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FOREWORD

Life sciences and biotechnology are widely regarded as one of the most promising frontier technologies for the coming decades. Life sciences and biotechnology are enabling technologies – like information technology, they may be applied for a wide range of purpose for private and public benefits.
On the basis of scientific breakthroughs in recent years, the explosion in the knowledge on living systems is set to deliver a continuous stream of new applications.

There is a huge need in global healthcare for novel and innovative approaches to meet the needs of an ageing populations and poor countries. There are still no know cures for half of the world's diseases, and even existing cures such as antibiotics are becoming less effective due to resistance to treatments.
Pharmacogenomics, which applies information about the human genome to drug design, discovery and development, will further support this radical change.

Stem cell research and xenotransplantation offer the prospect of replacement tissues and organs to treat degenerative diseases and injury resulting from strokes, Alzheimer's and Parkinson's diseases, burns and spinal – cord injuries.

In the agro-food area, biotechnology has the potential to deliver improved food quality and environmental benefits through agronomically improved crops. Food and feed quality may be linked to disease prevention and reduced health risks. Foods with enhanced qualities ('functional foods') are likely to become increasingly important as part of lifestyle and nutritional benefits.

Considerable reductions in pesticide use have been recorded in crops with modified resistance. Life sciences and biotechnology are likely to be one of the important tools in fighting hunger and malnutrition and feeding an increasing human population on the currently cultivated land area, with reduced environmental impact.

Biotechnology also has the potential to improve non-food uses of crops as sources of industrial feedstocks or new materials such as biodegradable plastics. Plant – based materials can provide both molecular building blocks and more complex molecules for the manufacturing, energy and pharmaceutical industries.

Under the appropriate economic and fiscal conditions, biomass could contribute to alternative energy with both liquid and solid biofuels such as biodiesel and bioethanol as well as to processes such as bio-desulphurisation. Plant genomics also contributes to conventional improvements through the use of marker – assisted breeding.

New ways to protect and improve the environment are offered by biotechnology including bioremediation of polluted air, soil, water and waste as well as development of cleaner industrial products and processes, for example based on use of enzymes (biocatalysis).

Through research, development and demonstration activities we hope to find new and more sustainable solutions.

In this respect, the International Symposium organized in Bucharest by the Faculty of Biotechnology, at the University of Agronomical Sciences and Veterinary Medicine Bucharest, offered a great opportunity for discussing latest findings on the topic to researchers, professors, lecturers, students and biotechnology specialists from various institutions.

The symposium has been organized in five sections consisting of oral presentations and posters, as follows: Section Agricultural Biotechnologies, Section Biotechnologies in Veterinary Medicine, Section Food Biotechnologies, Section Industrial and Environmental Biotechnologies, and Section Food Safety.
The symposium proceedings are published in English, in the present volume.

I would like to wish good luck in their research activities in this wonderful and interesting field of biotechnology, optimism, health and personal achievements to all the participants.

Professor Petru Niculita
Dean of the Faculty of Biotechnology

Bucharest,
November 2010

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BEHAVIOUR OF SEVERAL POTATO (*Solanum tuberosum L.*) VARIETIES WITH DIFFERENT PROTEIN CONTENT TO THE POTATO TUBER NECROTIC RINGSPOT DISEASE AFTER PVY^{NTN} INOCULATION

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Abstract. *Obtaining good quality for potato seed impose the improvement of identification's techniques of pathogen agents, knowing the biochemical composition of this kind of food, especially the components wich could affect its fitosanitary status. The goal of this research was to examine and evaluate the behaviour of 10 potato varieties to the desease caused by PVY^{NTN} and the protein content of these samples. Protein content of PVY^{NTN} health plants from potato varieties of potato (Solanum tuberosum L.), wich differ in their susceptibility or resistance to virus, was compared. Another goal of this study was to elucidate the biochemical basis responsables for different reaction to infection with PVY^{NTN} among the varieties: Christian, Romano, Roclas, Dacia, Tampa, Ostara, Agata, Hermes, Sante, Koretta. Excepting the cultivars Christian, Dacia and Sante, wich were very resistent and resistent to mechanical inoculation all the other varieties presented 62.5-100% infected plants. After 3 months from harvesting, the frequency of tubers with symptoms was between 6.6-22.2% for varieties Dacia, Agata, Romano, Roclas, Ostara and for Tampa, Koretta, Hermes varieties this percentage was higher (60-95.8%). The protein content was significantly higher to the samples resistents to PVY^{NTN} inoculation. In this case (cv. Sante and Christian), after 3 months from harvest, the stored tubers didn't have visible tuber necrotic ringspot disease symptoms.*

Keywords: potato virus Y, protein content, varieties, necrotic strain.

1. INTRODUCTION

Obtaining good quality for potato seed impose the improvement of identification's techniques of pathogen agents, knowing the biochemical composition of this kind of food, especially the components wich could affect its fitosanitary status. Among the known pathogen agents infecting potato, the viruses produce serious damages to potato quality. Potato virus Y (PVY) (*Potyviride*) is one of the most important potato's viruses, (*Solanum tuberosum L.*) (Ragsdale et

al. 2001). High PVY level can cause stand loss, reduced yields, undersized tubers and reduced quality (Beemster and de Bokx, 1987, Hane and Hamm 1999). Over the past 20 years, PVY has become an increasingly serious constraint to seed potato production in the world (Davis et al. 2008, Lorenzen et al. 2006). Thus, efforts to control PVY are essential when producing potatoes for market or seed.

PVY induced symptoms can be diverse and depend on the cultivar and the virus strain. Potato virus Y^{NTN} (PVY^{NTN}) is a very aggressive and virulent strain belonging to the necrotic group of potato virus Y wich causes the potato ring necrotic disease (PTRND) (Beczner et al. 1984, Le Romancer et al. 1994, Tribodet et al. 2005). Several researchers consider PVY^{NTN} as sub-strain of PVY^{N} and the identification of non-recombinant PVY^{N} inducing PTNRD revealed that the recombinant structure of the genome is not a necessary prerequisite for PTNRD phenotype (Glais et al. 2005, Nie and Singh 2003, Lorenzen et al 2008).

The potato tuber ring necrotic disease (PTNRD) reported in many european countries has been indentified also in our experimental plots of Bârsa depression-Braşov county. In the last 10 years on few varieties sporadicaly tubers with superficial necrotic ringspots or bows occured. Firstly these are fairly protruding and light brown, then later becoming fairly deep and brown-black and the skin could be cracked (normaly after harvesting)

The aime of the present work was to compare the protein content of PVY^{NTN} infected plants (primary infection) from potato varieties of potato (*Solanum tuberosum L.*), wich differ in their susceptibility or resistance to virus. Another purpose of this research was to evaluate the behaviour of 10 potato varieties to the desease induced by PVY^{NTN} and to estimate the correlation between this behaviour and the protein content of the tubers planted for the experience.

Judget on the basis of fresh weight the protein content of potato tubers is fairly low. Nevertheless, the protein content in dry matter is often comparable with that in grain and per unit area the potato is able to produce more preotein than this crop. Potatoes are a good source of amino acids (Talley et al. 1983). Approximately half of the potato tuber nitrogen is protein. Potatoes supply proteins which are rated higher in quality than those of soybeans by some nutritionists (Eppendorfer et al. 1979; Rhoades 1982).

The quality of potato has been judged practically only from the aspect of cooking quality and taste, while the content of biologically important substances as protein and aminoacids has been overlooked. Increased demands on quality indicate that the protein content and aminoacids composition will become of greater importance not least concerning the use of potatoes in the processing industry.

2. MATERIALS AND METHODS

2.2. Potato material

All the biological material (positive and negative controls) was obtained from the virus collection of our institute. The potato varieties tested in our research were the following:
- Christian, Romano, Roclas, Dacia, Ruxandra (roumanian cv.)
- Ostara, Desiree, Hermes, Sante, Koretta (foreign cv.)
Our researches regarding the behaviour of these varieties were done in green house conditions. From each variety, 8 pots (with 1 eye pieces) were planted. Plants were grown in 18 cm pots and were maintained at 18-22^{o}C with 14 hour day length. After emergence, plants have been mechanicaly inoculated, using an Y^{NTN} isolate (Vital variety). After the inoculation, disease symptoms were observed and ELISA tests have been made. The infection of this material was confirmed by using antiserum from Bioreba (Switzerland) and antiserum obtained in our laboratory (Cojocaru et al., 2009). The percentage of tubers with necrotic symptoms was estimated at harvesting time and later (after 3 months).

2.3.ELISA test

A press with smooth roles was used for preparation leaf samples. For the tuber testing, the sap was extracted, diluted and dispensed directly into the plate using the extractor Microlab 500B/C (Hamilton). We tested sprouting tubers after natural break of dormancy, when the sprouts were 2-3mm long. The antiserum and conjugated used for viruses detection were obtained in our laboratory (Cojocaru et al., 2009). Microplates-NUNC microplates were coated with antibodies for overnight incubation in the refrigerator. The analysis was performed following essentially the protocol described by Clark and Adams (1977). We used 100 µl from each reactives solutions in each well of the plate (Clark and Adams 1977, Badarau et al. 2009, 2010). All experiments were repeated four times. Rinsed microplates were filled with substrate solution (p-nitrophenylphosphate) incubated 60 minute and the absorbance values were estimated at 405 nm (A_{405}) on PR1100 reader. The samples having A_{405} values exceeding the cut-off (two times the average of healthy control samples) were considered virus infected.

2.4.Quality characteristics

Dry matter (thermoventilated oven at 105^{o}C), total protein content (Kjeldal method) were determined on healthy tubers before planting them in the pots. We choosed a representative sample of tubers per plot. The sample for these analysis were choosed from each 2 tubers (2 tubers/sample). The quality characteristics determination was made in four repetitions.

2.5.Statistical interpretation

Each set of comparable assay was conducted at the same time and with the same bulk sample. Analysis of variance (ANOVA) and Duncan's multiple range test were used to analyze the data. In the aim to illustrate the precision of the mean we use confidence interval (CI).

3. RESULTS AND DISCUSSIONS

After mechanical inoculation, about all of tested plants presented mosaic symptoms on leaves, associated with crinkling top leaves (Ostara, Desire, Tampa and Hermes) or with necroses and streak on leaves, veins, petioles and stems followed by wilting of leaves (Koretta, Roclas and Romano). Our results confirme the former investigations of Le Romancer and Kerlan (1992, 1994) that Y^{NTN} is more virulent than Y^{N} strains. In most of the plants the virus began to multiply in the inoculated leaves four to five days after inoculation, at the time when the first local lesions appeared. We evaluated the foliar symptoms from primary infections in a greenhouse conditions. The virus then spread to the stem, followed by the upper, green parts of the plants and the roots at the same time. The virus multiplied vigorously in the potato cv. Koretta and Tampa similary phenomen observed to the extremely susceptible variety Hermes, the percentage of infected plants being maximal in these situations (table 1). As espected, the virus did not multiply in the highly resistant cv. Sante. Excepting cv. Sante, Christian and Dacia which were very resistent and middle resistent to mechanical inoculation, all the other varieties presented 62.5-100% infected plants.

Table 1. Frequency of infected plants (after mechanical inoculation) and of tubers with potato ring necrotic disease (PTRND) symptoms

Variety	% plants infected after inoculation*	% tubers with symptoms**	
		At harvest	**After 3months**
Sante	0 (0/8)***	0	**0,0**
Christian	50 (4/8)	0	**0,0**
Dacia	50 (4/8)	3,3	**6.6**
Agata	100 (8/8)	4	**8,0**
Roclas	62,5 (6/8)	4,5	**9,0**
Romano	87,5 (7/8)	4	**12,0**
Ostara	62,5 (6/8)	0	**22,2**
Tampa	100 (8/8)	10	**60,0**
Koretta	87,5 (6/8)	53,1	**87,5**
Hermes	100 (8/8)	62,5	**95,8**

* ELISA test made after 3 weeks after inoculation

** Tuber symptoms characterized by raised or sunken necrotic lesions, were scored at harvest and after 3 months storage at 4-8^{o}C

*** In the brackets, the first number represents nr of plants found infected after inoculation, the following one represents the total number of plants inoculated for every variety.

At harvesting, symptoms could be indentify on the tubers from all the other varieties, excepting the cv. Sante, Christian and Ostara. Concerning the other varieties, the appearence and evolution of symptoms on tubers is going on imediatly after harvesting. After 3 months, the frequency of tubers with symptoms was between 6.6-22.2% for varieties Dacia, Agata, Romano, Roclas, Ostara and for Tâmpa, Koretta, Hermes varieties this percentage was between 60-95.8% (table 1).

Table 2. Biochemical analyses of tubers* used in the aim to estimate the behaviour of inoculated plants to potato virus Y^{NTN} (PVY^{NTN})

Variety	Dry matter (% f.w.)	**Proteins (% d.w.b.) ±SD****	N (% d.w.b.)	Proteins (% f.w.b)
Sante	21.2	**9.010±0.183 a*****	1.761±0.018	2.157
Christian	20.1	**8.790±0.115 b**	1.718±0.013	2.159
Dacia	19.8	**7.839±0.082 c**	1.573±0.013	1.947
Agata	20.6	**6.853±0.055 cd**	1.416±0.009	1.824
Roclas	19.6	**6.890±0.083 cd**	1.422±0.013	1.741
Romano	22.4	**6.020±0.155 d**	1.274±0.024	1.735
Ostara	20.8	**5.739±0.089 de**	1.230±0.014	1.655
Tampa	21.8	**5.636±0.177 de**	1.198±0.022	1.482
Koretta	22.6	**5.389±0.127 f**	1.181±0.004	1.638
Hermes	22.2	**5.273±0.104 f**	1.163±0.017	1.643

* These characteristics were made to the tubers before planting them in the pots. Tissue was taken from tubers stored at 6-8^{o}C six weeks after harvest. Half of every tuber was analysed and the other one was planted in the pot.

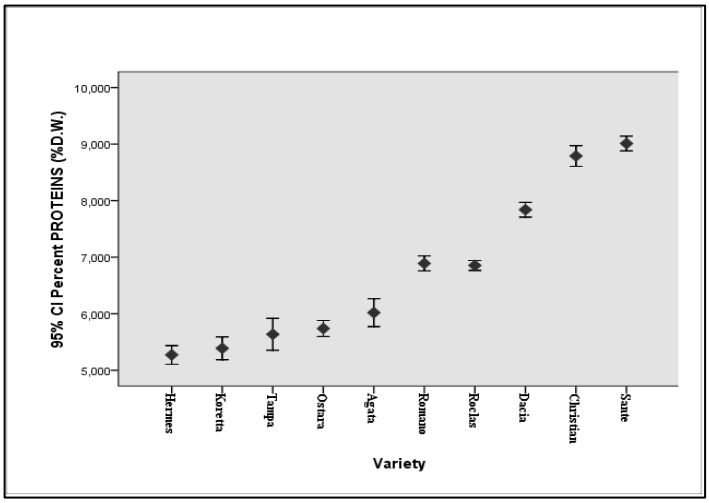
** Mean values for 4 repetitions ± standard deviation. Analyses were made on dried tuber tissue. The proteins % was determinated using the nitrogen % content (Kjeldal method).

*** Values not followed by the same letter are significantly different (P=0.05) according to Duncan's test.

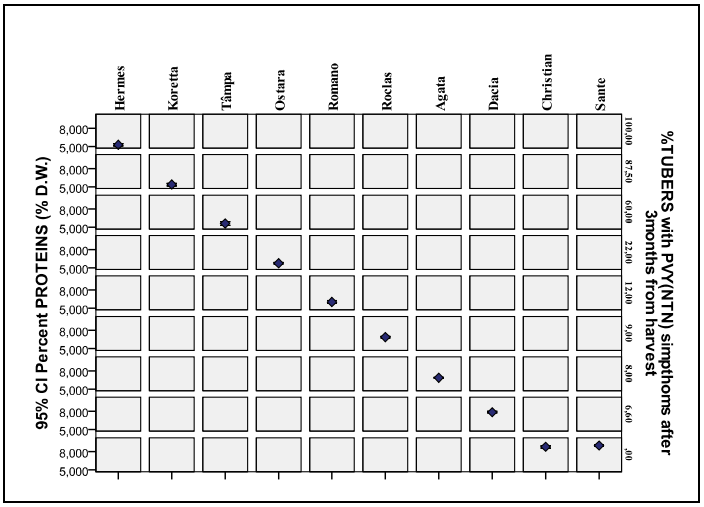
Abreviation: f.w.b. = fresh weight basis; d.w.b = dry weight basis; S.D.=standard deviation.

The proteins percentages (% from dry matter) of tubers planting in the pots were very different. As shown in tabel 2, these values were significantly high to the varieties resistent and very resistent to the inoculation like cv. Sante, Christian and Dacia comparated with the sensible cultivars Hermes, Tâmpa and Koretta. On our opinion, it is a strong correlation between the protein's content (% from dry matter) of tubers planted in the pots and the behaviour of inoculated material to Potato Tuber Ring Necrotic Disease. So, the variants wich started in vegetation with high percentage of proteins (% from dry matter) were resistent to the inoculation (Sante,

Christian, Dacia) (figure 1). Concerning these cultivars, the percentage of tubers with Y^{NTN} symptoms visible imediately after harvesting and after 3 months from the harvest was significantly lower (0.0%-3.3%) comparatively with the other varieties. Symptoms of potato tuber ring necrotic desease (PTNRD) on roumanian varieties visibles after 3 months from the harvest of the inoculated material are visible in the figure 2.



A.



B.

Figure 1. Percent of protein content (% dry weight basis) for the material planted in greenhouse fonction on the variety (A) and on the behaviour to the Potato Tuber Ring

Necrotic Disease (PTRND) induced by mechanic inoculation with potato strain Y^{NTN} (B). 95% CI= 95% confidence interval of the difference; (Error bars show 95% CI of mean).

The behaviour of cultivars Hermes, Koretta and Tâmpa (which started in vegetation with lower proteins content) was different. So, after 3 months from harvesting the inoculated plants, the percents of tubers with Potato Tuber Ring Necrotic Disease symptoms were the highest (60-95.8%) (figure 1).

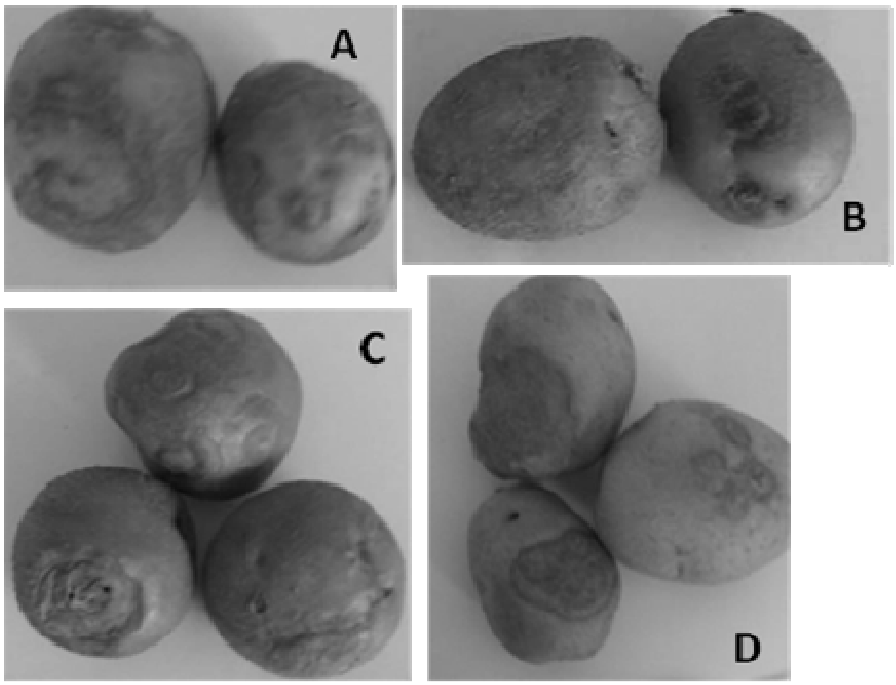


Figure 2. Symptoms of potato tuber ring necrotic desease (PTNRD) on roumanian varieties after 3 months from the harvest of the inoculated material from the roumanian varieties: Dacia (A); Roclas (B); Romano(C); Tâmpa (D)

3.CONCLUSION

The variety and the protein content (% dry weight) of tubers used for the experiment influenced the behaviour of the material after the inoculation with potato virus Y (Y^{NTN} strain- variety Vital).

Excepting the cultivars Sante, Dacia and Christian, wich were very resistent and resistent to mechanical inoculation all the other varieties presented 62.5-100% infected plants. The first symptoms on the leaves have been observed on Hermes, Tampa and Koretta varieties and later on cv. Ostara, Romano, Roclas and Agata.

At harvesting, Potato Tuber Ring Necrotic Disease symptoms could be indentified on the tubers from all the other varieties, excepting the cv. Sante, Christian and Ostara. Concerning the other varieties, the appearence and evolution of symptoms on tubers is going on imediatly after harvesting.

After 3 months from harvesting the inoculated material, the frequency of tubers with symptoms was between 6.6-22.2% for varieties Dacia, Agata, Romano, Roclas, Ostara and for cv. Tampa, Koretta, Hermes this percentage was very high (60.0-95.8%).

The samples with significantly higher protein content (cv. Sante and Christian) were resistent to PVY^{NTN} inoculation. In this case, after 3 months from harvest, the stored tubers didn't have visible tuber necrotic ringspot disease symptoms.

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DETERMINATION OF BIOACTIVE COMPOUNDS AND EVALUATION OF FREE RADICAL SCAVENGING POTENTIAL OF SOME EXTRACTS FROM NATIVE *LAMIUM* SPECIES – *LAMIUM ALBUM AND LAMIUM PURPUREUM* –

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Abstract: *Free radical scavenging properties of some vegetal extracts from Lamium album and Lamium purpureum were studied by DPPH free radical scavenging assay. The content of active principles was determined and correlated with antioxidant activity. This study shows the antioxidant potential of 4 extracts of Lamium album and Lamium purpureum, emphasizing the correlation between chemical composition and biological effect.*

Keywords: lamium, scavenger, extracts

1. INTRODUCTION

Antioxidants are important in disease prevention in both plants and animals, inhibiting or delaying the oxidation of biomolecules by preventing the initiation of propagation of oxidizing chain reactions. [1]

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. In both cases, there is a preference for antioxidants from natural rather than from synthetic sources. [2]

Synthetic antioxidants require extensive and expensive tests to ascertain their safety and for this reason, there fore the use of naturally antioxidants is preferred. The consequent search for natural replacements for synthetic antioxidants has led to the evaluation of a number of plant sources. [1]

The plant kingdom offers a wide range of natural antioxidants. Many herbal and plant infusions frequently used in domestic medicine have antioxidative and pharmacological properties connected with presence of phenolic compounds, especially flavonoids. Flavonoids very easily take part in oxidation reaction processes, both inside and outside cells. The antioxidant properties of flavonoids relies on their ability to interact with free radicals witch initiate oxidation reaction or which are produced during chain reactions, on the inhibition of oxidation processes, which diminishes the activity of oxidase enzymes, or on the complexation of transmition metal ions which catalyse oxidation reactions [3]

Now *Lamium* genus from Lamiaceae family is less studied and turned to account (valorized), comparing to other members of this family.
The genus *Lamium* (Lamiaceae) comprises about 40 species distributed in Europe, Asia and Africa. Some Lamium species have been used in traditional and folk medicine. The most popular is *L. album*. [4]

Besides this species, in Romania also grow *L. maculatum*, *L. bifidum* Cirillo ssp. *balcanicum* Vel, *L. galeobdolon* L., L. *amplexicaule*, *L. bithynicum* Benth., *L. bifidum* Cirillo ssp. *balcanicum* and *L. purpureum*. *Lamium purpureum* is less studied, but this species shows a special pharmacological potential due to the family that belong to.

This study intents to show the antioxidant potential of 4 extracts from native Lamium species -*Lamium album and Lamium purpureum*-, emphasizing the correlation between chemical composition and biological effect.

2. MATERIALS AND METHODS

Chemicals: Folin-Ciocalteu's phenol reagent, 2.2-Di(4-tert-octyphenyl)-1-picrylhydrazyl (DPPH), caffeic acid, chlorogenic acid, gallic acid, rutin, hyperoside were purchased from Sigma Chemical Co. Dried vegetal material was purchase from a local drugstore (*Lamium album, Lamium purpureum*)

Methanol, ethanol and acetone were used as dissolving/extracting solvents.

Ethyl acetate, formic acid, and distilled water (p.a) as TLC developants.

Preparation of plant extracts – Extracts were obtained by:

- succesive (repetitive) methanolic extraction

Lp1 *Lamium purpureum* (methanol 80%)

La1 *Lamium album* (methanol 30%)

and

- repetitive acetonic extraction

Lp2 *Lamium purpureum* (acetone 50%)

La2 *Lamium album* (acetone 50%).

vegetal material/ solvent rate -1/10 m/v at the boiling temperature of the solvent for 2 hours first time and 1 hour second time, under continuous stirring. After filtration, the reunited methanolic/ acetonic solution was concentrated under reduced pressure (72-74 mm Hg) and 50°C to a vegetal material/methanolic (acetonic) extract rate - 1/1.5 (m/v). The insoluble substances were removed by alcohool precipitation and after 24 hours repaos was centrifuged at 3000 rpm for 30 minutes. The resulted solution was concentrated under reduced pressure (72-74 mm Hg) and 50°C to a vegetal material/methanolic (acetonic) extract rate - 1/1 (m/v) and spray-dried.

Determmination of flavones: Qualitative analyses for flavones were carried out usig TLC methods [5]. Quantitative analysis for flavones were made usig a

spectrophotometric method based on their property of forming stable yellow compounds with Al^{3+}, having maximum absorbtion at 450nm [6]

Determination of total phenolics: Qualitative analyses for total phenolics were carried out usig TLC methods [5]. Total phenolics content was determined according to the Folin-Ciocalteu method using gallic acid, caffeic acid and chlorogenic acid as standards.[6]

DPPH assay: In eitch reaction 50 µL vegetal extract of different concentrations was mixed with 2950µL 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 517 nm. Rutin and caffeic acid were used as positive controls. Inhibition ratio (percent) was calculated from the following equation:

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

3. RESULTS AND DISCUSSIONS

Qualitative analyses for flavones and total phenols have been performed according to TLC Atlas - *Plant Drug Analyses* [20]:

Adsorbent: Silica gel 60F254 – percolated TLC plates (*Merck, Germany*);

Solvent system: Ethyl acetate-formic acid- acetic acid gl.– distilled water (100:11:11:26);

Reference compounds: *Sigma* polyphenols prepared as standard solutions 10^{-3}M solved into methanol p.a. - 10 to 20µl of these standard solutions applied at the starting line.

Identification: pulverization with NP/PEG (natural products reagent) followed by exposure at 366nm.

Obtained chromatograms for *Lamium album* revealed fluorescent (fl.) spots attributed as following:

-*Rf~0.49*- chlorogenic acid blue fl. spots;
-*Rf*~0.94 – rosmarinic acid blue fl. spot;
-*Rf*~0.96– caffeic acid blue fl. spots.

Obtained chromatograms for *Lamium purpureum* revealed fluorescent (fl.) spots attributed as following:

-*Rf~0.4* – rutin orange fl. spot;
-*Rf~0.49* / chlorogenic acid blue fl. spots;
-*Rf~0.62*- hyperoside orange fl.spot;
-*Rf*~0.95 – rosmarinic acid blue fl. spot;
-*Rf*~0.96– caffeic acid blue fl. spots.

TLC studies reveals yhat chlorogenic acid, rosmarinic acid and caffeic acid are the main polyphenols compounds present in indigenous *Lamium album*

extracts and rutin, hyperoside, chlorogenic acid, rosmarinic acid and caffeic acid are the main compounds in *Lamium purpureum extracts.*

Table 1. Phytochemical composition of *Lamium album* obtained by extraction with various types of solvents

Lamium album extract	Extraction solvent	Flavones expressed as rutin (%)	Total polyphenols expressed as caffeic acid (%)	Total polyphenols expressed as gallic acid (%)	Total polyphenols expressed as chlorogenic acid (%)
La1	Methanol 30%	0.584	2.591	1.838	4.806
La2	Acetone 50%	0.835	2.89	2.504	4.52

Table 1 shows the percent of total phenolics (expressed as gallic acid, caffeic acid and chlorogenic acid) and the percent of flavonoids (expressed as rutin) for *Lamium album* samples obtained by extraction with various types of solvent

Table 2. Phytochemical composition of *Lamium purpureum* obtained by extraction with various types of solvents

Lamium purpureum extracts	Extraction solvent	Flavones expressed as rutin (%)	Total polyphenols expressed as caffeic acid (%)	Total polyphenols expressed as gallic acid (%)	Total polyphenols expressed as chlorogenic acid (%)
Lp1	Methanol 80%	5.097	5.232	3.83	8.18
Lp2	Acetone 50%	4.729	5.256	3.54	7.364

Table 2 shows the percent of total phenolics (expressed as gallic acid, caffeic acid and chlorogenic acid) and the percent of flavonoids (expressed as rutin) for Lamium purpureum samples obtained by extraction with various types of solvents.

Acetonic extract La2 (from *Lamium album*) has the highest composition in total phenolic and rutin while methanolic extract Lp1 (from *Lamium purpureum*) has the highest content in total phenolics and rutin.

The *Lamium purpureum* extracts have the highest percent of biological compounds compared with the *Lamium album* extracts.

DPPH Radical Scavenging Activity

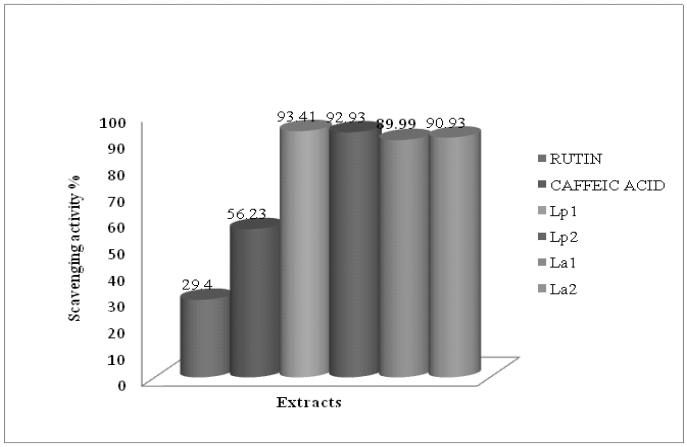

Figure 1. Scavenging activity on DPPH free radical at 0.1 mg/ml of the extracts

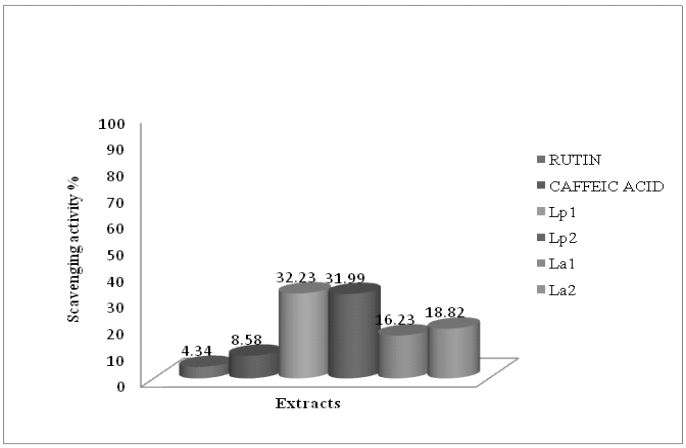

Figure 2. Scavenging activity on DPPH free radical at 0.01mg/ml of the extracts

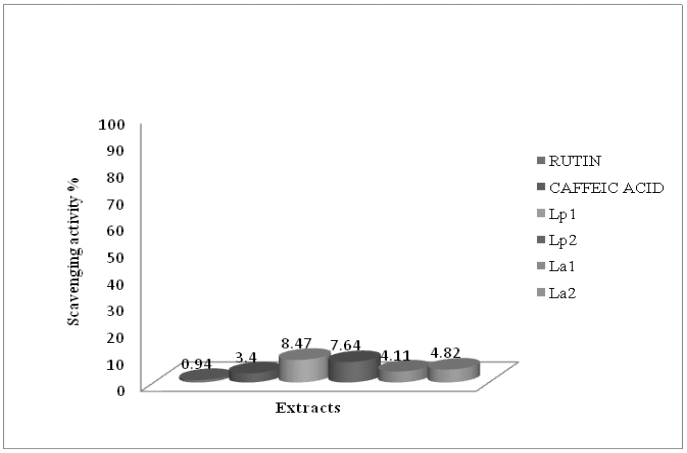

Figure 3. Scavenging activity on DPPH free radical at 1µg/ml of the extracts

Figures 1-3 illustrates a decrease of DPPH radicals concentration due to the dose-dependent scavenging ability of the extracts. At all concentrations, even at 1µg/ml, the Lamium purpureum extracts showes a stronger DPPH scavenging activity compared to Lamium album extracts and also all the vegetal extracts have higher activity than standard compounds, rutin and caffeic acid.

4. CONCLUSIONS

Oxidation processes are very important for living organism.The uncontrolled production of oxygen free radicals and the unbalanced mechanism of oxidation protection leads to many diseases and accelerate ageing.

In biochemistry and medicine, antioxidants are capable of counteracting the damaging effects of oxidation in animal tissue. (7)

This study shows the antioxidant potential of 4 extracts from two Lamium species - *Lamium album* and *Lamium purpureum*- by DPPH assay. All extracts showed a high DPPH scavenging activity, *Lamium purpureum* extracts showed a stronger activity compared with *Lamium album* extracts. The antioxidant activity was correlated with total phenolic and flavonoid content. The study proves that the *Lamium album* and *Lamium purpureum* extracts exhibit considerable antioxidant properties, expressed by their capability to scavenge DPPH.

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CO-TRANSFORMATION OF POTATO (*SOLANUM TUBEROSUM* L.) CV. DESIREE BY TWO SEPARATE T-DNAs CARRYING ORYZACYSTATIN GENES

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Keywords: potato, *Agrobacterium tumefaciens*, co-transformation, oryzacystatin I, oryzacystatin II

ABSTRACT

In an effort to develop plants with enhanced insect and pathogen resistance rice cystatin genes were introduced into potato via Agrobacterium-mediated transformation. Plants of Solanum tuberosum L. cv. Desiree were co-transformed using the mixture method with two separate Agrobacterium tumefaciens EHA101 strains carrying oryzacystatin I (OCI) or oryzacystatin II (OCII) genes. PCR analysis indicated integration frequency of 38.0 ± 2.9% and 42.0 ± 5.8% for OCI and OCII genes, respectively, while co-transformation frequency of both rice cystatin genes was 20.0 ± 2.9% of total number of analyzed transformants.

1. INTRODUCTION

Sterility and tetraploidy, together with a high degree of heterozygosity, significantly reduce the efficiency of conventional breeding as a methods for improving potato genotypes. *Agrobacterium*-mediated gene transfer has appeared as promising technique to introduce heterologous genes into potato since it was first reported by Ooms *et al.* (1986). A large number of agronomically useful genes have already been transferred to potato. Most of these genes play role in a resistance to potato predators/pathogens (Chue *et al.,* 2004; Naimov *et al.,* 2001; Urwin *et al.,* 2001; Lyapkova *et al.,* 2001; Hefferon *et al.,* 1997; Wierenga *et al.,* 1996) and modification of important metabolic and physiological pathways such as starch and sucrose synthesis (Edwards *et al.,* 1999; Wolters *et al.,* 1998).

Co-transformation with multiple *Agrobacterium* strains carrying different T-DNA is mainly used to study the mechanism of T-DNA integration (De Neve *et al.,* 1998; De Block *et al.,* 1991), to facilitate selectable marker removal (Park *et al.,* 2004; Vidal *et al.,* 2003) or for multiple gene enhancements via genetic engineering (Li *et al.,* 2009). This approach has not been widely used for potato, although it provides an effective method for rapidly stacking transgenes. Chang *et al.* (2002) reported a co-transformation efficiency of 24% for potato cv. Russet Burbank obtained using two plasmids cloned in separate *A. tumefaciens* LBA4404 containing the same selectable marker. Using same approach Morris *et al.* (2006)

obtained co-transformation frequency of about 10% when cv. Desiree was infected with two *Agrobacterium* strains.

The transgenes used in this study, rice cystatins cDNAs: *OCI* (Abe *et al.,* 1987) and *OCII* (Kondo *et al.,* 1990), showed potential in controlling pests relying on cysteine proteinases for digestive protein hydrolysis (Ninkovic *et al.,* 2007; Ribeiro *et al.,* 2006; Samac and Smigocki 2003; Leple *et al.,* 1995). Thus, introduction of these genes in potato could potentially enhance resistance to predators or pathogens.

2. MATERIAL AND METHODS

Plant material

Potato plants cv. Desiree were propagated *in vitro* on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and solidified with 0.6% agar. Plants were maintained under controlled light and temperature conditions with a photoperiod of 16 h (47 µmol m^{-2} s^{-1} white fluorescent light) at 25 ± 2°C.

Transformation vector and *Agrobacterium* strain

For genetic transformation, *Agrobacterium tumefaciens* strain EHA101carrying the pGV-GFP-OCII-4.2A7 and pGV-GFP-OCI-3.8(19) were used, kindly provided by Ann Smigocki (Plant Molecular Pathology Laboratory, USDA, ARS). Plasmids carry the rice *OCII* and *OCI* genes, respectively, fused to the pin2 promoter, as well as 35S-*GFP* reporter gene and nos-*nptII* selectable gene.

Transformation and plant regeneration

The leaf radial segments of about 5 mm^2, from 4 weeks old *in vitro* propagated plants, were incubated for 5-10 minutes in 1:1 mixture AGV4.2A7 and AGV 3.8(19) bacterial suspensions (~10^8 bacterial cells), blotted dry on a filter paper and cultured on CIM (callus-induction medium: MS supplemented with 3% sucrose, 2 mg l^{-1} BAP and 0.2 mg l^{-1} NAA). After 5 days of co-cultivation the explants were washed in sterile water with cefotaxime (1 gr l^{-1}), dried on filter paper and transferred onto CIM supplemented with 50 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime. After 2 weeks callus were removed from the explants and transferred on SIM (shoot-induction medium: MS supplemented with 1.5% sucrose, 2 mg l^{-1} BAP and 5 mg l^{-1} GA_3) plus 300 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin, and cultured until shoots were regenerated. Individual shoots 10-20 mm length (one shoot per explant) were excised and transferred on selective RIM (root-induction medium: MS supplemented with 300 mg l^{-1} cefotaxime and 75 mg l^{-1} kanamycin). Plantlets with well-developed roots were multiplied and used for further analyses.

PCR analysis

Genomic DNA from putative transformed and nontransformed control plants was isolated according to the method of Zhou *et al.* (1994). The presence of the

transferred *OCI* and *OCII* genes were confirmed by PCR technique using specific *pin*:*OCI* primers (5'- GGC TCC TCC GTC CAA TTA TA -3'and 5'-ATC GAC AGG CTT GAA CTC CT-3') and *pin*:*OCII* primers (5'-GGC TCC TCC GTC CAA TTA TA-3'and 5'-GGT GGC GTC GTC GAG GGG-3'). Reactions were carried out for 35 cycles of 95ºC for 1 min, 55°C for 1 min, and 72°C for 2 min (*OCI*) and 35 cycles of 95ºC for 1 min, 53°C for 1 min, and 72°C for 2 min (*OCII*). PCR amplified gene products were analyzed by electrophoresis on 1.2% agarose gels.

3. RESULTS AND DISCUSSION

Simple and rapid transformation protocol employed in this work was based on previously established potato transformation procedure (Cingel *et al.,* 2010). MS medium supplemented with BAP and NAA for callus induction and with BAP and GA_3 for shoot formation appeared to be suitable for plant regeneration from leaf explants. These results correspond to a number of published papers (Benchekroun *et al.,* 1995; Visser *et al.,* 1989; Wenzler *et al.,* 1989; Webb *et al.,* 1983).

Within 7 days after bacterial infection average 86% leaf explants cultivated on selective CIM manifested callus proliferation along the cut edge. Shoot bud regeneration was observed 10 days after calli were transferred on selective SIM. Within 3 weeks on SIM 74% calli produced an average 7.6 buds per explant (Table 1). After 10 days of culture on selective RIM 96% shoots developed roots. There were no apparent differences between shoot buds regeneration from control and transformed explants and all rooted plants exhibits normal phenotype.

Table 1: Co-transformation frequency and shoot bud regeneration efficiency of leaf explants.

	Number of explants that developed calli (%)*	Number of calli that developed buds (%) **	Number of buds/explants
control	95.0 ± 0.3	88.0 ± 0.6	8.5 ± 0.6
OCI/OCII	86.0 ± 0.4	74.0 ± 0.9	7.6 ± 0.4

Results are expressed as mean ± S.E. (No. of explants =100)
* On CIM, mean was calculated as No. of explants per petri dish with calli/No. of explants
** On SIM, mean was calculated as No. of explants per petri dish with developed buds/No. of explants.

PCR detection for two target genes showed that 100% of putative rooted transgenic plants carry either or both transgenes. Transformed plants produced about 800 bp or/and 1000 bp amplification products that were not detected in nontransformed control plants (Figure 1). Apparently, rooting on selective RIM with increased kanamicyn concentration to 75 mg l^{-1} was a good indicator of

transformation. Lower level of kanamycin (50 mg l^{-1}), adequate for control of nontransformed cell growth and efficient to support early shoot bud regeneration, allows some escapes (Cingel *et al.,* 2010; Wenzler *et al.,* 1989).

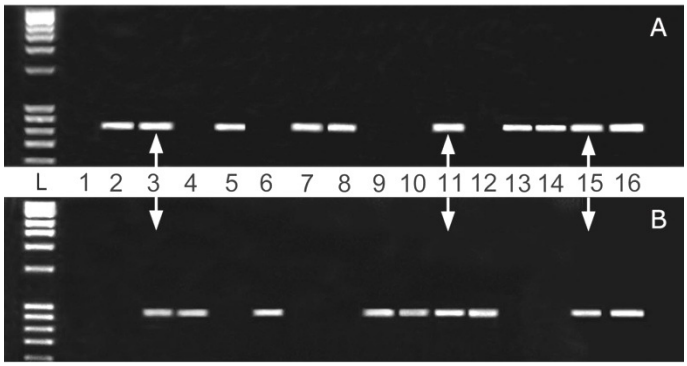

Figure 1. PCR analysis of *OCII* (A) and *OCI* (B) genes in putative co-transformed potato plants. L: 1Kb DNA ladder, lane 1: nontransformed control, lanes 2-15: putative OCI/ OCII transformed clones, lane 16: positive control pGV-GFP-OCII-4.2A7 (A) or pGV-GFP-OCI-3.8(19) (B). Arrows indicates presence of both *OCI* and *OCII* T-DNAs.

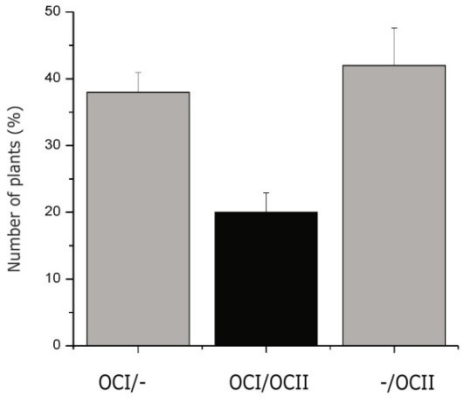

Figure 2. Co-transformation frequency of *OCI* and *OCII* T-DNAs.

Overall, there were no significant differences between *OCI* (38.0 ± 2.9%) and *OCII* (42.0 ± 5.8%) integration frequencies (Figure 2). This could be due the same effectiveness of bacteria-plant cell interactions because both plasmids were multiplied in EHA101 and an equal amount of both bacteria were used for co-cultivation. The co-transformation frequency for both transgenes of 20.0 ± 2.9% was similar to the frequencies of 10-24% previously obtained in potato co-transformation experiments (Morris *et al.,* 2006; Chang *et al.,* 2002) when the same selectable marker (*nptII*) were used for each transgene. Also, our results

correspond to co-transformation frequency of 18-27% reported for some other systems (Carrer *et al.,* 1995; Christou *et al.,* 1990; Uchimiya *et al.,* 1986).

4. CONCLUSIONS

This study demonstrated that an efficient co-transformation of two genes in potato can be achieved by cloning independent plasmids in separate *Agrobacteria*, using the same gene as the only selection marker. This approach provides an effective means of rapidly stacking transgenes and has the advantage of eliminating the need for introducing several antibiotic resistance genes.

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EXPERIMENTS ON LASER RADIATION INFLUENCE ON THE DEVELOPMENT AND GROWTH OF SEEDLINGS OF EGGPLANT (*SOLANUM MELONGENA* SP.-VARIETY" DRAGAICA")

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ABSTRACT

Additional red light illumination produced by laser diodes in continuous and different exposure times, was applied to „eggplant" seeds (Solanum melongena sp.) from Romanian type "Dragaica".

Sets of seeds were irradiated once in the first day of the experiment at different energy doses by changing exposure time. Thus, four dry seed lots were irradiated at different doses during the period from 2009 and 2010 corresponding variants V1-V4 (5-20 minutes). The experimental results were analyzed in parallel with a control group of seeds that did not treated with red laser diodes.

The capacity of germination and the growth rithm were studied for all seeds of eggplant Solanum melongena-variety "Dragaica by the determining the germination rates every two days for 3 weeks.

Results have shown a percentage of germination higher than that for the control group of seeds (75%) for all repetitions of variant V4 (95%), with the time exposure of 20 minutes (1.53 joules / cm [2]). In the treatment was used red light produced by 19 laser diodes.

To seedlings of eggplants resulted from seeds irradiated in an experimental series S1-2010 were made observations on the dynamics of formation of true leaves at 14 and 30 days after the onset of germination, compared with control group seedlings.

Key words: laser radiation, germination seeds, plant growth, eggplant.

