicate, and the results were expressed as mean ±SD values of the three sets of observations.

RESULTS AND DISCUSSIONS

The minimum time of colonization was nine days for the control realized on wheat straw and sorghum (Exp. 1). For dried clover and dried oat straw the average colonization time was by five days longer. Finally, FS 2 substrate was infected (for all experiments carried out) and was not taken into further consideration for interpreting the results. FS 5 formula determined a colonization period by one third longer compared to FS 1. From our point of view, the infection of all clover samples was determined by inadequate sterilization procedure, which was not compatible with this raw material. For such a situation it is necessary to use autoclavable bags with microfilter. The introduction of these stages makes clover unsuitable for cultivation oyster species. This behavior has been observed since the presentation of partial results (Stănescu & Vamanu, 2015).

Figure 1. Fructification phase

Substrate colonization had a medium propagation rate of 0.5 cm/24 h. Generally, after this period, the advance of mycelium into the substrate was of minimum 0.4 cm/24h. The trend was the increase of propagation rate by approximately 20 % in 24 hours. FS 4 substrate had a medium propagation rate which was constant in 24 h (data not shown). These values were not directly proportional to the total productivity (**Table 2**).

There were obtained up to six flushes, and calculated productivity had medium values that exceeded 50 g (**Figure 1**). From **Table 2** it is observed that the first two flushes had similar productivity, regardless of the used substrate formula. Mushrooms obtained after flush no. 3 cannot be used, but it is significant that, in constant environmental conditions, tested species may use the substrate at a maximum level. The mushrooms were inadequate because of the small size. The cap was below 5 cm in diameter. This productive behavior was not observed in previous studies on *P. ostreatus* M 2175 (Dinu & Vamanu, 2015).

Maximum productivity exceeded 350 g for substrate FS 3. This was similar to the control, FS 1, but also to the M 2175 species, when using poplar sawdust (Dinu & Vamanu, 2015). Biological efficiency was 45-50% for FS 3 being a novelty in the composition of substrate for the species cultivation (**Figure 2**). Productivity in this case was approximately

15% higher compared to other used formulas. The fastest fructification (**Figure 3**) was registered for flush 1, once the substrate was fully colonized.

Table 2. The average productivity obtained for M 2125 species

Substrate formula	Flush	Productivity (g)	Medium colonization time (days)
FS 1	1	313.33±34.99	9±3
	2	310.33±38.50	
	3	242±85	
	4	164±14	
	5	115±15	
	6	63±10	
FS 2	-	-	-
FS 3	1	373.33±50	14±5
	2	364.33±48	
	3	293.33±100	
	4	203.5±33.5	
	5	142.5±2.5	
	6	70.5±17.5	
FS 4	1	307±50	15±4.5
	2	305±47.5	
	3	239.66±110.5	
	4	145±5	
	5	115±0.00	
	6	62±0.00	
FS 5	1	297.66±8.32	12±3
	2	294.33±55.83	
	3	238.66±82	
	4	166±29	
	5	114±0.00	
	6	-	

Figure 2. Fructification phase on different substrates

It was noted that at least some of primordia appeared before this moment, which corresponded with substrate formulas FS 1 and FS 3. The transition from one flush to another generally decreases the fruitification time. Reduction of fructification time does not exceed two days. Also, from flush no. two, relatively constant period, of nine days was observed, regardless of substrate formula.

In the case of supplements (broken rice, for example) productivity was by 8% higher, which lead to a product formula for industrial cultivation (**Figure 2**), with the following composition: wheat straw and dried leaves stalks (1:1), supplemented with broken rice (10%).

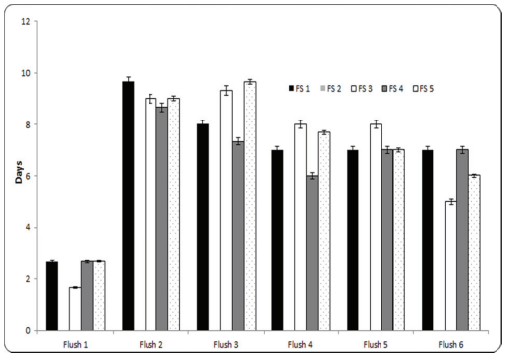

Figure 3. The medium fructification periods for M 2125 species

If using bags of 20 kg substrate in the first flush, an average of 1 kg of mushrooms was obtained. The first flush appeared after a period of 12 days from the substrate inoculation. In this case the first three flushes could be valorified. Primordia number significantly decreased by over 50 % after the third flush. Keeping adequate humidity was a parameter difficult to maintain in the absence of industrial cultivation system (**Figure 4**).

Figure 4. Bags cultivation

CONCLUSIONS

The substrate of dried leaves stalks lead to the best productivity (30 %), being used to create a new formula for industrial cultivation. Cultivation conditions proved that the species is a competitive one for the valorization of raw vegetal materials from agricultural industry in Romania. In addition, the species may be cultivated during periods of high temperatures. The substrate was also used as inoculum for colonization of stumps during winter time (**Figure 5**).

Figure 5. Substrate valorisation

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REFERENCES

Alananbeh K.M., Bouqellah N.A., Al Kaff N.S., 2014. Cultivation of oyster mushroom *Pleurotus ostreatus* on date-palm leaves mixed with other agro-wastes in Saudi Arabia. Saudi Journal of Biological Sciences, 21:616–625.

Dinu M., Vamanu E., 2015. Growing species *Pleurotus ostreatus* M 2175 on different substrates under household. Scientific Bulletin. Series F. Biotechnologies, Vol. XIX:364 – 368.

Konan Y.K.F., Kouassi K.M., Kouakou K.L., Koffi E., Kouassi K.N., Sekou D., Kone M., Kouakou T.H., 2014. Effect of methyl jasmonate on phytoalexins biosynthesis and induced disease resistance to *Fusarium oxysporum* f. sp. vasinfectum in cotton (*Gossypium hirsutum* L.). International Journal of Agronomy, vol. 2014, Article ID 806439, 11 pages.

Rahi D.K., Malik D., 2016. Diversity of mushrooms and their metabolites of nutraceutical and therapeutic significance. Journal of Mycology, vol. 2016, Article ID 7654123, 18 pages.

Stănescu D., Vamanu E., 2015. Cultivarea speciei *Pleurotus ostreatus* var. *florida* M 2125 pe diferite formule de substrat, în regim gospodăresc. Simpozionul Național Studențesc – „Biotehnologiile - prezent și viitor", 26 iunie, București.

Vamanu E., 2012. Biological activities of the polysaccharides produced in submerged culture of two edible Pleurotus ostreatus mushrooms. Journal of Biomedicine and Biotechnology, vol. 2012, Article ID 565974, 8 pages.

Zervakis G.I., Koutrotsios G., Katsaris P., 2013. Composted versus raw olive mill waste as substrates for the production of medicinal mushrooms: An assessment of selected cultivation and quality parameters. BioMed Research International, vol. 2013, Article ID 546830, 13 pages.

Yang W.J., Guo F.L., Wan Z.J., 2013. Yield and size of oyster mushroom grown on rice/wheat straw basal substrate supplemented with cotton seed hull. Saudi Journal of Biological Sciences, 20:333–338.