INTRODUCTION

Bodies known plant can not grow in the absence of light. In fact, light is electromagnetic radiation with wavelengths in the range 400 nm - 750nm. In non-protected areas, light reaching the plants is generated by the sun, while in protected areas light is generated by sources with certain spectral characteristics (*Anghel, S.,* et al., 1999; *Călugăreanu M.,* et al., 1999;).

Light is to plants, both a source of energy and information. It is a source of energy for photosynthesis and a source of information for photoperiodism (night / day), phototropism (light direction) and photomorphogenesis (quantity and quality of light). The light in the spectral range 640 nm - 660nm is very important considering that chlorophyll absorbs strongly in this range. Red field - about 660-670nm, is important to the absorption bands of chlorophylls, a 'and' b ' (*Quail, P.,H.,1991).*

Fluorescent lighting commonly used in protected areas emit very weak in this spectral domain. Laser diodes - semiconductor-lasers covering the spectral domain. Illumination of fluorescent radiation emitted simultaneously with the radiation emitted from laser diodes leads to more rapid and healthy development of plants.

Laser light has special properties: it is monochromatic, coherent and polarized. In the present experiments we used laser and semiconductor laser diode that is. Radiation emitted from it is 3-4 nm spectral width, the coherence length of several mm and the degree of polarization of 99:1(*Chita Monica Anca; Godeanu Marioara, 2004*).

Use of laser radiation on the seeds of positive stimulation leads to plant growth, reducing time to germinate, increasing the number of flowers per plant, qualitative and quantitative growth of production, reducing germination and development during all stages of vegetation (*Russ J., 1995*).

1. MATERIAL AND METHODS

As biological material the variety of eggplant seeds "DRAGAICA" were used, created by SCDL Buzau. After physiological characteristics is an early variety, with the vegetation period, from sunrise plants (sprung mass - 75%) at the beginning of technical maturity (10%), between 117 and 118 days, with seven or eight days earlier to control variety "raven". Reaction to diseases is good and the average total harvest is 51.5 t / ha (*Andronicescu D.,* et al., *1997; Indrea D., Isac, M., 1987 and Apahidean AL. S., 1995).*

Red laser radiation emitted by 19 laser diodes was used. Laser illumination was homogeneous on the all exposed surface (Petri dishes with vegetable seeds).

Experiments were conducted in the form of two experimental series:

S1(2009)-seeds of the genus *Solanum melongena* eggplant - variety "Dragaica additional illuminated continuously with red laser radiation (exposure times: 5, 10, 15, 20 minutes).

S2 (2010)--seeds of the genus *Solanum melongena* eggplant - variety "Dragaica additional illuminated continuously with red laser light (exposure times: 5, 10, 15, 20 minutes). For each experimental variation and repetition of series **S1** were used 20 seeds and **S2** were used 25 seeds.

Seeds were then placed in numbered Petri dishes for irradiation at 23 ° C and relative humidity of 55%. After this irradiation, the two experimental series were seeded immediately with a clamp on the surface of organic potting mix. The mixture of land used for sowing seeds of eggplant was previously sterilized and distributed in 10 cm diameter pots (**Table 1, Figure 1**).

Monitoring germination was performed daily, counting all germinated seedlings, regadless of size hypocotyls axis, or the appearance epicotyls leaf

formations. The average percentage of bacteria determined on day-3 of the corresponding value was considered as germination energy (EG). On Day 14 the germination process was considered finished, the percentage of bacteria results in the period between day 3 and day 14 was being considered as estimator of germination (FG)

Table 1 Time and the appropriate dose for the irradiation of a Petri dish with a diameter of 10 cm

T(min)	D (J/cm2)
5	0.38
10	0.75
15	1.15
20	1.53
30	2.3

a **b**

Figure 1. Experimental device unit attachment with diode laser irradiation (a) and seed treatments on *Solanum melongena* (b)

2. RESULTS AND DISCUSSIONS

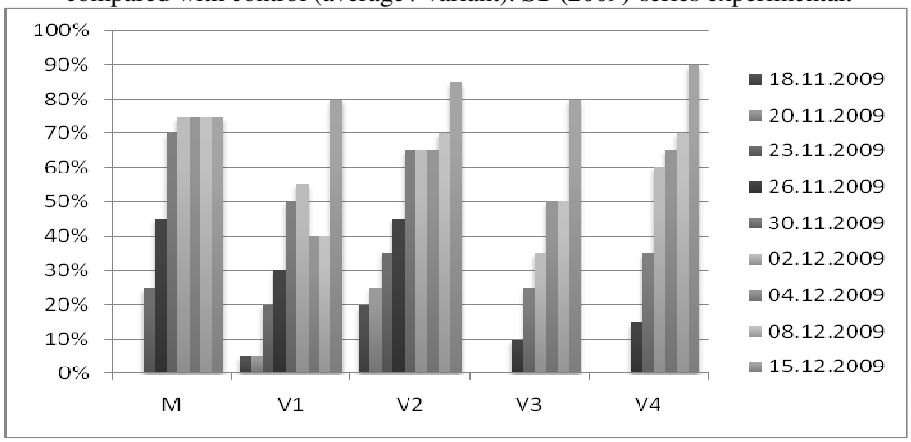
Observations made on the influence of laser radiation treatments with eggplant

For the **Series 1 (2009**), eggplant seeds additionally illuminated with red laser light, we have obtained the following results comparing the non-irradiated control sample:

- In case of eggplant seeds - *Solanum melongena*, variety "Dragaica, studying the germination of dry seeds irradiated in a **Series 1(2009)** showed that exposure to laser radiation initially causes a slight inhibition of germination compared to group processes control . Thus, after 14 days of the onset of germination, a percentage of 40-65% of germinated seeds for irradiated groups was mesured, comparing with a rate of 75% for the control group (values of germination energy). (**Graphic 1**). After another 10 days the number of plants germinated under the laser treatment,

the variant V4 (20 minutes of treatment with laser diodes) significantly exceeded the control sample. Final results of germination value for variants V1 (5 minutes) and V3 (15 minutes) were equal and easy proximity to witness the irradiated seeds.

Graphic 1 Seed germination of eggplant, Solanum melongena variety Drăgaica species compared with control (average / variant). **S1-(2009)-**series experimental**.**



Legend: Variants: **V1** (5 minutes of exposure time and dose of 0.38 joules / cm ²), **V2** (10 minutes of exposure time and dose of 0.75 joules / cm ²), **V3** (15 minutes of exposure time and dose of 1.15 joules / cm ²) **V4 (**20 minutes exposure time and dose of 1.53 joules / cm ²); **MV** (witness/control sample)

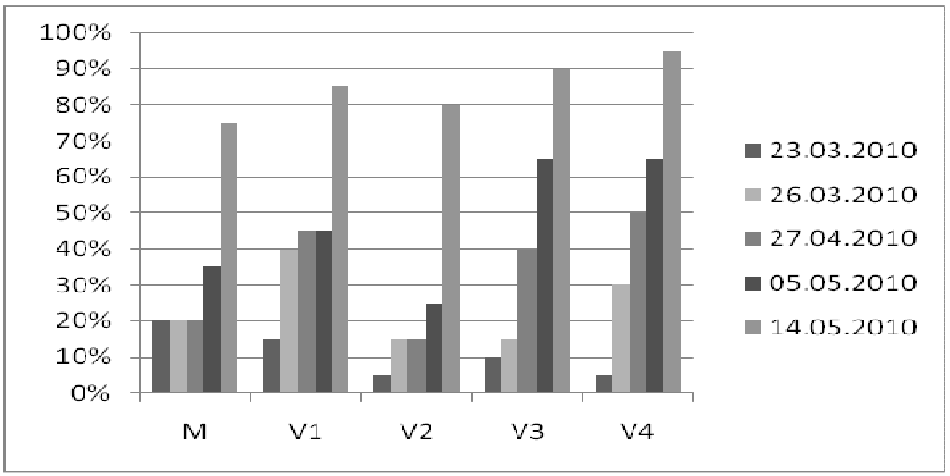
To confirm the stimulating effect due to applied treatments in the onset of germination we considered necessary in this case a repetition of the experiment in 2010.

In **Series 2 (2010),** red laser light treatment leads to the following results:

Recorded results of the testing process eggplant seed germination in dry irradiated Series 2 (2010), confirmed the values obtained in 2009 using the same exposure times under red laser illumination.

So, the observations have shown that the laser treatment causes a slight inhibition of germination processes comparing the control group, so that, after 14 days of the onset of germination, a percentage of 25-65% for germinated seeds from irradiated groups comparing a percentage of 35% in the control group (these values of germination energy). (**Graphic** 2).

Graphic 2 Observed germination values for irradiated seeds comparing to control eggplants (average / variant) - **S2 (2010)** -experimental series



Legend: Variants: **V1** (5 minutes of exposure time and dose of 0.38 joules / cm ²), **V2** (10 minutes of exposure time and dose of 0.75 joules / cm ²), **V3** (15 minutes of exposure time and dose of 1.15 joules / cm ²) **V4 (**20 minutes exposure time and dose of 1.53 joules / cm ²) **MV** (witness/control sample).

At 20 days after emergence of the first seeds germinated seedling, the variant V4 significantly exceeded (95%) the MV (75%). The final amount of germination for variants V1 (5 minutes) and V3 = 85% (15 minutes) = 90% were slightly closer to that of variant V4.

Table 2. Dynamics evaluation of the true leaves of irradiated eggplant plants compared with control sample (experimental series **S1 -2010**) on 14 and 30 days after irradiation (number/version)

Variant	Media rehearsals	After 14 days of irradiation			After 30 days of irradiation		
		Total Number of plants	Total number of leaf	Report Leaves /plant	Total number of plants	Total leaf	Report Leaves /plant
M	M1-M4	14	17	1,21	15	19	1,26
V.I.	R1- R4	9	20	2,22	16	37	2,31
V.II.	R1- R4	13	13	1,0	17	18	1,05
V.III.	R1- R4	8	14	1,75	16	40	2,5
V.IV.	R1- R4	10	23	2,3	18	41	2,27

Legend: V1 (5 minutes of exposure time and dose of 0.38 joules / cm ²), **V2** (10 minutes of exposure time and dose of 0.75 joules / cm ²), **V3** (15 minutes of exposure time and dose of 1.15 joules / cm ²) **V4 (**20 minutes exposure time and dose of 1.53 joules / cm ²), **MV** (witness/control sample).

As we can see, after evaluation of seed germination S2-2010 experimental series the observations were carried out on the dynamics of formation of true leaves

from plants of eggplant on 14 and 30 days after irradiation compared with control sample, the results being centralized and presented in **Table 2.**

3. CONCLUSIONS

The variety of eggplant "DRAGAICA" productive value chosen for experimentation is great and he was very appreciated by consumers but also by seedlings and fruit vendors near Buzau, area known for its importance in the production and marketing of vegetable products. It can however grow in all areas of the country in greenhouses and field crops.

Laser light irradiation device used in the experiments was realized in according to the dosage of irradiation required for performing experiments surfaces. The continue component of laser radiation, the distance from the laser source to the irradiated surface and the exposure times were taken into account.

For experimental series **S1 (2009**) and **S2 (2010),** seedlings from seeds of the variety of eggplant "DRAGAICA" additional illuminated once at the start of experiments with continuous laser light emission in red and have obtained the following results:

- The results recorded showed a higher maximum germination percentage for all rehearsals witness of variant V4, with exposure time of 20 minutes.
- Increasing the maximum germination obtained after laser treatment with red light was in the register on the day to 30th and was superior to control sample.
- We can say that variant V4, additional treated daily for 20 minutes by red laser light with a dose of J/cm^2, positively influences the college and eggplant seed germination energy, comparing with results for other variants irradiation and also for untreated control seeds.
- Other variants and repetitions have responded differently to treatment but the results of stimulation were located around the value of the untreated control;

So, the results after 20 -25 days are closely the same for all samples. This means that the germination period for the laser treated sample is shorter than that for untreated sample. So, it can say that the laser treatment decreases the germination period. But, now we must find the optimum doses for laser treatment in order to obtain the better results.

The obtained results are encouraging and we must continue our experiments.

Acknowledgments

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BIOTECHNOLOGIC STUDIES REGARDING THE METABOLIC ACTION OF SOME PHYTOHORMONES WITH INHIBITION EFFECT OVER THE PLANT DEVELOPMENT - *REVIEW*

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ABSTRACT

The discovery of substances with regulatory action over growth – phytohormones are growth stimulators, inhibitors, retardants – provided an efficacious, sensitive instrument for specialists in guiding and controlling the plant growth and development processes.

The fact that phytohormones increase the efficiency of using fertilizers leads to some specialists claiming the superiority of crops supervision through bioregulators, over against chemical fertilizers because they do not contribute to the pollution of the environment.

Following the basic mechanism of synthesis-transportation-action, the purpose of this paper is to establish the mechanisms through which phytohormones with inhibitor properties can operate on plant evolution processes.

Keywords: bioregulators, growth promoters, growth inhibitors.

1. INTRODUCTION

In a future "ecologic agriculture" that consider all environmental factors, phytoregulators have a great importance.

Besides external environment factors (water, nourishing substances, temperature, light) for plants to grow and develop they also need substances that form in their structure/ body – phytohormones. They influence the process of growth and morphogenesis and they adjust the physiological processes from different tissues and organs of the plant. They are synthesized in the chloroplast of young cells and they cumulate especially in the steams and roots growth zones, in seeds, pollen, buds, young tissues and others. [3]

By intensifying the cellular division and elongating the existent cells phytohormones make plants grow. This mechanism that causes plants to grow depends on the nature of the phytohormone. Chemically speaking, phytohormones are highly heterogenic substances, similar to vitamins and animal hormones. They are composed of cyclical rings (indolic, purinic, iononic, gibbanic and others), they contain ethereal bindings and different functional groups. They are largely spread in nature and they can be found in different tissues and organs in superior plants, in dregs, fungus, algae and in numerous microorganisms, in free form as well as associated with proteins. [2]

Regarding superior plants they are taken into consideration the main hormonal groups: auxins, gibberellins, citokinine, abscisic acid and ethylene. Auxins, gibberellins and citokinine are growth promoters and the abscisic acid and the ethylene are growth inhibitors.

During the plant evolution, if phytoromones stimulate the growth process and retardants stop it, inhibitors stop it completely or partially. Controlling the plant evolution mechanism by inhibiting the biochemical processes at the right moment can lead to obtaining vegetal products at the desired quality. Phytohormones with inhibiting properties can operate on the whole plant as well as on some tissues and organs causing the inhibition of some metabolic processes like: biosynthesis of nucleic acids, photosynthesis of chloroplasts and of some physiological processes, especially the division and elongation of cells and the buds opening. [12]

The abscisic acid is an inhibitor acid and alongside growth stimulating substances: auxins, gibberellins, citokinine controls the processes of plant growth and development. It is the promoter and it maintains the ageing process by hasting the fruit and leaves abscission. That is why it is called abscisic acid (the international abbreviation is ABA). [2, 5]

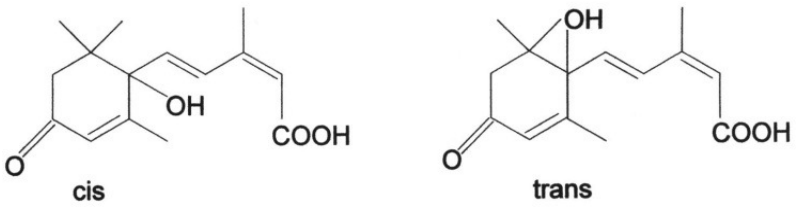
Ethylene has a large specter of biochemical and physiological action over the growth process of plants. It is the first gaseous hormone and it is also known as the hormone of fruit ripening. [2, 6]

The authors of this paper want to distinguish the mechanisms through which phytohormones with inhibiting properties operate on the plant evolution processes as well as the influence they have on the course of their action.

2. RESULTS AND DISCUSSION

Chemically speaking, the **abscisic acid** is a sesquiterpene because it consists of three isoprene units. It is composed of a (iononic) hexacycle and a lateral catena that has 5 carbon atoms with two double conjugated bounds. ABA contains an asymmetrical carbon atom (C -1) and that is why it forms 2 optic enantiomers (endogenous ABA is dextrogir). The double bound in the nucleus of the hexacycle determines two isomers to form: cis and trans. [8, 9]

In figure 1 there are presented the structures of the two geometric isomers of the abscisic acid.



(+) Acid abscisic (ABA)

Fig.1. The structures of the two geometric isomers of the abscisic acid.

The biosynthesis of ABA takes place in chloroplasts, through the general path of the synthesis of terpenoidic compounds and through the photooxidation of some carotenoids.

The catabolism of ABA is similar to the one of carotenoidic pigments and triterpenic derivatives. Under the action of some specific enzymes (oxidase, lipooxidase, peroxidase) the abscisic acid suffers oxidative changes which can take place at the hexacycle's level. The path of the biosynthesis of abscisic acid and the majority enzymes involved were identified (Fig. 2). [13]

The molecular action mechanism of ABA in vegetal organism is not fully elucidated. It is known that ABA has a biochemical and physiological action against substances that stimulate growth, forming a biochemical "balance" that causes the plant to grow and develop in different phenophases and seasons in the temperate zones.

The inhibition of the growth and development of plants takes place because the biosynthesis of the ribonucleic acids inhibit, especially the RNAm blocking the processes of transcription and translation of information from the DNA's molecule. The proteins biosynthesis is blocked (enzymes). The processes of photosynthesis, breathe, oxidative phosphorilation are reduced. [3]

Another biochemical action mechanism of ABA in plants is the flux of inorganic ions adjustment through the biological membrane. This mechanism is present in leaves, in the cells that cause the stoma to close and open by regulating the content of $K^{+.}$

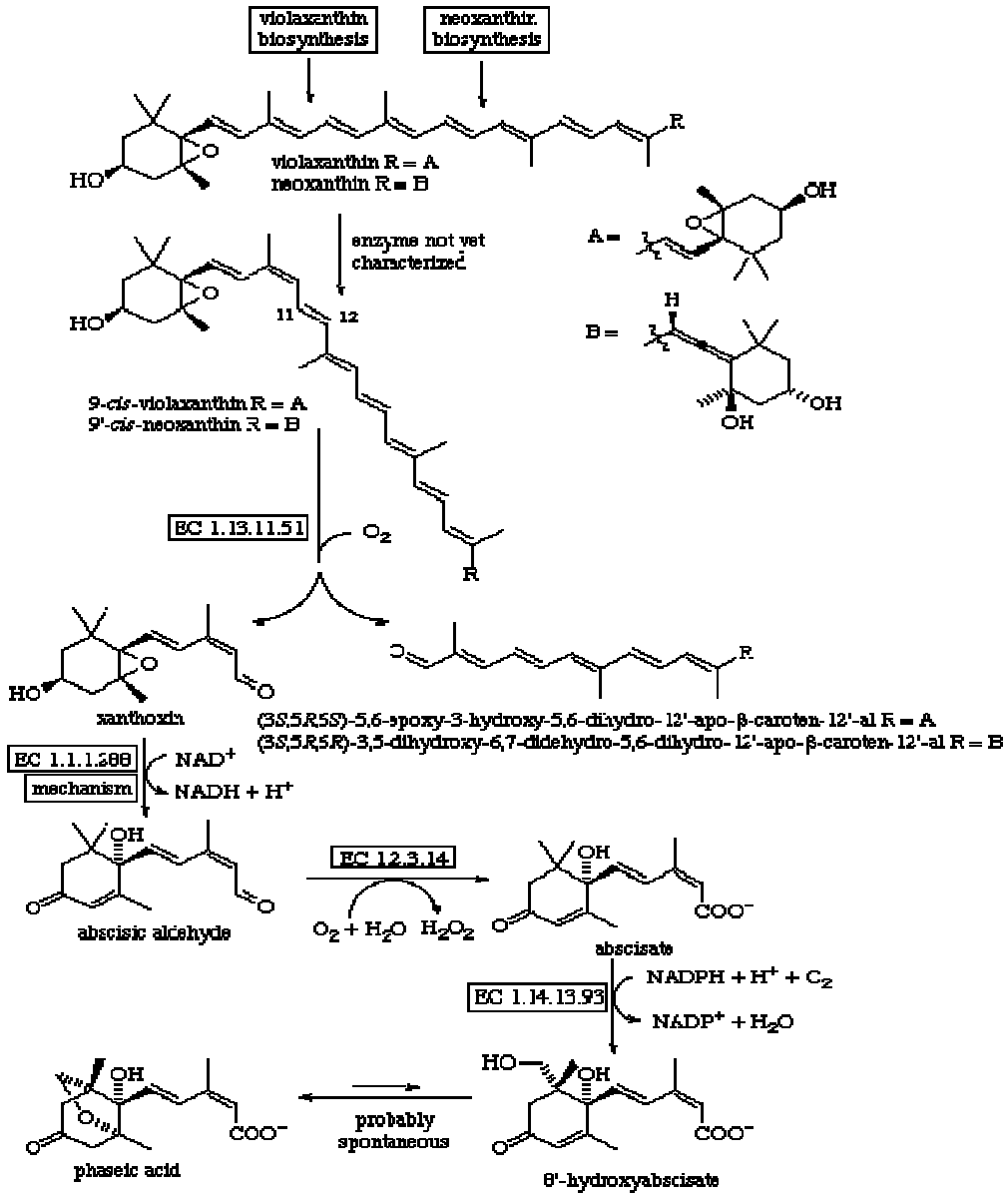


Fig. 2. The mechanism of the abscisic acid biosynthesis

During the time when plants are in the stage of stress, ABA accumulates in roots, causing the permeability of cellular membranes to increase, facilitating the processes of water and the mineral salts absorption. [3, 4]

Another action mechanism of ABA was noticed in the inhibition processes of seeds germination, potatoes germination – and that is the inhibition of enzymes involved in these processes (amylase, esterase, ribonuclease, protease). [9]

The fact that buds come to a stage of dormancy is due to the intensification of the biosyntheses of ABA in leaves. The seminal rest is also determined by the accumulation of ABA in fruit and seeds. [2]

A characteristic function of ABA is the production of abscission in fruit and leaves by creating a separation layer. The activity of some enzymes intensifies causing the median lamella (cellulase, pectinase) to deteriorate from the cellular membrane. By treating the fruit trees with ABA solutions it would biochemical weed out the fruit and flowers and it would increase their quality.

Abscisic acid delays seed germination in many plants. Has some effect on induction and maintenance of dormancy in general. It induces the synthesis of deposit proteins in seeds. It inhibits the effect of gibberellins on stimulating de novo synthesis of α-amylase. It impact decreases when seeds germinate. In desert plants, ABA found in the outer layer of the seeds must be washed away by rain in order for the seeds to germinate. [10, 11]

Abscisic acid inhibits stimulatory effects of other hormones. Inhibits shoot growth but will not have as much effect on roots or may even promote growth of roots. It inhibits cell division in the vascular cambium. It induces gene transcription especially for proteinase inhibitors in response to wounding. [6]

Ethylene acts where it is formed, it is not synthesizes in a certain place of the plant and then moved somewhere else, at the place of action, but it can spread through tissues.

Chemically, ethylene is an acyclic unsaturated hydrocarbon with 2 carbon atoms ($CH_2 = CH_2$) and it is the first compound of the alchene series. Though it has a small concentration in nature, ethylene is relatively vast spread because it is produced by almost all vegetal and animal organisms, being an endogenous metabolic compound. [3, 6]

The biosynthesis of ethylene takes place in more ways, in accordance with the precursors that have been used and the systems involved in these transformations. As precursors there are being used aminoacids (methionine, homoserine, β-alanine), organic acids (ethanol, propanol), aldehydes.

The biosynthesis of ethanol from methionine was intensely studied by using the marked atoms (C^{14}). The enzymatic transformation of methionine in ethylene was favored by the presence of Cu^{2+}, Fe^{3+} ions and peroxidase. [13]

The biosynthesis of ethylene in vegetal organism is influenced by the others phytohormones. [1] Cytokinines stimulate indirectly the biosynthesis of ethylene by stimulating the auxines absorption. The biosynthesis and degradation of ethylene in plants are influenced not only by the internal factors (phytohormones, enzymes), but also by several external factors (CO_2, O_2, temperature etc). [8]

In figure 3 it is presented the biosynthesis of ethylene in the mechanism of plants, having as precursor methionine. [13]

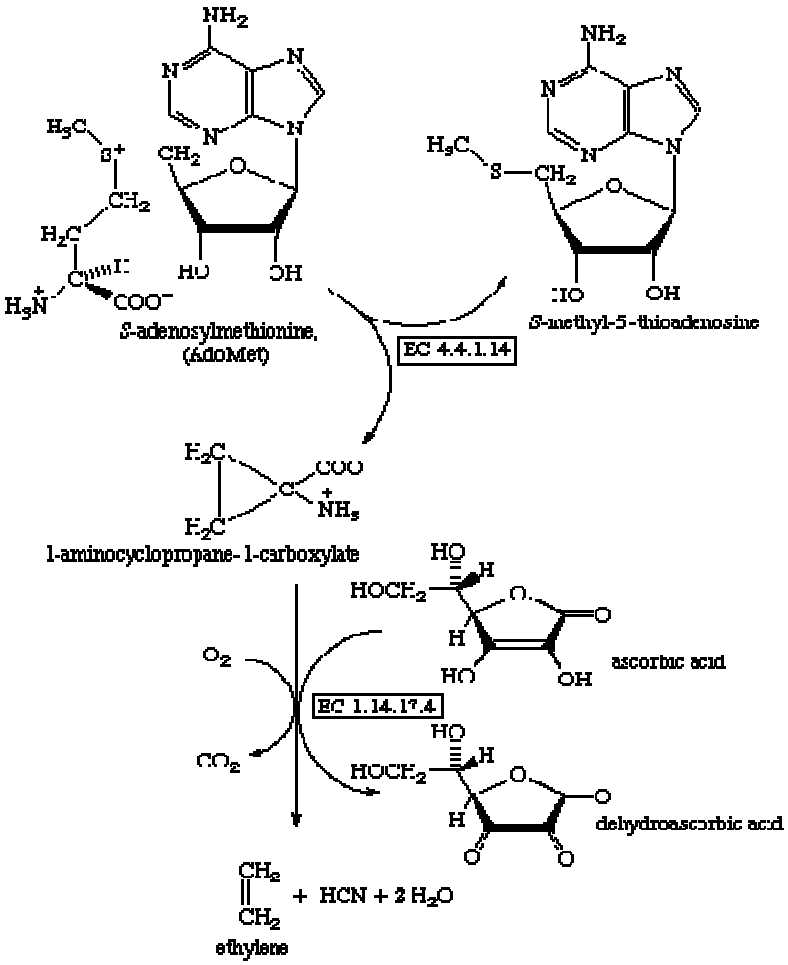


Fig. 3. The scheme of the methionine transformations in ethylene

For studying the *catabolisation of ethylene* there was used ethylene marked with C^{14}. These studies have shown that a part of the endogenous ethylene serves in the biosynthesis of other compounds (benzene, toluene), another part spreads in the atmosphere and another one is degrading in simpler metabolits (CO_2). [1].

Ethylene is not included in the classic hormonal scheme because, apparently, it does not suffer a directed transportation from where the biosynthesis takes place to the receptor (the place of action). It has synergic or antagonistic interactions with the others phytohormones, being an active participant at the endogenous regulation of the plant growth and development.

The multitude of research that has been made on the metabolism of plants have shown that ethylene is mostly involved in: fruit ripening, elongation, hypertrophy of inflorescence, expression of sex, the inhibition of growth and thickening of roots and steams, inhibition of cellular division, biosynthesis of nucleic acids. [2, 4]

Ethylene stimulates all these factors of fruit ripening: breakdown of chlorophyll and synthesis of other pigments; fruit softening by cellulase and pectinase, cell wall breakdown; formation of volatile compounds – attractants; conversion of starches and acids to sugars. [11]

Ethylene inhibits flowering in a lot of species, but promotes it only in few species. It also promotes senescence of flowering plants. An increase in ethylene production causes the breakdown of the middle lamella and this result in abscission.

The sex of flowers is determined by gibberellins and ethylene: for male flower by gibberellins, and for female flower by ethylene. Shaking increases ethylene production, which causes cells to grow short, which cause cells to form short, thick stems. [10]

Influencing the plant growth and development processes with the help of physiological active substances has built a permanent preoccupation for researchers, managing to establish the synergic and antagonistic effects between different chemical compounds. [7]

In biotechnological practice, phytohormones are used in small quantities – by order of ppm. They have a pronounced effect on the plant growth and development processes, especially on young organisms, if they are applied in the proper period of development. These characteristics will give phytohormones an important place in the agriculture of the future based on ecological systems. [12]

3. CONCLUSIONS

Using abscisic acid and ethylene as phytohormones – inhibitors in controlling the mechanisms of plant evolution - is a good alternative for an ecologic agriculture.

The existing studies over the mechanism of the abscisic acid action have shown its great importance as a bioregulator for plants.

It has been clarified its involvement in the following phases of the plant development: closure of stomata, bud dormancy and seed dormancy.

In the same time it has been proved the importance of the abscisic acid in the following processes: counteracts stimulatory effects, inhibits shoot growth, it inhibits cell division in the vascular cambium, induces gene transcription responsible with the synthesis of proteinase inhibitors.

Studies concerning the effects of ethylene as a bioregulator have shown its importance in the following phases of the plant evolution: fruit ripening, flowering, abscission, sex expression, stem elongation.

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HIGH INDIVIDUAL VARIATION IN REGENERATION RESPONSE OF SPINACH

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ABSTRACT

High variability in regeneration capacity among individual plants was observed in spinach (Spinacia oleracea L. cultivar Matador). Somatic embryos were induced from apical sections of axenic seedling's lateral roots (1 cm), cultivated on solid medium with Murashige and Skoog's mineral solution, 20 μM α-naphthaleneacetic acid and 5 μM gibberellic acid. In some individuals the regeneration commenced within the fourth week of culture and these individuals were superior in regeneration. By contrast, some individuals reacted poorly giving only few embryos during the second or even the third subculture. Regenerated plants flowered, self-pollinated and set seeds. The seeds were sown and the seedling's lateral roots were subjected to the induction of regeneration. The offspring's performance indicated that the regeneration capacity was under genetic control. The genotypes exhibiting high regeneration capacity gave offspring with similar potential, whereas genotypes that responded poorly gave also nonresponsive offspring. In a preliminary study, we selected a few lines with extremely high regeneration potential following three to four cycles of self-pollination. This system may be very useful in studying genetic control of somatic embryogenesis.

Keywords: ***Chenopodiaceae*, genotype, *in vitro*, somatic embryogenesis, *Spinacia oleracea*, seeds**

1. INTRODUCTION

Spinach has been considered as a species recalcitrant to *in vitro* regeneration for long period of time (Nešković and Radojević 1973). In later studies, we reported regeneration of the same spinach cultivar Matador (Zdravković-Korać and Nešković 1998, 1999), but the procedure was rather inefficient. Numerous reports showed high variation in regeneration response of different spinach cultivars (Al-Khayri *et al*. 1991; Komai *et al*. 1996; Knoll *et al*. 1997), and even high individual variations within a population of the same cultivar (Ishizaki *et al*. 2001). Accordingly, significant differences in regeneration response among inbred lines were shown in other species, such as maize (Rakshit *et al*. 2010), sunflower (Nestares *et al*. 2002) and sugar beet (Zhang *et al*. 2008). Chen *et al*. (1987) emphasized that highly productive individuals can be selected from poor cultivars of alfalfa, and Kumar *et al*. (1998) obtained somatic embryos in hybrids between cotton individuals selected for high frequency somatic embryogenesis and some completely irresponsive lines. Armstrong *et al*. (1992) showed that an elite maize inbred line with very low *in vitro* response increased regeneration capacity from 0.2% to 46% through breeding and selfing. Crossing regenerative and non-

regenerative lines of alfalfa, Wan *et al*. (1988) concluded that two complementary genes were responsible for regeneration *in vitro*. The same conclusion was drawn for melon (Molina and Nuez 1996) and *Cyclamen persicum* (Pueschel *et al*. 2003). All these findings suggest that the regenerative capacity is under genetic control.

Regeneration capacity in spinach varied among different types of explants (Komai *et al*. 1996) and most of authors showed high regenerative potential of spinach roots (Xiao and Branchard 1995; Komai *et al*. 1996; Knoll *et al.* 1997). Hence, we adopted a procedure of Knoll *et al.* (1997) in an attempt to increase regeneration response of cultivar Matador, which is well adapted to local climate and is a dominant cultivar on the Serbian market. Randomly chosen individuals of cultivar Matador exhibited high variation in regeneration success. Seeds obtained by *in vitro* self-pollination of some of these lines were use to test the offspring's performance. The goal of this study was to compare the parents and their offspring and to select lines with high regeneration capacity.

2. MATERIALS AND METHODS

Plant material

Spinach seeds of cultivar Matador were washed with a plenty of running water and a few drops of detergent (Fairy, Procter & Gamble), then immersed in 30% commercial bleach (4% NaClO, Panonija, Pančevo, Serbia) for 30 min, and in 15% bleach for 15 min. The seeds were rinsed with sterile distilled water, blotted dry on a piece of sterile filter-paper and planted in 90 mm Petri-dishes (20 seeds per dish) containing 25 ml of basal plant growth regulator (PGR)-free medium for germination. The Petri-dishes were sealed with Parafilm® M (Pechiney Plastic Packing, Chicago, IL, USA). Within the next 1–3 weeks the non-contaminated seedlings were picked out and collected on new Petri-dishes (three seedlings per dish) containing the same medium and grown for an additional few weeks, until the seedlings developed 4 leaves and the root system was well developed. Twenty randomly chosen seedlings were used for the experiment. These seedlings were marked as P-generation seedlings.

Basal medium

The basal medium contained MS (Murashige and Skoog 1962) mineral solution and 20 g/l sucrose, 100 mg/l myo-inositol, 2 mg/l thiamine, 2 mg/l pyridoxine, 5 mg/l nicotinic acid and 2 mg/l adenine, all purchased from Sigma-Aldrich (St. Louis, MO, USA). The media were solidified with 0.7% (w/v) agar (Torlak, Belgrade, Serbia) and pH was adjusted to 5.6 by using pH-meter before sterilization. The media were sterilized by autoclaving at 114ºC for 25 min.

Induction of regeneration from root sections and culture conditions

Procedure for the induction of regeneration was essentially as was described in Knoll *et al.* (1997), with some modifications. The lateral roots were isolated and 1 cm long apical sections were cut off and placed on solid MS medium with

20 μM α-naphthaleneacetic acid (NAA) and 5 μM gibberellic acid (GA_3). Explants were subcultured on the same medium at 4-week intervals.

Somatic embryos (SE) were isolated after recording at the end of each subculture and cultured on MS medium supplemented with 5 μM 6-furfurylaminopurine (kinetin, Kin) for rooting and multiplication.

The seedling's shoot apices were also isolated and cultured in test tubes (one plant per tube) with 10 ml of solid MS PGR-free medium for 12 weeks. Apical shoot and root sections of the same plant were marked with the same number.

All cultures were maintained under cool white fluorescent tubes with a photon flux density of 55 μmol m^{-2} s^{-1} and a 16 h day length at 25 ± 2ºC.

Second generation seedlings and the induction of regeneration

Second-generation seedlings were obtained by *in vitro* self-pollination of plants regenerated from somatic embryos and apical shoot-derived plants. The seeds were harvested aseptically and planted on MS PGR-free medium. The seedlings were designed S1-generation and marked with the same number as the parental genotype plus individual consecutive number. Root sections were isolated from S1-seedlings, and the induction of regeneration was performed as for P-seedlings.

Recordings and statistical analysis

All cultures were placed in a completely randomized design. For induction of regeneration, six samples (Petri-dishes) with five subsamples (root sections) (n = 30) were prepared for each genotype. Twenty P-seedlings and fifteen S1-seedlings were used for the experiment. The number of SE was recorded with the aid of a stereomicroscope at 4-week intervals over 12-week period.

Statistical analysis was performed using analysis of variance (ANOVA) and Duncan's post-hoc test at $P \leq 0.05$. Data were subjected to angular transformation before analysis and inversely transformed for presentation. The results were expressed as the frequency of root sections regenerating somatic embryos and the mean number of somatic embryos per regenerating root section during 12-week period. To take into consideration both values simultaneously, we used an index of somatic embryo-forming capacity (EFC), calculated as follows:

EFC = (mean number of SE per regenerating section) x (% of regenerating sections) / 100.

3. RESULTS AND DISCUSSION

Induction of regeneration

We induced SE regeneration from apical sections of seedling's lateral roots using PGR combination (20 μM NAA + 5 μM GA_3) and type of root fragments, which were optimal in a study of Knoll *et al*. (1997). Root sections began to proliferate during the first week of culture, but the proliferations were very limited and no voluminous calli formed. SE regenerated directly from these proliferations (Fig. 1a, b). This is in accordance with the results of Knoll *et al.* (1997), who

demonstrated direct regeneration from proliferating root epidermal and subepidermal cells, without a callus phase. However, morphogenic pathways in these two studies were different, somatic embryogenesis in this study and caulogenesis in the study of Knoll *et al*. (1997). Komai *et al*. (1996) also obtained SE from seedlings' root sections of cultivar Jiromaru using the same PGRs (10 μM NAA + 0.1 μM GA_3), but the process of somatic embryogenesis was through a callus phase. The differences obtained in the three studies may be attributed to different genetic background, since different cultivars were used in each study, as well as to different explant pretreatment and the stage of development. We used roots of 2-3 weeks old seedlings, as was done by Komai *et al*. (1996), while Knoll *et al*. (1997) used roots induced from primulae and hypocotyls on NAA-supplemented media.

Regeneration success of P-seedlings

Out of 20 randomly chosen seedlings, 16 were capable to regenerate during three subcultures, with variable regenerative success (Fig. 2.). The frequencies of regeneration of the responsive genotypes varied from 0.3% to 64.5% (Fig. 2a), and the mean SE number per regenerating explant ranged from 1 to 10.8 (Fig. 2b) The most responsive genotypes were P7, P11, P12, P13 and P18, which formed 94, 67, 88, 145 and 136 SE, respectively, for 12-week period (not shown). These genotypes exhibited both high frequency of regeneration and the mean SE number per explant. EFC indexes, which take into account both of these values, reached 1.88 - 4.26 (Fig. 2c). By contrast, some other genotypes formed only a few SE during this period, exhibiting EFC indexes just a little bit above zero. Dynamics of the regeneration process was also variable. During the first subculture only genotype P18 regenerated. In the second subculture nine new genotypes regenerated, and the remaining 6 genotypes regenerated until the end of the third subculture (not shown). Four genotypes were irresponsive. Our results are in accordance with Ishizaki *et al*. (2001), who demonstrated high individual variation in embryogenic capacity of spinach cultivar Nippon, although Nippon showed higher regeneration response than Matador, reaching the frequency of up to 90% in some individuals.

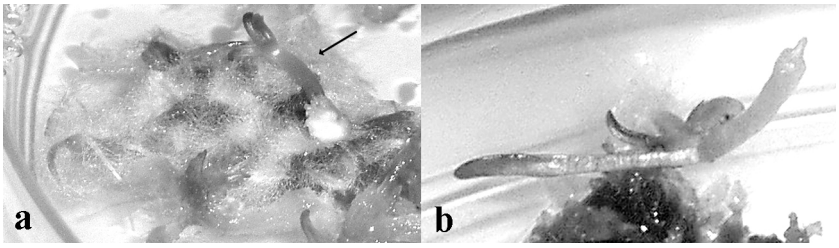

Fig. 1. - (**a**, **b**) Somatic embryo regeneration from spinach seedling's root sections cultivated on MS medium with 20 μM NAA + 5 μM GA_3 for 8 weeks.

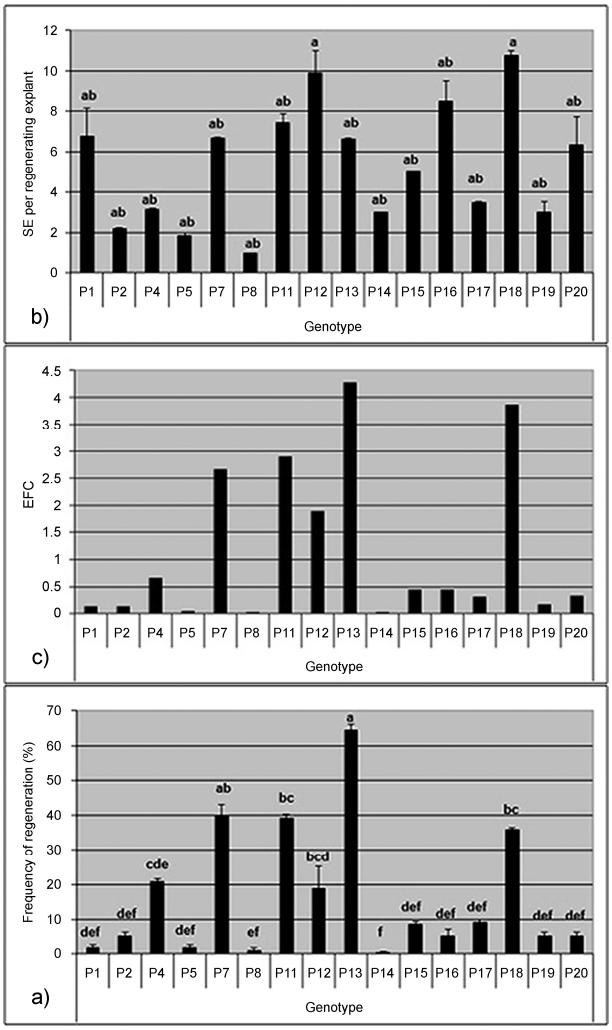

Fig. 2. - Somatic embryo (SE) regeneration from apical root sections of 20 randomly chosen spinach seedlings (P genotypes), cultured on MS medium with 20 μM NAA and 5 μM GA_3 for 12 weeks. **a**) Frequency of regeneration, **b**) the mean embryo number per regenerating explant, **c**) EFC (embryo-forming capacity). Data indicate the mean ± standard error (n = 30). Genotypes with no regeneration (P3, P6, P9 and P10) are not presented. Treatments denoted by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's test.

This finding explained the inefficiency of previous studies conducted with cultivar Matador (Nešković and Radojević 1973; Zdravković-Korać and Nešković 1998, 1999). Comparing to cultivars Nippon (Ishizaki *et al.* 2001), Longstanding

(Knoll *et al*. 1997, Zhang and Zeevaart 1999), Jiromaru, Nihon and Hoyo (Komai *et al*. 1996) cultivar Matador exhibited poor regeneration capacity.

In vitro seed production

Apical shoots isolated from all 20 seedlings and cultivated in test tubes for 12 weeks, developed into healthy plants, which attained maturity, flowered, self-pollinated and set seeds. Seeds did not require a cold treatment for germination, as was also noticed by Al-Khayri *et al*. (1992). Surprisingly, some seeds germinated while were still attached to the mother plant (vivipary). Eleven seeds were obtained from P5 plant, and all of them germinated, but only three (namely 5-2, 5-3 and 5-11) were suitable for the experiment.The rest of the seedlings failed to develop good root system and were discarded. P9 set four seeds; all of them germinated and three (9-1, 9-3 and 9-4) were used. P17 gave two good-quality seedlings (17-1 and 17-2). The rest of primulae-derived plants were dioecious or failed to self-pollinate. In addition, SE were grown on 5 μM Kin, which was shown to be beneficial for further SE development (Zdravković-Korać and Nešković 1998). Mature SE-derived plants flowered and set seeds. SE-derived plants of line P18 produced 14 seeds, and seven seedlings were suitable for the experiment (18-1, -2, -3, -4, -7, -9 and -14). In this study, high S1 seed germination of nearly 100% was attained. Similarly, seeds harvested from acclimatized spinach plants obtained by Knoll *et al*. (1997) germinated at 95%.

The selected S1 lines originated from parents of extremely different performance. While P18 exhibited high regenerative capacity (EFC = 3.86), P17 showed moderate regeneration success (EFC = 0.32), P5 was unsuccessful (EFC = 0.03), and P9 was completely irresponsive (EFC = 0) (Fig. 2c).

Regeneration success of the S1-seedlings

The S1-seedlings generally showed higher regeneration capacities than P-seedlings, in account of higher the frequencies of regeneration (Fig. 3a), while the mean SE number per explant was not significantly different among the lines (Fig. 3b). In spite of that, the relative differences observed among P-lines remained conspicuous for S1 lines. The offspring of P18 showed much higher regeneration capacities with EFC indexes of 2.73-8.07, followed by P17's offspring (EFC = 0.86-1.42), P5's offspring (EFC = 0.06-0.39) and P9's offspring (EFC = 0-0.01) (Fig. 3c). Consequentially, the overall SE number varied from a few to 226 per line (not shown). In addition, the S1 lines reacted in exactly the same meaner as the corresponding parent. Root sections of lines P5 and P18 developed a number of secondary roots, whereas P9 formed almost no secondary roots (not shown). So, all P5-and P18-derived lines developed numerous secondary roots, whereas P9-derived lines developed no or only a few secondary roots. Concerning dynamics of the process of somatic embryogenesis, only lines 18-1, 18-2, 18-9 and 18-14 regenerated during the first 4-week subculture, exactly as the parent P18.

Preliminary results obtained with S2-, S3- and S4-generation seedlings, selected for the best regenerative capacity, indicate dramatic increase of both the frequencies (nearly 100%) and the mean SE number (above 20 per explant) (In preparation for publication). Besides, time necessary for *in vitro* regeneration was shortened to 4-6 weeks. This is consisted with a conclusion of Chen *et al*. (1987) who stated that highly productive individuals could be selected from poor cultivars like Matador.

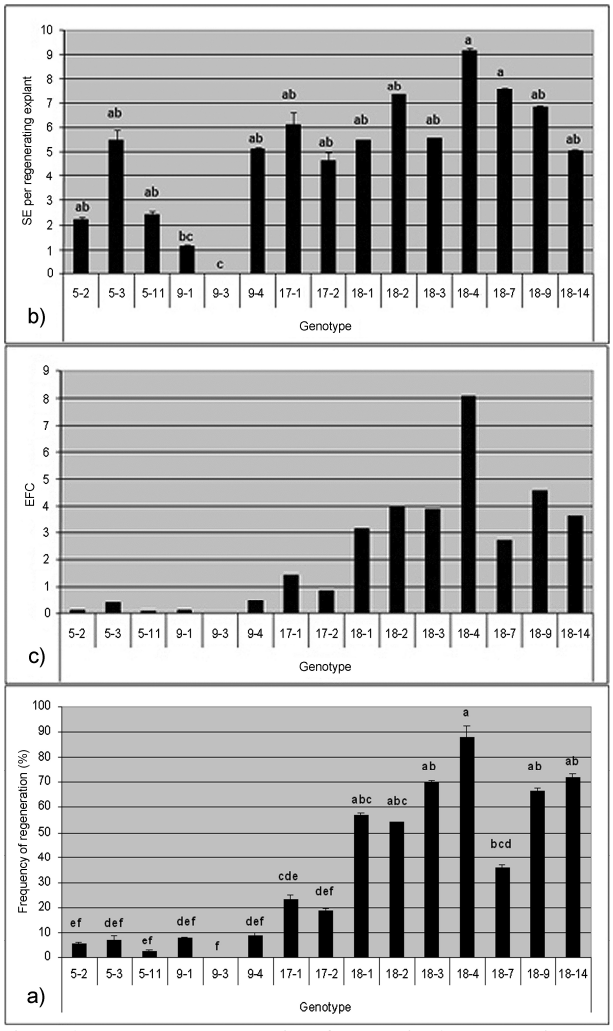

Fig. 3. - Somatic embryo (SE) regeneration from apical root sections of 15 seedlings, obtained by *in vitro* self-pollination of P-plants, and cultured on MS medium with 20 μM NAA and 5 μM GA_3 for 12 weeks. **a**) Frequency of regeneration, **b**) the mean embryo number per regenerating explant, **c**) EFC (embryo-forming capacity). Data indicate

the mean ± standard error. Six samples, each with five subsamples (n = 30), were used per genotype. Treatments denoted by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's test.

4. CONCLUSIONS

In this study we showed high variability of regeneration capacity within a population of spinach seedlings of cultivar Matador, which explained the inefficiency of previous attempts with this cultivar. Selecting for highly responsive individuals and then subjecting them to self-pollination dramatically increased regeneration response. These highly responsive lines are suitable for genetic transformation for the variety improvement and other fundamental studies.

ACKNOWLEDGEMENTS

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IMPACT OF PHOTOPERIOD ON SPINACH REGENERATION CAPACITY

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ABSTRACT

High variation in regeneration capacity of spinach at individual level masked the differences between regeneration responses in long days (LD) and short days (SD) in previous studies. In this study, we exposed explants isolated from the same individuals to both SD (8/16h light/dark period) and LD (16/8h light/dark period). Apical sections (1 cm) of seedling's lateral roots were used for the experiment. Root sections were placed on solid medium with Murashige and Skoog's mineral solution, 20 μM α-naphthaleneacetic acid and 5 μM gibberellic acid. A half of root sections isolated from a single seedling has been exposed to SD, and the other half of root sections has been exposed to LD. In all of thirty seedlings used for the experiment, somatic embryos were noted in LD a few weeks earlier than in SD. Both the frequency of regeneration and the mean number of somatic embryos were significantly higher in LD than in SD. Some of genotypes regenerated in LD only. We assume that the process of somatic embryogenesis in spinach is triggered and controlled by photoperiod.

Keywords: ***Chenopodiaceae*****, photoperiod, somatic embryogenesis,** ***Spinacea oleracea*****, tissue culture**

1. INTRODUCTION

Spinach is a vegetable crop used for human consumption, grown for leaves, which are rich in vitamins and other essential nutrients. At short days (SD) spinach plants force growth of vegetative organs, forming rosette, while in long days (LD) spinach plants bolt and flower. Eight continuous cycles of at least 12h of light are required for the induction of flowering (Ćulafić 1973), but spinach plants could be induce to flower in SD conditions in response to different environmental stimuli, such as high temperature and egzogenously supplied GA_3 (Ćulafić and Nešković 1980).

In spite of this, almost all researches dealing with spinach tissue culture chose LD photoperiod for culture growth. In a preliminary study, we intended to cultivate spinich in SD, to prevent precocious flowering, but the induction of regeneration was lower in SD than in LD, although the differences seemed statistically insignificant. However, variations in regeneration response were observed among different spinach cultivars (Al-Khayri *et al*. 1991a; Komai *et al*. 1996; Knoll *et al*. 1997), and even within population variations were reported for cultivars Nippon (Ishizaki *et al*. 2001) and Matador (Milojević *et al*. 2009). So, we assumed that high individual variations in regeneration response masked the differences caused by photoperiod. In this study, we assesed regeneration response of root sections of

the same seedling exposed to both SD and LD and found high statistical significance for both photoperiod and genotype at individual level.

2. MATERIALS AND METHODS

Plant material

Spinach seeds of cultivar Matador were washed with running water and a few drops of detergent, then immersed in 30% commercial bleach (4% NaClO) for 30 min, and in 15% bleach for 15 min. The seeds were rinsed with sterile distilled water, blotted dry on a piece of sterile filter-paper and planted in 90 mm Petri-dishes (20 seeds per dish) containing 25 ml of basal plant growth regulator (PGR)-free medium for germination. The cultures were exposed to a 16 h day length at photon flux density of 55 μmol m^{-2} s^{-1}. Non-contaminated seedlings were collected on new Petri-dishes (three seedlings per dish) containing the same medium and grown for an additional 1-2 weeks, until the seedlings developed 4 leaves and the root system was well developed.

Basal medium

The basal medium contained MS (Murashige and Skoog 1962) mineral solution and 20 g/l sucrose, 100 mg/l myo-inositol, 2 mg/l thiamine, 2 mg/l pyridoxine, 5 mg/l nicotinic acid and 2 mg/l adenine. The media were gelled with 0.7% (w/v) agar (Torlak, Belgrade, Serbia) and pH was adjusted to 5.6 before sterilization, by using pH-meter. The media were sterilized by autoclaving at 114ºC for 25 min.

Induction of regeneration from root sections and culture conditions

Procedure for induction of regeneration was essentially as was described in Knoll *et al.* (1997), with some modifications. Seedling's lateral roots were excised and 1 cm long apical sections were cut off and placed on basal medium with 20 μM α-naphthaleneacetic acid (NAA) and 5 μM gibberellic acid (GA_3). For every seedling, one half of root sections was exposed to short days (SD) whereas the other half was exposed to long days (LD). Explants were subcultured on the same medium at 4-week intervals.

All cultures were maintained at 25 ± 2ºC, under cool white fluorescent tubes with a photon flux density of 55 μmol m^{-2} s^{-1} and two photoperiods: SD (8*h* light/16*h* dark period) and LD (16*h* light/8*h* dark period).

Recordings and statistical analysis

For induction of regeneration, 30 randomly chosen seedlings were used. Four Petri-dishes with 5 root sections, were prepared for each of the two treatments (SD and LD photoperiods) for each seedling. One seedlings was referred as one genotype throughout the text. The number of somatic embryos (SE) was recorded with the aid of a stereomicroscope at 4-week intervals over 12-week period. SE were harvested and removed at the end of each subculture.

The effects of photoperiod and genotype on SE induction were evaluated using standard two-factor factorial Analysis of Variance (ANOVA). The difference

between SD and LD response of the same genotype was tested using t-test for independent samples. Percentage data were subjected to angular transformation and SE number data to square root transformation before analysis and inversely transformed for presentation. The results were expressed as the frequency of root sections regenerating SE and the mean number of SE per regenerating root section during 12-week period. An index of somatic embryo-forming capacity (EFC) was used to evaluate cumulative effect of both the mean SE number and frequency of regeneration, and it was calculated as follows: EFC = (mean number of SE per regenerating section) x (% of regenerating sections) / 100.

3. RESULTS AND DISCUSSION

Seedling's root sections cultivated on both photoperiods began to proliferate after a week of culture, and first differences were observed at the end of the first subculture. SE appeared only from roots of genotype 110 exposed to LD. The regeneration of this genotype was delayed in SD for 2-3 weeks. During the second subculture 23 new genotypes regenerated, and 12 of these regenerated only in LD. Finally, two genotypes regenerated in the third subculture in LD only, and four genotypes were irresponsive. By the end of the experiment, out of 26 responsive genotypes, 9 regenerated only from explants exposed to LD. The frequences of regenerating root explants and the SE mean number were higher in LD than in SD (Fig. 1a,b). ANOVA confirmed that both genotype and photoperiod significantly affected the regeneration process ($P \leq 0.00001$), as well as their interaction. Hence, t-test for dependent samples was performed for each genotype to asess the differences between two photoperiods. This analysis confirmed the previous notion. The frequencies of regeneration and the SE mean numbers of all genotypes were higher in LD than in SD (Fig. 1a,b). The differences were even more conspicuous when the EFC indexes were calculated (Fig. 1c). They were dramatically higher for LD, with no exceptions. The overall SE number, calculated for the whole period of 12 weeks, also varied significantly among genotypes and between photoperiods (not shown).

The highest SE number was attained in genotypes 107, 110, 115, 119 and 125, which produced 243, 480, 220, 232, 352 SE respectively in LD, and 9, 22, 14, 34 and 63 SE respectively in SD. The poorest response was in genotypes 102, 103, 105, 128 and 130, which produced 15, 21, 19, 9 and 9 SE, respectively in LD, and among them only genotype 102 regenerated 2 SE in SD.

High variability among randomly chosen seedlings obtained in this study is in agreement with the results obtained in cultivar Nippon by Ishizaki *et al*. (2001). The later study was performed in LD only. However, Geekiyanage *et al*. (2006) obtained higher regeneration response of cultured cotyledons in SD then in LD, but only at high light intensity of 90-100 μmol m^{-2} s^{-1}. We assume that high variability

among the treatments in that study may be also a consequence of individual variations.

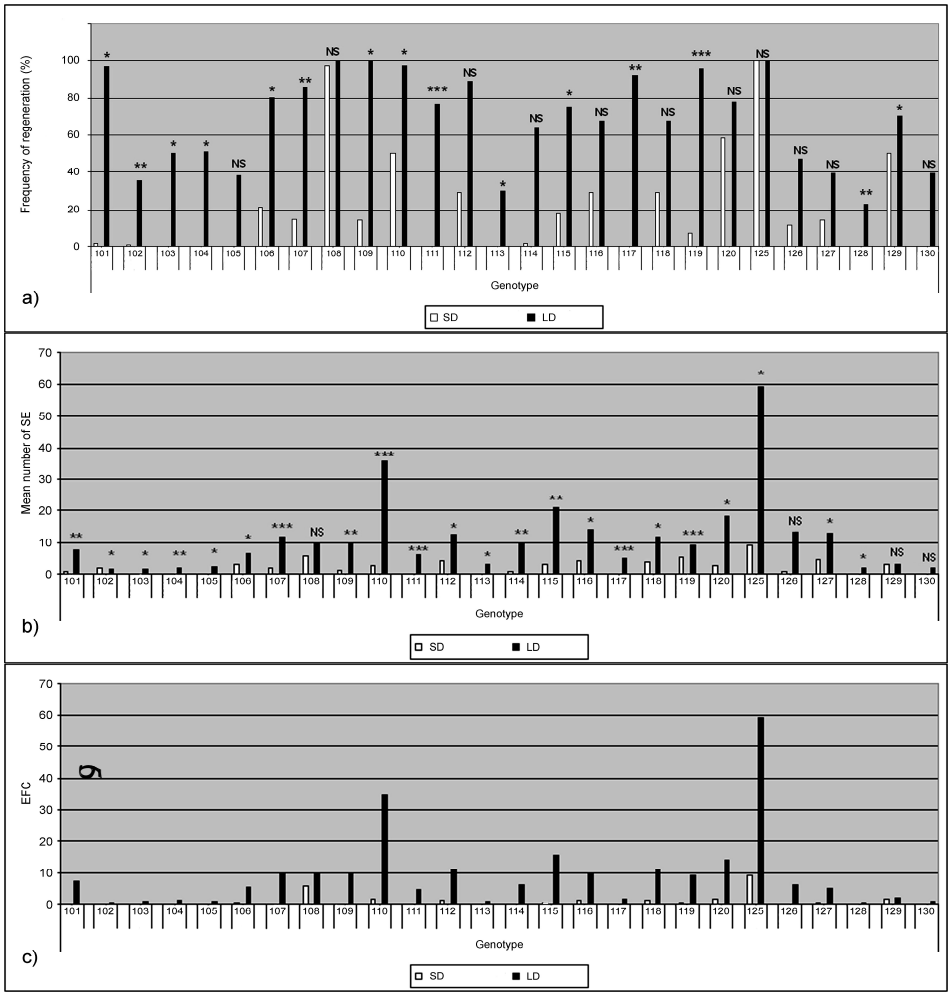

Fig. 1. - Somatic embryo regeneration from root sections of spinach seedlings cultivated on MS medium with 20 µM NAA and 5 µM GA_3, exposed to short day (SD) and long day (LD) photoperiod for 12 weeks. **a**) Frequency of regeneration, **b**) the mean SE number per regenerating explant, **c**) EFC (embryo-forming capacity). Thirty seedlings were used for the experiment and roots of each seedling were exposed to both photoperiods. Genotypes with no regeneration (121-124) are not presented. Data indicate the means (n = 20). T-test with dependent samples was used to asess SD/LD pairs within each genotype. Symbols used to denote significance between SD/LD pairs: NS - not significant, * significant at 95% level, ** significant at 99% level, *** significant at 99.9% level.

GA_3 plays an important role in spinach plant regeneration. From the earliest report (Nešković and Radojević 1973) to the modern days authors empasized its indispensability (Al-Khayri *et al*. 1991a, 1991b, 1991c, 1992; Molving and Rose 1994; Xiao and Branchard 1995; Knoll *et al.* 1997; Komai *et al.* 1996; Ishizaki *et al*. 2001). Ishizaki *et al*. (2001) pointed out that some highly responsive spinach lines did not regenerate in the abscence of GA_3, and that GA_3 increased the regenerative capacity of all lines in a dose response manner. All these findings may be in a correlation with fact that some stages in biosynthetic pathways of gibberellins were controlled by photoperiod. Biosynthesis of ent-kauren, first stabile precursor of gibberellins, was enhanced in LD condition (Zeevaart and Gage 1993), as well as oxidation of $GA_{53} \rightarrow GA_{44}$ and $GA_{19} \rightarrow GA_{20}$ (Gilmour *et al.* 1986). Wu *et al*. (1996) demonstrated enhanced expression of gene coding for GA_{20}-oxidase in spinach in LD. So, higher regeneration response of explants in LD could be explained by higher level of endogenous gibberellins, probably different of GA_3. GA_1, whose level is higher in LD than in SD in spinach (Wu *et al*., 1996) is active *per se* (Zeevaart *et al*. 1993) and it may influence the process of regeneration.

4. CONCLUSIONS

We indubitably demonstrated LD-stimulation of the process of somatic embryogenesis from root sections of spinach and confirmed very high individual variability of regeneration response. This study is a valuable contribution to the efficiency of spinach regeneration, usable in breeding programs for developing new varieties. On the other hand, as gibberellin biosynthetic pathway was readily studied in spinach and the gene sequences, their expression and enzymes activities are well known, this system may be valuable in highlighting the role of gibberellins in the process of regeneration in spinach.

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INFLUENCE OF POLYETHYLENE GLYCOL (PEG) CONCENTRATIONS ON WATER STRESS INDUCING

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Keywords: plantlets, concentration, growth, height, leaves

ABSTRACT

Drought causes changes in growth of plants. Tissue culture is useful in studying the mechanism of tolerance to water stress "in vitro" conditions. Culture technique "in vitro" reduced variation due to medium (the nutria medium, controlled conditions). Polyethylene glycol is used in simulating water stress for plants and as such, and the influence of PEG was studied to NIRDPSB Brasov. It had been experienced over the plantlets "in vitro", on Romanian cultivars. Influence of modified media supplemented with polyethylene glycol on growth in height of plantlets and number of leaves/plantlets obtained "in vitro" was different in the two cultivars tested.

INTRODUCTION

Drought is one of the most common environmental stresses affecting plant growth and productivity (Boyer, 1982). Plant cell and tissue culture has been an useful tool to study stress tolerance mechanisms under *in vitro* conditions (Bajji et al., 2000). Water deficit is a common stress in potato production, which leads to decrease in tuber quality and yield. Because of potato susceptibility to drought (Hassanpanah et al, 2008), preparing sufficient water is very important for increasing potato quality and quantity.

It is very important to study about tolerance of different potato cultivars against water deficit stress. Water deficit decreased number of leaves, plant water potential (Frensch, 1997), leaf area, stem height, ground coverage, tuber number, growth and yield.

In this study, our aim is to determine the potential reactions of plants to drought "in vitro" by inducing water stress using polyethylene glycol (PEG).

MATERIAL AND METHOD

In Plant Tissue Culture Laboratory of NIRDPSB Brasov, in 2009, was fitted the experience that followed the influence of polyethylene glycol PEG's on the plantlets, experience bifactorial, with 10 options, divided into three repetitions.

To achieve the experience, have been studied experimental variants presented in next table:

Table 1. Experimental variants depending of PEG concentration (NIRDPSB Brasov, 2009)

Var.	Cultivar (a)	Basic medium used	PEG concentration	Component of layers (b)	Number of layers
V_1	Christian (a_1)	MS+ sucrose (20g/l)+ Phyto - Agar (8 g/l)	-	MS (b_1)	1
V_2			0,006 M PEG	MS + 0.006 M PEG (b_2)	1
V_3			0,006 M PEG	- bottom layer: MS + 0.006 M PEG - upper layer: MS (b_3)	2
V_4			0.012 M PEG	MS + 0.0012 M PEG (b_4)	1
V_5			0.012 M PEG	- bottom layer: MS + 0.012 M PEG - upper layer: MS (b_5)	2
V_6	Roclas (a_2)	MS+ sucrose (20g/l)+ Phyto - Agar (8 g/l)	-	MS (b_1)	1
V_7			0,006 M PEG	MS + 0.006 M PEG (b_2)	1
V_8			0,006 M PEG	- bottom layer: MS + 0.006 M PEG - upper layer: MS (b_3)	2
V_9				MS + 0.0012 M PEG (b_4)	
V_{10}			0.012 M PEG	- bottom layer: MS + 0.012 M PEG - upper layer MS (b_5)	1

Bifactorial experience axb (5x2) 10 variants was performed using the following graduations:

- experimental factor a, cultivar, with 2 graduations:
  - a_1- Christian
  - a_2- Roclas
- experimental factor b, nutrition medium, with 5 graduation:
  - b_1- M1 medium witness, Murashige – Skoog, enriched with vitamins, 20g/l sucrose, 8g/l agar;
  - b_2- M2 contains a single layer: Murashige - Skoog, enriched vitamins 20 g/l sucrose,

8g / l agar, 0.006 M PEG

  - b_3-M3- contains double layer: bottom layer medium was Murashige - Skoog,

enriched with 20 g / l sucrose, 8g / l agar, 0.006 M PEG, and the upper layer was without PEG (which is poured after the first layer was solidifiat)

- b_4-M4- contains a single layer
- b5-M5- - contains double layer: bottom layer medium was Murashige - Skoog,

enriched with 20 g / l sucrose, 8g / l agar, 0.012 M PEG, and the upper layer was without PEG.

In his experience was working with Romanian varieties: Christian and Roclas.

Polyethylene glycol (PEG) with molecular weight of 6000, was used as a simulator of water stress "in vitro". Have been used two PEG concentrations (0.006 M, 0.012 M). The investigations were made both on a single layer of polyethylene glycol, and dual-layer (bottom layer: MS and PEG, and the upper layer: MS).

There have been measurements of the height of plantlets, number of leaves, at six to eight weeks from inoculation to determine the response to drought.

Fig.1. Plantlets with control medium

Fig.2. Plantlets with PEG concentrations, reduced height and reduced number of leaves

RESULTS AND DISCUSSION

Results on the influence of modified media supplemented with polyethylene glycol on the height of plantlets obtained "in vitro", for the varieties tested

Analyzing the growth of plantlets (Table 2) for Christian cultivar on the five culture media is observed that the presence of PEG-site to varying degrees of water stress simulated the growth of plantlets was inhibited. These results are shown in fig 3, where V1 is apparent that the control has not hampered the growth of plants, resulting from experiments height is 12.53 cm. The lowest height of the plantlets is observed for the variant with addition of 0.012 M PEG which strongly inhibited growth, the resulting plantlets height was 2.27 cm.

If we examine the variations in terms of number of layers used in culture media may indicate that variants 2 and 4 containing a single layer of culture medium enriched with 0.006 M, 0.012 M PEG of PEG that have directly

influenced the plant height resulting in the lowest values that differ from the control of 12cm and 10.26 cm respectively.

Table 2. Plantlets height variation in the number of leaves in the varieties tested "in vitro" experimental variants

Var.	Cultivar, a	Plantlets height (cm)	Number of leaves/ plantlets
1	Christian a1	12,53	15,27
2		2,53	3,53
3		4,43	8,33
4		2,27	2,27
5		3,60	5,13
6	Roclas a2	10,33	13,40
7		2,27	3,33
8		4,27	6,47
9		1,53	2,47
10		3,43	4,47

In case of Roclas cultivars, fig. ., it also notes that the version control, V1 has obtained the highest plants, with an average height of 10.33 cm and the smallest heights were obtained in variant 4 with an average height of 1.53 cm.

We can see that the presence of PEG-site at a rate of 0,012 m of water stress inhibited growth ensuring.

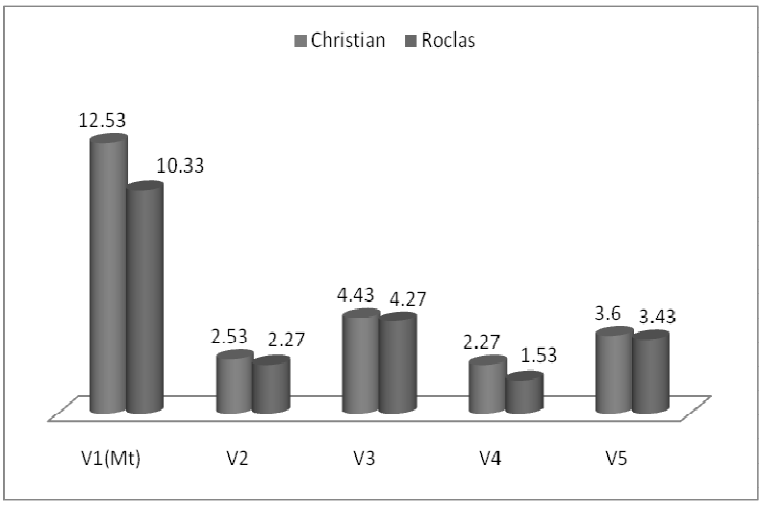

Fig.3. Variation of plantlets height for the cultivars tested(NIRDPSB Brasov, 2009)

If we compare the two varieties Roclas and Christian, the height of plantlets (Fig. .) for Christian cultivar can say that has responded better than getting Roclas potato plantlets (plantlets height was bigger in all cases).

The statistical interpretation of results of the plantlets height

From assessment results, it is noted that the Christian cultivar exceeded the average value of Roclas cultivar, the difference was not statistically, insignificant, negative, -0,661 for Roclas cultivar (Table 3).

Table 3 . Influence of variety on growth in height of plantlets (NIRDPSB Brasov, 2009)

Cultivar	Height		Dif. (cm)	Significance
	(cm)	(%)		
Christian (a_1) (Ct)	5,027	100,00	-	-
Roclas (a_2)	4,366	86,85	-0,661	Insignificant

DL 5% = 2,7663 cm
DL 1% = 4,1841 cm
DL 0,1% = 6,7216 cm

Overview of the plantlets height (Table 4) of the two cultivar have very significant negative results in the same variety, but insignificant between the two varieties, which shows that the culture medium influenced the very significant growth in both cultivars but plantlets results were not significantly influenced by self-chosen variety.

Table 4. Combined influence of cultivar and culture medium on the growth of plantlets height (NIRDPSB Brasov, 2009)

Cultivar (a) Medium (b)	Christian (a_1)			Roclas (a_2)			Dif. between cultivars
	(cm)	Dif. (cm)	Signif.	(cm)	Dif. (cm)	Signif.	a_2-a_1 (cm)
b_1(Ct)	12,53	-	-	10,33	-	-	2,20 insignif.
b_2	2,53	-12,00	ooo	2,27	-8,06	ooo	0,26 insignif
b_3	4,43	-8,10	ooo	4,27	-6,06	ooo	0,16 insignif
b_4	2,27	-10,26	ooo	1,53	-8,80	ooo	0,74 insignif
b_5	3,60	-8,93	ooo	3,43	-6,90	ooo	0,17 insignif

DL 5% = 3,13 cm
DL 1% = 4,24 cm
DL 0,1% = 5,65 cm

DL 5% = 4,144 cm
DL 1% = 5,231 cm
DL 0,1% =6,012 cm

Looking at plant height using regression (Fig. 4) between the concentration of PEG and increasing the height of plantlets can be said that the use of polynomial regression coefficients of two terms, show highly significant correlations of 0.9803 and 0.9812 for the Roclas cultivar and Christian cultivar.

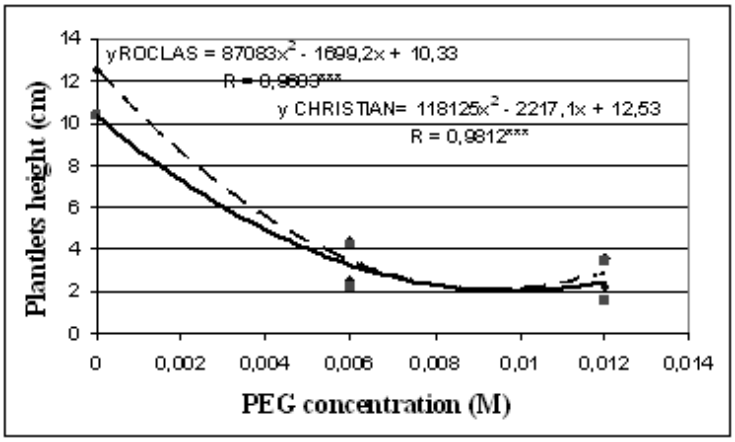

Fig. 4. Influence of the addition of PEG on the plantlets height

Results on the influence of modified media supplemented with polyethylene glycol on the number of leaves of plantlets obtained "in vitro", for the varieties tested

For Christian cultivar, unmodified culture medium without addition of PEG, determining obtaining an average number of leaves on plantlets of 15.27. On versions with modified culture media decreased the number of leaves, the lowest number being recorded in for variant 4 with 0.012 m with an average PEG of 2.27.

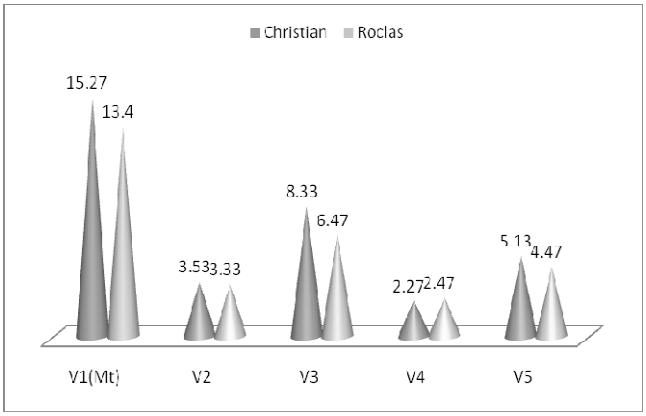

Fig.5. Variation of number of leaves on different culture medium to experimental varieties (NIRDPSB Braşov, 2009)

For Roclas cultivar, the highest number of leaves on the environment has been medioum control, medium without added PEG, the value being 13.4; the lowest number of leaves on plantlets was obtained variant 4 of medium which had a PEG concentration of 0,012 M (fig. 5).

Statistical interpretation of results obtained by the number of leaves on plantlets of varieties tested

In the case of the average values obtained from the analysis of cultivar, it is noted that the differences are small by -0.872 leaves and were statistically significant as distinct, but negative (Table 5).

Table 5. Influence of variety on the average number of leaves per plantlets for the two varieties (NIRDPSB Braşov, 2009)

Var.	**Cultivar**	**Number of leaves/ plantlet**		**Dif.**	**Significance**
		Nr.	**%**		
A1 (Mt)	Christian (a_1)	6,900	100,00	-	-
A2	Roclas (a_2)	6,028	87,36	- 0,872	oo

The statistical interpretation of the combined influence of these two factors the cultivar and the culture medium (PEG's presence in the substrate) shows that differences between cultivars are statistically distinct and significant for the control variant 3 and are not statistically the other variants (Table 6).

Table 6. Combined influence of variety and culture medium over emergence of leaves on plantlets
(NIRDPSB Braşov, 2009)

Cultivar, a/ Medium, b	**Christian (a_1)**			**Roclas (a_2)**			**Dif. Medium**
	Nr.	**Dif.**	**Signif.**	**Nr.**	**Dif.**	**Signif.**	**a_2-a_1**
b_1(Mt)	15,27	-	-	13,40	-	-	+ 1,87 **
b_2	3,53	- 11,74	ooo	3,33	- 10,07	ooo	+0,20 ns
b_3	8,33	- 6,94	ooo	6,47	- 6,93	ooo	+1,86 **
b_4	2,27	- 13,00	ooo	2,47	- 10,93	ooo	- 0,20 ns
b_5	5,13	- 10,14	ooo	4,47	- 8,93	ooo	+ 0,66 ns

DL 5%= 0,3534 (leaves /plantlets) DL 5%= 0,8390 (leaves /plantlets)
DL 1% = 0,4867 (leaves /plantlets) DL 1% = 1,6198 (leaves /plantlets)
DL 0,1% =0,6701 (leaves /plantlets) DL 0,1% =1,9466 (leaves /plantlets)

Regression statistical analysis based on the two cultivars have different coefficients. Thus Christian cultivar is highly significant regression coefficient, statistically, so there is a very significant correlation between the concentration of PEG and the number of leaves produced. In case of Roclas cultivar is only

distinctly significant regression coefficient, means that the concentration of PEG in the substrate influenced the number of leaves beside the cultivar factor (fig. 6).

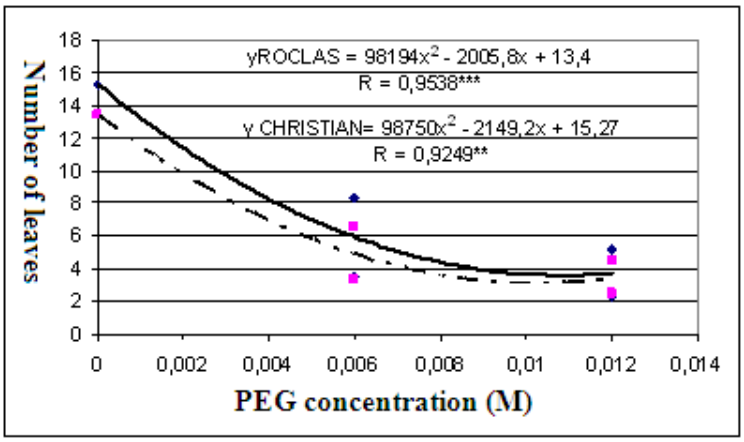

Fig.6. Influence of PEG addition on leaves numbers per plantlets (NIRDPSB Braşov, 2009)

CONCLUSIONS

- control V1 (constitution without the addition of PEG) has not hampered the growth of plants, height resulting from experiments was 12.53 cm and 10.33 cm of the Christian and Roclas cultivars; the lowest height of plantlets is observed in variant with the addition of 0.012 M PEG which strongly inhibited height growth, the resulting plantlets were 2.27 and 1.53 cm for the Christian and Roclas cultivars.
- Christian and Roclas cultivars differ in the height of plantlets obtained from the Roclas cultivar, with an average difference of - 0.661 cm from the Christian variety.
- results on the number of leaves produced on substrates, indicating that variants 2 and 4 with the substrates to which PEG was added directly, the number of leaves were decreased for both cultivars, with values of 3.53 and 2.27 for the Christian cultivar and 3.33 and 2.47 for the Roclas cultivar.

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RESEARCH ON THE AVERAGE NUMBER OF POTATO MINITUBERS OBTAINED INDUSTRIAL SUBSTRATES

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Key words: clay, perlite, plantlets, minitubers

ABSTRACT

In the years 2009 was a series of experiences polifactorials mounted in protected areas belonging to the National Institute of Research and Development for Potato and Sugar beet Brasov. For culture substrates, soil was used as a witness, and perlite and clay balls were used as industrial substrates and biological material was composed of minitubers and plantlets obtained „in vitro". At the end experiences were recorded minitubers number. Number of minitubers produced per plant, varied depending on different factors.

The potato minituber production is the classical intermediate stage enabling field use of potato materials with an in vitro origin. Conventional methods for producing basic and certified potato seeds begins with the production of pre-basic material. Plants are produced in vitro using meristem culture and after an acclimatization period in greenhouse, are transferred to glasshouse where tubers are produced. The health risk at this stage depends in part upon the growth substrate used. Soil disinfestations generally is done with chemicals such as methyl bromide, which eliminates both pathogenic and beneficial microorganisms. This technique usually results in low yield of minitubers per plant, generally five or less, and is a serious constraint to the production (Daniels et al., 2000). The development of more efficient and productive systems with lower environmental impact would improve the quality of the material used by the potato growers, directly affect the crop yields and reduce the production costs (Pereira and Fortes, 2003; Pereira and Fortes, 2004). The use of hydroponic systems offers an excellent alternative to reduce the potential risks of root and tuber contamination by the soil pathogens and also for eliminating the need of chemical disinfectants, generally very harmful to the human health and environment (Medeiros et al., 2001; Pereira and Fortes, 2003).

By routing very precise, rigorous correlation of all factors of vegetation, based on high technological level of crops without soil substrate industry, is seeking obtaining minitubers of high biological value.

Our **aim** is getting minitubers - prebase material coming from vitroplantlets transplantation experiments mounted in the substrate industry since 2009.

The research objectives was to follow precocity influence on the ability to produce minitubers.

1. MATERIAL AND METHOD

Biological material: we used Christian, Roclas (figures 1,2) and Ostara cultivars;

Other materials: culture trays for pots with industrial substrate made of galvanized sheet, with sides of 0.9 m and height of tray of 10 cm provided with plug for drain and refresh with new solution;

-pots with volume of 1.0L;

-compound garden with soil 1:1, for setting up the potato culture (for soil control);

-solid substrates for plant roots in hydroponic system consisting of inert inorganic materials resulting from industrial processes simpler like, expanded clay, perlite;

-fertilizers

- crop nutrient solutions without soil - nutrient solution was prepared on the basis of soluble fertilizer, ,, Universol " (produced by Scott - Netherlands);

- foliar fertilizers: Cropmax, Agroleaf;

- calcium nitrate, the correct response to nutrient solution was applied to the use of calcium nitrate containing 15.5% total nitrogen (of which 1.1% ammonium nitrogen, calcium 19.0% Ca);

- pesticides:

- ELECTIS 75 WG - mildew treatment;

- ACTARA 25 WG - pest control.

Trays were made of galvanized sheet, with sides of 0.9 m and height of tray of 10 cm provided with drain plug of solution obsolete.

Pots used were of medium size, with volume of 1litru.

Solution of the product was prepared from Univesol. On the first step was watched the higher concentration of nitrogen, then the second stage higher concentration of phosphorus.

For better growth of plant, was applied to start a foliar fertilization Cropmax product (the dose 1l/ha), when plants were 15-20 cm high. When treatment was applied for first mildew, Electis product (1.5 kg / ha) was applied and Agroleaf product (5kg/ha); before flowering Agroleaf P (3kg/ha-) was applied; two weeks after flowering Agroleaf K (5kg/ha) was applied. Vegetation interruption was performed at 70 days of plants emergencing formed from minitubers, planted in pots, with the transfer of plantlets from the laboratory. With

two weeks before the harvest, were cut the creeping stems, then perform harvesting, counting and sizing minitubers trained.

Fig.1 Roclas cultivar on clay

Fig.2 Christian cultivar on perlite

2. RESULTS AND DISCUSSIONS

a. Results on the reaction of potato cultivars on soil

Analyzing the average number of tubers observed, results showed that the Christian cultivar present 7.13 minitubers number and 6.38 Roclas to use a number of plantlets. Ostara had the worst results both variants use minitubers (4.40 buc.) and for plantlets (3.44 buc.) (fig. 3).

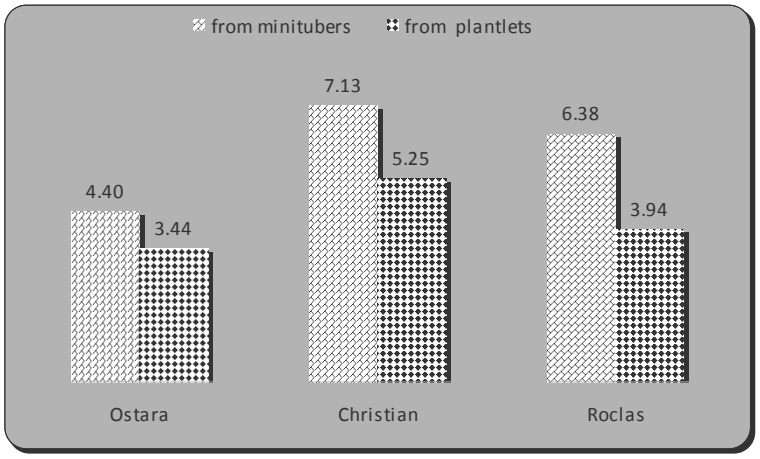

Fig.3. Average number of tubers harvested from experimental variants (NIRPSB Brasov, 2009)

Were analyzed trifactorial number of tubers produced by soil control in the three cultivars. The analysis results per cultivar influence overall results, show that very significant compared with the control Ostara (table 1).

Table 1. Influence of variety on the number of tubers derived from experience on soil (NIRPSB Brasov, 2009)

Cultivar	Average number of tubers obtained		Difference +/-	Significance
	No.	%		
Ostara	3,920	100,00	-	-
Christian	6,190	157,90	+1,579	***
Roclas	5,16	131,63	+1,240	***

DL 5% =0,3601 tub. DL 1% =0,5453 tub. DL 0,1% =0,8761 tub.

The influence of biological material used per global experience shows that the differences are insignificant statistically assured (table 2).

Table 2. Influence of biological material on the number of tubers derived from experience on soil (NIRPSB Brasov, 2009)

Cultivar	Average number of tubers obtained		Difference +/-	Significance
	no	%		
minitubers	5,97	100,00	-	-
plantlets	4,21	70,51	-1,76	ooo

DL 5% =0,6236 tub. DL 1% =0,8968tub. DL 0,1% =1,3190tub

b. results on the reaction of potato cultivars on perlite

Perlite is an inert substate, which is sometimes used in hydroponic culture as such does not affect plant growth and development.

Average number of tubers obtained from the three varieties and two types of biological material, it is noted that when used minitubers Christian variety produced the highest number of tubers by 8.63 and 6.44 variety made Roclas tubers lowest number (fig. 4).

Finally, was accomplished bifactorial analyzed of tubers number produced by three cultivars. First factor was cultivar, the second biological material. From point of view of influence of cultivar, by comparison with Ostara, Christian cultivar had very significant results about the number of tubers obtained and Roclas cultivar accomplished obtaining of very significant results (table 3).

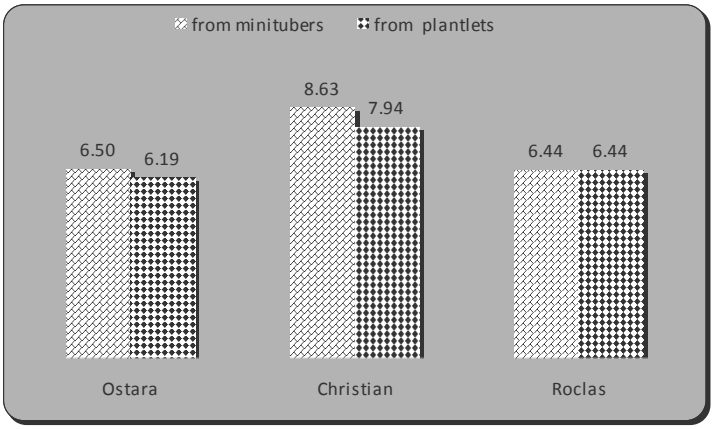

Fig 4. The number of tubers produced in the experimental variants perlite substrate (NIRPSB Brasov, 2009)

Table 3. Influence of cultivar on the number of tubers derived from experience perlite (NIRPSB Brasov, 2009)

Cultivar	**Average number of tubers obtained**		**Difference + /-**	**Significance**
	No.	**%**		
Ostara	6,34	100,00	-	-
Christian	8,28	130,59	+1,94	***
Roclas	6,44	101,57	+0,10	ns

DLa 5% =0,1009tub. DLa 1% =0,1528tub. DLa 0,1% =0,2455tub.

If biological material influences the results are very significant for Christian and Roclas (table 4).

Table 4. Influence of biological material on the number of tubers derived from experience perlite (NIRPSB Brasov, 2009)

Cultivar	**Average number of tubers obtained**		**Difference + /-**	**Significance**
	No.	**%**		
minitubers	7,19	100,00	-	-
plantlets	6,8566	95,36	-0,3334	ooo

DLb 5% =0,0739 tub. DLb 1% =0,1062 tub. DLb 0,1% =0,1563 tub.

c. Results on the reaction of potato varieties on expanded clay

Another substrate used was expanded clay from Hungary. Experience has also included three cultivars, biological material and is formed either minitubers or from plantlets.

From the experience made on expanded clay is observed that the number of tubers produced range from 6.83 tub to Ostara cultivar of biological material plantlets in vitro by 9.38 tub. to Roclas minitubers biological material (fig. 5).

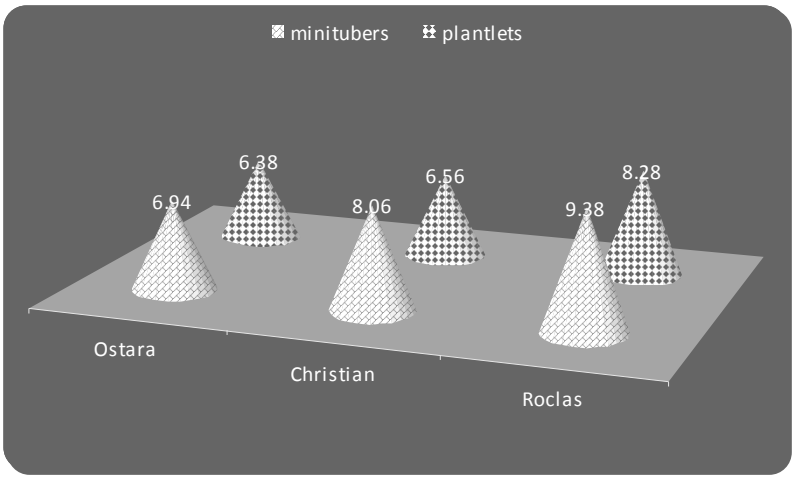

Fig. 5. Number of tubers obtained from experienced cultivars
(NIRDPSB Brasov, 2009)

In terms of cultivar, good results were obtained with the Roclas cultivar with a number of 9.38 tubers from minitubers and 8.25 tubers from the plantlets and by 8.06 tubers from minitubers Christian and 6.56 tubers to seedlings.

If we compare the values of the two types of biological material is noted that all cultivars are more productive when biological material is minitubers.

Finally we compare the influence of cultivar, range and biological material used in obtaining the number of tubers obtained.

If the number of tubers variety influence the results obtained are provided statistically very significant and insignificant to Roclas and Christian (table 5).

Table 5. Cultivar influence on the number of tubers derived from experience expanded clay
(NIRPSB Brasov, 2009)

Cultivar	**Average number of tubers obtained**		**Difference +/-**	**Significance**
	No.	**%**		
Ostara	6,65625	100,00	-	-
Christian	7,3125	109,85	+0,6563	ns
Roclas	8,8125	132,39	+2,1563	***

DLa 5% =0,7100tub. DLa 1% =1,0751tub. DLa 0,1% =1,7272tub.

If biological material influences the results are very significant for Christian and Roclas (table 6).

Table 6 . Biological material influence on the number of tubers derived from experience expanded clay (NIRPSB Brasov, 2009)

Biological material	**Average number of tubers obtained**		**Difference +/-**	**Significance**
	No.	**%**		
minitubers	8,1266	100,00	-	-
plantlets	7,0633	86,91	-1,0633	ooo

DLb 5% =0,3595 tub. DLb 1% =0,5170tub. DLb 0,1% =0,7604tub.

Analyzing the combined influence of the cultivar and biological material used for planting (table 7) it's observed that the biological material plantlets by comparison with minitubers the results for every cultivar are either insignificant or negative significant but nevertheless may indicate that Roclas and Christian cultivars were able to very significant results by comparing two biological material between them.

Table 7. Cultivar and biological material influence on the number of tubers derived from experience expanded clay (NIRPSB Brasov, 2009)

Cultivar Biological material	**Ostara**		**Christian**		**Roclas**		**Dif.**		
	No.	**Dif. +/- Sign.**	**No.**	**Dif +/- Sign.**	**No.**	**Dif +/- Sign.**	**a2-a1**	**a3-a1**	**a3-a2**
minitubers	6,94	-	8,06	-	9,38	-	+1,12 *	+2,44 ***	+1,32 **
plantulets	6,38	-0,56 ns	6,56	-1,50 ooo	8,25	-1,13 oo	+0,18 ns	+1,87 ***	+1,69 ***

DLa 5% =0,6226 tub. DLb 5% =0,7890 tub.
DLa 1% =0,8953 tub. DLb 1% =1,1345 tub.
DLa 0,1% =1,3168tub. DLb 0,1% =1,6686 tub.

3. CONCLUSIONS

Number of minitubers produced per plant, varied depending on the cultivar, the substrate used and the biological material used for planting.

Minitubers largest number is obtained at Roclas cultivar (8.81 minitubers) on clay substrate and the lowest number is obtained from Ostara (3.92 minitubers / plant) on soil, used as control.

The number of tubers produced per substrate soil was between 7.13 tubers / plant using minitubers from Christian cultivar, and the lowest number was 3.44 minitubers tubers / plant using the plantlets Ostara cultivar.

The number of tubers produced on perlite substrate using minitubers planting was between 8.63 to Christian cultivar and 6.44 to Roclas cultivar (equal for both planting materials).

The number of tubers produced on clay substrate was between 9.38 tubers / plant for plantlets to Roclas cultivar using minitubers and 6.38 to Ostara cultivar using plantlets.

In case of expanded clay substrate showed a high average number of tubers from minitubers to Christian cultivar (8.06 tubers), to Roclas using minitubers (9.38 tubers) and to Roclas, using plantlets (8.25 tubers).

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BIOTECHNOLOGY OF ORGANIC CULTIVATION OF EDIBLE MUSHROOMS ON WINERY AND VINEYARD WASTES

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ABSTRACT

Huge amounts of wine and vine wastes cause serious environmental damages in vineyards as well as nearby winery factories, for instance, by their burning on the soil surface or their incorporation inside soil matrix. The optimal and efficient way to solve these problems is to recycle these biomass wastes as main ingredients in nutritive composts preparation that could be used for edible mushrooms cultivation. In this respect, the main aim of this work was to establish the best biotechnology of winery and vine wastes recycling by using them as natural substrata for growing of edible mushrooms. According to this purpose, two mushroom species of Basidiomycetes, namely Lentinula edodes as well as Pleurotus ostreatus were used as pure mushroom cultures in experiments. The experiments of inoculum preparation were set up under the following conditions: constant temperature, 23°C; agitation speed, 90-120 rev min^{-1}; pH level, 5.0–6.0. All mycelia mushroom cultures were incubated for 120–168 h. In the next stage of experiments, the culture composts for mushroom growing were prepared from the lignocellulosic wastes as vineyard cuttings and marc of grapes in order to be used as substrata in mycelia development and fruit body formation. The tested culture variants were monitored continuously to keep constant the temperature during the incubation as well as air humidity, air pressure and a balanced ration of the molecular oxygen and carbon dioxide. In every mushroom culture cycle all the physical and chemical parameters that could influence the mycelia growing as well as fruit body formation of L. edodes and P. ostreatus were compared to the same fungal cultures that were grown on poplar sawdust used as control samples.

Key words: biotechnology, edible mushrooms, organic cultivation, winery and vineyard wastes

INTRODUCTION

The agricultural works as well as the industrial activities related to vine crops and wine processing have generally been matched by a huge formation of wide range of waste products. Many of these lignocellulosic wastes cause serious environmental pollution effects, if they are allowed to accumulate in the vineyards or much worse to be burned on the soil (*Altieri*, 1995). The solid substrate fermentation of plant wastes from agro-food industry is one of the challenging and technically demanding of all biotechnologies known to humankind (*Wainwright*, 1992). The major group of fungi to degrade cellulose and lignocellulose are the edible mushrooms of Basidiomycetes Class (*Carlile & Watkinson*, 1996).

The main aim of this work was focused on establishing the best biotechnology of recycling the winery and vineyard wastes by using them as natural growing sources for edible mushrooms and, last but not least, to protect the vineyard ecosystems (*Petre & Petre*, 2008). Taking into consideration that most of the edible mushrooms species requires a specific micro-environment including

complex nutrients, the influence of physical, chemical and biological factors upon fungal biomass production and mushroom fruit bodies formation were investigated by testing new biotechnological procedures (*Verstraete & Top*, 1992).

MATERIALS AND METHODS

According to the main purposes of this work, two fungal species of Basidiomycetes group, namely *Lentinula edodes* (Berkeley) Pegler (folk name: Shiitake) as well as *Pleurotus ostreatus* (Jacquin ex Fries) Kummer (folk name: Oyster Mushroom) were used as pure mushroom cultures isolated by authors from the natural environment and now being preserved in the local collection of the University of Pitesti. The stock cultures were maintained on malt-extract agar (MEA) slants (20% malt extract, 2% yeast extract, 20% agar-agar). Slants were incubated at 25°C for 120-168 h and stored at 4°C. The pure mushroom cultures were expanded by growing in 250-ml flasks containing 100 ml of liquid malt-extract medium at 23°C on rotary shaker incubators at 110 rev min^{-1} for 72-120 h. To prepare the inoculum for the spawn cultures of *L. edodes* and *P. ostreatus* the pure mushroom cultures were inoculated into 100 ml of liquid malt-yeast extract culture medium with 3-5% (v/v) and then maintained at 23-25°C in 250 ml rotary shake flasks. The designed experiments for inoculum preparation were set up under the main following conditions: constant temperature, 25°C; agitation speed, 90-120 rev min^{-1}; initial pH, 5.5–6.5. All the seed mushroom cultures were incubated for 120–168 h. After that, the seed cultures of these mushroom species were inoculated in liquid culture media (20% malt extract, 10% wheat bran, 3% yeast extract, 1% peptone) at pH 6.5 previously distributed into rotary shake flasks of 1,000 ml. During the incubation time period, all the spawn cultures were maintained in special culture rooms, designed for optimal incubation at 25°C. There were prepared three variants of culture compost made of marc grapes and vineyard cuttings. Then, they were mechanically pre-treated by using an electric grinding device to breakdown the lignin and cellulose structures in order to make them more susceptible to the enzyme actions (*Carlile & Watkinson*, 1996). All the culture compost variants made of ground vineyard and winery wastes were transferred into 1,000 ml glass jars and disinfected by steam sterilization at 120°C for 60 min. When the jars filled with composts were chilled they were inoculated with the liquid spawn already prepared. Each culture compost variant for mushroom growing was inoculated using liquid spawn having the age of 72–220 h and the volume size ranging between 3–9% (v/w). During the period of time of 18–20 d after this inoculation, all the mushroom cultures had developed a significant mycelia biomass on the culture substrata made of vineyard cuttings and marc of grapes (*Petre & Teodorescu*, 2009).

RESULTS AND DISCUSSION

According to the registered results of the performed experiments the optimal laboratory-scale biotechnology for edible mushroom cultivation on composts made of marc of grapes and vineyard cuttings was established (Fig. 1).

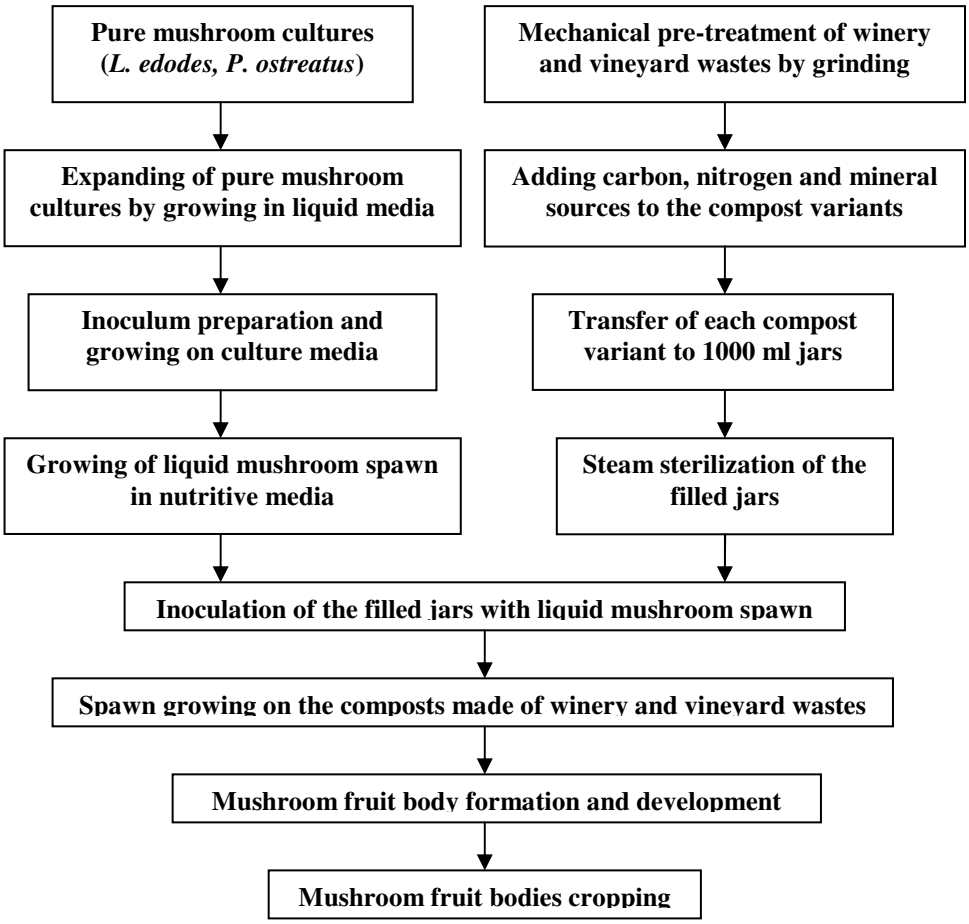

Fig. 1. Scheme of laboratory-scale biotechnology for edible mushroom production by recycling winery and vineyard wastes

The effects induced by the composts composition, nitrogen and mineral sources as well as the inoculum amount upon the mycelia growing during the incubation period were investigated. There were made three variants of composts which were tested by comparing them with the control sample made of poplar

sawdust. The first variant of compost composition was prepared from vineyard cutting, the second one from a mixture between marc of grapes and vineyard cuttings in equal proportions and eventually, the third one was made only from marc of grapes as full compost (*Petre & Petre*, 2008).
The experiments were carried out for 288 h at 25°C with the initial pH 6.5 and the incubation period lasted for 168-288 h. All data are the means of triple determinations carried out on the variants of composts made of vineyard cuttings and marc of grapes.
As it can be noticed in figure 2, the registered results show that from all tested compost variants the most suitable substratum for mycelia growing was that one prepared from marc of grapes, because it showed the highest influence upon the mycelia growing and fresh mushroom production (32–35 g%). This compost variant was followed by the mixture prepared from marc of grapes and vineyard cuttings in equal amounts (20-23 g%) and finally by the variant made of only vine cuttings (12-15 g%).

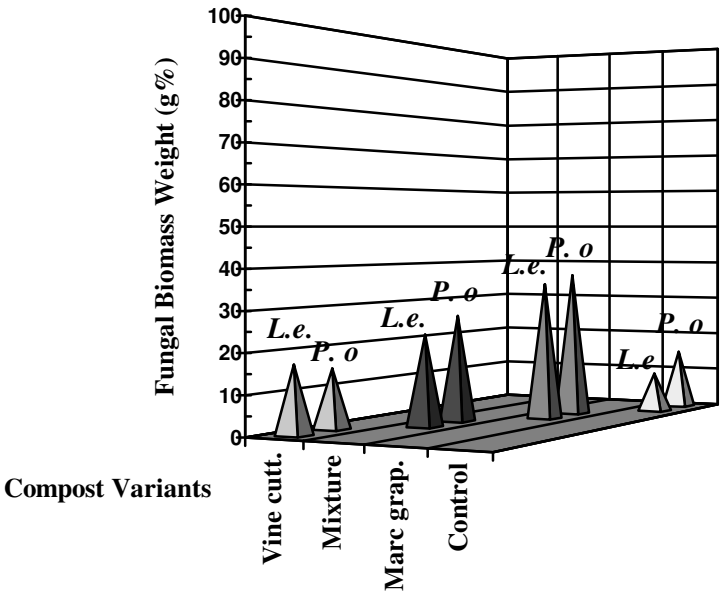

Fig. 2. Comparative effects of composts composition upon mycelia growing of *P. ostreatus* (*P.o.*) and *L. edodes* (*L.e.*)

The effects of nitrogen sources were registered as they are shown in figure 3. All registered data are the means of triple determinations. From the tested

nitrogen sources, barley bran was the most efficient upon the mycelia growing and fruit mushroom producing at 35-40 g% fresh fungal biomass weight, being closely followed by rice bran at 25–30 g%. Wheat bran is also a well known nitrogen source for fungal biomass synthesis but its efficiency in these experiments was relatively lower than that ones induced by the barley and rice bran added as natural organic nitrogen sources (*Petre & Petre*, 2008; *Petre & Teodorescu*, 2009; *Carlile & Watkinson*, 1996).

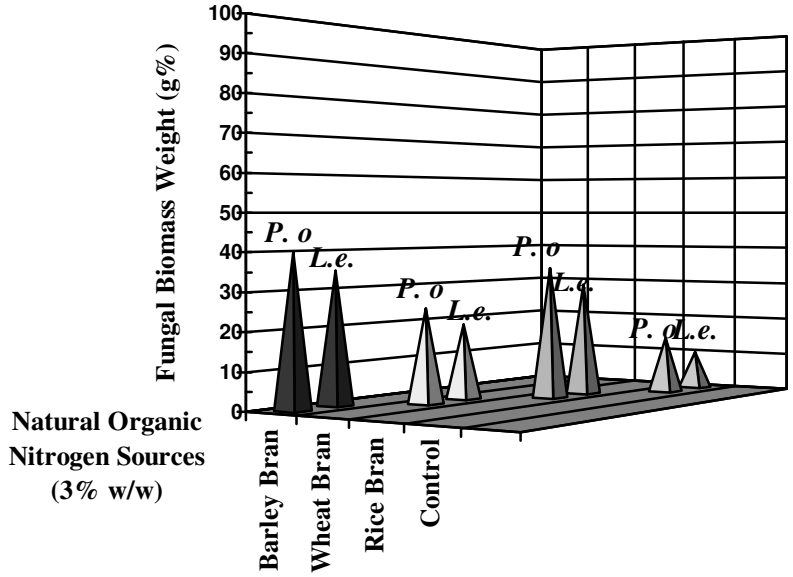

Fig. 3. Comparative effects of natural organic nitrogen sources upon mycelia growing of *P. ostreatus* (*P.o.*) and *L. edodes* (*L.e.*)

Among the various mineral sources examined, natural calcium carbonate ($CaCO_3$) from marine shells yielded the best mycelia growing as well as fungal biomass production at 28-32 g% and for this reason it was registered as the most appropriate mineral source being followed by natural gypsum ($CaSO_4 \cdot 2\ H_2O$) at 20-23 g%, as it is shown in figure 4. The mineral source hepta-hydrate magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$) showed a quite moderate influence upon the fungal biomass growing as other researchers have already reported so far (*Gliessman*, 1998; *Zarnea,* 1994; *Lamar et al*., 1992; *Leahy & Colwell*, 1990). All data are the means of triple determinations.

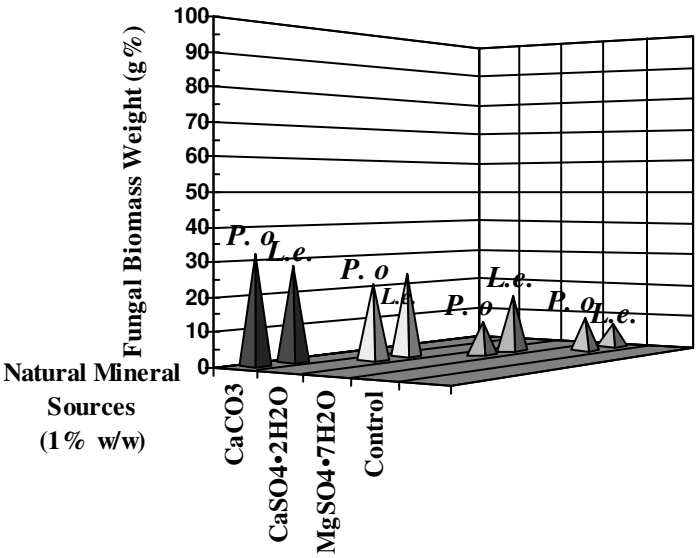

Fig. 4. Comparative effects of mineral sources upon mycelia growing of *P. ostreatus* (*P. o.*) and *L. edodes* (*L.e.*)

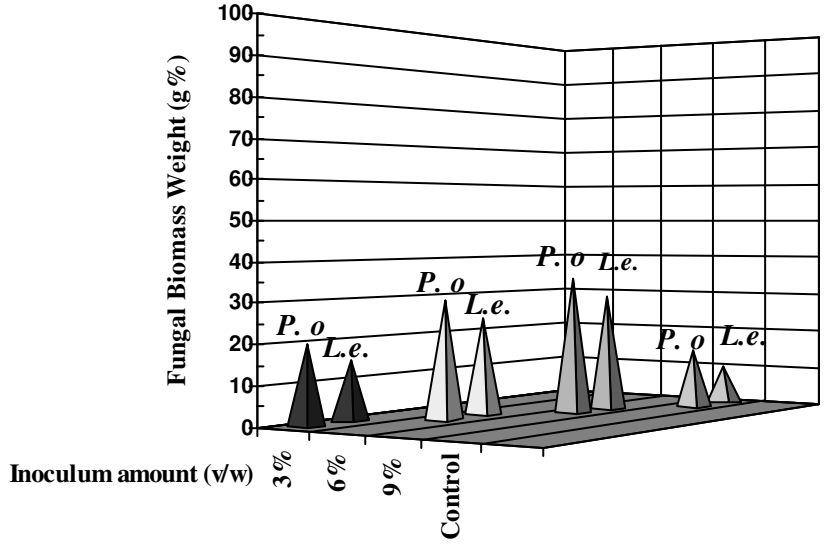

Fig. 5. Comparative effects of inoculum amount upon mycelia growing of *P. ostreatus* (*P. o.*) and *L. edodes* (*L.e.*)

In figure 5 the effects of inoculum amounts added to the composts for mycelia growing and mushroom fruit bodies producing can be noticed. Thus, the best results were registered at the variants of 6% and 9% of inoculum (v/w) with insignificant differences between them. The variant of 3% showed the lowest influence upon the mycelia growing. The control sample was established at 1% (v/w) inoculum added to each compost variants. All data are the means of triple determinations.

Almost similar experiments concerning such techniques of mushroom cultivation were made by *Stamets* (1993) as well as other researchers, but the culture substrata were different (*Carlile & Watkinson*, 1996; *Moser*, 1994; *Moo-Young*, 1993).

The whole period of mushroom growing from the inoculation to the fruit body formation lasted between 30–60 d, depending on each fungal species used in experiments. During the whole period of fruit body formation, the culture parameters were set up and maintained at the following levels, depending on each mushroom species: air temperature, 15–17^{o}C; the air flow volume, 5–6m^{3}/h; air flow speed, 0.2–0.3 m/s; the relative moisture content, 80–85%, light intensity, 500–1,000 luces for 8–10 h/d.

The final fruit body production of these mushroom species used in experiments was registered between 1.5–2.8 kg relative to 10 kg of composts made of vineyard and winery wastes.

CONCLUSIONS

1. The registered data revealed that by applying this biotechnology, the winery and vineyard wastes could be recycled as useful raw materials for culture compost preparation in order to get edible mushrooms, the best results being obtained by using the compost made from marc of grapes.
2. Most suitable substratum for mycelia growing was prepared from marc of grapes, showing the highest influence upon the mycelia growing and fresh mushroom production of 32–35 g%.
3. From the tested nitrogen sources, barley bran was the most efficient upon the mycelia growing and fruit mushroom producing at 35-40 g% fresh fungal biomass weight, being closely followed by rice bran at 25–30 g%.
4. Among the various mineral sources examined, natural calcium carbonate ($CaCO_3$) from marine shells yielded the best mycelia growing as well as fungal biomass production at 28-32 g% and for this reason it was registered as the most appropriate mineral source being followed by natural gypsum ($CaSO_4 \cdot 2\ H_2O$) at 20-23 g%.
5. Concerning the effects of inoculum amounts added to the composts for mycelia growing and mushroom fruit bodies producing the best results were registered at

the variants of 6% and 9% of inoculum (v/w) with insignificant differences between them.

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DORMANCY OF SEEDS AND HIS IMPORTANCE FOR WINTER WHEAT CROPS

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ABSTRACT

*The movement of water into harvest-ripe grains of dormant and non-dormant genotypes of wheat (**Triticum aestivum** L.) was investigated using observation methods and analysis of climacterically data of regions of crops wherefrom the samples were taken. Observation of the grain were collected at intervals after the start of ISTA – germination method and used to reconstruct a picture of the different grain tissues and changes over time. In closely related genotypes, with either a dormant or a non-dormant phenotype, neither the rate of increase in water content nor the pattern of water distribution within the grain was significantly different until 18 h, when germination became apparent in the non-dormant genotype. Water entered the embryo and scutellum during the very early stages of imbibition through the micropyle and by 2 h water was clearly evident in the micropyle channel. After 12 h of imbibition, embryo structures such as the coleoptile and radicle were clearly distinguished. Although water accumulated between the inner (seed coat) and outer (pericarp) layers of the coat surrounding the grain, there was no evidence for movement of water directly across the coat and into the underlying starchy endosperm. By analyses of germination of five varieties of winter wheat into laboratory, it could be observed that one variety is going to start germination earlier than others, in constant climacteric values of temperatures and rain during the harvest season, but, could be noticed that during the years with alternates values of temperatures and rains during the ripening time, could be observed different reactions of genotypes to dormancy of germination and head sprouting into the head before harvesting. The best germination have been observed by Dropia variety (92%) and the last place among analyzed varieties have been taken by Dor variety (83%). To the Crina variety, where have been founded 7% of sprouting seeds into the samples, has as laboratory results a germination of 88%, which confirm that the sprouting seeds could produce normal germs during ISTA germination test into laboratory.*

Keywords: Dormancy, germination, Triticum aestivum**, water, winter wheat**

INTRODUCTION

The movement of water into ripe and dry grains is a critical step in germination, preharvest sprouting, expression of dormancy, and processes such as conditioning for optimum milling performance. Imbibing and maintaining grains under warm, moist conditions is the only means of determining the dormancy or germinability of wheat grains. Variation in the rate or the pathway of water movement may affect measurement of the dormancy phenotype and might also be involved in the dormancy mechanism. Preharvest sprouting, or the germination of grain in the spike, can occur prior to harvest maturity if the crop is exposed to wet, humid conditions. The majority of wheat-growing climates throughout the world experience rain during harvest, which can result in sprouted grain and a loss of

quality premiums. Although there are a number of management techniques and varietal differences that can reduce the risk of sprouting, these are largely ineffective if adverse environmental conditions persist. Grain dormancy is the most reliable protection against preharvest sprouting in a broad range of environments and will significantly protect against preharvest sprouting.

Dormancy in some plant species is controlled, at least in part, by differential permeability to water of the seed coat between dormant and non-dormant genotypes (Bewley and Black, 1982). The possibility that differences in grain water uptake or seed coat water permeability could be associated with dormancy in wheat, as in other species (Finch-Savage and Leubner-Metzger, 2006), has not been discounted. Wheat caryopses, commonly called grains, like the caryopses of other cereals and grasses, are indehiscent fruit having a single seed surrounded by a seed coat and, in turn, enclosed within a thin, adherent pericarp (fruit coat) (Bradbury **et al.**, 1956; Black **et al.**, 2006). In this investigation, the term coat is used to refer to the tissues, pericarp (inner and outer) and the true seed coat (testa and nucellus), that surround the germ (embryo, scutellum, and epiblast) and endosperm (aleurone and starchy endosperm) of the wheat grain. In wheat, therefore, the pericarp and/or the seed coat could affect water permeability and water entry into the germ.

In white-grained wheat, dormancy is relatively rare, but genetic studies have indicated that there appears to be more than one factor which contributes to dormancy (Mares, 1999). According to the model proposed by Mares (1999), there is at least one gene expressed in the embryo that controls a transient sensitivity to abscisic acid (ABA) which, on its own, gives rise to an intermediate dormant phenotype. Another unknown factor controlled by a gene that is expressed in the coat of dormant genotypes, can interact with the sensitive embryo to produce a stronger complete dormant phenotype (Mares, 1999). The coat factor could involve germination inhibitors, a reduced permeability of the seed coat to water or oxygen, or other unknown mechanisms.

The aim of this investigation was initially employed over a period of 0–18 h after the start of imbibition, i.e. prior to the rupture of the seed coat and the first physical evidence of germination in the non-dormant genotype, to address the question of whether dormancy involved differences in grain water uptake or seed coat permeability and to determine the pattern of water movement into imbibing grains.

MATERIAL AND METHODS

Grain of the following bread wheat (**Triticum aestivum** L.) genotypes, representing the extremes in the range of genetic variation for grain dormancy in winter wheat, was used: Dropia, Boema, Glossa a genetically dormant (sprouting resistant) and genetically non-dormant (sprouting susceptible) varieties Crina and

Dor. Large samples of grain were harvested from field plots of each genotype at harvest-ripeness by gentle hand-threshing this time period.

Temperatures and volumes of rain during ripening of grains of cereals have been registered by meteorologically station of Craiova using international standards methods.

Measuring of temperatures during germination into germination thermo-cabinet has been made, automatically by the thermometers incorporated into thermo-cabined, which have been calibrated by National Metrological Authority.

The samples of grains were taken, carried into laboratory and primary have been observed, counted and registered the sprouted seeds. After that have been homogenized and counted four repetitions of 100 seeds of pure grains for every varieties of Dropia, Boema, Glossa, Dor and Crina which have transferred to germination laboratory. The samples were lied to un wet substrate (filter paper), using method between paper. .

Due to the seed's dormancy of grain of wheat, which could be longer or shorter accordingly with genetics of varieties and influences of environmental condition during harvest season, the samples have been physically thermal treated by pre-cooling at 10°C for 4 to 8 days up to getting of coleoptiles and seminal roots from 70% of seeds.

The time of pre-cooling (4 to 8 days) is not counted as germination time, accordingly to the ISTA norms for germination of cereals seed determination.

The germination substrates (filter paper with neutral pH) have been moist with municipal pipe-water, with neutral pH, both of them been free of fito-toxic substances. The filter paper have a good capacity to water retention in order to assure the moist necessary to seeds to germinate throw the all garminative period (8 days).

The all repetition have been introduced into germination cabinet, into plastics bag, uncolored and transparent, with dimensions large enough to permit development of germs.

The results have been interpreted using international ISTA methods for determination of cereal's seed germination.

RESULTS AND DISCUSSION

The records have been made from year 2005 in Şimnic area, from begining of grain formation up to harvest time. Could be observed o constant rising from one day to the next along the entire periods without thermical shocks. The average daily temperatures recorded have values between 11.9°C and 23.6°C, with an amplitude of 11.7°C (Figure 1). The rainfall recorded for these periods, not very often in these seasons, have been with high values, having an influence of full

ripening time. For entire periods analised, the amount of rainfall recorded were of 170.30 mm, due to 134.3 mm in last ten days before harvesting (Figure 2).

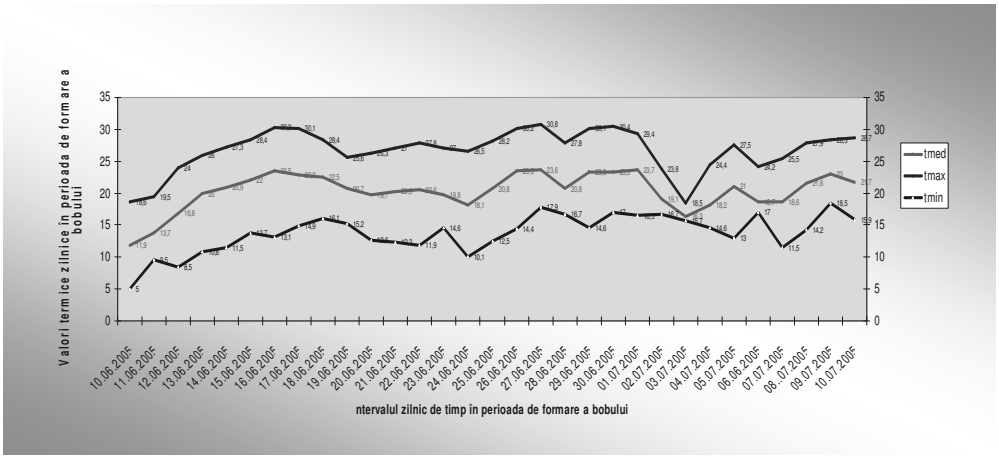

Figure 1. Temperatures recorded in summer of 2005 during ripening of wheat (SMH Craiova, 2005)

The last vegetative period has been caracterised by high values of dayly variaton of temperatures and rainfall.

The bahavior of these two factors, temeprature and rainfall could start the complex biochemical processes of activation of grows's hormons and to spiklets' sprouting phenomena.

Table 1. Sprouting seeds in spikelets before harvesting founded into analized samples (four repetition of 100 seeds)

Variety	**Spikelet's sprouting seeds (nr.)**				**Sprouting seeds (%)**
	Repetition R1	Repetition R1	Repetition R1	Repetition R1	
DOR	2	6	4	1	3.25
CRINA	4	8	7	9	7.00
GLOSA	4	1	2	4	2.75
BOEMA	4	1	2	0	1.75
DROPIA	1	2	3	1	1.75

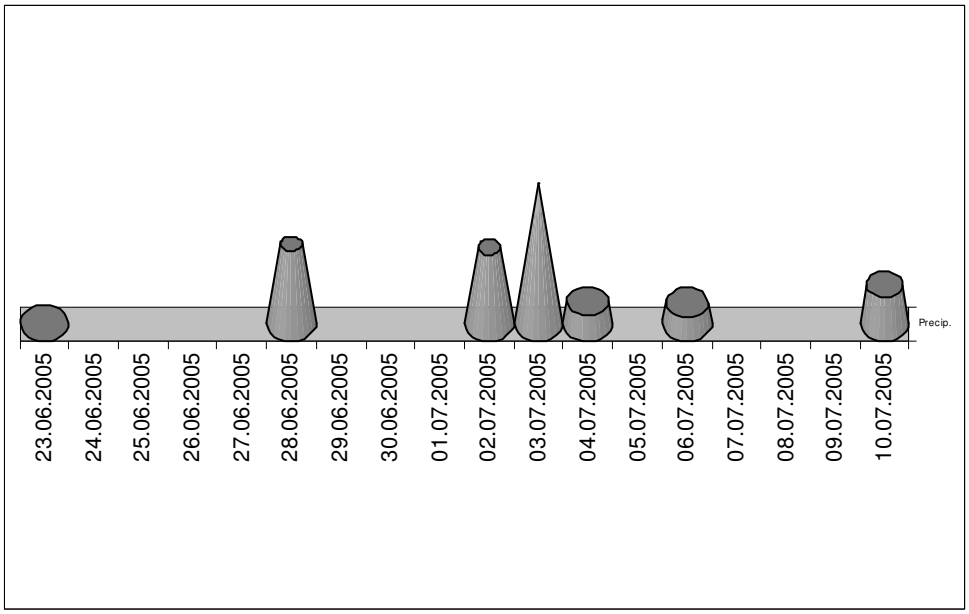

Figure 2. Rainfalles recorded in summer of 2005 during ripening of wheat (SMH Craiova, 2005)

The primary observation into laboratory show that among five varieties of winter wheat analyzed, two varieties shows that are genetically non-dormand (Crina and Dor) and three varieties present genetically characteristics as dormand varieties (Glosa, Boema and Dropia). From sample's analyzed, the Crina variety present 7.00 % of seeds sprouted into spikes. The Boema and Dropia varieties show a resistence to spike's sprouting in climatic condition of summer 2005 in Craiova area (Table 1), but into the field have been founded some plants which present strong phenomenon of sprouting into spikes (Figure 3).

After the analyses of germination after 48 h, 72 h and 96 h, into laboratory could be seen that the highest germination have been obtained by Dropia variety with 92 % of normal germs after 96 h, followed by Boema variety with 90% of normal germs developed after 96 h. The smallest germination have been obtained by Dor variety with 83% of normal germs developed after 96 h. Could be seen also that after 48 h, Dor variety have developed more sprouts than Glosa variety, but at the end of germination time, Dor variety have developed 83 normal germs comparative with Glosa variety which developed 85 normal germs.

From sample's analyzed, the Crina variety present 7.00 % of seeds sprouted into spikes, but after 48 h of germination developed 27 sprouts, more than Glosa variety which have developed 18 sprouts and presented 2,75% of sprouting

seeds in spikes, and more than Dor variety which have developed 19 sprouts after 48 h of germination and which have presented 3.25% of sprouting seeds in spikes. After 72 h of germination the highest sprouting level have been obtained by Dropia with 92 sprouts, followed by Glosa variety with 81 sprouts, Crina variety with 77 sprouts, Boema variety with 73 sprouts and Dor variety with 70 sprouts.

Figure 3. Sprouted seeds into spikes to Dropia variety in condition of Simnic , 2005

Table 2. Germination of wheat's seeds determined at temperature of 20°C

Variety	**Time of germination (h)**		
	48	**72**	**96**
	Sprouts (%)		**Normal germs (%)**
DOR	19	70	83
CRINA	27	77	88
GLOSA	18	81	85
BOEMA	29	73	90
DROPIA	33	92	92

After 96 h of germination the Crina variety could rich higher value of normal germs (88%) than Glosa variety (85%) and than Dor variety (83%), even if have registred an higher percent of spikes sprouting than these varieties.

CONCLUSIONS

Dormancy of wheat's seeds is an important characteristic of variety with major impact in faculty of germination of seeds, with importance in producing of seed

certificates technologies. In the other way the spike's sprouting present an importance for quality of wheat grains and also for producing of certificate seed of wheat, especially in regions with high rainfalls during harvesting season, and high level of relative humidity of air.

The mainly effects of sprouting into wheat spikes are: reducing of a high amount of carbon hydrates into the grains, reducing of volumes weight, reducing biological activity of grains by infestation with some parasitic fungi which could produce mycotoxines.

Resistivity to spike's sprouting for the wheat varieties results by combination of effects of factors which have influences in water absorption in the spikelets, loosing of moist of spikes during ripening, dormancy capacity of wheat varieties and degradation rate of chemical compounds of seeds during germination process.

The total effect of these factors is calling *robur contra pluvium*, the resistance to effect of rainfall.

Dormancy of wheat' seed in a genetical character and have an influence in limits the effects of spikes' sprouting. In the other way in some regions where the time between harvest and sowing is shorter than 60 days, could have an negative effect by limiting the germination capacity of the seeds into soil.

In Simnic area in summer 2005 seasons the five varieties of winter wheat analyzed shown that the sprouting into the spikes of some varieties due to moist condition during harvest and pre-harvest time has no major influence on germination of seeds of these varieties. The Crina variety's seeds have a higher level of germination, determined by ISTA methods in comparation with Glosa variety's seeds which have been more resistant to spike's sprouting than Crina variety.

The higher level of dormancy of wheat' seeds has not always as effects the high level of seeds germination, e.g. the Boema variety's seeds which has an dormant genotypes has an comparative germination with Crina's seeds which has an non–dormant genotypes.

As a consequence the level of sprouting spike's seeds is not a references into ISTA norms for seed certificates of wheat.

After analizes into laboratory could not be exactly determined the time necessary for differt genotipes of wheat for finish the dormancy period. The dormancy time could be longer or schorter, depending not only by genotypes but also by wheather condition during pre-harvest and harveting time. The spike's sprouting, after dormancy in the rainy years, could be as results of interactions of some factors as biological, mechanical, phisiological and which could establish some desequilibrium at functional level of biological activities of seeds.

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PROBLEMS RELATED TO RECULTIVATION OF MARITSA-IZTOK COAL MINE DISTURBED TERRAINS OF AGRICULTURAL LAND

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Key words: recultivation, problems, solutions

ABSTRACT

An analysis is made on problems related to recultivation of Maritsa-Iztok coal mine disturbed terrains of agricultural land. New decisions for direct implementation are described for recovery of soil fertility of disturbed lands and problems related to the different technological measures. On analysing the conditions for reclamation with agricultural purposes, it was concluded that the most important aspects are of technological and climatic character. We recommend that corrections are made in the technological activities of the technical and biological recultivation. The development of a special agricultural system is required and technologies to grow siderate crops.
Any amelioration practice which does not reflect the actual conditions may cause a risk to the whole technology of reclamation of disturbed lands.

In the region of Maritsa-Iztok coal mine in the open mining large areas of arable land are destroyed. The opening lands covered so far the more than 10000 ha of agricultural land. To use these areas for farming or reforestation of forest types it is necessary to restore their fertility through the application of technical and biological reclamation.

The technical recultivation includes placement of materials on their fitness, leveling the ground and adjusting the area. Biological reclamation involves the selection of plant species, treatments, fertilization, introduction of regular sowing-rotation and other agricultural activities (Treykyashki, Hristov, 1982).

Along with the approved reclamation regulation in recent years new solutions are searched for reclamation of damaged areas, primarily in bio-remediation stage.

In reclamation making more acute arises a number of problems. Compliance therewith is mandatory, and finding adequate solutions to overcome them is related to the successful conduct of operations.

The purpose of this report is to mark the attendant problems of implementing new technology solutions for the reclamation of damaged areas in the region of Maritsa - Iztok.

A number of technological solutions is developed and proposed for implementation in practice:

- Use of siderates – "Alternative biotechnological method for the recovery of soils "

- Agricultural and industrial waste - "Participation in organizing the production tests of non-humus rehabilitation technology" with the use of sludge of pig breeding farm and "Impact the gypsum of SCI of "Maritsa Iztok 2" TES on the reclamation of external dumps" through a composite filling of cinder-ashes and gypsum.

Currently the studies of the use of the gypsum from SCI of Maritsa Iztok TES completed as the meliorant of saline soils and reclamation of disturbed land in mixtures of ashes and with clay.

In conducting scientific research and innovatory work in developing the above technology solutions, the same problems observed connected to the specific mode of conducting the agro-melioration processes as the same technical and biological reclamation.

We'll list some of the more important of them.

In the overlapping of clay materials and their leveling on the practice accepted now, trucks and bulldozers are uses, which owing to their big weight and putting pressure on the soil surface substrate re-pack significantly the soil horizons.

It must be replaced by graders and long-basis levelling devices and the materials filling to be occurred only by soil moisture content, where the further hardening of the area is kept to a minimum size. This moisture content ranges from 14% to 19% by a.s. soil weight depending on the clay materials texture.

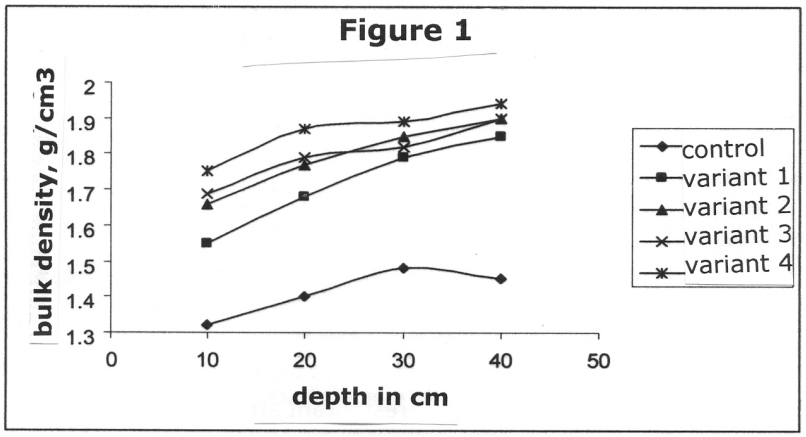

Fig. 1 -Bulk density of tests making in Mednikarovo and Staroselets dumps

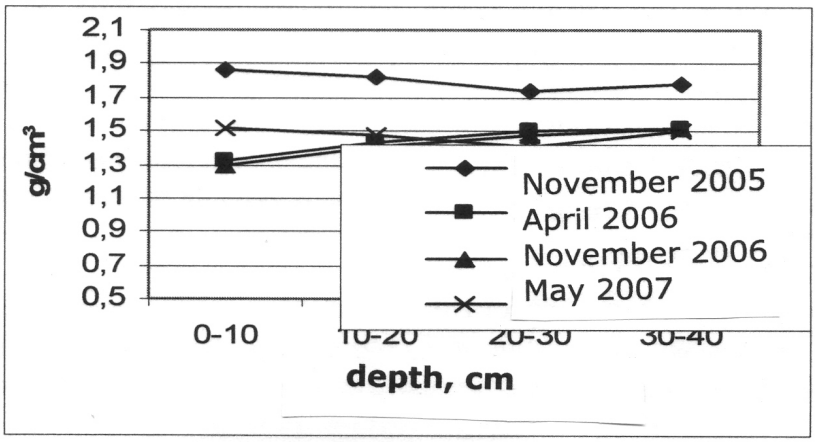

Fig. 2 - Density of tests making in Mednikarovo and Staroselets dumps

Another problem of technological nature is that it can not be obtained the reclaimed land's micro-relief stability. In conducting field experiments in the area of Mednikarovo and Staroselets dump it was obtained a lap in the field and a great leveling of soil surface. In order the effect of alignment to be not brief, the bulk clay materials must be with humidity below 60% of the marginal soil moisture and the layers piling to be accompanied by a gradual rolling of the freshly covered areas, rather than simultaneously.

A third problem is that the standard treatments of soil substrate can not achieve good mixing and conversion of arable horizon due to the thickness of the layer from meliorated components. Recommended by some researchers area trench is not suitable for the recultivated land because there is a top layer of clay remains without organic matter and it is unsuitable nutrition environment for seeds sown. These conclusions were confirmed by the test with the inclusion of gypsum from SCI in the soil substrate held on Staroselets dump. The results showed that during the first year is the necessary conducting two major processes - plowing to a depth of 23-25 cm and a loosening of 45-50 cm, which takes place after the subsidence of the plowed layer.

The water deficit in soil influences the quality of sowing areas preparation for winter cereals. As a result of deteriorating physical condition of the soil it can not be achieved the desired depth of processing and structure of soil aggregates. The use of siderate crops contributes to further plowing the crop residues and creates conditions for their rapid decomposition.

Poor physical conditions are identified as the most limiting factor for a successful crops growing on mining dumps. The problems of their optimization can be solved by using other methods of technical reclamation. Compact nonhumus

reclaimed soils are deprived of valuable agronomic structures of natural soils, which are created by decades. Until then it must be performed by soil treatment.
Due to heavy mechanical composition, clays are susceptible to compaction and the volume of the bulk density of the soil substrates is too high. The soil treatments reduce these values, but for more permanent loosening a period of several years is needed.

Improving the structure of soil substrates also leads to lower values of the bulk density. It was observed a trend for soil loosening in introducing siderate crop residues and including gypsum from SCI.

In the past 10-15 years a big problem, attended biological reclamation is relatively intensive climate aridization, which leads to inability of successfully growing of a number of traditional for the area spring crops. Table 1 shows data of soil water reserves for the three years of test conducting on the technology for nonhumus reclamation. Except for 2002 the summer droughts are quite perceptive, in 2000 for the months July and August there is no receipt of rainfall water, as the water reserve in 0-80 cm layer is about 1.5 times smaller than that in May 2002 (Table 1).

Table 1. Water reserve (lt) in the layer up to 80 cm of the soils substrates

Layer depth in cm	Variations of soil substrates				Variations of soil substrates			
	1	2	3	4	1	2	3	4
	2000				2002			
0 –10	15	13	12	10	27	23	23	19
10 – 20	21	18	20	16	30	30	29	23
20 – 30	23	17	19	20	32	32	33	25
30 – 40	25	22	21	22	31	31	29	25
40 – 60	54	52	52	50	62	63	60	47
60 – 80	51	54	55	52	64	63	65	48
Сума	189	176	179	171	245	242	239	186

Changed climatic conditions require development of a new structure of the crops used for reclamation. Prolonged summer drought many years upset the sunflower crop, which is considered as a drought resistant crop. It is necessary to study the possibilities for growing profitable crops which to be included in sowing-circulation with winter cereal crop growing almost as a monoculture. Such crops are coriander, rape, winter pea, chickpeas, etc. They use autumn-winter soil moisture reserves and ensure sustainable production.

The analysis made of the conditions of conducting the reclamation for agricultural purposes in the region of "Maritsa Iztok" and the prospects for its successful development through nonhumus ways ascertained that:

- There are many problems, the most significant is the importance of their climatic and technological nature.
- - It is advisable to make adjustments in the technological operations in technical and biological reclamation. To develop a specific system of farming for biological reclamation purposes by growing siderate crops, species and variety structure of field crops, consistent with the changed climatic conditions and criteria for the maintenance of the parameters of soil substrates in optimal limits according to the requirements of the occupational cultures.
- - Any agro-ameliorative event unsuitable for the specific conditions leads to the risk of failure of the whole technology of disturbed land reclamation of the land disturbed by the open coal mining in “Maritza-Iztok" area.

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INFLUENCE OF MIXED GROWING PEA AND TRITICUM-SECALE ON THE DEVELOPMENT OF ROOT SYSTEM, TUBER-FORMATION AND THE AMOUNT OF PLANT RESIDUES FOR IMPROVING SOIL FERTILITY

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ABSTRACT

The mixtures are intense dynamic biological systems. Various factors as the ratio of the components and emerging relationships between them, growing conditions, timing of sowing and others exert influence on their development and effective functioning.

The experimental work was conducted during 2006-2009 in EFC-Pleven. The wintering pea from Mir kind and the triticum secale from Vihren kind are used. Two field tests have been made (on the method of fractional plots and the chessmethod). The period of sowing and sowing rate ratio in mixed growing of wintering pea and triticum secale influence the development of the root mass, the tuber formation and the quantity of post-harvest residues.

The mixed seed sown in the period 25-30 October, which corresponds to the maximum formation of post-harvest residues (1913.3 kg / ha) has the most weight of the root system (6.69 g). As regard the tuber-formation the optimum sowing is in the period 10-15 October (36.7 number of tubers / plant). Later sowing periods are characterized by high nitrogen and carbon content of the soil, determined by the larger quantity of root and post-harvest residues.

Most favorable conditions for development of mixed agro-phytocenoses are created at a ratio of pea: triticum-secale 60:40 and 50:50 - respectively formed 1070.1 and 885.1 kg / ha post-harvest residues. Increasing the share participation of legume component (70:30 and 80:20) in the sowing rate at the expense of cereals leads to decreasing the values of the above-mentioned index by on average 56.4 and 40.3%. Notwithstanding the different ratio of cereal and legume component in the sowing rate, the grain component develops better than legumes. The quantity of post-harvest residues and weight of the root mass in triticum secale is higher than this of pea on average with 121.4 and 311.4%.

Key words: mixed growing, pea, triticum-secale, root system, tuber formation, soil fertility

Growing annual crops in a mixture has many advantages over individual crops and is important for the development of modern agriculture and livestock. Participation of plants with different morphological structure and arrangement of bodies creates conditions for the formation of a dense grass composition, better utilization of environmental factors and sowing as the system functions more actively. Therefore the mixtures are usually more highly productive than individual crops. Very important is the interaction of plants of the various groups. Legume crops through the root system help the wheat providing them nitrogen through the nitrogen fixation. Thus the wheat plants grow better. After harvesting the mixtures 6-8 kg / da nitrogen remained in the soil which is used by subsequent crops in rotation (Kostov and Pavlov, 1999).

The mixtures are intense dynamic biological systems. Various factors as the ratio of the components and emerging relationships between them, growing conditions(Kostov and Pavlov, time of sowing (Kalaydzhieva and Petrakieva, 1963) and others.1999) exert influence on their development and effective functioning

The purpose of the present study is to determine the influence of mixed growing of wintering pea and triticum secale (in the conditions of different time of sowing and ratios in sowing norm) on the development of root system, tuber-formation and the amount of plant residues.

MATERIAL AND METHODS

The experimental work is conducted during the period 2006-2009 in EFC-Pleven. A wintering pea kind Mir and triticum secale kind Vihren are used. Soil type is poorly leached black earth. Two field tests are made (on the method of fractional plots and the chess method), repeated in four variants of size and a size of the reporting plot 10 m^2. Phosphorus (60 kg / ha) and potassium fertilizer (40 kg / ha) have been introduced before the primary soil tillage and nitrogen (50 kg / ha) – in early spring.

First test - sowing is done in three calendar terms: 25-30 September, 10-15 October (optimum time for sowing of wintering pea in the region of Central Northern Bulgaria) and 25-30 October. Mixed seed is sown in sowing rate 18 kg / da in the weight ratio of legume and cereal component 80:20.

Second test - Mixed crop is sown during the period October 10-15 at the following ratios between the legume and cereal component: 50:50, 60:40, 70:30 and 80:20.

A small-size planter was used for accurate sowing of seeds in sowing. During the vegetation pesticides are not imported. Mowing is done in butonization phase -25% flowering, with powered BCS mower.

The first sampling was carried out in butonization phenophase -25% flowering, and the second - 10 days after completion of flowering, taking into account the following indicators: weight of the root mass, post-harvest residues and tuber-formation. In the same phase soil samples are taken from all the variants to determine pH changes in soil (potentiometry, the aqueous extract and in KCl, by Atanasov and others, 1979; Velchev and others, 1982; Totev and oters, 1987), mineral nitrogen content - ammonium and nitrate form (by Kornfiyld), phosphorus (by Egner-Riym) and organic carbon (by Kononova and Belchikova, 1965; Laktionov, 1985).

RESULTS AND DISCUSSION

The investigation data for the three years of the test are shown in Table 1. In the terms of I test the biggest weight of the root system (6.69 g) has the mixture

of the last third period of sowing (25-30 October), which corresponds to the maximum amount of post-harvest residues (1913.3 kg / ha). As regard the tuber-formation the optimal is the sowing during the period 10-15 October, the number of tubers is 36.7 pcs / plant.

In the terms of II test the most favorable conditions for development of mixed agro-phitocenosis are created at a ratio legume: cereal 60:40 and 50:50 - 1070.1 respectively formed and 885.1 kg / ha post-harvest residues. Increasing the legume component share participation (70:30 and 80:20) in the sowing rate at the expense of cereals leads to decreasing the values of the above mentioned index by on average with 56.4 and 40.3%.

Although the mixed crops are planted in different ration of the legume and cereal component in the sowing rate, grain component develops better than legumes, which is found in studies of other authors (Kostov and Pavlov, 1999). On average with 121.4 and 311.4% the quantity of post-harvest residues and the weight of the root mass in triticum secalr is higher than this of pea. No significant differences observed in the number of tubers formed of a plant under the influence of different involvement of pea in sowing rate.

Table 1. The effect of different periods of sowing and sowing rate ratios of mixed sowing pea: triticum-secale on tuber-formation, root mass formation and plant residues content – on average for the period

Variants	Crop components	Root dry weight, g / plant	Tuber formation, tuber number / plant	Post-harvest residues kg/ha
		I sampling – sowing terms		
25-30.09	pea	1.13	18.45	430.7
	triticum-secale	2.47	**-**	387.2
10-15.10	pea	2.42	36.70	346.9
	triticum-secale	2.70	**-**	279.1
25-30.10	pea	0.34	33.30	457.9
	triticum-secale	6.35	**-**	1455.4
		II sampling – sowing terms ratio		
50:50	pea	1.05	43.5	111.3
	triticum-secale	5.87	**-**	773.8
60:40	pea	1.52	41.7	841.2
	triticum-secale	3.56	-	228.9
70:30	pea	0.98	39.7	268.9
	triticum-secale	4.40	-	380.0
80:20	pea	1.36	43.2	218.6
	triticum-secale	1.50	-	364.9
LSD $_{5\%}$		0.16	3.84	**57.24**

Mixed growing of wintering pea and triticum secale, as the different planting dates affect the agrochemical composition of the soil (Table 2). Compared with the control option in mixed crop pH remains constant (7.37 in KCl) or slightly increases (8.24 in H2O).

The soils, where are harvested mixed crops are characterized by high content of mineral nitrogen, phosphorus and organic carbon in soil compared to the control resulting from the activity of tuber bacteria. Later sowing periods are characterized by high nitrogen and carbon content of soil determined possibly by the bigger quantity root and post-harvest residues in these terms. As regards the amount of phosphorus the opposite trend is established.

Table 2. Agrochemical soil analysis – on average for the period 2006 – 2009

Variants	pH		Mineral N mg/1000 g soil			$P_2 0_5$ mg/100 g soil	limited C
	H_2O	KCl	NH_4-N	NO_3-N	сума		
control*	**8.07**	**7.38**	**0.47**	**0.15**	**0.62**	**6.83**	**1.00**
25-30.09	8.09	7.36	10.22	5.04	15.26	12.28	1.02
10-15.10	8.14	7.35	11.20	5.32	16.52	10.32	1.33
25-30.10	8.50	7.40	11.48	5.60	17.08	6.00	1.10
on average for the three sowing period	**8.24**	**7.37**	**10.97**	**5.32**	**16.29**	**9.53**	**1.15**

* - the control is without fertilization, no plants

CONCLUSIONS

The period of sowing and sowing rate ratio in mixed growing of wintering pea and triticum secale influence the development of the root mass, tuber-formation and the amount of post-harvest residues.

The biggest weight of the root system (6.69 g) has the mixed seed sown in the period 25-30 October, which corresponds to the maximum formation of post-harvest residues (1913.3 kg / ha). As regard the tuber-formation the optimum sowing is in the period 10-15 October (36.7 number of tubers / plant). Later sowing periods are characterized by high nitrogen and carbon content of the soil, determined by the larger quantity post-harvest and root residues.

Most favorable conditions for development of mixed agro-phitocenosis are created at a ratio legume: cereal 60:40 and 50:50 - 1070.1 respectively formed and 885.1 kg / ha post-harvest residues. Increasing the legume component share participation (70:30 and 80:20) in the sowing rate at the expense of cereals leads to decreasing the values of the above mentioned index by on average with 56.4 and 40.3%. Notwithstanding the different ratio of cereal and legume component in the sowing rate, the grain component develops better than legumes. The quantity of

post-harvest residues and weight of the root mass in triticum secale is higher than this of pea on average with 121.4 and 311.4%.

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RHODIOLA ROSEA L. *IN VITRO* CULTURES PECULIARITIES

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Key words: ***medicinal plant, Rhodiola, golden root, propagation, in vitro response, phytoregulators***

ABSTRACT

Rhodiola rosea L. (Golden Root, Roseroot) (Crassulaceae) is not widely spread, living at high altitudes in Europe and Asia. The plant is rich in biologically active substances: rosavin (2.1 %), salidrosid (0.8 %), rhodiolin, antraglikosides, β-sitosterin, monoterpenoids and tanins (16 – 18 %). Rhizome extracts are used in traditional medicine to decrease depression, to enhance work performance, to prevent cancer diseases, and to treat consequences of physical and psychological stress.

The aim of the paper was to study the conditions for optimal development in vitro of Golden root.

Rhodiola rosea seeds were collected from Rila Mountain. Seeds were subjected to decontamination following several schemes depending on their origin. Seed germination was studied on four variants of Murashige and Skoog basal medium (MS), supplemented with different concentration of gibberellic acid. Seedlings development started on the 7^{th} day and continued until the 40^{th} day. MS medium enriched with 2 mg/l zeatin, 0.2 mg/l IAA and 0.4 gibberellic acid (GA_3) was used for in vitro development of the seedling explants. The coefficient of propagation varied during the different seasons. Highest level of proliferation was observed in May-June, when the shoots per explant were 6.78 ± 0.10. Rooting was induced on half-strength MS medium using 8 variants of supplements with different concentration of IBA, NAA or IAA. Development of in vitro clones differed being influenced by medium type. Differences were observed in rooting parameters and micropropagation rate. Adaptation of obtained plants was done under controlled conditions in a phytotrone room and in a greenhouse. Acclimatization of rooted Golden root plantlets under greenhouse conditions was successful up to 70 %.

INTRODUCTION

Medicinal plants utilization in pharmaceutically practices is increasing according to the recent investigations of the World Health Organization (WHO) (Tripathi and Tripathi, 2003; Matthys et al., 2007). At present about 20 000-plant species are used in two basic spheres: modern phototherapy and modern allopathic medicine (Farnsworth and Soejarto, 1991). Bulgaria is one of the world leading countries in wild medicinal plants export. Annual export is about 10 000 tones of herbs, and more than 50 % of them are harvested in the mountains (Vitkova and Evstatieva, 2000). Bulgarian famous medicinal plants are among those with highest world quality due to specific soil and climatic country conditions (Varabanova, 2002).

Rhodiola rosea L. (Golden root, Roseroot) (*Crassulaceae)* is extremely valuable for the Bulgarian genefund, but natural resources continue to decrease dramatically. Wild habitats restoration is limited because of the low seed germination and the inefficient vegetative propagation (Platikanov and Evstatieva 2008). *Rh. rosea* roots are rich in biologically active substances from six chemical groups. Rosavin, rosarin, rosin and salidroside, as well as, p-tyrosol, rhodioniside, rhodiolin and rosiridin, are of greater pharmaceutical use (Ganzera et al. 2000). They have antidepressant, anticancer, cardio and central nervous system protective effect (Kelly 2001; Brown et al., 2002). They are used in prevention and therapy of important diseases.

Medicinal plants including Roseroot have been in the focus of the plant biotechnology for long years. However, *in vitro* cultivation of *Rh. rosea* appeared not very easy task, as far as successful tissue cultures depend on many factors. When studied the callogenesis and regeneration ability of three ecotypes of *Rh. rosea* from Higher Altai and South Ural regions Ishmuratova (1998) observed the importance of the genotype in addition to the importance of culture media composition and the explant type. High efficiency of shoot multiplication can be reached if benzyl amino purine (0.2 mg/l) and indolyl acetic acid (0.1 mg/l) are added to Murashige and Skoog medium. Other authors reported about the crucial effect of some phytoregulators and the explant nature for the successful establishment of callus and shoot cultures (Kaftanat, 1988; Kirichenko et al., 1994; Yin et al., 2004). Micropropagtion, rooting and plant adaptation are also difficult in most of the cases (Ishmuratova, 1998; Ishmuratova, 2002)

In vitro culture establishment of Golden root has started in Bulgaria several years ago (Dimitrov et al., 2003) and resulted in development of efficient systems for plant regeneration, shoot multiplication and micropropagation followed by adaptation in natural condition and development of regenerants in wild environment (Tasheva and Kosturkova, 2010). These plants produced biologically active substances during the first and second year, proved by HPLC analysis (data to be published).

The objectives of the present investigations are to study the effect of different factors having impact on the successful *in vitro* propagation of *Rhodiola rosea.*

Relationship between tissue culture conditions during *in vitro* propagation from one side and rooting and survival during acclimatization, from another side, will be discussed. It will be demonstrated that the *ex vitro* plant performance can be much improved by optimizing the *in vitro* rooting conditions.

MATERIALS AND METHODS

Donor plants and initial in vitro plant material. Seeds from *Rhodiola rosea* L wild plants growing in their natural environment were used. Donor ecotype was

from Rila Mountain National Park of Bulgaria. Plant material was kindly supplied by Dr. L. Evstatieva, Institute of Botany, Bulgarian Academy of Sciences. "Fresh" and "old" seeds were used to study germination efficiency in *in vitro* conditions. "Fresh" seeds were cultivated *in vitro* in a short period (few days to 2 weeks) after harvesting. "Old" seeds were used after being stored at room temperature from 6 to 12 months.

Explants for *in vitro* propagation were obtained from twenty days old seedlings by cutting the seedlings and obtaining apical buds (6-8 mm in size) and stem segments (8-10 mm in size) with a leaf node and two adjacent leaves. Fifty to 100 seeds or 80-120 explants were tested for each experimental variant. Statistical analysis was according to Sigma Plot 3.1. (Systat Software Inc – SSI, A scientific Data Management Company).

Decontamination. Seeds were subjected to decontamination by different time of treatment with various sterilizing agents applied in single or in a consecutive manner according to the following five schemes:

(1) 96° C_2H_5OH for 5 min;

(2) 70° C_2H_5OH for 3 min; 20 % [v/v] bleach (trade mark Ache produced by Procter & Gamble Co., USA containing 5 % of active chlorine) for 15 min;

(3) 70° C_2H_5OH for 1 min; bleach for 5 min;

(4) 70° C_2H_5OH for 1 min; bleach for 10 min;

(5) 70° C_2H_5OH for 1 min; 0.2 % $HgCl_2$ for 10 min.

A drop of Twin-20 (Sigma-Aldrich) was added into the decontamination solution.

Each procedure was followed by triple rinse in autoclaved distilled water for 5 min, 10 min and 15 min.

Media composition for in vitro cultivation. Seeds were germinated on filter paper soaked in distilled water (control conditions) or on Murashige and Skoog (1962) basal medium (MS) supplemented with 10 - 100 mg/l gibberellic acid, 30 g/l sucrose and 6 g/l agar-agar (Table 1). MS basal medium enriched with 2 mg/l zeatin, 0.2 mg/l IAA, 0.4 mg/l GA_3, 30 g/l sucrose and 7 g/l agar-agar for shoot development. Rooting media contained half strength MS salts, 10 g/l sucrose and 6 g/l agar-agar and different phytoregulators (Table 1). The pH of all media was adjusted to 5.7 - 5.8 prior to autoclaving at 1.1 kg cm^{-2}, 121° C, for 20 min.

Conditions for in vitro cultures and plant adaptation. In vitro materials were cultured in a phytothrone room at 16 h photoperiod, temperature of 18-21° C and 40 $\mu Mm^{-2}s^{-1}$ (fluorescent lamps type FL-40 W-1, Svetlina Ltd., Bg). Regenerated plantlets after formation of roots *in vitro* were transferred to small pots containing different substrates (soil, peat and perlite only or in combinations) and were grown in chambers under different conditions (humidity from 60 % to 90 %, temperature from 18° C to 21°C, light intensity 60 $\mu Mm^{-2}s^{-1}$).

Table 1. Composition of culture media for Bulgarian *Rhodiola rosea in vitro* seed development, shoot multiplication, and rooting of plant regenerants.

Media variants	Concentration of phytoregulators and supplements [mg/l]					
	Zea	IAA	NAA	IBA	2,4-D	GA_3
G1[1]						10
G2[1]						25
G3[1]						50
G4[1]						100
Z-5[1,2]	2.0	0.2				0.4
V-1				1.0		1.0
V-2			1.0			1.0
V-3				2.0		1.0
V-4		0.2		2.0		1.0
V-5				0.5		1.0
V-6				0.25		1.0
V-7				0.1		1.0
V-8		0.2		2.0		0.4

Legend: [1]Sucrose is 30 g/l instead of 20 g/l; [2]Agar-agar is 0.4 % instead of 0.6 % as in other media

RESULTS AND DISCUSSIONS

Decontamination of Rhodiola rosea seeds.

Ethyl alcohol treatment (Table 2, scheme 1) was insufficient for sterilization of 'old' and 'fresh' seeds. The latter needed longer time treatment with bleach in addition (scheme 2) for 100 % disinfection and high development ability (96 %). When scheme 3, 4 and 5 were used, the "old" seeds' decontamination was 100 %, however, without germination. Within 2-3 weeks bacterial contamination appeared around the seeds. On the same three schemes (number 3, 4, and 5) low germination rate (26 %, 23.7 %, and 22.5 %, respectively) was recorded for "fresh" seeds.

Table 2. Decontamination efficiency of *Rhodiola rosea* L seeds.

Plant material	Scheme №	Cultivated [number]	Decontamination [%]	Development of seeds and explants [%]
Seeds – 1 year stored				
	1	60	0	0
	2	60	0	0
	3	80	100	0
	4	80	100	0
	5	80	100	0
Seeds – "fresh"				
	1	60	0	0
	2	100	100	96.0
	3	100	100	26.0
	4	80	100	23.7
	5	80	100	22.5

In vitro seed germination and development of seedlings. Seeds germinated in all tested media. Germination started on the 7th day of cultivation and lasted until the 40th day reaching from 37.5 % to 97.0 % depending on media composition (Table 3). Culture media G3 and G4 containing gibberellic acid in high concentrations (50 mg/l or 100 mg/l, respectively) were the most efficient with 91.11 % and 93.0 % on the 20th day and 95.5 % and 97.0 % on the 40th day, respectively. Seedling size on the 20th day varied between 14 mm (G3 medium) and 20 mm (G2 medium). This size was appropriate to obtain explants (apical buds and stem cuttings) for propagation on Z-5 medium.

Table 3. *In vitro* seed germination and development of *Rodiola rosea*

Nutrition medium variant	Cultivated seeds [number]	Seed development		
		on the 20th day		on the 40th day
		Germination [%]	Seedling size [mm]	Germination [%]
G0	80	35.00	17.0 ± 0.09	37.5
G1	100	53.00	15.0 ± 0.08	66.0
G2	100	66.00	20.0 ± 0.06	72.0
G3	90	91.11	14.0 ± 0.06	95.5
G4	100	93.00	19.0 ± 0.07	97.0

Micropropagation of Rhodiola rosea from seedlings. Seedlings developed *in vitro* were cut to obtain apical buds and stem segments (Fig. 1) for propagation on Z-5 medium. The latter was chosen having in mind the effectiveness of zeatin in our previous experiments (Tasheva and Kosturkova, 2010). On this medium propagation rate of the both types of explants was not very significant.

Stem segment with a leaves node formed 78 % new shoots while apical buds - 75 %. The mean number of shoots per explant was 2.19 ± 0.16 and 2.00 ± 0.12 for the first and for the second type of explants, respectively. The shoot size was similar – 15.1 ± 0.11 mm and 14 ± 0.47 mm (on 15th days). The coefficient of propagation varied during the different seasons. Highest level of proliferation was observed in May-June, when the number of shoots per stem explant was 6.78 ± 0.10.

During cold seasons (October-March) multiplication was relatively lower - 2.11 ± 0.07 shoots per explant.

Fig 1. Explants from *in vitro* plants *Rhodiola rosea*.

Further, in our experiments, the effect of phytoregulators on the processes of propagation and rooting of regenerants obtained

from seeds germinated on different media, multiplied on Z-5 medium and rooted on various nutrient media.

Rooting. Plant regenerants 2.5 to 3.5 cm in size were transferred for rooting on eight different nutrient media containing GA_3 in combination of three auxins. Rhizogenesis of more than 58 % was observed on the all variants except of V2 forming callus. This medium contained NAA in contrast to the other media containing IBA or IAA. The process of rhizogenesis induction, root length and formation of new shoots from an explant was recorded for the different variants of culture media.

The most efficient rhizogenesis concerning percentage of root induction (higher than 85 %) and root length (longer than 2 cm) was observed when the first scheme (seed germination on G1 medium, seedling explants development on Z-5 medium and induction of rhizogenesis on all rooting media (V1, V3-V8 variants) (Table 4).

Table 4. Effect of culture media composition on the processes of rooting and propagation.

Rooting media	Scheme I: Seedling growth on G1 medium, explant development on Z-5, transfer of shoots on rooting media:			Scheme II: Seedling growth on G2 medium, explant development on Z-5, transfer of shoots on rooting media:			Scheme III: Seedling growth on G3 medium, explant development on Z-5, transfer of shoots on rooting media:			Scheme IV: Seedling growth on G4 medium, explant development on Z-5, transfer of shoots on rooting media:		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
V1	92	2.0± 0.03	4.0± 0.13	76	2.2± 0.06	3.0± 0.11	90	1.8±0. 10	4.4± 0.06	78	1.5± 0.12	2.6±0. 13
V2	Calli											
V3	85.7	1.63	2.26	43.6	2.07	1.27	86.7	1.14	1.18	65	1.08	2.65
V4	96	2.5± 0.09	6.0±0. 11	93	2.5± 0.09	3.4± 0.13	92	1.6±0. 10	4.5± 0.09	88	1.4± 0.07	6.5±0. 17
V5	85	1.6± 0.07	5.5± 0.13	67	2.0± 0.03	2.5± 0.12	78	1.5±0. 08	4.0± 0.03	72	1.8± 0.05	5.0±0. 19
V6	87	1.5± 0.09	5.0± 0.10	72	1.2± 0.09	4.0± 0.05	76	1.5±	4.5± 0.08	64	1.8± 0.14	5.0±0. 01
V7	92	1.9± 0.10	4.5±0. 03	95	1.0± 0.03	4.8± 0.14	48	1.8±0. 16	2.5± 0.08	42	1.2± 0.09	3.0±0. 02
V8	96	2.3± 0.05	6.0± 0.10	88	2.0± 0.03	5.5± 0.06	98	2.4±0. 08	6.5± 0.04	97	2.2± 0.12	7.4±0. 09

Legends: (1) Rooting [%]; (2) Root Length [cm]; (3) Propagation rate

Higher rooting efficiency of more than 85 % in four out of seven cases and relatively long roots was recorded for Scheme III where seed germination was on G3 medium and rooting of shoots was on V1, V4 and V8 media. Schemes II and IV were less efficient. Concerning the best rooting media – V8 and V4 were ranked at the first two places (Fig. 2). The both media contained IBA 2 mg/l, IAA 0.2 mg/l and GA_3, however, in different concentration – 1 mg/l for V4 and 0.4 mg/l for V8 medium.

Along with rhizogenesis, shoot formation continued bringing to regeneration of plants with different propagation rate depending on media composition for seedling development (G1-G4) and for rooting of shoots (V1-V8). The highest multiplication coefficient was recorded for Scheme I followed by IIIrd, IInd and IVth schemes.

Fig 2. *Rhodiola rosea* i*n vitro* rooting and new shoot formation on V8 medium.

Adaptation. Adaptation of obtained *in vitro* plants into their natural environment is one of the most delicate and difficult stages in elaborating micropropagation procedures. Usually regenerants formed well-developed root system. However, they quickly loose their turgor after transfer to soil. Their leaves withered and dried. These plants underwent stress due to the changes in humidity and culture medium. Our results showed that high air humidity of 80 % to 90 % during the first 5-10 days of acclimatization is very important for further plant survival (Table 5). The type of rooting substrates (soil, peat and perlite) has dramatic impact on plantlets development. Used in a single manner each of the substrates is ineffective (variants 1-4, Table 5). Combination of soil and perlite or peat brought to survival of 10-22 %. Best combination is soil, peat and perlite in ratio of 2:1:1 (variant 9). The same rooting medium is much more efficient when air humidity is very high – from 80 % to 90 % (Fig. 3).

Fig 3. Plant adaptation on rooting substrates (variant 10).

In this environment, survival of plantlets is nearly 60 % and their further development in wild nature in the mountains was possible.

Table 5. Survival of *Rhodiola rosea* plants regenerated *in vitro* after transfer to rooting substrates.

Variant	Rooting substrates (components and proportions)	Air humidity [%]	Survival [%]
1	Soil	60 - 70	4
2	Soil	80 - 90	2
3	Peat	80 - 90	2
4	Perlite	60 - 70	Roots get rot
5	Soil + perlite (3:1)	60 - 70	10
6	Soil + perlite (3:1)	80 - 90	12
7	Soil + peat (3:1)	60 - 70	10
8	Soil + peat (3:1)	80 - 90	22
9	Soil + peat + perlite (2:1:1)	60 - 70	35
10	Soil + peat + perlite (2:1:1)	80 - 90	57

CONCLUSIONS

Golden root seed germination started on the 7th day of cultivation and lasted until the 40th day reaching from 37.5 % to 97.0 % depending on media composition. Highest level of proliferation on Z-5 medium was observed in May-June, when the number of shoots per stem explant was 6.78. During cold seasons multiplication was relatively lower - 2.11 shoots per explant. The most efficient rhizogenesis concerning percentage of root induction (higher than 85 %) and root length (longer than 2 cm) was observed on the first scheme (seed germination on G1 medium, seedling explants development on Z-5 medium and induction of rhizogenesisi on all rooting media (V1, V3-V8 variants). Best combination for plant adaptation was soil, peat and perlite in ratio of 2:1:1 and very high air humidity (80 % to 90 %). In this environment, survival of plantlets is nearly 60 % and their further development in wild nature in the mountains was possible.

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HYGROMYCIN STIMULATES SOMATIC EMBRYOGENESIS FROM ROOT SECTIONS OF SPINACH

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ABSTRACT

Hygromycin (hyg) has been widely used as a selective agent for transformed cells selection. Thus, optimization of its concentration is essential. Somatic embryos were regenerated from root sections (1 cm) of highly responsive line of spinach (Spinacia oleracea L. cv. Matador). Root sections were placed on solid medium containing Murashige and Skoog's (MS) mineral solution, 20 μM α-naphthaleneacetic acid, 5 μM gibberellic acid and various concentrations of hyg (0, 1, 2.5, 5 and 7.5 mg/l). Hyg at 1 mg/l stimulated somatic embryogenesis comparing to control (11.9 versus 4.9 somatic embryos on average per root section). The frequency of regeneration was doubled in the presence of 1 mg/l hyg. The number of somatic embryos decreased to 4.2 at 2.5 mg/l hyg and kept decreasing with the hyg concentration. At 7.5 mg/l only solitary somatic embryos were formed from a few root sections. The embryogenic capacity was estimated to be 4 times higher at 1 mg/l hyg then in control.

Keywords: *Chenopodiaceae*, hygromycin, somatic embryogenesis, *Spinacia oleracea*, tissue culture

INTRODUCTION

The choice of a selective agent and optimization of its concentration is crucial for successful plant regeneration from transformed cells. Due to toxic effect, high concentrations of a selection agent may cause malformations or even inhibit regeneration. Numerous reports have demonstrated inhibition of morphogenesis *in vitro* provoked by kanamycin (Eapen and George 1990; Yepes and Aldwinckle 1994; Humara and Ordas 1999) even at concentrations as low as 5 mg/l. However, in *A. cepa* kanamycin was inefficient as selective agent even at 200 mg/l (Eady and Lister 1998).

Hygromycin B (hyg) is a widely used selective agent for the selection of transgenic cells. However, there are reports showing its cytokinin-like activity, which promoted *in vitro* regeneration at lower concentrations. Park *et al*. (1995) reported on stimulation of bud regeneration from potato leaves by hyg at 1 mg/l. In *Allium cepa* it was efficient in the induction of regeneration in the range of 10-30 mg/l and it was better than control at 20 mg/l (Eady and Lister 1998).

Many authors reported beneficial effect of other antibiotics on morphogenesis *in vitro*. Cefotaxime stimulated regeneration from immature embryos of *Sorghum bicolor* (Rao *et al*. 1995), barley (Mathias and Mukasa 1987) and wheat (Mathias and Boyd 1986), and bud regeneration from apple leaves (Yepes and Aldwinckle

1994), while timentin stimulated morphogenesis from tomato cotyledons (Costa *et al*. 2000) and epycotyls of *Citrus sinensis* (Da Silva Mendes *et al*. 2009).

MATERIALS AND METHODS

Plant material

Spinach (Spinacia oleracea L. cv. Matador) line 238-6-3-1, highly responsive to induction of regeneration, obtained by 3 cycles of self-pollination, was used for the experiment (Milojević et al. 2009). Donor plants were acquired by somatic embryogenesis from seedling's root sections. Somatic embryos (SE) were germinated and the plantlets were maintained on medium supplemented with 5 μM 6-furfurylaminopurine (kinetin, Kin).

Basal medium

The basal medium contained MS (Murashige and Skoog 1962) mineral solution and 20 g/l sucrose, 100 mg/l myo-inositol, 2 mg/l thiamine, 2 mg/l pyridoxine, 5 mg/l nicotinic acid and 2 mg/l adenine. The media were gelled with 0.7% (w/v) agar (Torlak, Belgrade, Serbia) and pH was adjusted to 5.6 before sterilization, by using pH-meter. The media were sterilized by autoclaving at 114ºC for 25 min.

Induction of regeneration from root sections and culture conditions

Procedure for induction of regeneration was essentially as was described in Knoll et al. (1997). Roots were isolated from donor plants and 1 cm long apical root sections were cut off and placed on basal medium with 20 μM α-naphthaleneacetic acid (NAA) and 5 μM gibberellic acid (GA_3) for a week, and then transferred to media of the same composition supplemented with hygromycin (hyg) at 0, 1, 2.5, 5 and 7.5 mg/l .

To assess tolerance of SE to hyg, SE (3-5 mm) obtained from root sections cultured on hyg-free medium were subcultivated on MS medium with variable concentration of hyg (0, 1, 2.5, 5, 7.5, 10, 12.5, 15 and 20 mg/l).

The cultures were maintained under cool white fluorescent tubes with a photon flux density of 55 □mol m^{-2} s^{-1} and a 16 h day length at 25 ± 2ºC.

Recordings and statistical analysis

All cultures were placed in a completely randomized design. For SE induction, experiment was performed in three replicates (Petri-dishes) with 20 samples (root sections per Petri dish) (n=60) per treatment. The number of SE per regenerating root section was recorded after 4-week culture period with the aid of a stereomicroscope.

For SE multiplication, each treatment consisted of three replicates with 15 samples (SE) per treatment (n = 45). The number of secondary somatic embryos per SE was counted following 4 weeks of treatment, using a stereomicroscope. Necrotic explants were also recorded.

Percentage data were subjected to angular tansformation and SE number data to square root transformation before analysis. The data were subjected to standard

analysis of variance (ANOVA), and the means were separated using Duncan's test at $P \leq 0.05$. For presentation, the data were inversely transformed. An index of somatic embryo-forming capacity (EFC) was used to evaluate cumulative effect of the mean SE number and frequency of regeneration, and it was calculated as follows: EFC = (mean number of SE per regenerating section) x (% of regenerating sections) / 100. For secondary regeneration from SE, we used an index of secondary somatic embryogenesis (SSE), calculated in the same manner as EFC.

RESULTS AND DISCUSSION

Induction of SE regeneration

In this experiment we used highly responsive line 238-6-3-1, which regenerated so efficiently that the whole root section's tissue turned into SE (Milojević *et al*. 2009). It showed early regeneration and produced no secondary roots. For this reasons, the results were recorded at the end of the first subculture, so the procedure was less laborious.

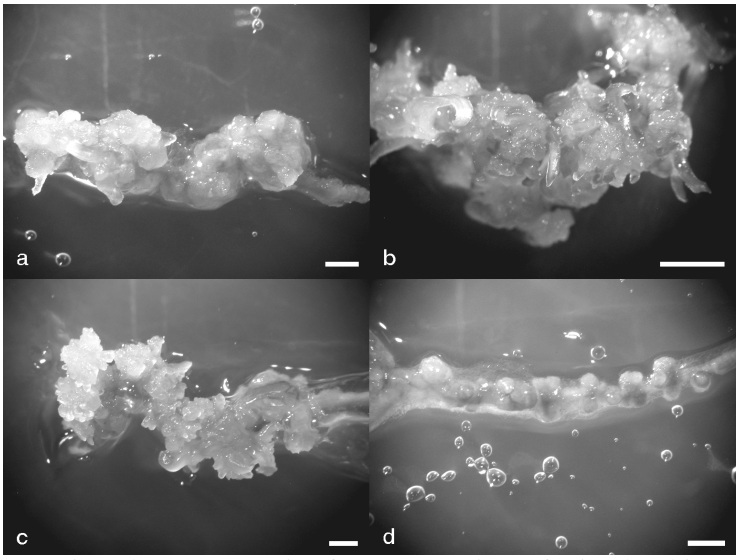

Fig. 1. - Somatic embryo regeneration from root sections of spinach cultivated on MS medium with 20 μM NAA, 5 μM GA_3 and **a**) 0 mg/l hyg, **b**) 1 mg/l hyg, **c**) 2.5 mg/l hyg and **d**) 7.5 mg/l hyg. a-d bar = 1 mm.

Spinach root fragments cultivated on media with variable hyg concentration proliferated, but the zones of proliferation were very limited and no voluminous calli were formed (Fig. 1). The proliferations formed from roots cultivated on 0,

1 and 2.5 mg/l hyg were not necrotic until the end of the experiment (Fig. 1a, b, c). At 5 mg/l hyg some proliferations became necrotic, while at 7.5 mg/l hyg all proliferations were necrotic (Fig. 1d). The roots themselves did not show any sign of necrosis in all media (Fig. 1 a-d).

Surprisingly, root sections cultured on medium with 1 mg/l hyg showed higher frequency of SE regeneration comparing to control (32.4% vs. 18.9%), as well as higher the mean SE number per root section (11.9 vs. 4.9) (Table 1.). EFC index was four times higher for 1 mg/l hyg then in control. At 2.5 mg/l hyg both values dropped to the control level, and kept increasing, so that at 7.5 mg/l hyg only few solitary SE were formed from a few root sections.

Table 1. - Regeneration of somatic embryos from root sections cultured on MS medium with 20 μM NAA, 5 μM GA_3 and hygromycin at 0, 1, 2.5, 5 and 7.5 mg/l.

Hygromycin concentration mg/l	Frequency of regeneration Mean ± SE	No.of somatic embryos per regenerating section Mean ± SE	EFC
0	18.9 ± 6.4 b	4.9 ± 1.5 b	0.93
1	32.4 ± 0.07 a	11.9 ± 3.1 a	3.85
2.5	21.8 ± 0.4 b	4.2 ± 0.7 bc	0.91
5	2.6 ± 0.9 c	1.5 ± 0.5 bc	0.04
7.5	0 c	0 c	0

Data indicate the mean ± standard error. Three samples, each with 20 subsamples (n = 60), were used per treatment. Treatments denoted by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's test. EFC- embryo-forming capacity, SE - standard error.

Although proliferations were visible even at 10 mg/l hyg, they became necrotic after ten days of culture and at 20 mg/l hyg proliferation was completely inhibited.

Our results are in accordance with Park *et al*. (1995), who also found 1 mg/l hyg stimulative for induction of regeneration in potato. Eady and Lister (1998) demonstrated hyg-stimulated regeneration in *A. cepa*, but at much higher concentration (20 mg/l).

Tolerance of SE to hygromycin

Spinach SE, which were induced on hyg-free medium, were assessed for tolerance to hyg. As for the induction of regeneration, hyg stimulated proces of secondary somatic embryogenesis in a very narrow range of concentrations (Table 2.). All values were the highest for 1 mg/l hyg, and the frequency of necrosis was similar to the control (Table 2.). The explants were healthy, without any sign of necrosis (Fig. 2), except they were a little malformed at hyg above 2.5 mg/l (Fig. 2c). These embryos were harvested and cultivated on MS medium with 5 μM Kin and all developed into normal plants, which rooted readily, attained maturity and flowered. SE necrosis was conspicuous starting from 5 mg/l hyg.

Table 2. - Secondary somatic embryogenesis from spinach somatic embryos cultured on MS medium with 20 µM NAA and 5 µM GA_3 and variable levels of hygromycin (hyg).

hyg (mg/l)	Frequency of secondary somatic embryogenesis (%) Mean ± SE	No. of secondary somatic embryos per prymary embryo Mean ± SE	SSE	Necrotic explants %
0	87.5 ± 0.9 ab	4.36 ± 0.05 b	3.81	6.1 ± 1.6 c
1	95.3 ± 1.8 a	6.29 ± 0.05 a	5.99	8.9 ± 2.4 c
2.5	81.7 ± 0.2 ab	5.35 ± 0.06 a	4.37	6.1 ± 1.6 c
5	75.9 ± 1.9 ab	2.73 ± 0.05 bc	2.07	30.2 ± 1.7 bc
7.5	69.1 ± 2.6 b	2.11 ± 0.04 c	1.46	53.4 ± 3.3 b
10	57.8 ± 1.1 bc	0.71 ± 0.03 d	0.41	98.1 ± 0.6 a
12.5	31.2 ± 2.2 cd	0.21 ± 0.02 de	0.06	100 a
15	25.0 ± 1.8 cd	0.11 ± 0.01 de	0.03	99.2 ± 0.8 a
20	7.4 ± 1.3 d	0.01 ± 0 e	0	100 a

Data indicate the mean ± standard error. Two samples, each with 15 subsamples (n = 30), were used per treatment. Treatments denoted by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's test. SSE - secondary somatic embryogenesis, SE - standard error.

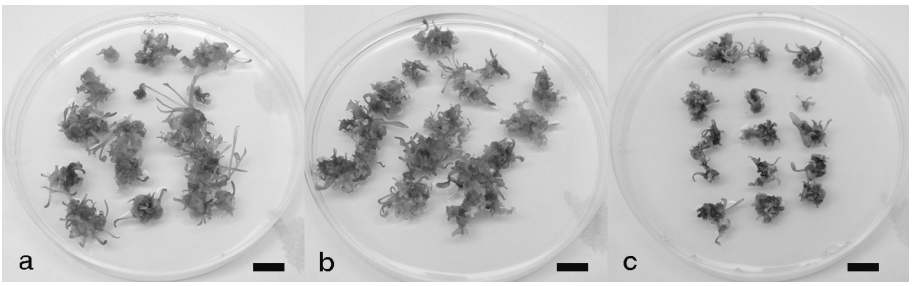

Fig. 2. - Secondary somatic embryo regeneration from somatic embryos cultured on MS medium supplemented with 20 µM NAA, 5 µM GA_3 and **a**) 0 mg/l hyg, **b**) 1 mg/l hyg and **c**) 7.5 mg/l hyg. a-c bar = 1 cm.

This study suggests 10 mg/l hyg as suitable level for selection of transgenic cells, at least under these experimental conditions. However, complete absence of proliferation was attained at 20 mg/l hyg. Chin *et al*. (2009) used this concentration to select transformed cells from calli induced from spinach leaf fragments.

Nevertheless, we suggest stepwise increase of hyg concentration, to benefit the stimulative effect of hyg on SE regeneration, as non-transformed spinich SE were intolerant to hyg and starting from 10 mg/l showed almost 100 % necrosis.

The effect of some penicillins was explained by a discovery of Holford and Newbury (1992), who found that these substances break down to give physiologically active levels of the auxin phenylacetic acid. However, to the best of our knowledge, this kind of research was not reported for hygromycin.

CONCLUSIONS

We reported here on stimulation of SE regeneration by lower doses of hyg. The results indicated 10 mg/l hyg as suitable level for selection, as the regeneration of non-transformed tissue and its multiplication were completely inhibited at this concentration. This study will be useful for genetic transformation of this responsive spinach line.

ACKNOWLEDGEMENTS

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SECTION II: BIOTECHNOLOGY IN VETERINARY MEDICINE

THE INFLUENCE OF PRO-PREBIOTIC BIOMIN IMBO ON FODDER CONSUMPTION AND NUTRIENT DIGESTIBILITY BY WEANING PIGS

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Key words: piglets, probiotics, weight gain, fodder consumption, nutrient digestibility

ABSTRACT

The aim of the trial was to determine the influence of the optimum level of Biomin IMBO introduced into the mixed fodder for the young breeding pigs on fodder consumption and nutrient digestibility. According to the target of the scientific experiment four groups of piglets were selected. The trial was carried out at the enterprise for pig breeding "Moldsuinhibrid".

The obtained results showed that the introduction of probiotic Biomin IMBO into the fodder for pigs in quantities of 0.5 - 2.0 kg/t did not significantly influence the weight gain. At the same time, it was revealed a tendency for fodder consumption unit to decrease. The survey experiment demonstrated that under the influence of probiotic Biomin IMBO the digestibility of some nutrients in the young pigs in groups LE_2 and LE_3, which were administered the preparation in quantities of 1.5 - 2.0 kg/t, improved.

INTRODUCTION

Probiotics can be defined as live micro organisms in nutritive supplements, which have a beneficial effect on the host animal by improving intestinal microbial balance and health in general (Jensen, 1998, Houdijk et.al., 1999, Corcionivoschi et.al., 2009).

Many studies have demonstrated that probiotics have positive effects on performance, health, vitality, intestinal ecology and digestibility, although the mode of action of probiotics is still not fully explained (Smirnov, 1993, Fialho et. al.)

The main mechanisms of probiotics' action are: the reduction of intestinal pH, which makes it unsuitable for pathogens and conditional pathogens (Salmonella spp, E.coli, etc.); the reduction of intestinal colonization by pathogenic organisms; the stimulation of endogenous enzymes production and the increase of rate absorption in the small intestine; the stimulation of body immunity by increasing the number of antibodies and macrophages's activity (Evans, 1988, Taracanov, 1998, Nozdrin et.al., 2009).

From this point of view, probiotics should be considered as part of rational potential of animals that will help to maintain their health and to obtain production of high quality, safe both in bacterial and chemical terms (Ouwerhand et.al., 2002).

For a better understanding of the action mechanisms of probiotics in pigs, and nutrient availability and digestion, we studied the preparation pro-prebiotic Biomin IMBO.

The main objective of the investigation was to improve the knowledge about the influence of probiotics on bio parameters in pigs and their ability to be used as growth promoters in conditions of Moldova.

MATERIALS AND METHODS

The aim of the scientific researches was to establish the optimum level of Biomin IMBO addition in the fodder for piglets in the zone conditions of the Republic of Moldova. The change of growth indicators, the development of piglets, and the fodder consumption under the influence of probiotic supplement were also studied. During the period 18.03.2009- 24.08.2009 a scientific experiment was carried out at the State Research and Production Enterprise "Modsuinhibrid" that breeds pigs.

According to the research scheme (tab.1) four groups of piglets aged 35 days were chosen according to the principle of pairs-analogues (the same bred, age, body mass; Ovseannicov, 1976, Pocerneaev, 1977).

The peculiarity of feeding experimental piglets – LE_1, LE_2, LE_3 groups – consisted in Biomin IMBO supplementation in the basic fodder at the level indicated in the scheme.

Table 1. Scheme of the trial

Group	Number of pigs in a group, head	Feeding features
LM - control	10	BF (basic fodder)
LE_1 - experimental	10	BF+1.0, 0.5 kg/t of probiotic Biomin IMBO
LE_2 - experimental	10	BF+1.5, 1.0 kg/t of probiotic Biomin IMBO
LE_3 - experimental	10	BF+2.0, 1.5 kg/t of probiotic Biomin IMBO

During the scientific and practical experiment a digestibility test was conducted over a period of 13 days beginning on 18.05.2009 at the enterprise "Moldsuinhibrid' (according to the method described by Pocerneaev and others, 1979; Pop and others, 2006).

For this experiment twelve young pigs, similar by age, sex and body weight, were used which were divided into four groups each with 3 heads. A group was selected as a witness one (LM), the rest of them were experimental groups (LE_1, LE_2, LE_3).

The young pigs were fed in accordance with the regulatory requirements (Kalashnicov, et.al., 2003) and the feeding technology at the enterprise. It was investigated the effect of supplementation of mixed fodder with the preparation Biomin IMBO (a complex of four active ingredients: probiotic "Enterococcus faecium", prebiotic "Inulin", fragments of cell walls, ficofitic substances, seaweed extract) in a concentration of 1.0 kg / tonne, 1.5 kg / tonne and 2.0 kg / tonne on the nutrient digestibility (tab.2).

Table 2. Scheme of the digestibility experiment

Group	Number of pigs in a group, head	Feeding features
LM	3	BF (basic mixed fodder)
LE_1	3	BF + Biomin Imbo (1.0 кg/t)
LE_2	3	BF + Biomin Imbo (1.5 кg/t)
LE_3	3	BF + Biomin Imbo (2.0 кg/t)

Samples of fodder, faeces and urine were conditioned and analyzed chemically according to Petuhova, et.al. (1989).

The statistical interpretation of obtained results was performed according to the methodology described by Ovseanicov (1976), Cucu, et.al., (2004).

RESULTS AND DISCUSSION

The trial lasted 145 days, of which 10 days constituted the preliminary period; the testing period was divided into sub periods. According to the requirements of feeding norms, and taking into consideration the age differences and body mass change, the experimental piglets were given the fodder produced at the enterprise.

Table 3 The mixed fodder composition used in the experiment

Ingredients	%, by mass	
	I	II
Grain corn	7.5	27.5
Extruded corn	15.0	-
Extruded wheat	17.0	17.0
Extruded peas	8.0	8.0
Extruded barley	19.0	19.0
Soya grouts	14.1	-
Sunflower grouts	4.0	13.1
Fish meal	5.0	5.0
Powdered milk	5.0	5.0
Premix 2231	2.0	2.0
Soya oil	2.0	2.0
Salt	0.5	0.5
Chalk	0.9	0.9

This study suggests 10 mg/l hyg as suitable level for selection of transgenic cells, at least under these experimental conditions. However, complete absence of proliferation was attained at 20 mg/l hyg. Chin *et al*. (2009) used this concentration to select transformed cells from calli induced from spinach leaf fragments. Nevertheless, we suggest stepwise increase of hyg concentration, to benefit the stimulative effect of hyg on SE regeneration, as non-transformed spinich SE were intolerant to hyg and starting from 10 mg/l showed almost 100 % necrosis.

The effect of some penicillins was explained by a discovery of Holford and Newbury (1992), who found that these substances break down to give physiologically active levels of the auxin phenylacetic acid. However, to the best of our knowledge, this kind of research was not reported for hygromycin.

The concentration of nutritional substances in 1 kg of fodder according to the first period was 1.02 NEU, 1.15 OKE or 12.51 MJ and 150.9 g of the digestible protein; in the second period respectively 1.13 NEU, 1.0 OKE or 11.37 MJ and 150.6 g (tab.4).

Table 4 The mixed fodder nutritional characteristics

Issue	Quantity	
	I	II
Energy fodder units	1.02	1.00
Fodder units	1.15	1.13
Exchange energy, MJ	12.51	11.37
Dried matter, kg	0.84	0.84
Crude protein, g	177.64	178.40
Digestible protein, g	150.90	150.55
Crude fibber, g	33.81	35.09
Lysine, g	9.29	9.11
Methionine + cysteine, g	5.48	5.52
Sodium salt, g	0.50	0.50
Calcium, g	9.00	8.81
Phosphorus, g	5.61	5.80
Iron, mg	139.00	138.37
Copper, mg	6.30	6.28
Zinc, mg	30.56	31.63
Manganese, mg	19.35	19.17
Cobalt, mg	0.11	0.11
Iodine, mg	0.28	0.28

During the scientific experiment the piglets were weighed individually (tab.5, fig.1).

The data obtained from weighing of the piglets showed that with the same live weight, at the beginning of the experiment this indicator had been changing according to the age periods and to the level of probiotic Biomin IMBO supplementation of the fodder.

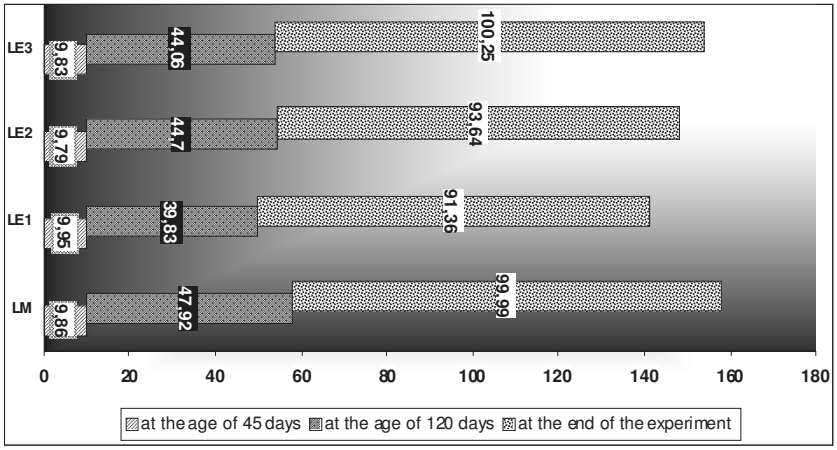

Fig.1. The average weight gain of piglets in the experiment, kg

Thus, at the early stages of the piglets' development the introduction of probiotic in the fodder was the best at the level 2.0 kg/t in the second experimental group; then the same level of supplement was more efficient at the end of the experiment in the LE_4 experimental group.

Table 5 Average weight gain of piglets

Lot	The average weight of piglets, kg		
	at the beginning of the experiment	at the end of the II nd period of the experiment	at the end of the experiment
LM	9.86 ± 0.069	47.92 ± 1.819	99.99 ± 1.986
LE1	9.95 ± 0.070	39.83 ± 1.775	91.36 ± 3.640
LE2	9.79 ± 0.083	44.70 ± 1.702	93.64 ± 1.678
LE3	9.83 ± 0.075	44.06 ± 2.260	100.25 ± 1.917

The live weight of the piglets in group LE_2 and LE_3 which got probiotic supplement at the level of 1.5-1.0 and 2.0-1.5 g/t, according to the stages of the experiment, was a little higher – by 0.41 % (100.40 kg) – than in the control group (LM); in group LE_1 the piglets weight was 91.36 during the experiment, while in group LE_2 the weight was 93.64 kg, that is much lower than the weight of the piglets in the control group.

During the trial the average daily weight gain was of 0.609; 0.550; 0.567 and 0.611 kg/head respectively in LM and LE_1, LE_2, LE_3 experimental groups.

Table 6 The fodder consumption by young pigs in the trial

Month	Groups			
	LM	LE_1	LE_2	LE_3
April	389.88	358.30	365.87	361.85
May	595.13	558.22	581.09	557.69
June	799.37	693.21	785.70	794.76
July	875.92	819.20	843.12	856.95
August	800.10	680.41	689.53	788.99
Total over the trial	3460.04	3109.34	3265.31	3360.24

The administered fodder and its consumption were registered every day. The analysis of the fodder consumption data according to the periods of the piglet's growth (tab.6, fig.2) showed, that during the first month of growing, the highest fodder consumption was observed in LM-control group, and it constituted 389.88 kg per month, while in the experimental groups the fodder consumption was lower by 8.10; 6.16 and 7.19 % respectively in LE_1, LE_2 and LE_3 groups.

The same regularity was observed during the experiment, and the total fodder consumption was as follows according to the groups: in LM – 3460.4 kg, in LE_1 – 3109.34 kg in LE_2 – 3265.31 kg and in LB_3 _ 3360.24 kg. Thus, the fodder consumption was lower in comparison with LM respectively with 10.15, 5.64 and 2.89 %.

The efficiency of probiotic Biomin IMBO introduction into the fodder for piglets was established according to the size of live mass gain and to the fodder use for gain unit.

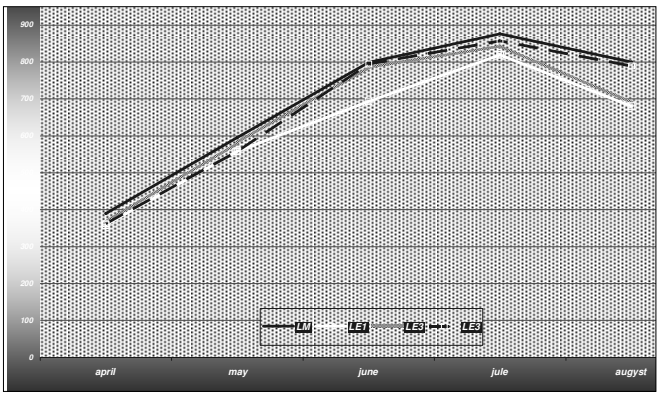

Fig.2 Fodder consumption during the trial

The feed consumption per unit of live weight of piglets was slightly lower in the groups that were administered the probiotic Biomin IMBO and constituted respectively in the groups LM-LE_3 – 3.84; 3.82; 3.89 and 3.72 kg. Thus, the level of probiotic addition in the fodder for growing pigs was the most efficient in the first period of growing – 2.0 kg/t, and 1.5 kg/t in the second trial period of growing.

During the digestibility test daily measurements of fodder and water intake and the quantity of faeces and urine were recorded (tab.7).

Table 7 Record data during the test on digestibility (average per head)

Group	On average over 24 hours			
	Ingestion		Excretion	
	mixed fodder, кg	water, l	faeces, кg	urine, l
LM	1,380	3,077	0,478	1,398
LE_1	1,515	3,227	0,535	1,486
LE_2	1,510	3,371	0,487	1,744
LE_3	1,533	4,805	0,521	2,570

The analysis of food and eliminated products record data showed that the young pigs, whose ration was supplemented with the preparation pro-prebiotic Biomin IMBO of different levels, consumed a greater quantity of mixed fodder in comparison with LM: by 9.78%, 9.42% and 11.09% respectively for LE_1, LE_2, and LE_3. At the same time it was observed that faeces eliminations were higher in LE_1 and LE_3 in comparison with LM respectively with 11.9 and 9.0%, and 1.9% for the young pigs in LE_2.

During the test there was a trend towards increased consumption of water in the experimental piglets, and the same trend towards growth and urine discharges. As it was already mentioned, throughout the whole experimental period faecal samples were collected and conditioned which were chemically analyzed (tab.8) according to the laboratory methodology described by Petuhova (1989).

Table 8 Chemical composition of faeces eliminated during the digestibility experiment, g

Specification	Group			
	LM	LE_1	LE_2	LE_3
Dry matter	145,42	163,62	151,76	159,62
Organic substance	113,09	125,88	111,89	121,37
Crude ash	32,34	37,74	39,87	38,25
Crude protein	30,00	33,41	32,27	32,18
Crude fat	6,48	7,25	6,02	7,05
Crude fiber	43,03	48,61	42,68	46,58
Gross SEN	33,58	36,62	30,92	35,56

The coefficients of digestibility of nutrients in the mixed fodder were determined according to the records of fodder consumption and faeces discharges during the digestibility testing and to the primary chemical analysis.

Researches on nutrient use showed that the supplementation of the basic rations for young pigs in LE_1 with the preparation pro-prebiotic “Biomin IMBO” at the recommended level did not significantly affect the digestibility of nutrients in the basic mixed fodder (tab.9).

Table 9 Digestibility of nutrients, % $\overline{X} \pm S_{\overline{x}}$

Specification	Group			
	LM	LE $_1$	LE $_2$	LE $_3$
Dry matter	87,6±0,41	87,3±1,18	88,2±0,74	87,8±0,56
Organic substance	89,8±0,25	89,7±0,95	90,8±0,54	90,2±0,32
Crude ash	47,2±3,35	43,9±5,74	40,5±4,96	43,8±5,18
Crude protein	84,2±0,40	84,0±1,58	84,5±1,00	84,7±0,82
Crude fat	78,3±0,91	77,8±0,85	81,5±1,58	78,7±2,92
Crude fiber	49,9±1,76	48,4±6,28	54,5±4,58	51,1±3,76
Gross SEN	95,9±0,10	95,9±0,48	96,5±0,01	96,0±0,30

The data shows that there are not significant differences on the coefficients of digestibility of dry and organic substances in the experimental groups compared to the control group. Digestibility coefficient (CD) of dry matter in LE_1, LE_2 and LE_3 ranging respectively from 0.3 in LE_1 to 0.6 and 0.2 in LE_2, LE_3.

The results of balance of experiment showed that the piglets in LE_2 and LE_3 that received fodder supplemented with pro-prebiotic at the level of 1.5 kg / t and 2.0 kg / t achieved a slightly higher digestibility compared with the control group. Therefore, the young pigs in LE_2, the ration of which was supplemented with the preparation at a level of 1.5 kg/t, improved their protein digestibility by 0.3%, the fat digestibility by 3.2%, and respectively the piglets in LE3 improved their protein digestibility by 0,5% and the fat digestibility by 0,4% compared with LM.

The supplementation of the basic ration with pro-prebiotic preparation influenced the digestibility of crude fibre in the piglets in LE2 which was by 4.6%, and in LE3 by 1.2% higher as compared with LM.

The non nitrogenous extractive substances in LE1 were in the quantity of 95.9%, being at the same level as in LM. The digestibility in the piglets in LE2 was of 96.5%, which was 0.6%, and respectively in LE3 – 0.1%, higher than in LM and LE1. During the digestibility test it was observed a negative trend of fodder digestibility of crude ash. The lowest coefficient of digestibility was observed in the piglets in LE2 at a level of 40.5% which was 6.7% less than in LM. The value of the digestibility coefficient in LE1 and LE3 was within the limits 43.9 and 43.8% which represented a decrease from 3.3 to 3.4% respectively compared to LM.

CONCLUSIONS

- The introduction of Probiotic Biomin IMBO into the fodder for piglets didn't influence much the change of their live weight.

- As a result of probiotic supplement Biomin IMBO introduction into the fodder for the piglets in the experimental groups, there was registered a decrease of fodder consumption by 8.10, 6.16 and 7.19 % respectively in LB_1, LB_2 and LB $_3$.

- The use of fodder per unit of live weight gain of the piglets was insignificantly lower in the groups that were administrated the probiotic supplement Biomin IMBO and it constituted in LM-LB_3 groups respectively: 3.84, 3.82, 3.89 and 3.72 kg.

- The supplementation of the basic rations with pro-prebiotic Biomin IMBO in the dose of 1.5 kg / t in group LE_2 and 2.0 kg / t in LE_3 during the growing period of the piglets increased the cellulose digestibility by 4.6 and 1.2%, the non nitrogenous extractive substances digestibility by 0.6 and 0.1%, the protein digestibility by 0.3 and 0.5%, and the fat digestibility by 3.2 and 0.4% respectively.

- The optimum level of the preparation Biomin IMBO administration was determined to be equal to 1.5 kg/t.

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SOME RESULTS ON EFFECT OF NACN ON THE UPTAKE IN VITRO OF A PARTICULAR AMINO ACID BY INTESTINAL PREPARATIONS FROM A CERTAIN SPECIES OF FISH

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ABSTRACT

The topic of this paper is determining effect of NaCN on the uptake in vitro of a particular amino acid by intestinal preparations from a certain species of fish, through one of the most used statistical methods, analysis of variance. Three preparations were made for each of four fish. We presented certain theoretical results in the literature in connection to the analysis of variance in testing significance of regression, and then, we applied this method to study the experimental data. Finally, we established a report on the relationship between the variance due to differences between fish and between replicates

Keywords: analysis of variance, sum of square of deviations, degrees of freedom, standard error of a treatment mean

1. INTRODUCTION

The analysis of variances is a good technique for the study of the properties of an experimental situation, having multiple applications in medicine, biology, agriculture, engineering, economics, as well as other domains. In many problem by reality, we want determine the effect of variability factors acting on a character of a statistical population, wich can be calculated experimentally and we propose establish the differences between the means of more than two samples. It is well known that for solve these situations, we must organize the observational data for determine certain indicators. The statistical test as: Fisher test, Tukey test and Multiple-range test, using shortest significants ranges (S.S.R.) are only a few techniques that findings may be relevant

2. METHOD

We suppose that the character (X) of a statistical population, which in terms of probabilities is a random variable normally distributed with mean μ and dispersion σ^2, one factor (A) actings simultaneously, his influence evidenced by mean of character X.

It considers that factor A has $A_1, A_2, ..., A_m$ - m versions. The experience gives us m observation made for each experiment x_{ij}, $i = \overline{1,m}$, $j = \overline{1,n}$.

The observational data can be arranged in the table below:

Table 1- The observational data for *m* version and *n* experiments

y / A	A_1	A_2	...	A_m
1	x_{11}	x_{21}	...	x_{m1}
...	...	...	...	...
n	x_{1n}	x_{2n}	...	x_{mn}
$\overline{x_i}$	$\overline{x_1}$	$\overline{x_2}$	...	$\overline{x_m}$

We want to establish how the factors *A* acts on the character *X* by applying Fisher test. This involves the calculation of value based on experimental observation which compares with a table value corresponding to a particular confidence probability. If the experimental value is greater than the tabular, then the influence of factor on studied character is significant.

The sizes involved in the problem of analysis of variance lead to finding the experimental value, noted F_{exp} can be arranged in a table, as follows:

Table 2- The table of analysis of variance

Sources of variation	Sum of square	Degrees of freedom	Variances
A	SPA_A	GL_A	S_A^2
R	SPA_R	GL_R	S_R^2
T	SPA_T	GL_T	

and they have the following meanings:

1. The sum of squares of deviations (*SPA*)

- Sum of squares of deviations due to factor *A*:

$$SPA_A = m\sum_{i=1}^{m}\left(\overline{X_i} - \overline{X}\right)^2 . \quad (1)$$

- Total sum of squares of deviations:

$$SPA_T = \sum_{i=1}^{m}\sum_{j=1}^{n}\left(X_{ij} - \overline{X}\right)^2 . \quad (2)$$

- Sum of squares of deviations of error (residual):

$$SPA_R = SPA_T - SPA_A . \quad (3)$$

2. The degrees of freedom (*GL*)
- Degrees of freedom of factor *A*:

$$GL_A = m-1. \tag{4}$$

- Total degrees of freedom:

$$GL_T = mn-1. \tag{5}$$

- Degrees of freedom of error (residual)

$$GL_R = GL_T - GL_A = m(n-1). \tag{6}$$

3. The variances (S^2)
- Variance of factor *A*:

$$S_A^2 = SPA_A / GL_A . \tag{7}$$

- Residual variance:

$$S_R^2 = SPA_R / GL_R . \tag{8}$$

The influence factor *A* is highlighted by applying Fisher test to variable $F_{\exp A} = S_A^2 / S_R^2$. If this value is equal to or greater than tabulated value, we conclude that the samples have been drawn from populations with different means; or, in other words, that there are significant differences between the treatment means.

To establish the connection strength between factor *A* and character *X*, the correlation index is calculated by formula:

$$I_C(A) = \sqrt{SPA_A / SPA_T} \tag{9}$$

If this index is close to value 1, then the correlation between factor *A* and character *X* is very significant.

When the preliminary analysis of variance has indicated that there are significant differences between the treatment means, we apply multiple range test using S.S.R First, we compute the standard error of a treatment mean $\sqrt{S^2/m}$. Then, we look up values of Q (in the table of "Studentized range") for the number o degrees of freedom or error and for $n, n-1, ...2$ treatments. We compute S.S.R. for groups of $n, n-1, ...2$ as $Q\left(\sqrt{S^2/m}\right)$.

Having arranged the treatment means in order of magnitude, we test the total difference between the largest and smallest mean by comparison with the S.S.R. for m means. If this is non-significant, we stop testing. If this difference is significant, we test the differences between the largest and next-to- smallest mean, and the next-to-largest and the smallest mean, by comparison with the S.S.R. for $m-1$ means. We continue in this way for as long as differences prove significant but if a difference proves to be non-significant we do not test any differences within it.

Another method for compare the variant averages is the Tukey test. It consists in making a table in which the first column are ordered descending circles and lines, ascending. Then, it calculates the differences between these values, keeping only the positive values and thus, it obtains the average difference triangle. It determines the amplitude, by formula:

$$A_{\alpha} = \sqrt{S_R^2 / m} \times T_{tab} \qquad (10)$$

were S_R^2 is the sum of squares of deviations of error, and T_{tab} is the table value of Tukey test, according to α and m, the number of the averages variants. If the average difference values of the triangle are smaller than the amplitude, then the difference of the average is insignificant.

Further, we solve a problem proposed in the paper [] using statistical methods outlined above.

In an investigation of the effect of NaCN on the uptake in vitro of a particular amino acid by intestinal preparations from a certain species of fish, it was found that each fish would give only about six preparations. Since it would be necessary to use more than one fish in each experiment, a preliminary test was carried out to examine the variation between preparations from different fish. Three preparations were made for each of four fish.

The results obtained, expressed as μ mol g^{-1} dry weight per 20 min period, were as follows:

Table 3- The table of experimental data

version/fish	A_1	A_2	A_3	A_4
1	2,53	2,02	1,66	1,36
2	2,04	1,92	1,92	1,15
3	2,34	2,03	1,47	1,16

3. RESULTS AND DISCUSSIONS

First, we calculate the variant averages:

$$\overline{x_1} = 2,30\,;\ \overline{x_2} = 1,99\,;\ \overline{x_3} = 1,68\,;\ \overline{x_4} = 1,22$$

The total mean is $\overline{X} = \frac{\sum_{i=1}^{m} \overline{x_i}}{m} = 1,80$.

Now, we complete the table of variance analysis and we calculate the sum of the squares of deviations, the degree of freedom, options to ultimately reach the experimental value of Fisher test. We shall use formulas (1) - (8).

Table 4- The table of analysis of variance for experimental data

Sources of variation	Sum of square	Degrees of freedom	Variances
A	$SPA_A = 2,48$	$GL_A = 3$	$S_A^2 = 0,82$
R	$SPA_R = 0,38$	$GL_R = 8$	$S_R^2 = 0,04$
T	$SPA_T = 2,1$	$GL_T = 11$	

The experimental value of Fisher test is $F_{\exp A} = S_A^2 / S_R^2 = 20,5$.

The between-fish variance is about 20 times that between replicates for the same fish. The value table for Fisher test with $(3,8)$ degrees of freedom exceeds the experimental value for a probability of 0,001. In this case, the null hypothesis is accepted, i.e. the variant averages are not significantly different.

We calculate the correlation index, by formula (9).

$$I_C(A) = \sqrt{SPA_A / SPA_T} = 1,08 \approx 1.$$

We conclude that correlation between effect of NaCN on the uptake in vitro of a particular amino acid by intestinal preparations is very significant.

We determinate now the variance of *x* by formula:

$$S^2 = M(x^2) - M^2(x) = 3,41 - 3,24 = 0,17,$$

and the standard error of a treatment mean is $\sqrt{S^2/m} = 0,23$. Than, we compute the shortest significant ranges, i.e. S.S.R. for groups of 3 treatments and $GL_R = 8$, as $Q(0,23) = 4,04$ and for 2 treatments and $GL_R = 8$ as $Q(0,23) = 3,26$. The

difference between them is 0,78 and we compare it with the total difference between the largest and smallest mean, i.e. $\overline{x_1} - \overline{x_4} = 2,30 - 1,22 = 1,08$. Because $0,78 < 1,08$, we can say that it non-significant and we stop testing.

We observe that we obtain the same conclusion as Fisher test.

We apply the Tukey test to see if it reaches the same conclusion that if the Fisher test. For this, we calculate the triangle of difference averages:

Table 4- The table of Tukey test

$\overline{X_i}$	1,22	1,68	1,99	2,30
2,30	0,08	0,62	1,08	
1,99	0,77	0,31		
1,68	0,46			
1,22				

In this case, the amplitude, determined by formula (10) is:

$$A_\alpha = \sqrt{S_R^2 / m} \times T_{tab} = \sqrt{0,38 / 3} \times 3,82 = 1,35 .$$

We observe that the average difference values of the triangle are smaller than the amplitude, then the difference of the averages is insignificant.

4. CONCLUSIONS

Analyzing experimental data and more statistical tests, we reached the same conclusion: the diference of the variant averages are insignificant and we can say that the variance due to differences between fish and that due to differences between replicate preparations from the same fish.

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SECTION III: FOOD BIOTECHNOLOGY

RESEARCH ON THE ROLE OF BIOTEHNOLOGIES IN IMPROVING THE QUALITY AND TYPICAL OF WINES OBTAINED IN OLTENIA VINEYARDS

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Researches conducted for over a decade in the hilly of Oltenia vineyards showed that the use of biotechnology tools is a feature of modern winemaking. The obtaining of wines with compositional and sensory characteristics that harness the true potential as varieties and growing areas requires control of biophysical and biochemical phenomena that occur in the transformation of gravy into wine - maceration, alcoholic and malolactic fermentation. The use of selected yeasts and lactic acid bacteria with suitable physiologo-biochemical traits for type of wine who wants to be obtained, the fermentation activators and enzyme preparations have a strong influence on color, aroma and gustatory balance of wine as well as on his evolution during the maturation and aging.

Key words: biotechnologies, wines, alcoholic fermentation, winemaking

INTRODUCTION

Among all aliments and drinks that the human being consumes, the wine is one with the most complex composition, containing hundreds of constituents whose numbers increase continuously due to improvement of investigation methods (Stoian, V., 2001). The wine constituents in its impressive numbers classifies after 3 criteria: the high temperature behavior, chemical nature, and source. Based on the last criteria, distinguish constituents which come from grapes, constituents created during the alcoholic fermentation or other biochemical processes and constituents created during the wine evolution phases (Teodorescu, Şt., 1970). The chemical composition of wine is radically different from the must composition comes from. From some must chemical constituents, during its transformation in wine, under the action of yeasts enzymatic systems, form other constituents with different traits and chemical structures (Gheorghiţă, M., 2006).

The alcoholic fermentation and the other biophysical and biochemical complex phenomena that occur during the transformation of must in wine – maceration and malolactic fermentation – are the origin of hundreds of wine constituents, very different among them as chemical structure, proportions and sensorial importance (Băducă Cîmpeanu, C., 2008). The alcoholic fermentation is the phase when the wine is born and any defects of composition or sensory properties occurred in this phase is hardly to correct or may be cannot be corrected over the next wine evolution phases (Băducă Cîmpeanu, C., 2003). That's why the stringency of controlling the alcoholic fermentation and the other biochemical

processes that occur during the winemaking represents a characteristic of modern Oenology (Blouin J., Peynaud E., 2006).
For the Oltenian vineyards, one of the most important Romanian wine region (Popa. A., 2008), the biotechnological possibilities of stimulation and control of biochemical and biophysical phenomena represent an important opportunity to increase the quality, expressivity and the typical of wines.

MATERIALS AND METHODS

This work has been finalized based on the research made over the past decade at the University of Craiova, Faculty of Horticulture, Department of Oenology in the main vineyards from the hilly Oltenia, with the purpose of improving the white and red winemaking technologies. The targeted technological sequences have been mainly the based on the biophysical and biochemical complex phenomena, which occur during the transformation of must in wine – maceration, alcoholic fermentation and malolactic fermentation. From the multitude of biological, biochemical and technological factors involved in the control of mentioned processes, for this work it has been retained the role of yeasts, bacteria and enzymes as biotechnological tools of the winemaker.

The research made have been done in micro-winemaking conditions in the Oenology laboratory of Faculty of Horticulture or at the pilot station of Craiova University, as well as in production conditions, in famous wine cellars from Samburesti, Dragasani, Dealurile Craiovei, Vinju Mare vineyards, with the purpose of making superior quality wines from indigenous or foreign varieties cultivated in mentioned vineyards. Therefore, this work represents a synthesis of research made at the Oenology Department of Faculty of Horticulture, materialized in research contracts, doctorate thesis, university manuals and scientific works published in country or abroad from 1997 until today.

RESULTS AND DISCUSSIONS

Results and discussions concerning the role of the yeasts

In wine microbiology, the yeasts are considered agents of alcoholic fermentation, being responsible for the transformation of sugar in alcohol. By their technological characteristics and physiological-biochemical traits, the yeasts have a significant influence on the main composition parameters and sensory properties traits of wines. In the last 2-3 decades, the use of selected yeasts in winemaking has witnessed a great expansion due to many advantages. Despite this, it persists a series of controversies regarding the utility of selected yeasts in relation with the indigenous yeasts. The research made in many Oltenia vineyards in the last 15 years comes to a series of clarification regarding these controversies. In Table 1 is presented a synthesis of results regarding the influence of these two types of yeasts on starting and ongoing of alcoholic fermentation. The first analyzed aspect, the

duration of pre-fermentative phase, shows that in all cases when it has been used selected yeasts, the alcoholic fermentation started more rapidly due to higher density of initial population. The differences are more important in white winemaking, where the higher doses of sulphur dioxide and the must clarification decrease significantly the initial level of indigenous yeasts population. While the white must, regardless of variety, vineyard or wine year, from debourbage to the beginning of decrease of must density, takes between 30 to 72 hours at fermentation with indigenous yeasts, by addition of selected yeasts, the must start fermenting in less than 30 hours. Most of the times, the start of fermentation occurs in less than 24 hours. This tendency has been noticed also on red winemaking mentioning that the differences are little bit smaller due to maintaining of yeasts from indigenous micro flora in contact with the must.

Table 1 The influence of different types of yeasts on the ongoing alcoholic fermentation

Type of yeasts	Wines	Duration of pre-fermentative phase, hours	Duration of fermentation, days	Fermentation efficiency g/% vol. alcohol
Indigenous yeasts	White	30 – 72	10 – 18	16.8 – 17.4
	Red	18 – 48	8 – 14	17.2 – 17.8
Selected yeasts	White	18 – 30	8 – 12	16.4 – 17.1
	Red	12 – 24	6 – 9	16.7 – 17.2

The second aspect analyzed is the duration of alcoholic fermentation, depending on type of wines (white or red) and the winemaking conditions, especially the temperature control. In the case of selected yeasts, the duration of fermentation is 2-3 up to 8-10 days shorter, a very important aspect from economic and oenological point of view. The start and faster ongoing of alcoholic fermentation, eliminating the slowly and stop of fermentation represent one of the major advantage and the main argument in favor of using selected yeasts in winemaking.

Another aspect analyzed related to the yeasts fermentative capacity is the fermentation efficiency. The data from Table 1 show that the selected yeasts consume less sugar to produce 1% volume of alcohol, whatever the type of wine. So, in the white must, the selected yeasts consume between 16.4 – 17.1g of sugar while the indigenous yeasts consume between 16.8 – 17.4g of sugar to produce 1% alcohol. In red must, the consumption is little different and here the selected yeasts have better fermentation efficiency.

The superior fermentative capacity of selected yeasts is reflected in chemical composition of wine, as it can be seen from Table 2 where is represented a synthesis of results regarding the modification of main composition parameters of fermented wines with selected yeasts comparing to indigenous yeasts. From several

hundreds of fermenting experiences with both types of yeasts shows that the wines made with selected yeasts for fermentation contain 0.2 up to 3.5% vol more alcohol, 0.1 up to 1.8 g/l more glycerol, 2.4 up to 54 g/l less residual sugar and a volatile acidity lower with 0.1 up to 0.6 g/l acetic acid comparing to wine made from same must but fermented with indigenous yeasts.

Table 2 The influence of selected yeasts on main parameters of Oltenia wine composition

Parameters	Differences +	Differences -
Alcohol, % vol.	0.2 – 3.5	
Glycerol, g/l	0.1 – 1.8	
Residual sugar, g/l		2.4 – 54
Volatile acidity, g/l acid acetic		0.1 – 0.6

The data from Table 2 represent the limits in differences of wines. Regarding the alcohol content, most of the times the differences are less than 1% vol, the cases when the difference are much higher being very rare and those when the differences are over 2.5% vol are accidental. Despite these, the superior fermentative power of selected yeasts is very important for the quality and typical of wines made in Oltenia vineyards due to specific of the region. Based on the natural climate and terroir specific conditions, the Oltenia vineyards have a high grade of favorability for making quality red wine. These are dry wines, so that the complete fermentation of sugar is mandatory and the selected yeasts offer a better guarantee in this respect. For a 0.3% vol alcoholic strength corresponds over 5 g/l residual sugar, which may compromise the screening of red wine in the dry red wine category. Regarding the white wines, in all Oltenia vineyards, these have a lower acidity and a greater pH, so that it is very important to make them dry in order to have a better stability and a better gustatory balance.

Even if the technological advantages of alcoholic fermentation with selected yeasts are obvious, it still persists many controversies regarding their utility in the case of expressivity and typical of wines, elements that are very important in the overall quality of wines and especially demanded by the connoisseurs. Most of the time, the sensory properties of the wines are different in the case of using selected yeasts due to the fact that the yeasts are made by foreign companies from other wine regions. In this case, the region typical is practically canceled in the case of the wines fermented with selected yeasts.

Results and discussions concerning the role of the lactic acid bacteria

For the white wine the alcoholic fermentation is the only one normal microbiological phenomenon. In the case of red wine a new type of fermentation called secondary fermentation or malolactic fermentation follows winemaking the alcoholic fermentation. This type of fermentation is indubitable for red wine

winemaking being a characteristic for this type of winemaking with many compositional and organoleptic implications.
The great progresses made in the wine microbiology field drive in the last decade to the use on large scale of starter cultures of selected lactic bacteria to trigger the malolactic fermentation and for a better ongoing of it. Despite this, the use of selected lactic bacteria did not reach the same scale of use as the selected yeasts in alcoholic fermentation. In our country the starter cultures of lactic bacteria are used very little. At least in Oltenia, the winemakers consider that is not justified to use selected lactic bacteria when the malolactic fermentation goes pretty well with indigenous lactic bacteria, the indigenous micro flora being very well structured.
In Table 3 is presented the influence of spontaneous malolactic fermentation by the indigenous bacteria versus selected lactic bacteria for 3 types: Cabernet Sauvignon from Simburesti, Cabernet Sauvignon from Vinju Mare and Novac from Dragasani. Also, it has been tracked the influence of spontaneous malolactic fermentation on the red wine aroma for over 40 types of wines made from different varieties in 2008 and 2009.
The data from Table 2 show that after the malolactic fermentation a series of aromas get enforced, others diminish as intensity but the intensity of these modifications depends most on the type of bacteria which make the malolactic fermentation. So, in case of triggered malolactic fermentation with selected lactic bacteria, at all varieties the aromas of butter, banana and warm bread have enforced. At the two types of Cabernet Sauvignon, which come from two different areas located at over 200 km, distance, besides the above mentioned, the aroma of geranium, cranberries and currant have been enforced.

Table 3 The influence of malolactic fermentation on the red young wines aromas

		Aroma which are enforced	Aroma which are diminished
Malolactic fermentation triggered with selected lactic bacteria	CS Smb.	Butter, banana, geranium, pears, quince, warm bread, cranberries, currant	Pepper, mint, bunches, fern
	CS Vj.M.	Butter, banana, geranium, warm bread, cranberries, currant, strawberry, cacao	Pepper, bunches, fern, clover
	N	Butter, banana, core bread, black bread, strawberry jam	Red cornel, brushwood, nettle, mowing hay
Spontaneous malolactic fermentation with indigenous lactic bacteria	CS Smb	Butter, geranium, pears, currant	Pepper, mint, bunches
	CS Vj.M.	Butter, banana, geranium, currant, cacao	Pepper, bunches, corn
	N	Butter, gingerbread, strawberry, raspberry	Red cornel, nettle, brushwood

In the case of spontaneous malolactic fermentation, at all type of wines including both Cabernet Sauvignon the aroma of butter, currant and geranium has been enforced. In the case of Novac, only the aroma of strawberry can be find at the selected lactic bacteria.
Regarding the aroma which are diminished, it can be underlined the diminish of pepper and bunches aroma at all Cabernet Sauvignon wines whatever the geographical origin and the bacteria which make the malolactic fermentation. At both types of Novac, the aroma of red cornel is diminished.

Results and discussions concerning the role of the enzymes

The use of pectolitic enzymes represents an important stimulation and control factor of phenolic and aromatic compounds extractive phenomenon during the maceration. In the cellar environment, it has been made possible in many Oltenia cellars to compare wine from same type of grapes using pectolitic enzymes versus not using them.

The wines analyzed came from the main varieties of red wines cultivated in Oltenia vineyards: Cabernet Sauvignon, Merlot, Pinot noir, Feteasca neagra, Sangiovese, Novac, Negru de Dragasani cultivated at Stirmina, Vinju Mare, Oprisor, Dragasani.
After the taste of the above mentioned varieties, it become clear that the use of pectolitic enzymes represents a very important tool for the winemakers for better control of maceration and the optimization of phenolic compound extraction depending on characteristics of grapes and the type of wine that it has to be make.

The result of taste in conjunction with the environment of experiments drives to a series of very important conclusions regarding the utility of pectolitic enzymes in red winemaking. It is revealed that the use of pectolitic enzymes brings an important qualitative improvement of wine regarding the polyphenolic composition and the chromatic structure of wines but also the organoleptic traits.

In all situations, the add of the pectolitic enzymes in maceration determined a consistent plus in color, even in the case of a shorter maceration time so that it can be clearly asserted the pectolitic enzymes determine a faster extraction of anthocyans which for the wines made for consumption as young wines is very advantageous.
In Picture 1 and 2 is clearly seen the influence of pectolitic enzymes on the aroma of red young wines from the variety Negru de Dragasani and Merlot. At both wines it can be emphasize the fact that when it has been used the pectolitic enzymes (Vinozym G), despite the fact that the maceration period of time was identical (4 days for Merlot and 5 days for Novac), the color intensity of the wine is significantly greater and the color is brighter. Regarding the taste, there is an important difference between wines, the type made with pectolitic enzymes having

more body, balance being less astringent. It can be emphasized also the enforcement of the fruitful and floral aromas and a diminish of the intensity of the vegetal aromas.
These sensory properties differences allowing a faster moving to consumption of red wines comparing to the wines made in a classical way which need more time to diminish the strength of astringency and the asperity of taste in order to improve their aromas.

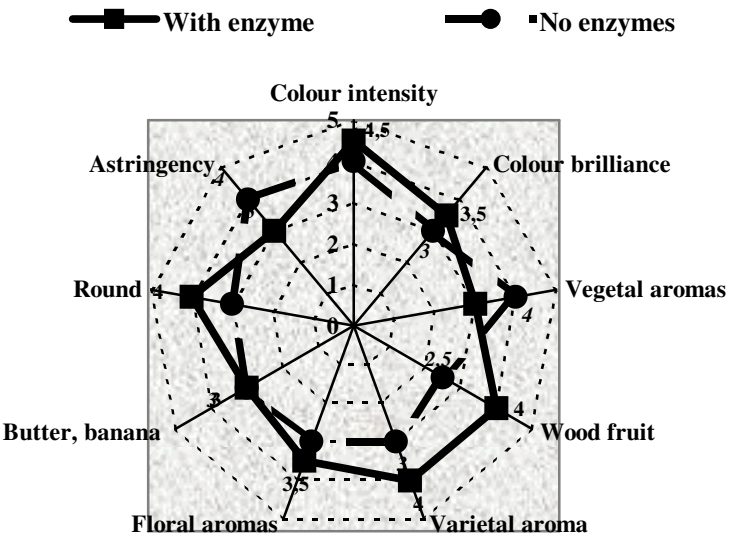

Fig 1 – The sensorial profile of Cabernet Sauvignon made with or without pectolitic enzymes

The enzymes became more and more an usual presence in white winemaking where are used first of all at must clarification, one of the most important pre-fermentative operation in white winemaking. In the last years, in Oltenia vineyards, for the varieties such as Muscat Ottonel, Tamaioasa romaneasca, Sauvignon the enzymes are used more and more including here the extraction of the aroma precursors. But the results are variable, the efficiency of using these enzymes being dependent on many factors, one of them being the greater diversity of enzymes existent in the market.

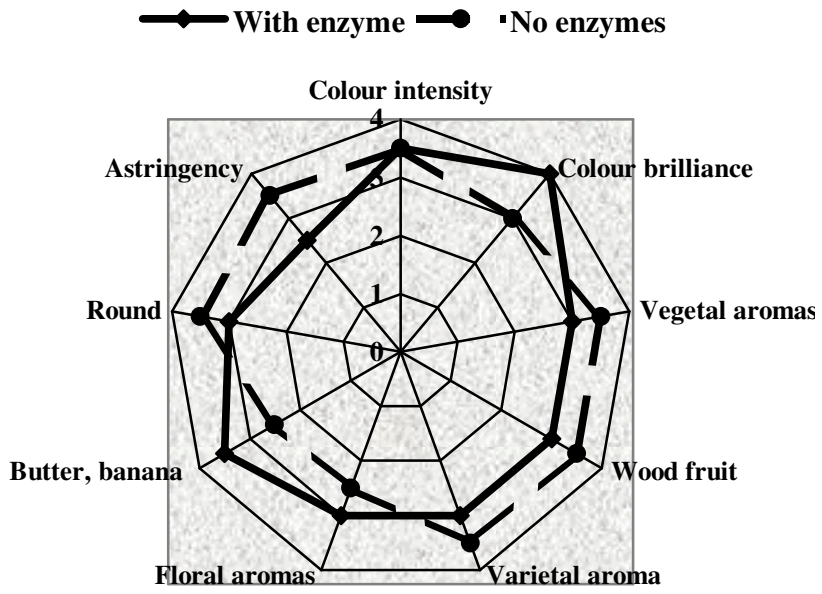

Fig 2 – The sensorial profile of Merlot made with or without pectolitic enzymes

CONCLUSIONS

In last decades, the winemaking technologies evolved very much and the modern biotechnologies represent one of the most important results of scientific progress in Oenology. The making of quality wines became unthinkable today without a rigorous control of maceration, alcoholic fermentation or malolactic fermentation, which means all technological sequences where the biotechnologies have a decisive role.

The vocation of hilly Oltenia vineyards for making high quality wines is unanimously recognized. The natural conditions of clime and terroir, in conjunction with the varieties cultivated allow making a high qualitative level of grapes production but for making quality wine are necessary performing winemaking technologies which are using biotechnologies. The use of starter cultures of yeasts and selected lactic bacteria and the control of phenolic and aromatic compounds extraction with the enzymes represent a characteristic of winemaking in hilly Oltenia area and the guarantee of making of wines with compositional and sensorial characteristics which reflect the qualitative potential of the varieties and wine areas.

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RESEARCHES ON THE BIOTECHNOLOGY POSSIBILITIES FOR INCREASING THE NATURAL RANK OF WINE

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ABSTRACT

Wine is a food for human consumption, in which composition are found many substances with positive effects on health but is not totally devoid of risks for the consumers. Dose reduction of antiseptics and antioxidants is a specific requirement of food safety rules for wine. The main substance used for antiseptic and antioxidant protection of the wine is sulfur dioxide. Dose reduction of SO2 in wine is only possible by rigorous control of alcoholic fermentation. The use of selected yeasts and activators fermentation are the main biotechnological possibilities of obtaining complete fermentations and, in this way, valuable wines as part of compositional and sensory and microbiologically stable.

Key words: biotechnologies, yeasts, alcoholic fermentation, malolactic fermentation

INTRODUCTION

Wine is the first alcoholic beverage produced and consumed by humans. Over time many testimonies have been reported on the health benefits of moderate wine consumption. Pasteur L. (1876) wrote, the wine is healthy and hygienic of beverages "and Renaud S. (1991) made famous expression," French paradox ". The most important benefit attributed to moderate wine consumption is linked to which it provides protection against heart disease, the leading cause of mortality in developed countries (PL Teissedre, P.L. et all. 1996).

For about a decade in the medical literature began to appear studies showing a possible association between moderate wine consumption and reduced risk of various cancers. A study published by Greenwood W. (2001), shows positive effects of wine consumption, combined with a Mediterranean-type diet may reduce cancer risks, for both men and women. The other authors have concluded that moderate wine consumption reduces the risk of cancer, such as the R. Curtis Ellison (1998) and Mollerup S. (2001), which found a lower risk of lung cancer among wine consumers compared with those who consume other alcoholic beverages, and Bozetti C. (2000) found a reduced risk of esophageal cancer in consumers of wine in Italy.

Wine is a foodstuff that stands out not only by its sensory properties and nutritional value but, by increasing body of useful substances, which gives a hygienic value as food, like no other alcoholic drink. At the same time, moderate wine consumption is a component of a rational and balanced lifestyle, a factor that shows the degree of culture and civilization of a people (Baduca Campeanu, C. 2008).

On the other hand, it is equally true that wine is not a completely safe food for consumers. Besides the risks of alcohol abuse, arising from excessive consumption therefore attributable to the consumer, first, can not be overlooked in the presence of wine composition of substances that can harm consumers, such as ethyl carbamate, Ochratoxin A well as some auxiliary and adjunct aids used for the stabilization and storage of wine, the potassium ferrocyanide, especially sulphur dioxide. The conditions under which these substances come to represent a potential risk for mistakes are bound exclusively to major technology in the development, preparation and storage of wine.

Although some of these substances are particularly dangerous, especially Ochratoxin A, which is known to be carcinogenic (Crespy, A., 2005) or ethyl carmamat, animal carcinogen and potential human carcinogen (Béland, A., 2005), they are still a minor risk to consumers because they rarely meet in the wine and doses, most often insignificant. Therefore, in the general context of concerns about food safety, increasing the wine naturally implies, firstly, the reduction of content on all those constituents that may pose risks to health and comfort of honest consumers. One of these substances is sulfur dioxide, widely used in winemaking.

MATERIAL AND METHODS

The research behind this work focused on microbiology and biotechnology opportunities to increase the biological stability of wine, as a prerequisite for reducing the dosage of SO2. Since only microbiological processes that are useful in winemaking are alcoholic fermentation, in some cases, malolactic fermentation, biological stability is closely related to the control of the two fermentations. Therefore, research has been directed to study the possibilities for inducing and stimulating biotech alcoholic fermentation - all kinds of wine - and malolactic fermentation - red wines - produced in the vineyards of Oltenia. In this sense, the past five years, we conducted several experiments in red and white wine that I sought to stimulate and control of alcoholic fermentation and malolactic fermentation using modern biotechnological tools in different enological conditions. Thus, in experiments we used both musts of good quality wine produced in favorable years, well-ripened grapes and healthy but also poor quality musts obtained in unfavorable years from less ripened grapes affected by mold.

For alcoholic fermentation, we organized two experiments on white grape musts and one red. The first experience, I used a white grape with 245 g / l sugar, from well-ripened and healthy grapes. The experience has included four types of fermentation, including two indigenous yeasts and two selected yeast each having two versions: with and without fermentation activator based cell walls. The second experience was carried out on a must with 182 g / l sugar from grapes attacked by gray rot and included 5 different faces and a selected fermentation yeasts indigenous variant. The third experience was performed on a wine must made

from Merlot grapes, with 224 g / l sugar and included three types of fermentation with selected yeasts and two indigenous yeasts.

For malolactic fermentation, the investigations were conducted in 2004 – 2007 in several vineyards of Oltenia (Sîmbureşti, Drăgăşani and Dealurile Craiovei), following its progress in different years with indigenous lactic acid bacteria and lactic acid bacteria selected.

RESULTS AND DISCUSSIONS

The main action of sulphur dioxide in winemaking is wine antiseptic protection. In Enology, the role of microorganisms is essential in two moments: alcoholic fermentation and malolactic fermentation, but the latter is not necessary in all wines. Normally, wine is a much less favorable growth environment for wine microorganisms compared to wine must, because of the presence of alcohol, which has a strong selective action on the wine microflora. However, some species of yeasts and bacteria in wine can multiply under certain conditions related mainly to the absence of SO2, low in alcohol content, higher residual sugar content, etc.. Wines with high growth potential of yeasts and bacteria contamination, thus biologically unstable, are those resulting from disrupted by the action of alcoholic fermentation of various factors.

Table 1 presents results of the first experiences with the alcoholic fermentation and it is found that under a very rich grape sugars, the risk of stopping the fermentation is considerably high even if it comes from healthy grapes.

Table 1 Timetable of alcoholic fermentation of grape must obtained from healthy

Variant	Duration of fermentation, days	Alcohol, % vol.	Residual sugar, g/l	Glycerol, g/l	Volatile acidity, g/l acetic acid	SO_2 combined/ SO_2 total, %
Indigenous yeasts	15	13.0	22.8	11.0	0.38	89.5
Indigenous yeasts + Activator	13	13.4	15.6	11.4	0.34	85.8
Selected yeasts	11	13.9	7.8	11.6	0.30	86.2
Selected yeasts + Activator	9	14.2	3.1	11.9	0.27	80.1

Thus, according to the type used and the use of an asset or fermentation the duration was 9 to 15 days, with 2up to 4 days less in the variants fermented with selected or in witch was used a fermentation activator. The chemical composition of wines obtained show significant differences between types of yeast. Fermentation with selected yeasts resulted in increases in alcohol content of about 1% volume and decreases of the residual sugar content of 12-15 g/l. The increase

of proportion of fermentable sugars is also reflected in higher glycerol contents in 0.5-0.6 g/l. A very important element to consider is that the wines fermented with selected yeasts present value of maximum volatile acidity of 0.3 g/l acetic acid, lower by almost 100 mg/l to those fermented with indigenous yeasts. Consequently, the proportions of SO2 are 3-5% lower, which is particularly important for biological stability of wines and can maintain a lower dose of antiseptic.

The second experience of alcoholic fermentation, conducted on a wine must with 182 g / l sugar, from moldy grapes, included five variants of fermentation with selected yeast from different strains, denoted A, B, C, D, E and a version control, fermented with indigenous yeasts. In this case, the must sulphitation dose was 120 mg/l and for debourbage we also used 100 g/l bentonite. In these circumstances, the indigenous microflora was much reduced in numbers, both because of the health status of the grapes, as well as by treatment with bentonite and sulphitation in behalf of deburbage. Therefore, indigenous yeast fermentation was more difficult, as noted in Table 2, where are the results of this experiment are presented.

Table 2 Influence of the type of yeast on a fermentation coming from moldy grapes must

Yeast strains	Pre-fermentative phase, hours	Duration of fermentation, days	Alcohol, % vol.	Residual sugar, g/l	Glycerol, g/l	SO_2 combined
A	72	8	10,4	4,6	8,1	88,4
B	48	7	10,5	3,0	8,3	86,2
C	48	7	10,5	2,8	8,5	87,4
D	60	7	10,5	2,8	8,4	85,1
E	36	6	10,6	1,3	8,8	82,4
Indigenous	96	12	10,7	9,3	7,8	91,2

Thus, indigenous yeast version had the longest prefermentative phase of 96 hours between debourbage and must start the fermentation, while versions with selected yeast fermentation started between 36 and 72 hours, i.e. 1-3 days faster. The explanation of this important decalogue is represented by the poorness of must in the indigenous yeasts microflora and in nitrogenous substances by operations prefermentative, energetic, applied to a moldy grapes must. Sowing must with selected yeasts significantly increased density of yeast population and allowed more rapid start of fermentation.

The duration of alcoholic fermentation is one of the most visible differences between selected and indigenous yeasts. Indigenous yeast fermentation

lasted 12 days, while the variants seeded with selected yeast finished fermentation over 6 to 8 days, so I had a shorter fermentation of 4-6 days. If we take into consideration the fact that the onset of fermentation was slower, it is clear that if a must from grapes affected by mold seeding - indigenous yeast fermentation at high risk of late start and even stop premature. However, there are important differences even between the selected yeasts. As in prefermentative phase, there was strain E, which showed the shortest duration of fermentation. This suggests that the strain E was best adapted to the difficult fermentation conditions from this must.

Analyses performed on the wine confirm the necessity of using selected yeasts in a must difficult to be fermented. Under a low sugar content (182 g/l), selected yeast fermentation caused an increase in alcohol content of wine 0.3-0.5 g/l and lower residual sugar content 1.5 - 8 g/l. Of the five strains of selected yeast strain E stands still, who led to the wine with biggest alcoholic strength (10.6% vol) and lowest residual sugar content (1.3 g/l). Contrary, strain A, after it caused the late onset of fermentation (72 hours) and longest duration of fermentation (8 days) of selected yeasts, led to the lowest alcohol content (10.4% vol) and was one of the five selected strains of yeast that has led to a wine with more than 4 g/l residual sugar. The fact that strain A was the least adapted to fermentation of this must the worth is clear in glycerol content (8.1 g/l), the youngest of all the selected yeasts. Highest content in glycerol (8.8 g/l) strain E presented with 1 g/l more than wine fermented with indigenous yeasts. The same hierarchy of yeasts is kept in the case where the percentage of SO_2 is combined from the total SO_2.

Last experience was focused on alcoholic fermentation in red wine experience, the fermented must made from ripe grapes of Merlot and healthy, with 224 g/l sugar, we tested three strains of selected yeast, plus two variants with indigenous yeast, one with active yeasts, coming form a must in full fermentation. The results of this experiment, presented in Table 3, show that the red wine-grape seeding with selected yeasts triggers a more rapid start and completion of alcoholic fermentation, which is very important for malolactic fermentation, which must follow. It also confirms other aspects found in white wine making and proving the superior fermentation ability of selected yeasts compared with indigenous yeasts. This experience brings a new element, however, with the fact that the addition of same variety of must, in full fermentation process, over the fresh Merlot must, has effects comparable to those of the addition of selected yeasts, by starting the alcoholic fermentation faster but wine composition parameters changes little.

The malolactic fermentation is a biologically desacidification process of wine made by lactic bacterium that transforms the malic acid into lactic acid and CO_2. Normally, this change takes place of the alcoholic fermentation, therefore is called secondary fermentation. As a result of this transformation there are

important changes in the chemical composition of the wine (the diminution of the total acid, increases of pH because the dicarboxilic acid is replaced by the monobarboxilic acid); the taste and the aroma of the wine (the malic acid, more aggressive for the gustative papilla, is replaced by one acid that stamps suppleness to the wine; the diminution of vegetal aroma, grassy and the appearance of new tastes, more agreeable); the biologically stability of the wine(the malic acid unstable biologically, is replaced by the lactic acid, biologically stable).

Table 3 Influence of the indigenous yeasts and selected alcoholic fermentation in red winemaking

Yeasts	Prefermementative phase duration, hours	Duration of fermentation, days	Alcohol, % vol	Residual sugar, g/l	Glycerol, g/l
A	24	9	13,0	2,8	11,7
B	18	8	13,1	1,8	11,9
C	30	9	12,9	3,8	11,5
Indigenuous	36	11	12,8	5,4	11,3
Indigenous active	18	9	12,9	3,7	11,2

Concerning the malolactic fermentation, the researches were effectuated between 2004–2007, in the main vineyards of Oltenia hilly areas, situated in South-West of Romania: Sâmbureşti, Drăgăşani şi Dealurile Craiovei. Foe this study we used 4 varieties of grapes for high quality red wines: 2 varieties very knowable in international plane Cabernet-Sauvignon and Merlot, and also 2 autochthonous varieties Fetească neagră and Novac. The period of researches include the different years like climatic conditions. So, year 2005 was for Romanian viticulture the worst in the last 50 years, very rainy and cold. Those conditions influenced the grapes and wines production, quantitative and also qualitative. The years 2004 and 2006 was a good vine-growing years, the climatic conditions was favorable for viticulture. The year 2007 was excellent for vine-growing by point of view of climatic conditions during the vegetation period and also during the ripening and over ripening.

During the years have existed visible differences upon the length of time of the malolactic fermentation among the fermentated variant with lactic bacteria resulted from the indigene microflora of the grapes and the fermentated variant with selected lactic bacteria, but the differences where variable, depending on the chemical composition of the wine, as a result of the climate conditions of the viticultural year. In the good viticultural years (2004 and 2006), the length of time of the spontaneous malolactic fermentation was between 45 and 60 days while the variants with selected lactic bacteria, the duration was, on an average, 10 days shorter, between 35 and 50 days. In drought-stricken years and very warm (2007), the length of time of the malolactic fermentation was between 35 and 45 days in

case of the produces fermentation. On unfavorable climate conditions, in an rainy cold year (2005), the differences were big: 80–120 days in the case of spontaneous fermentation and 45-60 days in case of the produces fermentation, so with 1–2 months shorter. Also, it has to be mentioned that in 2005, some red wines stopped the malolactic fermentation after 90–120 days without finishing the conversion of the malic acid into lactic acid. These accidents happened in wines that presented totally contains of SO_2 over 100 mg/l (fig. 1).

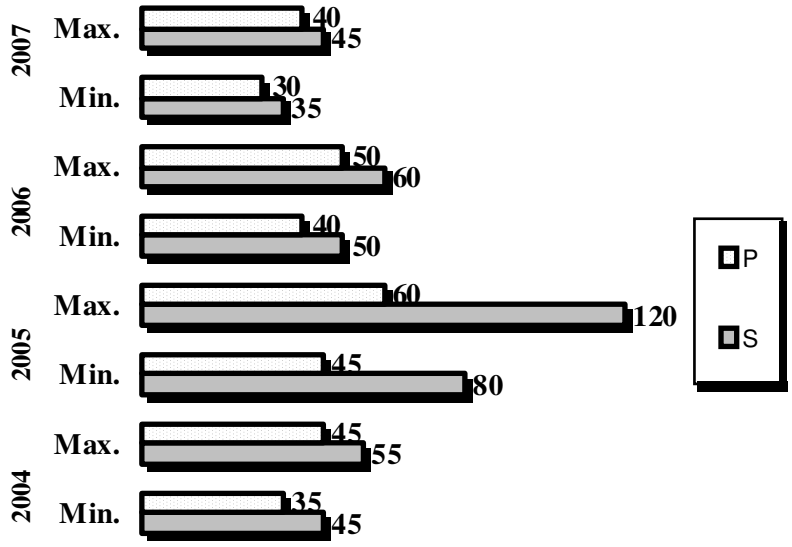
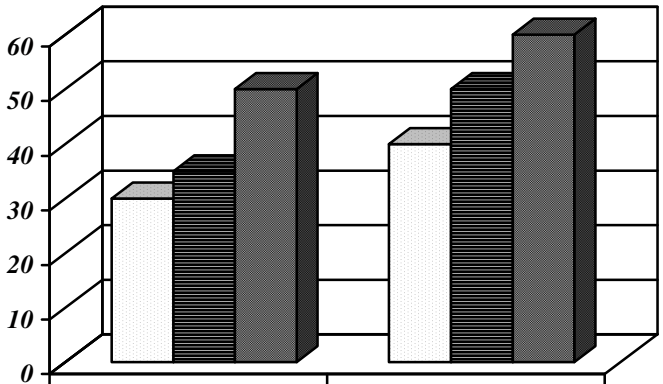


Fig 1. The length of time of the malolactic fermentation (days) after the type of lactic bacteria S – MLF spontaneous, with the bacteries from the indigene microflora of the grapes;
P – MLF induced with selected lactic bacteria

A study made in 2006 on red wines from the vineyard "Dealurile Craiovei" pointed out the important differences concerning the length of time of the malolactic fermentation, depending on the moment of the inoculation of the lactic selected bacteria. Thus in all the cases in witch the inoculation was made in unfermented wine, the length of time of the malolactic fermentation was between 30 and 36 days. In the case of the inoculation of the lactic bacterium into wine, the length of time of the malolactic fermentation was between 36–48 days, in case of the spontaneous malolactic fermentation, without lactic selected bacterium the length of time was between 42–60 days (fig. 2).



	Min.	Max.
Must	30	40
Wine	35	50
MLF spontaneous	50	60

Fig 2. The length of time of the malolactic fermentation (days) after the inoculation time of lactic bacteria

Therefore, the inoculation of the lactic bacterium into unfermented wine determined saves of time between 6 and 12 days confronted by de variant of the culture inoculation starter the lactic bacterium in wine and of 6 to 30 days unlike the cases when the malolactic fermentation occurred without selected lactic bacterium. This economies in time are very important because they offer the real possibility of realizing malolactic fermentation on secure during the first weeks adder the end of the alcoholic fermentation.

CONCLUSIONS

Extensive use of sulphur dioxide in winemaking is due to its many properties of enological interest in all phases of winemaking and wine storage, being the only substance that provides a double protection - antiseptic and antioxidant. Dosage reduction of SO_2, without affecting the stability and guarantee the preservation and development in good conditions is only possible finding microbiological and technological solutions so that doses are not necessary needed in addition to those normally used.

One of those possibilities is the strict control of alcoholic fermentation to prevent premature fermentation stops at the wine presses. Complete fermentation of sugars to obtain dry wine is the first condition to increase the biological stability of wine for two reasons: first, increase the alcoholic strength of wine and alcohol is

itself a protective factor against the propagation of undesirable microorganisms in the wine, in the second of all residual sugar remained unfermented forced to stop due to the fermentation medium is the main energy for bacteria and fungi contamination. Therefore, the use of selected yeasts with physiological and biochemical properties suitable type of wine to be obtained and encouraging their activities biotechnological fermentation is the first opportunity to reduce the dosage of SO2 in wines.

At all red-wines but sometimes white-wines winemaking the alcoholic fermentation is followed by a secondary fermentation called malolactic fermentation. During favorable winemaking years, when grapes were healthy, malolactic fermentation can be carried out in satisfactory conditions under the action of lactic bacteria in grapes indigenous microflora. Spontaneous malolactic fermentation has the advantage that it is a simple procedure that does not involve additional costs and inconvenience related to its associated risks. These risks relate to slow triggering and stop the premature onset, which creates conditions for development of micro-contamination, with negative consequences on the stability of biological and sensory characteristics of wine. In the absence of favorable environmental conditions, the multiplication of lactic acid bacteria is slowed down, which entails extending the lag phase until the onset of malolactic fermentation. This delay may be several weeks long or even months, and in these conditions the risks of developing micro-contamination are even greater when the lag phase is extended further. The most important microbiological such incidents occur when more than delay the onset of malolactic fermentation 4 – 6 weeks after the end of alcoholic fermentation, because is no longer possible to conclude it by the coming winter and spring reactivation of lactic bacteria is accompanied, almost every time, by the deviations of the emergence of microbial and the occurrence of unwanted tastes and odors as a result of prolonged contact with the wine yeasts deposit. In addition, delayed and complicated is the entrance of the wine in the process of normal evolution, especially those destined for maturation and aging.

Given the necessity to avoid uncertainties and incidents related to malolactic fermentation, the use of starter cultures of selected lactic bacteria constitutes a biotechnological possibility that must be taken into account for the increased biological stability and, this way, the degree of naturalness of wine.

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THE INFLUENCE OF THE CHEMICAL COMPOSITION OF THE GROWING ENVIRONMENT OVER THE ALCOHOLIC FERMENTATION OF THE WINE YEAST *SACCHAROMYCES BAYANUS*

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Key words: must malt, fermenter, Saccharomyces bayanus

ABSTRACT

In the wine making technology they use more and more the selected yeasts so that the alcoholic fermentation has an increased efficiency and to be produced faster. The yeast cell has the property of fermenting the glucides and oligoglucides due to its enzymatic equipment. According to this, the yeasts must have superior biotechnological properties, and the used yeasts to have the ability to multiply as fast as possible. The yeasts which were selected for this study were isolated from inland grapes and were subject to the alcoholic fermentation in the Sartorius bioreactor which was equiped with sensors for: temperature, biomass, CO_2 and oxigen. The three yeast strains of Saccharomyces bayanus (SB1,SB2,SB3) were monitored through the biomass accumulation which was resulted from alcoholic fermentation in standard enviroment and also in vitaminized and minerals enriched enviroments. The study pointed the strain with most biotechnological properties so that this strain can be used for industrial processes.

INTRODUCTION

In wine making technology, the used yeasts form the base agent which leads to physio-chemical, bio-chemical and microbiological processes which are implied in grapes must transformations. The yeasts can be found usually in soil and, by the means of physical and biological factors, they end up in vine, strains and grapes. The wasps and the bees, vinegar flies (*Drosophilla melanogaster*) provide the transfer of the yeasts from the soil to the bunch and contribute in forming of their life cycle.The climatic conditions play an important role in the quality and the quantity of the the yeasts on the grapes The grapes are the raw material in the wine making technology, and also for the obtaining of distilled products, juices or must.

Taking into account the big amount of yeast species which are in the grapes must and also the diversity of the actions, there has been questioned the isolation of the most active yeasts, which are called selected yeasts. In the case of using these yeasts, there is the advantage of increasing the speed of the fermentation, of the conversion of sugar into alcohol in an efficient way.

In order to select the yeasts for the wine making technology, the following properties are considered: fermentation speed, alcoholic power, resistance to SO_2 (sulfitoresistance), the endurance to low and high temperatures, tolerance to high concentrations of sugar (osmotolerance), behavioural in the presence of organic

wine acids. Usually in the process of must fermenting there are more yeasts that participate. In the case when the grapes must ferments with just a selected yeast strain, the wine is not sufficiently smooth.

Lately the reseach regarding the grapes micro-flora has been increased, so that we can now isolate yeasts with superior qualities which are needed for the alcoholic fermentation of the grapes must.It was observed that the yeasts which are present natively on the grapes do not always lead to an efficient fermentation, especially the ones from the rainy or cold autumns.

MATERIALS AND METHODS

The research in this study were using three Saccharomyces bayanus (SB1, SB2, SB3) yeast strains which were isolated from the grapes types Iordana, White Feteasca and Royal Feteasca from the Sebes-Apold vineyard. The growing environments we used were for the refference strain (M1) - must malt, M2 – must malt in which we added thiamine in quantity of 0,05 mg per liter, M3 – must malt in which we added magnesium sulphate ($MgSO_4x7H_2O$) 0,5 g per liter.

The biological strains which we considered were monitored during 10 days in the Sartorius fermenter (equipped with sensors for: temperature, biomass, CO_2, oxygen) at the temperature of 22°C.

RESULTS AND DISCUSSIONS

The results emphasized a series of modifications in the development curve of the three types of yeasts, depending on the chemical composition of the growing environment. The M1 environment (refference one) presents a minimum amount of biomass comparing with the M2 and M3 environments. The below graphics show this variation during the entire development and multiplication cycle of the yeast cells. The M2 environment in which we added thiamine, is the optimum one, especially during the time interval 5^{th} to 7^{th} fermentation days.A definitive rols plays also the addition of the $MgSO_4x7H_2O$ in the growing environments, the accumulation of biomass being faster than in the case of the refference environment, providing stability to the technological process and also providing the possibility to obtain higher quality wines, or for improvement of the secondary fermentation process.

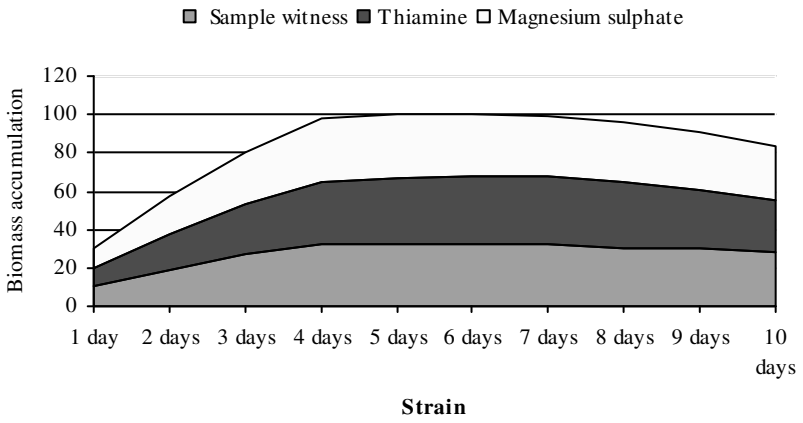

Fig 1. Biomass accumulation using the SB1 strain under the fermentation process in the growing environments M1, M2 and M3 during 10 days

Watching the evolution of the parameters in figure 1, we may see that there are high biomass accumulation values for the interval from 5th to 8th days when we add thiamine of 0,05 mg per liter. Compared to this, the addition of magnesium sulphate helps in producing the biomass faster during the time interval from the 4th to the 6th day in the fermentation cycle. The refference sample, the one that contains must malt provides to the fermentation a growing equilibrum with a lag phase in the interval from 4th to 7th day from this cycle.

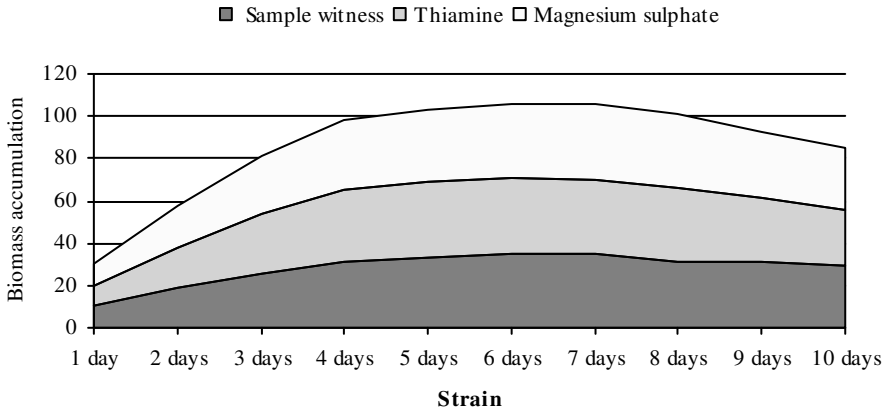

Fig 2. Biomass accumulation produced by the SB2 strain under fermentation in the growingenvironments M1, M2 and M3 during 10 days

The maximum biomass accumulation is shown in the figure 2 in the time interval from 5th to 6th day from the fermentation cycle of the growing environment which contains thuamine. In the case of the environment enriched with magnesium sulphate, the maximum biomass accumulation is in the time interval from 5th to 8th day from the fermentation cycle.

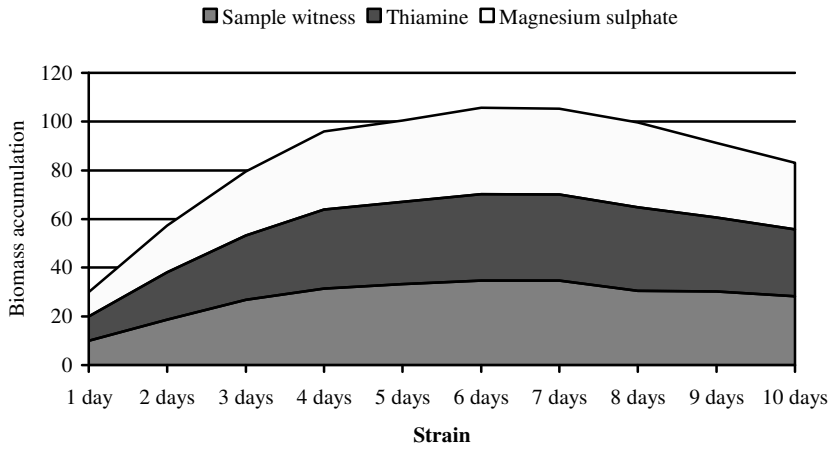

Fig 3. Biomass accumulation produced by the SB3 strain under the fermentation in the growing environments M1, M2 and M3 during 10 days

The SB3 strain shows a maximum biomass accumulation during the 6th day from the fermentation cycle in the thiamine enriched environment. Very close values are also shown in the same day in the magnesium sulphate enriched environment

CONCLUSIONS

It can be observed that the fermentative processes are conditioned by the composition of the growing environment for the same type of strains.

The thiamine plays an important role in increasing the biotechnological qualities of the wine yeast Saccharomyces bayanus, regarding the biomass accumulation and the multiplication speed. Both thiamine and magnesium sulphate are a stimulation factor of the fermentation process having a positive influence in the biomass accumulation. It was observed that after the action of the thiamine there is a synchronization effect which results from the division of an important amount of cells and and maintain successive fermentative cycles.

Both the vitamines (thiamine) and the minerals (magnesium sulphate) play a significant role in obtaining of biotechnological derivates with superior qualities.

AKNOWLEDGEMENTS - This work was cofinanced from the European Social Fund through Sectoral Operational Programme Human Resources Development 2007-2013, project number POSDRU/89/1.5/S/63258 "Postdoctoral school for zootechnical biodiversity and food biotehnology based on the eco-economy and the bio-economy required by eco-san-genesys".

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MONITORING THE FERMENTATIVE PROCESSES OF THE WINE YEASTS IN GLYCEROL ENRICHED ENVIRONMENTS

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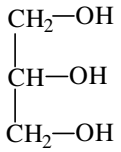
Key words: must malt, glycerol, wine yeasts, mathematical model

ABSTRACT

The fermentative processes happen under the influence of the wine yeasts resulting etilic alcohol, carbon dioxide and yeast biomass. Our study has the purpose of increasing the efficiency of the selected wine yeast under the influence of the chemical factors, using the enrichment of the growing environment with controlled quantities of glycerol. The glycerol added in the growing environment leads to increased and activated cellular metabolism during the process of optimal growing of the wine yeasts. The glycerol is phosphorilated by the ATP to L- glycerol- 3- phosphate, and the resulted ADP is converted, with the help of pyruvate kinase, into ATP forming the pyruvate. The glycerol contributes to the fermentative activity, a strong correlation exists between them and the processes which takes place in the environments with superior osmotic pressure. During this study we observed that the glycerol is absorbed during the first hours very quickly resulting an equilibrum between the intra and extra cellular glycerol. Following the evolution of the samples, we can determine a mathematical model depending on the cronological set of events.

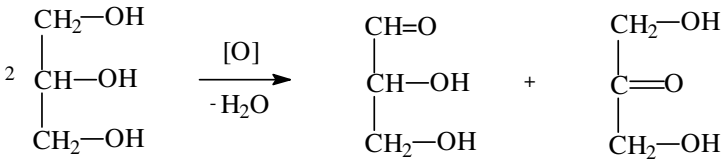
INTRODUCTION

An important component which influences the preservation of the yeast is the glycerol. The glycerol or glycerine is a tri-alcohol. It is also called 1,2,3-propantryol, having the following chemical structure:



Glycerol has the form of a crystalline liquid with t.p. 20^0C, colorless, syrupy, hygriscopic, sweet, participating in the following biochemical reactions, calalyzed by the enzymes.

1. Mild oxidation

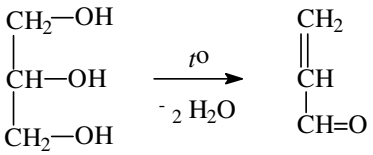


glycerol aldehide *dihydroxiacetone*

This reaction takes place inside the organism during the carbohydrates metabolis.

2. *Thermal de-composition*

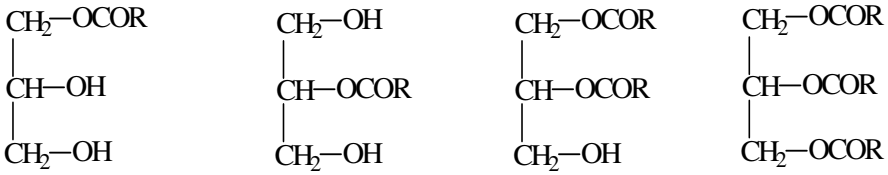
Under the action of high temperatures, the glycerol decomposes in acrolyne, a toxical product in high volume. This reaction takes place under high treatments of the fatty foods.



Acrolyne

3. *Esterification*

Through esterifaction, the glycerols transforms into glycerides, which are the main fatty foods. This reaction takes place during the lipids metabolism, under the action of the enzymes.



1-monoacilglycerol 2-monoacilglycerol 1,2-diacilglycerol 1,2,3-triacilglycerol

MATERIALS AND METHODS

The growing environment YPG (Yeast – Peptone – Glucose), which contains 1% yeast extract, 2% glucose, 2% peptone, sterilized for 15 minutes at 120°C, M1/refference.

Growing environment YPG (Yeast – Peptone – Glucose), which contains 1% yeast extract, 2% glucose, 2% peptone, 2,5% glycerol, sterilized for 15 minutes at 120°C, M2.

Growing environment YPG (Yeast – Peptone – Glucose), which contains 1% yeast extract, 2% glucose, 2% peptone, 5% glycerol, sterilized for 15 minutes at 120°C, M3.

The research in this study were using three Saccharomyces bayanus (SB1, SB2, SB3) yeast strains which were isolated from the grapes types Iordana, White Feteasca and Royal Feteasca from the Sebes-Apold vineyard.

The fermentation of the yeast strains was monitored by the Startorius fermenter equiped with sensors for: temperature, biomass, CO_2, oxygen, during 10 days at 22°C.

RESULTS AND DISCUSSION

Glycerol is phosphorilated by the ATP to L – glycerol – 3 – phosphate (reaction calized by glycerolkinasis – GK), and the formed ADP is converted to ATP by phosphoenolpyruvate (PEP) by the help of pyruvatkinasis (PK), forming the pyruvate. In the presence of L-lactic dehydrogenasis enzyme (L-LDH), the pyruvate is reduced to L-lactat by NADH, with the oxydation of NADH to NAD^+.

The absorbance differences are calculated for the refference sample and for the samples to be analyzed, reading the parameters at 340 nm. The glycerol quantity can be computed using the formula:

$$c = \frac{VxMW}{\varepsilon \times d \times v \times 100} x \quad \Delta A \ (g/L)$$

V – final volume (ml);
v – volume of the sample (ml);
d – optical path (1cm);
MW – mollecular mass of the product to be analized;
ε – extinction quotient of the NADPH at: 340 nm= 6,3 (1 x $mmol^{-1}$x cm^{-1}).

For glycerol,

$$C = \frac{3,020 \times 92,1}{\varepsilon \times 1,00 \times 0,100 \times 1000} x \ \Delta A = \frac{2,781}{\varepsilon} \times \Delta A \ [g \text{ glycerol / L sample}]$$

The final result is computed by the weighted mass, and the values of hte glycerol form the arithmentic average of the two measurements which are performed successively on the same samples; in the case when the differences between the two measurements was no greater than 5%.

glycerol content = (C glycerol / weight of sample) x 100 (g/100 g s.u.)

As it results from the figure 1, the biomass accumulation reaches the maximum level in the interval 4-7 from the fermentation cycle of the environment in which as added 2.5 % glycerol. In the case of adding 5% glycerol, the biomass quantity triples itself in the interval 4-8, the maximum value is reached in the 5th day.

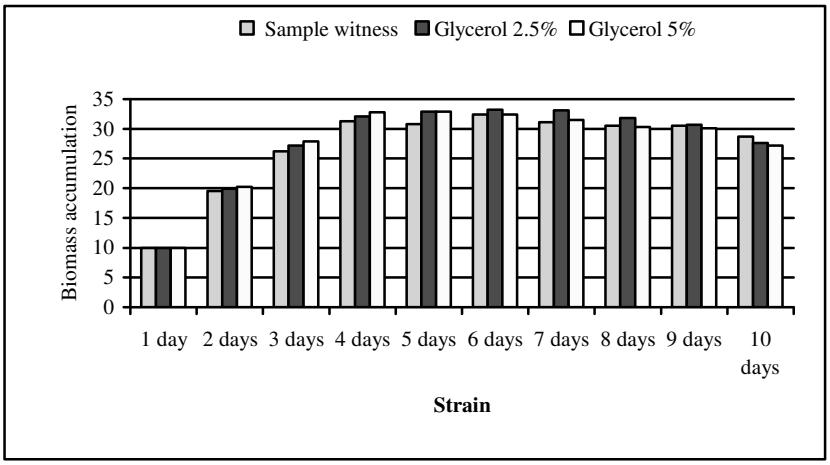


Fig 1. Biomass accumulation produced by the SB1 strain under the fermentation in the growing environments M1, M2 and M3 during 10 days

In the case of the SB2 strain, the maximum biomass level is in the interval 5-7 of the fermentation in the environment enriched with 2.5% glycerol. The environment which contains 5% glycerol reached the maximum biomass level in the interval 7-8 from the fermentation process. In this case a lag phase can be ovserved starting with the 4th day till the 8th day.

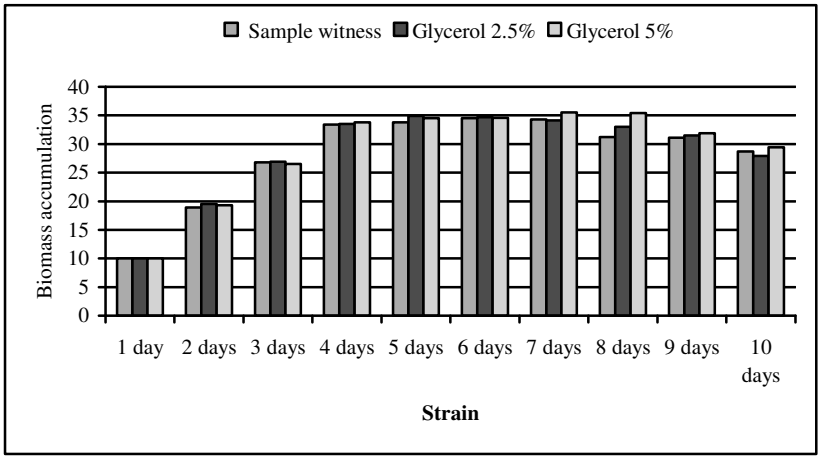


Fig 2. Bioamass accumulation produced by the SB2 strain under the fermentation in the growing environments M1, M2 and M3 during 10 days

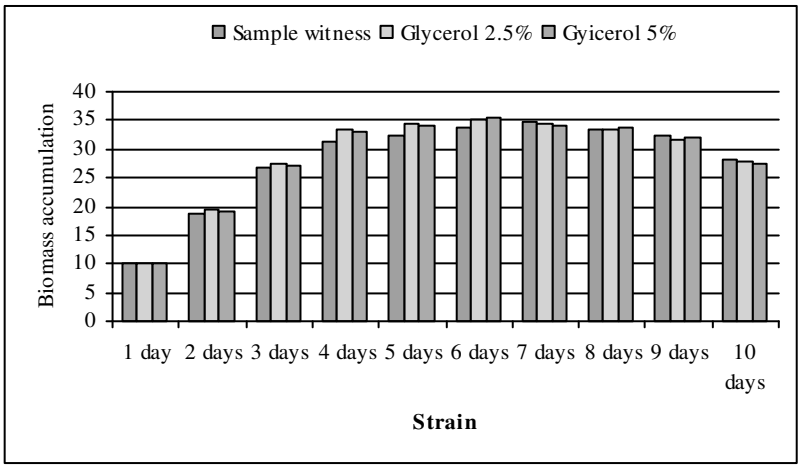


Fig 3. Biomass accumulation produced by tye SB3 strain under the fermentation in the growing environments M1, M2 and M3 during 10 days

The SB3 yeast strain has a behaviour close with SB1 regarding the interval of biomass accumulation. The interval 4-9 constitutes the optimum area for the refference sample. When adding 2.5% glycerol, the yeast SB3 reaches the maximum level in the 6th day decreasing slightly until the 8th day and then substantially. Also in the 6th day, we may observe it reaches the maximum value for the biomass accumulation in the environment enriched with 5% glycerol. In this case the lag phase is predominantly in the intervall 4-8 from the fermentation cycle.

Matematically, we may appreciate that the connection between the three types of environments (named X for M1 , Y for M2 and Z for M3) reported to the influence over the biomass accumulation of the injected yeast may be expressed using the co-variance and correlation quotient. If we name Dxi the accumulated biomass for SB3 strain from the X environment, Dyi the accumulated biomass from the environment Y, and Dzi the accumulated biomass of SB3 in Z environment, wheree i=1:3, and M(Dx) , M(Dy) M(Dz) the average values of the accumulated biomass in the three types of environments, (computed as strain averages), we have:

M(Dx)=28,1 ; M(Dy)= 28,7 M(Dz))= 28,6

$$\operatorname{cov}(X,Y,Z)=\frac{1}{3}\sum_{i=1}^{3}(Dx_i-M(Dx))(Dy_i-M(Dy)(Dz_i-M(Dz))=-0{,}33$$

The intensity of the connection can be determined using the corelation quotient. First we compute mean square deviation on the three types of environments:

$$\sigma(X) = \sqrt{\frac{1}{3}\sum_{i=1}^{3}(Dx_i - M(Dx))^2} = 6{,}34 \text{ and}$$

$$\sigma(Y) = \sqrt{\frac{1}{3}\sum_{i=1}^{3}(Dy_i - M(Dy))^2} = 6{,}58$$

$$\sigma(Z) = \sqrt{\frac{1}{3}\sum_{i=1}^{3}(Dz_i - M(Dz))^2} = 6{,}56$$

The correlation quotient will be:

$$\rho_{XYZ} = \frac{\text{cov}(X,Y,Z)}{\sigma(X)\cdot\sigma(Y)\sigma(Z)} = -0{,}002$$

CONCLUSIONS

It can be observed that the added glycerol contributes to the biomass accumulation in the time when it is assimilated by the yeast cells. Basicaly the added glycerol reaches an equilibrum intra and inter cell after aproximately 6 days.

The growing environments which are enriched with glycerol provide to the fermentation a higher biomass quantity with 10-12% comparaed with the refference sample. Temporary we observed also a modification regarding the color and the consistency of the biomass. As the osmotic pressure modifies, these transformations diminished, the biomass returning to its initial aspect.

Watching the three strains in this fermentative cycle, we may observe that SB3 strain has biotachnological properties which are superior compared to the strains SB1 and SB2.

From the mathematical point of view, we may say that the SB3 has a negative covariation (cov(X,Y,Z) = - 0,33 < 0).

The biomass accumulation has trends of variation in the other direction, the correlation quotient reaching the value of -0,002 which indicated the trend of the SB3 strain to evolve independently (the quotient is generally defined in the interval -1; 1), in the three types of culture.

AKNOWLEDGEMENTS - This work was cofinanced from the European Social Fund through Sectoral Operational Programme Human Resources Development 2007-2013, project number POSDRU/89/1.5/S/63258 ”Postdoctoral school for zootechnical biodiversity and food biotehnology based on the eco-economy and the bio-economy required by eco-san-genesys”.

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HIGH NUTRITIVE BIOMASS OF EDIBLE AND MEDICINAL MUSHROOMS PRODUCED BY SUBMERGED FERMENTATION OF CEREAL BY-PRODUCTS

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ABSTRACT

The main aim of this research work was focused on testing the most efficient food biotechnology to be applied in leading and controlling the submerged fermentation of edible and medicinal mushrooms on substrata made of by-products that resulted from the cereal processing of food industry. To carry out the experiments, a suspension of activated spores of the mushroom strains of Ganoderma lucidum (Curt.:Fr.) P. Karst and Lentinus edodes (Berkeley) Pegler, picked up in aseptic conditions, was prepared in order to be inoculated in the culture media previously poured and sterilized inside the culture vessel of a 20 l laboratory scale bioreactor, at 121^{0}C, 1.1 atm., for 15 min.. All culture media used in experiments were prepared from different sorts of bran and broken seeds resulted from the industrial food processing of wheat, barley and rye seeds. The submerged fermentation was set up at the following parameters: constant temperature, 23°C; agitation speed, 70-90 rev. min^{-1}; pH level, 5.7–6.0 units; dissolved oxygen tension within the range of 30%-50%. During a period of submerged fermentation lasting between 120-180 h, the fungal biomass in the shape of small fungal pellets was developed inside the culture media. The edible mushrooms G. lucidum and L. edodes were grown by submerged fermentation in controlled cultivation cycles to increase the fungal metabolism by developing and multiplying mycelia. The developed fungal pellets as well as all free mycelia fraction from the total biomass were microscopically studied. The chemical composition of the fungal biomass was analysed to determine and compare the chemical contents of those two mushroom species.

Key words: biomass, biotechnology, *Lentinus edodes, Ganoderma lucidum,* submerged fermentation

INTRODUCTION

The submerged cultivation in liquid media of mushroom mycelium is a promising method which can be used in novel biotechnological processes for obtaining pharmaceutical substances of anticancer, antiviral, immuno-stimulatory, and anti-sclerotic action from fungal biomass and cultural liquids and also for the production of liquid spawn (*Wainwright*, 1992; *Stamets*, 1993). The research works made to get nutritive supplements from the biomass of *Ganoderma lucidum* species (Reishi) have shown that the nutritive value of its mycelia is own to the huge protein content, carbohydrates and mineral salts (*Hobbs*, 1996; *Mizuno et al*., 1995; *Breene*, 1990). Also, it is well known the anti-tumor activity of polysaccharide fractions extracted from the mycelia of *Lentinus edodes* species (Shiitake) which is a good source of proteins, carbohydrates and mineral elements with beneficial effects on human nutrition (*Moser*, 1994; *Wasser & Weis*, 1994).

As a result of other recent studies, the continuous cultivation of edible and medicinal mushrooms was applied using the submerged fermentation of different natural by-products of agro-food industry that provided a fast growth as well as high biomass productivity of the investigated strains (*Petre & Petre*, 2008).

MATERIALS AND METHODS

The biotechnological procedure of controlled cultivation of edible mushrooms was established and tested in different variants of culture media made of cereal by-products that could influence the fast growth as well as high biomass productivity of the fungal strains belonging to *G. lucidum* and *L. edodes* species.

The main stages of such biotechnology of getting high nutritive fungal biomass by controlled submerged fermentation were the followings:

I. Preparation of culture media and pouring them into the cultivation vessel of the bioreactor (Applikon type).

II. Steam sterilization of the cultivation vessel at the temperature of 121° C, pressure of 1.1 atm. for 20 min.;

III. Inoculation of sterilized culture media inside the bioreactor vessel for submerged cultivation, using the sterile air hood, laminar flow with rings of mycelium from pure cultures of selected strains of fungi.

IV. Running submerged cultivation cycles under controlled conditions: temperature 23 ± 2°C, speed 70 rpm and continuous aeration at 1.1 atm.

V. Collecting, cleaning and filtering the fungal pellets obtained by the submerged fermentation of substrates made from by-products resulted from cereal grains processing.

Table1. Chemical composition of the nutritive culture media used in submerged fermentation of *G. lucidum* and *L. edodes*

Ingredients	Variant I (g/l)	Variant II (g/l)	Variant III (g/l)	Variant IV (g/l)	Control (g/l)
Barely bran	470	-	-	170	-
Wheat bran	-	430	-	230	-
Rye bran	-	-	450	150	-
Malt extract	30	25	35	10	35
Yeast extract	3	3.5	3	1	3.5
Peptone	1	1.5	1	1	1

According to the purpose of this work, three mushroom species belonging to Basidiomycetes Class, namely *G. lucidum* (Curt.:Fr.) P. Karst and *L. edodes* (Berkeley) Pegler were used as pure cultures in experiments. The stock cultures were maintained on malt-extract agar (MEA) slants. Slants were incubated at 25°C for 5-7 d and then stored at 4°C. The fungal cultures were grown in 250-ml flasks containing 100 ml of MEYE (malt extract 20%, yeast extract 2%) medium at 23°C on rotary shaker incubators at 110 rev min^{-1} for 5-7 d (*Petre*, 2008; *Stamets*, 1993; *Moo-Young*, 1993).

The fungal cultures were prepared by aseptically inoculating 100 ml in three variants of culture media by using 3-5% (v/v) of the seed culture and then cultivated at 23-25°C in 250 ml rotary shake flasks. The chemical composition of these culture media is shown in table 1.

The biotechnological experiments were conducted under the following conditions: temperature, 25°C; agitation speed, 120-180 rev min^{-1}; initial pH, 4.5–5.5. After 10–12 d of incubation the fungal cultures were ready to be inoculated aseptically into the glass vessel of 20 l laboratory-scale bioreactor, that was designed to be used for controlled submerged cultivation of edible and medicinal mushrooms on substrata made of wastes resulted from the industrial processing of cereal grains (Fig. 1).

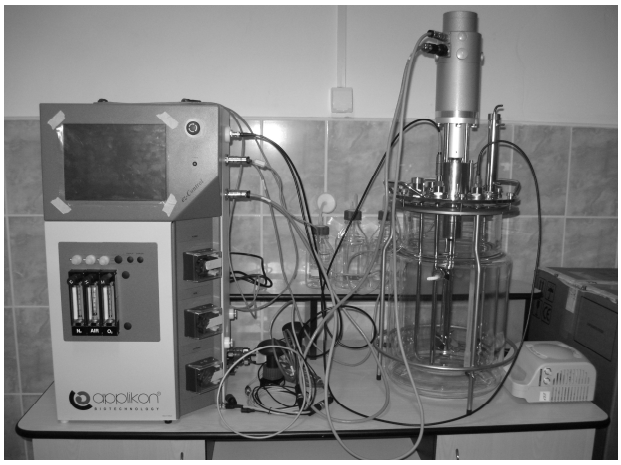

Fig. 1 – Laboratoy-scale bioreactor for submerged fermentation

For the mushroom growing, special culture media were prepared by using different liquid nutritive broths as can be seen in table 1. After the steam sterilization at 121^0C, 1.1 atm., for 15 min. each nutritive broth was transferred aseptically inside of the culture vessel of a laboratory scale bioreactor. This kind of culture medium was aseptically inoculated with the previously activated spores of

G. lucidum and *L. edodes* species. After inoculation into the bioreactor vessel, a slow constant flow of nutritive liquid broth was maintained inside the nutritive culture medium by recycling it and adding from time to time a fresh new one (*Carlile & Watkinson*, 1996). The submerged fermentation was set up at the following parameters: constant temperature, 23°C; agitation speed, 80-100 rev. min $^{-1}$; pH level, 5.7–6.0 units; dissolved oxygen tension within the range of 30-70%. After a period of submerged fermentation lasting up to 120 h, small mushroom pellets developed inside the nutritive broth (*Petre et al*., 2010).

RESULTS AND DISCUSSION

Fermentation process was carried out by inoculating the growing medium volume (10,000 ml) with secondary mycelium inside the culture vessel of the laboratory-scale bioreactor (Fig. 1). The whole process of growing lasts for a single cycle between 5-7 days in case of *L. edodes* and between 3 to 5 days for *G. lucidum*. The strains of these fungal species were characterized by morphological and cultural stability, manifested by its ability to maintain the phenotypic and taxonomic identity. Observations on morphological and physiological characters of these two tested species of fungi were made after each culture cycle, highlighting the following aspects:

► sphere-shaped structure of fungal pellets, sometimes elongated, irregular, with various sizes (from 2 to 5 mm in diameter), reddish-brown color – *G. lucidum* specific culture (Fig. 2).

Fig. 2 – Biomass of *G. lucidum* as fungal pellets

► globular structures of fungal pellets, irregular with diameters of 4 up to 7 mm or mycelia congestion, which have developed specific hyphae of *L. edodes* (Fig. 3).

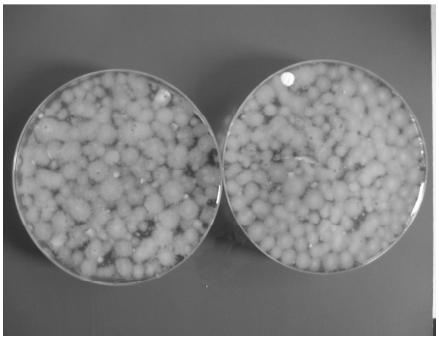

Fig. 3 – Fungal pellets of *L. edodes* biomass

Experiments were carried out in three repetitions. Samples for analysis were collected at the end of the fermentation process, when pellets formed specific shapes and characteristic sizes. For this purpose, fungal biomass was washed repeatedly with double distilled water in a sieve with 2 mm diameter eye, to remove the remained bran in each culture medium.

Biochemical analyses of fungal biomass samples obtained by submerged cultivation of edible and medicinal mushrooms were carried out separately for the solid fraction and extract fluid remaining after the separation of fungal biomass by pressing and filtering. The percentage distribution of solid substrate and liquid fraction in the preliminary samples of fungal biomass are shown in table 2, together with the percentage of substance remaining (which was measured for dry matter content) and the percentage level of liquid fraction (resulted after pressing the solid substrate).

Table 2. Percentage distribution of solid substrate and liquid fraction in the preliminary samples of fungal biomass

Mushroom species	Total volume of separated liquid per sample (ml)	Total biomass weight per sample (g)	Water content after separation (%)
L. edodes	83	5.81	83.35
L. edodes	105	7.83	82.50
L. edodes	95	7.75	82.15
L. edodes	80	5.70	79.55
G. lucidum	75	7.95	83.70
G. lucidum	115	6.70	82.95
G. lucidum	97	5.45	80.75
G. lucidum	110	6.30	77.70

Also, the most obvious sensory characteristics (color, odor, consistency) were evaluated and presented at this stage of biosynthesis taking into consideration that they are very important in the prospective view of fungal biomass using as raw matarials for nutraceuticals producing.

In each experimental variant the amount of fresh biomass mycelia was determined. Percentage amount of dry biomass was determined by dehydration obtained at a temperature of 70° C, until constant weight. Total protein content was determined by biuret method, whose principle is similar to the Lowry method, this method being recommended for the protein content ranging from 0.5 to 20 mg/100 mg sample (*Bae et al*., 2000; *Lamar et al*., 1992).

In addition, this method requires only one sample incubation period (20 min) and using them is eliminated interference with various chemical agents (ammonium salts, for example). The principle method is based on reaction that takes place between copper salts and compounds with two or more peptides in the

composition in alkali, which results in a red-purple complex, whose absorbance is read in a spectrophotometer in the visible domain (λ - 550 nm). In tables 3 and 4 are presented the amounts of fresh and dry biomass as well as the protein contents for each fungal species and variants of culture media.

Table 3. Fresh and dry biomass and protein content of *L. edodes* grown by submerged fermentation

Culture variants	Fresh biomass (g)	Dry biomass (%)	Total proteins (g % d.w.)
I	20.30	5.23	0.55
II	23.95	6.10	0.53
III	22.27	4.79	0.73
IV	20.10	4.21	0.49
Control	4.7	0.5	0.2

According to registered data, using wheat bran strains the growth of *G. lucidum* biomass was favored, while the barley bran led to increased growth of *L. edodes* mycelium and *G. lucidum* as well. In contrast, dry matter content is significantly higher when using barley bran for both species used. Protein accumulation is more intense when using barley bran compared with those of wheat and rye, at both species of mushrooms.

The sugar content of dried mushroom pellets collected after the biotechnological experiments was determined by using Dubois method. The mushroom extracts were prepared by immersion of dried pellets inside a solution of NaOH pH 9, in the ratio 1:5. All dispersed solutions containing the dried pellets were maintained 24 h at a precise temperature of 25^0C, in full darkness, with continuous homogenization to avoid the oxidation reactions.

Table 4. Fresh and dry biomass and protein content of *G. lucidum* grown by submerged fermentation

Culture variants	Fresh biomass (g)	Dry biomass (%)	Total proteins (g % d.w.)
I	25.94	9.03	0.67
II	22.45	10.70	0.55
III	23.47	9.95	0.73
IV	21.97	9.15	0.51
Control	5.9	0.7	0.3

After the removal of solid residues by filtration the samples were analyzed by the previous mention method (*Wasser & Weis*, 1994). The nitrogen content of mushroom pellets was analyzed by Kjeldahl method. All the registered results are related to the dry weight of mushroom pellets that were collected at the end of each biotechnological culture cycle (Table 5).

Table 5. The sugar and total nitrogen contents of dried mushroom pellets

Mushroom species	Culture variant	Sugar content (mg/ml)	Kjeldahl nitrogen (%)
L. edodes	I	5.15	6.30
L. edodes	II	4.93	5.35
L. edodes	III	4.50	5.70
L. edodes	IV	4.35	5.75
	Control	0.55	0.30
G. lucidum	I	4.95	5.95
G. lucidum	II	5.05	6.15
G. lucidum	III	5.55	6.53
G. lucidum	IV	4.70	5.05
	Control	0.45	0.35

Comparing all the registered data, it could be noticed that the correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushroom species. From these mushroom species that were tested in biotechnological experiments *G. lucidum* – culture variant III showed the best values concerning the sugar and total nitrogen content. In order, on the very next places, *L. edodes* – culture variant I and *G. lucidum* - culture variant II could be mentioned from these points of view. These registered results concerning the sugar and total nitrogen contents have higher values than those obtained by other researchers (*Bae et al*., 2000; *Moo-Young*, 1993).

The nitrogen content in fungal biomass is a key factor for assessing its nutraceutical potential, but the assessing of differential protein nitrogen compounds requires additional investigations.

CONCLUSIONS

1. The cereal by-products used as substrata for growing the fungal species *L. edodes* and *G. lucidum* by controlled submerged fermentation showed optimal effects on the mycelia development in order to get high nutritive biomass.
2. The dry matter content of fungal biomass produced by submerged fermentation of barley bran was higher for both tested species.
3. The protein accumulation is more intense when using barley bran compared with those of wheat and rye, at both fungal species.
4. The correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for each tested mushroom species.
5. *G. lucidum* - culture variant III showed the best values of sugar and total nitrogen contents, being followed by *L. edodes* – culture variant I.

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STUDY OF THE CORRELATIONS BETWEEN GLUTEN INDEX AND GLUTEN DEFORMATION INDEX IN ROMANIAN WHEAT

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ABSTRACT

Correlation between Gluten deformation index and Gluten index was studied for 650 samples of Romanian wheat crops of 2005-2009. The research was conducted on wheat characterized by the following quality parameters: Hectolitric mass 76 065 ± 3460 kg / hl, Moisture 12.256 ± 1.214%, Wet gluten 31.216 ± 6.702%, Gluten deformation index 9.754 ± 7.145 mm ± 329 048, Falling number 77.176 seconds, Gluten index 46.958 ± 25.708, Protein content 14.086 ± 2.003%.

Results showed that despite a significant negative correlation (r = -0.61) between the two quality parameters, the coefficient of determination is low (R^2 = 0.360). In addition, correlation becomes negligible within the ranges characterized by high values of both parameters (over 80 for Gluten index and 20 mm for Gluten deformation). This suggests that the two quality parameters of gluten can not replace each other in quality assessment procedures. They should be utilized together.

Key words: wheat, crop quality, gluten index, gluten deformation, correlation index

INTRODUCTION

Wheat production in Romania is characterized by large variations in crop quality from one year to another, especially the variation of some parameters which reflect the enzymatic activity (falling number, strain index). Although it has been claimed the lack of adaptation of the Romanian varieties to new climatic conditions for higher quality variations, certain studies have shown that the main causes are: the inadequate cultivation technologies for the wheat requirements, inappropriate harvesting time, improper storage and handling of crops [2].

We also found that the assessment of gluten quality is done through the excessive use of a single descriptor, namely the index of deformation, due to lack of popularity among wheat manufacturers and processors of other methods for evaluating the quality of gluten, as Zeleny index or gluten index [1]. The assessment type is widely used, given that there are studies showing that the gluten index parameter can be a good predictor for a range of rheological parameters of dough, such as: area under the extensografic curve, the extensografic resistance (*Anne Ingver and Reine Kopp, 2004*), the alveografic work - W, mixograph peak time (*Gaines et al., 2006*), the alveografic parameters Resistance (P), Extensibility (L), the extensibility index of the gluten (G), the Elasticity index (Ie) and the farinographic parameter named Hydration capacity (*NC Popa et al., 2009, 2010*) [3, 4, 5, 6].

Unlike the deformation index, which significantly reflects the level of the attack of bed bugs (*Eurygaster sp.*), the gluten index parameter is influenced to a large extent by the genetical heritage of wheat varieties (*Kostyukovsky and Zohar, 2004*; *Rashed et al*., 2007) [7,8].

The optimal values for the deformation index parameter are situated in the range 6-13 mm. A lower value than 6 shows a tenacious gluten, and a value greater than 20 mm a low gluten, characterized by a very rapid process of proteolytic degradation. The gluten index parameter can take values between 0 and 100. the optimal value is considered between 65 and 80. Values greater than 80 describe a strong gluten. Values less than 65 describe a gluten having an initial moderate proteolytic activity until 40 and very strong below 40 [9].

In this context, we considered as necessary this study, in order to investigate the interrelation between the most widely used parameters for assessing the quality of Romanian wheat, namely the gluten deformation index and gluten index.

MATERIALS AND METHOD

There have been analyzed 650 wheat samples from the harvests 2005 to 2009, taken from the south region of the country, in order to determine the main physical and chemical parameters: hectolitric mass (ISO 7971-2), moisture (ICC Standard No . 202), wet gluten and gluten index (ICC Standard No 155), protein content (ICC Standard No. 202), ash content(AACC Standard No. 08-21), falling number (ISO 3093:1997) and deformation index (STAS 6283/1-83). The tests have been made in the laboratory of the Research - Development Department of SC FARINSAN SA. The results have been processed by statistical analysis, using the specialized software programs COHORT and STATISTICS.

RESULTS AND DISCUSSION

The average results obtained from the laboratory tests of the 650 wheat samples are presented in Table 1.

Table 1. The estimates of the quality parameters variability of the wheat samples

Parameter	X ± sx	CV (%)	min	max
Moisture (%)	12.246 ± 1.216	9.930	7.200	19.910
Hectolitric Weight (kg/hl)	76.279 ± 3.199	4.194	66.400	83.700
Protein content (%)	14.011 ± 1.926	13.746	9.360	20.490
Wet gluten (%)	31.217 ± 6.654	21.315	10.10	53.900
Gluten index	46.787 ± 25.820	55.187	0.000	98.000
Falling number (sec)	329.641 ± 76.523	22.304	62.000	587.000
Gluten deformation (mm)	9.752 ± 7.094	72.744	1.500	40.000

The analyzed wheat was characterized by relatively low values of the gluten index and average values of deformation index. In terms of the reference values expressed in the specialized literature, the two parameters present a moderate to high proteolytic activity. Despite the fact that the gluten index parameter is described in the specialized literature as being primarily dependent on the genotype, its coefficient of variation has been very high (55.187%), similar to the variation level of the deformation parameter index (72.744%). This value may suggest that the genetic determinism of this parameter is modulated to a significant extent by external factors (phenotype).

Table 2 summarizes the values of the correlation coefficients between the analyzed parameters for the 650 wheat samples.

Table 2. The correlation coefficients for the quality parameters of wheat samples

	M	**HW**	**PC**	**WG**	**GI**	**FN**	**GD**
M	1						
HW	-0.25***	1					
PC	-0.06	-0.44***	1				
WG	-0.19***	-0.32***	0.90***	1			
GI	-0.01	0.39***	-0.27***	-0.25***	1		
FN	-0.28***	0.28***	0.06	0.03	0.16***	1	
GD	-0.22***	-0.38***	0.32***	0.25***	-0.61***	0.11***	1

*M - Moisture, HW - Hectolitric Weight, PC - Protein content, WG - Wet gluten, GI – Gluten Index, FN – Falling Number, GD – Gluten Deformation; *significant (p<0.050), **distinct significant (p<0.010), *** very significant (p<0.001)*

From Table 2 we can see that both the deformation index, as well as the gluten index, highlight the same correlations except the correlation between deformation index and humidity (r = -0.22). The correlations established between the two parameters may be similarly explained from technical point of view. Thus, the deformation index of gluten is smaller and gluten index is higher for the wheat having higher hectolitric mass. This is explained by the fact that the hectolitric mass is an indicator of the phyto technology apllied to the culture, as well as an indicator of the purity degree and phytosanitary health of grain. A large value of the hectolitric mass may be associated with a minimum level of the phenotypic variations that may adversely influence the two quality parameters of wheat. Wet gluten is higher for the wheat with higher proteolytic activity (indicating higher deformation index and lower gluten index), because it is sticky and therefore it is more difficult to be washed / dried. This correlation may be more an artifact of the method of analysis, than the expression of a genuine link between gluten index and deformation index on the one hand and wet gluten on the other side.

Likewise, the protein content increases with the increasing of the proteolytic activity, being higher for the wheat with high deformation index and low gluten index.

However, the relationship of the two parameters with the falling number parameter was not similar, when the falling number was associated with the wheat amylasic activity. The increasing of the proteolytic activity, expressed by the deformation index, was correlated with a decreasing of the amilolytic activity, while increasing the proteolytic activity expressed by the gluten index was correlated with an increase of the amilolytic activity. This apparent contradiction suggests that, at least one of the two parameters studied by us was characterized by significant variability, derived from other factors than the proteolytic activity.

The existence of a significant relationship between proteolytic activity and the amylase of wheat is controversial. Although it seems logical that proteolytic enzymes assist to increase exposure of starch granules through degradation of its surrounding protein matrix, more recent studies have shown that in wheat infected by Aelia sp. and Eurygaster sp., the matrix protein in the area of the insect sting is absent and starch granule remains intact. Basically, the two enzymatic activities, proteolytic and amylase, are not necessarily correlated [10, 11].

Gluten deformation index increased significantly with the decrease of the parameter value of gluten index (Figure 1). Although both parameters express proteolytic activity of wheat, variation of one parameter explains variation of the other only to an extent of 37% (index of determination R2 = 0.366). If we accept the assumption that each of the two gluten quality parameters, capture different aspects of the technological characteristics of wheat, exhaustive use of only one of them becomes a problem, because the assessment leaves out most of the factors that determine quality.

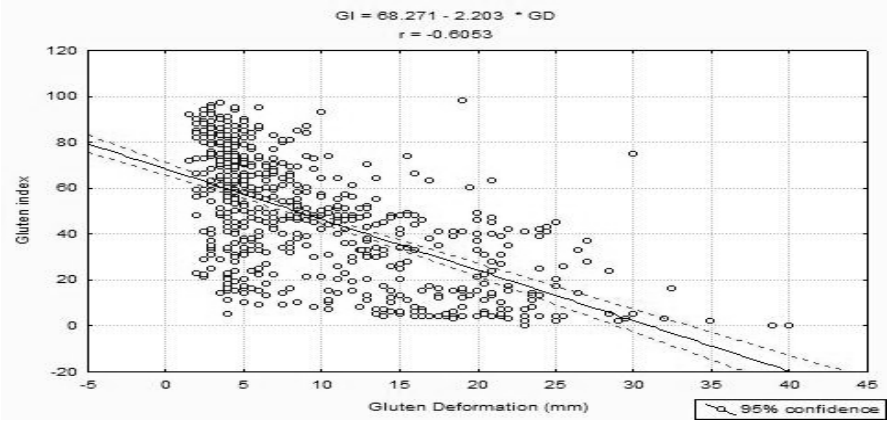

Fig 1. The regression gluten index –wheat deformation index

The lack of a significant relationship between the two parameters suggested the need of a study on intervals of variation. Table 3 highlights eight classes of ranges, four corresponding to the gluten index (0-30, 31 -60, 61-805 and 81-100) and four corresponding to the deformation index (0.0 - 6.0 mm, 6.5 - 12.0 mm 12.5 to 20 mm and 20.5 - 40.0 mm). For each of these intervals there were determined correlation coefficients gluten index - index of deformation and protein content - wet gluten.

Table 3. Correlation coeficients (r) in the range of the parameters gluten index and deformation index

Parameter	No. of samples	Range of variation	Coefficients of variation (%)		r GI – GD	r PC –WG
			GI	GD		
Gluten Index (GI)	**650**	**0 - 100**	**55.187**	**72.744**	**-0.61*** **	**0.90*** **
	176	0 – 30	60.957	52.035	-0.46***	0.83***
	253	31 -60	18.299	60.995	-0.24***	0.91***
	144	61 - 80	8.178	64.057	-0.20*	0.93***
	77	81 - 100	5.160	58.046	0.14 ns	0.91***
Deformation index (GD, mm)	**650**	**0 - 40**	**55.187**	**72.744**	**- 0.61*** **	**0.90*** **
	290	0 -6.0	37.822	26.224	-0.24***	0.93***
	160	6.5 – 12.0	39.129	19.418	-0.30***	0.88***
	120	12.5 – 20	71.317	14.797	-0.20*	0.91***
	80	20.5 - 40	90.146	17.051	-0.16 ns	0.75***

significant (p<0.050), **distinct significant (p<0.010), * very significant (p<0.001), ns - insignificant*

Table 3 shows that the ranges of correlation coefficients decrease as the values of two parameters increase. In the case of values exceeding the limits of 20.5 mm for the deformation index and 81 for gluten index, the correlation between the two parameters becomes insignificant. Correlation between protein content and wet gluten remains highly significant, regardless of the range of variation (Figure 2).

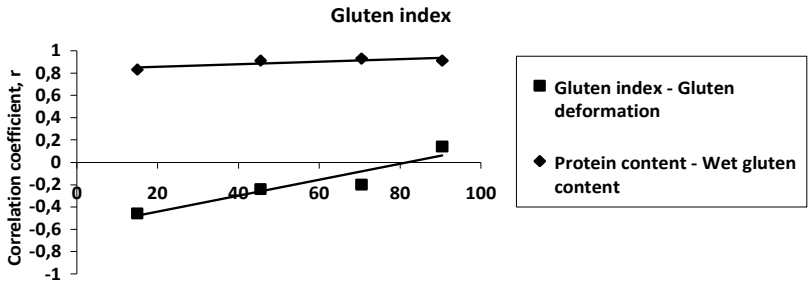

Fig 2. Evolution of the correlation coeficients (r) gluten index – deformation index and protein content – wet gluten, depending on the ranges of the gluten index parameter

The value of the correlation coefficient between gluten index and the deformation index decreases from very significant to insignificant and even changes its sign for gluten index values greater than 81. This trend suggests that at high levels (over 61), the gluten index parameter expresses to a low extent the proteolytic activity of wheat.

Analysis of correlation coefficients gluten index - index of deformation, within the ranges, as a function of the index of deformation, shows that proteolytic activity is not characterized by both parameters to the same extent. Thus, at high levels of deformation index, the correlation between parameters becomes insignificant (Figure 3). Moreover, small variations of the deformation index correspond to high variations of gluten index (90% over the range 20.5 - 40 mm).

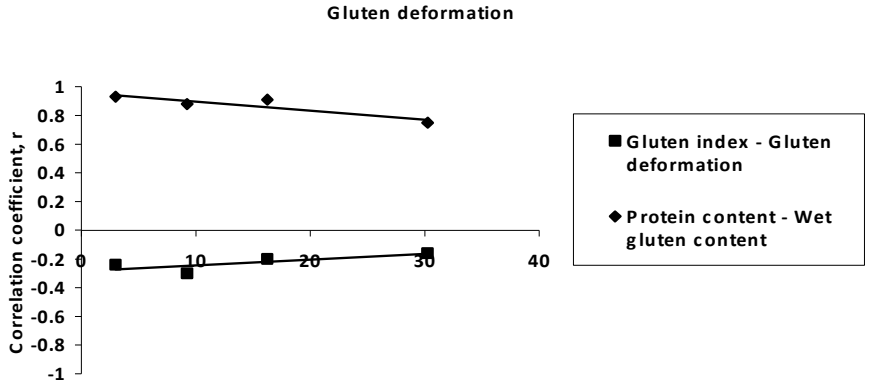

Fig 3. Evolution of the correlation coefficients (r) gluten index - deformation index and protein content - wet gluten, depending on the ranges of the gluten deformation index

The coefficients of variation associated to the deformation index tend to decline as the value of this parameter increases. Consequently, this parameter more accurately describes the effects of the proteolytic activity on gluten.

CONCLUSIONS

Our results suggest that the two parameters, namely the deformation index and gluten index, are not interchangeable in evaluating the quality of wheat. Although there is a very significant correlation between the two parameters, the coefficient of determination is low. The phenomenon is especially pronounced for extreme values, superior to their range of variation.

The wheat quality evaluation should include both quality parameters, because none of them covers all aspects of gluten quality. The deformation index seems more appropriate for the assessment of the proteolytic activity

(phenotypically influenced) and gluten index seems more appropriate to evaluate the overall quality of gluten (genotype + phenotypic influences).

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VARIATION OF AROMATIC COMPOUNDS ON *MUSCAT OTTONEL* WINE DEPENDING ON THE GROWING REGION

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ABSTRACT

For years flavor compounds in wine have been the subject of extensive research, much work in this area referring to the group Muscat wines.

Muscat flavor is determined especially by terpenes, namely linalool, but also by other volatile compounds (esters, aliphatic alcohols) that gives flavor to this group of grape varieties.

Lately it was observed that the aromatic potential of grapes and therefore, wines, varies according to their geographical area of origin, an important variation suffering terpenes that gives flavour to aromatic and semi-aromatic varieties (Muscat, Tămâioasă, Sauvignon, etc.).

Our research focused on physical, chemical and analytical analysis (GC/MS) of Muscat Ottonel wines from two wine regions: Stefanesti Arges and Murfatlar, aiming, in particular the variation of flavors depending on the region of origin.

Results showed that the growing region influence largely the organoleptic characteristics of wines, especially their aromatic content, making easier their recognition when tasting. Variation of chemical compounds in wines is even stronger if the growing areas of origin are different in terms of climate and ecopedological conditions.

In case of Muscat wines from noted vineyards it was observed a variation of terpenes, especially linalool, reaching a value almost double in Murfatlar area.

Key words: flavor compounds, *Muscat Ottonel* wine, muscat flavor, terpenes

1. MATERIALS AND METHODS

Muscat Ottonel wines have been produced in the Murfatlar and Ştefăneşti-Arges, vineyards, in 2007.

Variants, MI (*Muscat Ottonel* wine, Ştefăneşti-Arges vineyard) and M II (wine *Muscat Ottonel,* Murfatlar vineyard) were analyzed in terms of physico-chemical: alcoholic strength (vol% alcohol), sugar content (g/l sugar), total acidity (g/l sulfuric acid), total dry extract (g/l), volatile acidity (g/l acetic acid) analysis was performed by standardized methods.

Chemical tests were followed by organoleptic analysis (tasting laboratory) analysis and GC/MS (gas chromatography coupled with Mass Spectrometry) for identification and determination of volatile compounds.

Chromatographic conditions

A Carlo Erba GC 8000 gas chromatograph equipped with split/ssplitless injector was used. The separation of volatiles was achieved on a Supelcowax 10 fused silica capillary column (Supelco, Belefonte, USA) of 60 m x 0,32 mm i.d. x 0,5 μm film thikness. The oven temperature was programmed from 3°C (5min.) to 250°C (20min.) with 5°C /min. Hydrogen was used as carrier gas with an inlet pressure of 60 Kpa. The temperature of the injection port was 250°C and the detector (FID) temperature was set at 260°C. For data acquisition a Spectra Physics

DP700 integrator was used. Compounds identification was performed on the same capillary column installed in a FISONS INSTRUMENTS MD 800 gas chromatograph/mass spectrometer equipped with split/splitless injector, using the same oven temperature program with helium as carrier. Data acquisition was performed with MassLab software for the mass range from 10 to 350 a.m.u. with a scan rate of 1 scan/sec. The ionization energy was set at 70 eV. Recordered spectra were compared with NIST (National Institute for Standardization, USA) and Wiley (6^{th} edition – 220 000 spectra) mass spectral libraries. Compounds identification was confirmed by Kovats retention time indices on Carbowax 20M capillary columns. Standard substances were also used for compound confirmation.

2. RESULTS AND DISCUSSION

I. Chemical analysis of wines

The two *Muscat Ottonel* wines M I and M II were analyzed in terms of physico-chemical parameters and the main results are presented in Table 1.

Table 1 - The main physico-chemical parameters analyzed wines

Wine	total acidity (g/l H_2SO_4)	sugar content (g/l)	alcoholic strength (vol%)	glycerol (g/l)	total dry extract (g/l)	volatile acidity (g/l CH_3COOH)
Muscat Ottonel ***Ştefăneşti-Argeş M I***	3,7	38	11,5	9	26	0,5
Muscat Ottonel ***Murfatlar M II***	3,2	40	12	11	26	0,6

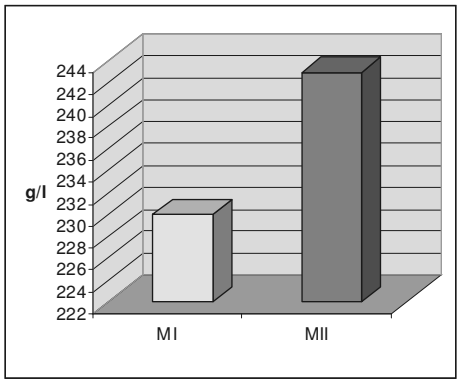


Fig. 1 - Sugars (g /l) accumulated at harvest of grapes Muscat Ottonel

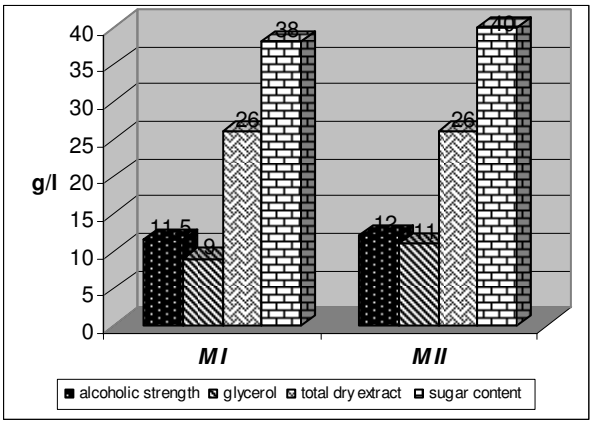


Fig. 2 - Composition parameters of Muscat Ottonel wines

II. Organoleptic characterization of the wines

Muscat Ottonel wine is a white wine of high quality obtained with the remaining sugar with a high alcoholic strength, because of the high accumulation of sugars both in Ştefăneşti-Arges and Murfatlar vineyard.

In this growing area due to favorable climatic conditions, some varieties of grapes (including *Muscat Ottonel*) manifest their full varietal potential, so that accumulate large amounts of sugars that allow oenologists to produce both alcoholically and sweet wine.

In terms of accumulation of sugars in the vineyard of Murfatlar *Muscat Ottonel* grapes registered 243 g / l sugar at harvest so wine is produced with a high alcohol content based on a quantity of 40 g / l residual sugars.

While both *Muscat Ottonel* wines have a specific flavor, the Murfatlar wine's flavor is more pronounced, leading to a wine with more personality.

III. Characterization of the potential of aromatic wines

In the wines, by GC/MS analysis, were identified 19 aromatic compounds: 5 esters, 7 alcohols, 2 aromatic alcohols, 2 terpene alcohols, a lactone and two aldehydes.

Among the esters predominates $C_4H_8O_2$ ethyl acetate, which is a colorless liquid with a fruity fragrance and sensitive to moisture. This compound is the most important ester in wines. Its presence is questionable only in excessive content, when wine is highlighted in a spicy smell, like, similar to that of pepper.

It is formed in small quantities under the action of yeast during fermentation, but can also occur at high doses as a result of intervention of acetic bacteria, which is common especially during the barrel maturation. It is present in large amounts in wines that already have a high volatile acidity, which correlates with the volatile acidity of wine *Muscat Ottonel* which is 0.6 g / l H_2SO_4.

Of the esters were identified:

•2-hydroxy-ethyl propionate in higher concentration than the ethyl acetate;

• ethyl hexanoate ($C_8H_{16}O_2$) from colorless to straw yellow liquid with the smell of green apple, in this wine being into the smallest amount;

• ethyl butanoate ($C_6H_{12}O_2$) with bubble gum and fruit odor.

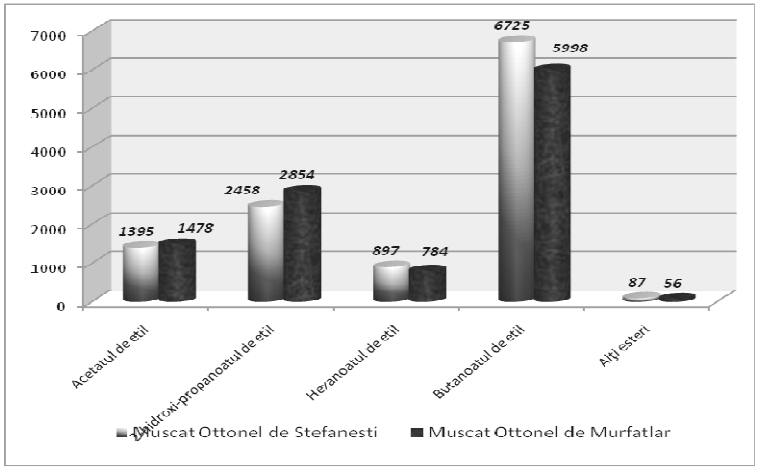
Higher alcohols:

• 3-methyl-1-butanol or isoamyl alcohol ($C_5H_{12}O$) a colorless liquid with a bittersweet chocolate odor coming from the enzymatic degradation of leucine;

• 2-methyl-1-propanol ($C_4H_{10}O$) a colorless liquid with mild odor. The wine is the amount of 15-140 mg / l normally;

• May appear in *Muscat Ottonel* wine and 1-butanol, 1-hexanol odor of fruit and herbs and 5-nonalol.

Fig. 3- Ester



concentration in the wines of Muscat Ottonel

In the aromatic alcohol class there is Benzethanol (ethanol-phenyl-2) with a roses odor.

Among terpenes in *Muscat Ottonel* wine have been identified two terpene alcohols, namely:

- Linalool ($C_{10}H_{18}O$), - 1 and 1.9 mg /l, with a flowers odor. The average amount of 0.8 mg /l is present in every bite, whose participants mostly from the grape flavor;

- α-terpineol – with a lilac odor.

Linalool is the one that gives the muscat flavor and can be observed in a high concentration in this compound.

Regarding the difference between wines produced in different wine regions can be seen that indeed zone of origin interferes in the concentration of products

but is limited to the aromatic flavor potential of the variety.

IV. Results obtained from the analysis of GC/MS *MUSCAT OTTONEL*

The Hexanol is a compound that gives flavor of raw green and, as we have seen in literature, its concentration is different depending on the vineyard.

Table 2 - Concentration of aliphatic alcohols in wine *Muscat Ottonel* (M I, M II)

Compound	Concentration (µg/l) M I	Concentration (µg/l) M II
2-metil-1-propanol	22	15
2-metil-1-propanol	13	8
3-metil-1-butanol	3100	2500
1-butanol	0,05	0,25
3-etoxi-1-propanol	0,12	0,14
1-hexanol	5	1
5-nonanol	6	8

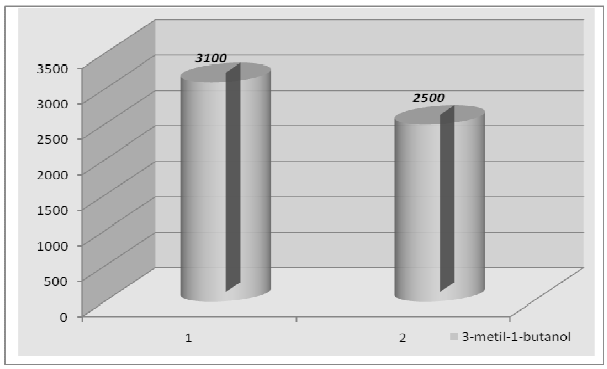


Fig. 4 - Concentration Muscat wines in 3 methyl butanol

Table 3 - Concentration of aromatic alcohols in wine *Muscat Ottonel*

Compound	Concentration (µg/l) M I	Concentration (µg/l) M II
Benzmetanol	2	2
Benzetanol	850	1250

Table 4 - Concentration of terpenes in *Muscat Ottonel* wine

Compound	Concentration (µg/l) M I	Concentration (µg/l) M II
Linalool	1,0	1,9
α-terpineol	0,5	0,7

Regarding the difference between the Linalool concentrations in the wines produced in the two wine regions, it is noted that the differences are significant, almost double in Murfatlar than in Ştefăneşti Arges. This reinforces the opinion that the vineyard influences the concentration in the wine flavor, inside the limits of the the aromatic potential of varieties.

Table 5 - Lactone concentration in the wine *Muscat Ottonel*

Compound	Concentration (µg/l) M I	Concentration (µg/l) M II
γ-butirolactonă	213	190

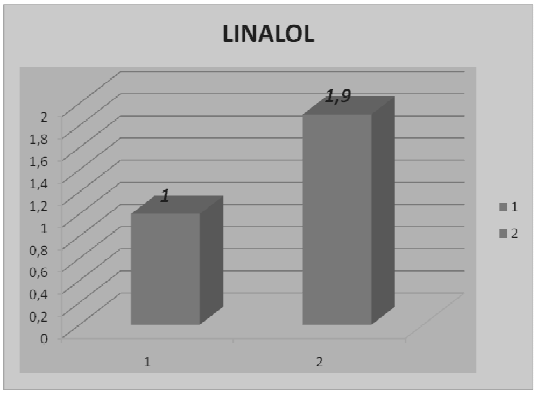


Fig. 5 - Linalool concentration in those two wines, Muscat Ottonel (M I, M II)

3. CONCLUSIONS

1. *Muscat Ottonel* wines studied are within the aromatic wines category of a high quality and were obtained in two different areas, namely: the vineyard Ştefăneşti Arges and Murfatlar

2. In terms of physico-chemical properties of wines, they are within the limits allowed by Romanian law on alcohol concentration, total and volatile acidity, glycerol, dry extract content and sugar content

3.As result of the organoleptic analysis of the *Muscat Ottonel* wines, has been

noticed the wine obtained in Murfatlar showing a strong aroma, full of personality and a touch of softness due to low total acidity;
4. Aromatic character of Muscat is printed by Linalool, which is found in greater quantity in these wines
5. Aroma compounds showed variations in the two wines (MI and M II), especially terpenes (Linalool), these being found in almost double the amount of wine produced in Murfatlar than the one produced in Stefanesti Arges.
6. Changes in aromatic compounds resulted in wines of distinct, easily recognizable by taste, wines, variant M II having a stronger flavor.

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SECTION IV: INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

STUDY THE BEHAVIOR OF TWO SPECIES OF LACTIC BACTERIA DURING MALOLACTIC FERMENTATION AT VARIOUS ENVIRONMENTAL CHANGES

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[3]*Romanian Academy of Sciences*

ABSTRACT

Malolactic fermentation was induced by inoculation with Leuconostoc oenos MLF Quick and MaloStart$^+$ of red wine which had been adjusted to seven different factors of environment. Malolactic fermentation brought about pH increases as great as 0,2 unit; the increases were greater at the higher initial pH Malolactic fermentation is the bacterial conversion of malic acid to lactic acid which often occurs in wine sometime after alcoholic fermentation. In vines, which normally have a pH between 3 and 4, one would expect the higher pH's to have a positive influence on biological activity of the bacteria present; and among the many factors said to influence malolactic fermentation rate. The work presented here was initiated to show this relation and to investigate other effects which temperature, SO_2 and pH might have on malolactic fermentation.

Keywords: wine, malolactic fermentation (MLF), lactic acid bacteria, organic acids, pH, theoretical temperature of saturation (T_{TS}).

INTRODUCTION

Decarboxilation of malic acid in wine in lactic acid and carbon dioxide under the action of lactic bacteria is a biological process of deacidification known in practice as the malolactic fermentation of wine, abbreviated as MLF, which disappears after one of the acidic functions of malic acid. In order to optimize the pattern of MLF, below are several attempts to achieve MLF using two strains of bacteria *Oenococcus oeni* at different environmental changes.

Actuality of theme: obtaining a balanced product as required by the modern consumer.

MATERIALS AND METHODS

They used two strains of lactic acid bacteria *Oenococcus oeni* selected: MLF Quick and MaloStart$^+$. As a control sample was accepted the Merlot red wine, harvest year 2007, with the following composition, (Table 1).

Table 1. Physicochemical characteristics of red dry wine Merlot vintage 2007

Product name	Alc. (% vol.)	Sugar (g/L)	Total acidity, (g/L)	Volatile acidity, (g/L)	SO_2, (mg/L)	Tartaric acid, (g/L)
1	2	3	4	5	6	7
Merlot	12,3	2,2	6,4	0,50	70/15	4,0

Physicochemical characteristics of red dry wine Merlot vintage 2007

Product name	Malic acid, (g/L)	Lactic acid, (g/L)	Anthocyanins, (mg/L)	pH	T_{TS} KHT, (^oC)	T_{TS} CaT, (^oC)
1	8	9	10	11	12	13
Merlot	0,7	< 0,1	445	3,45	14,4	20,5

Preparation of experimental samples. In all samples subjected to analysis were inoculated lactic acid bacteria MaloStart$^+$ and MLF Quick, two samples of each, for any variant of testing. Was made the following changes of various physical and chemical parameters in both samples sown with lactic acid bacteria cultures mentioned above:

Variant 1: FML at T = + 18,5 °C;
Variant 2: FML at T = + 8,0 °C;
Variant 3: FML at t = + 18,5 °C, SO_2 total/free = 73/31 mg/L;
Variant 4: FML at t = + 20 ^{o}C, pH 3.2;
Variant 5: FML at t = + 22 °C, SO_2 total/free = 124/41 mg/L;
Variant 6: FML at t = + 32 °C;
Variant 7: FML at t = + 22 °C, pH = 2,95 (acidification).

RESULTS AND DISCUSSIONS

During the completion of experiments with MLF (about 40 days) in all samples was determinate the change of main indices and composition, using traditional methods of analysis and reference methods for comparison. In Table 2 are presented physicochemical transformations of the examined samples.

Below, are shown graphically, change of the compositional characteristics of the concentration of tartaric, malic and lactic acids during MLF, made by two species of lactic acid bacteria studied in different environmental conditions (Figures 1, 2, 3).

Table 2. Variation of the compositional characteristics of Merlot red wine, vintage 2007 during the MLF, sown with lactic acid bacteria MLF Quick (I) and MaloStart^{+}(II)

Variant/ Sample		Total acidity (g/L)		Volatile acidity (g/L)		Tartaric acid (g/L)		Malic acid (g/L)	
		1		2		3		4	
		I	**II**	**I**	**II**	**I**	**II**	**I**	**II**
V.1	P_i	6,4	6,4	0,50	0,50	4,0	4,0	0,7	0,7
	P_f	5,8	5,7	0,59	0,92	2,3	1,3	0,39	0,41
V. 2	P_i	6,4	6,4	0,50	0,50	4,0	4,0	0,7	0,7
	P_f	5,6	5,9	0,53	0,69	2,4	2,0	0,45	0,38
V. 3	P_i	6,4	6,4	0,50	0,50	4,0	4,0	0,7	0,7
	P_f	5,4	5,8	0,73	1,02	1,8	2,1	0,42	0,40
V. 4	P_i	17,4	17,4	0,50	0,50	4,0	4,0	0,7	0,7
	P_f	15,8	15,2	0,59	0,73	1,9	0,8	0,74	0,68
V. 5	P_i	6,0	6,0	0,53	0,53	4,0	4,0	0,7	0,7
	P_f	5,8	5,9	0,60	0,79	1,9	1,7	0,47	0,42
V. 6	P_i	6,0	6,0	0,53	0,53	4,0	4,0	0,7	0,7
	P_f	5,6	5,9	0,58	0,69	1,7	1,8	0,64	0,43
V. 7	P_i	16,2	16,2	0,53	0,53	4,0	4,0	0,7	0,7
	P_f	6,1	5,8	0,73	1,02	1,6	1,4	>>	>>

Note: P_i – initial sample,
P_f – final sample.

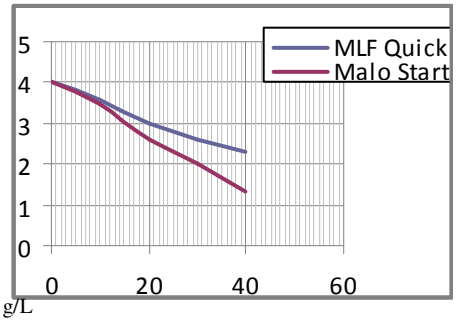

Fig1. Evolution of concentration of tartaric acid during MLF
both lactic bacteria cultures (V I)

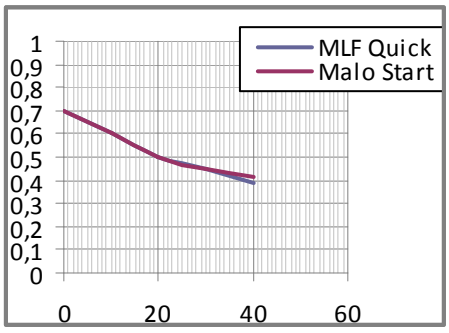

Fig 2. Evolution of malic acid concentration during MLF
both lactic bacteria cultures (V I)

Table 2 (continue)Variation of the compositional characteristics of Merlot red wine, vintage 2007 during the MLF, sown with lactic acid bacteria MLF Quick (I) and MaloStart^{+}(II)

Variant/ Sample		Lactic acid (g/L)		Anthocyanins (mg/L)		T_{TS} KHT (^{o} C)		T_{TS} CaT (^{o} C)	
		5		6		7		8	
		I	**II**	**I**	**II**	**I**	**II**	**I**	**II**
V.1	P_i	<0,1	<0,1	445	445	14,4	14,4	20,5	20,5
	P_f	0,39	0,73	418	386	18,1	21,2	15,4	11,3
V.2	P_i	<0,1	<0,1	445	445	14,4	14,4	20,5	20,5
	P_f	0,36	0,83	429	412	18,1	20,1	15,2	9,9
V.3	P_i	<0,1	<0,1	445	445	14,4	14,4	20,5	20,5
	P_f	0,62	0,815	402	334	19,5	19,5	16,4	12,1
V.4	P_i	<0,1	< 0,1	445	445	14,4	14,4	20,5	20,5
	P_f	1,88	2,2	416	408	20,1	22,4	17,6	18,2
V.5	P_i	<0,1	< 0,1	445	445	14,4	14,4	20,15	20,15
	P_f	0,82	0,66	391	328	19,2	18,8	14,6	12,1
V.6	P_i	<0,1	< 0,1	445	445	14,4	14,4	20,15	20,15
	P_f	0,33	0,69	422	400	18,1	20,1	15,2	9,9
V.7	P_i	<0,1	< 0,1	445	445	14,4	14,4	20,15	20,15
	P_f	0,26	0,21	432	408	21,3	20,1	10,2	16,2

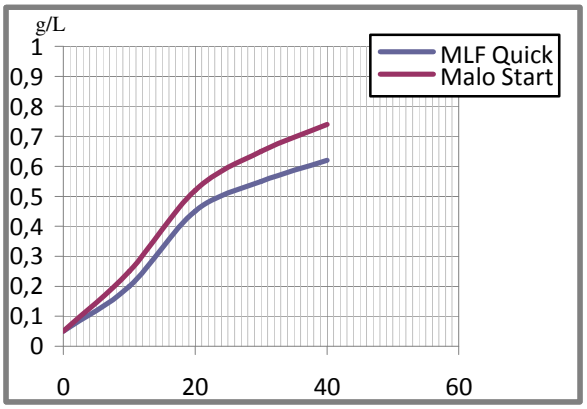

Fig 3. Evolution of lactic acid concentration during MLF both lactic bacteria cultures (V I)

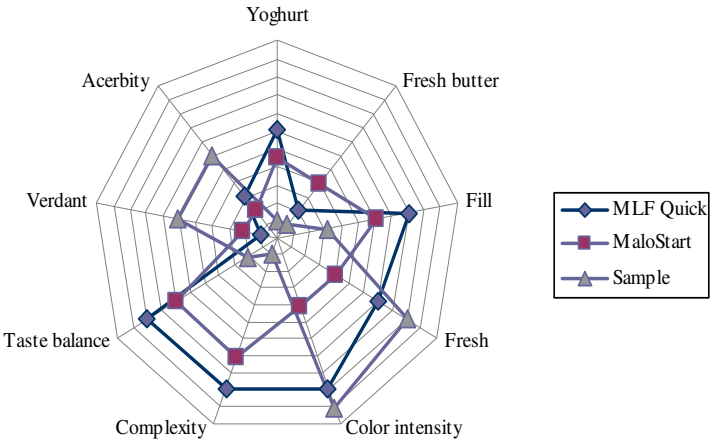

Fig 4. Results of sensory aroma-taste (V I).

CONCLUSIONS

1. Using cultures of selected lactic acid bacteria *Oenococcus Oeni* bring to improving the organoleptic qualities of red wines in general.
2. Based on the experimental results obtained are recommended for use in practical rational culture MLF Quick.
3. Basic condition of obtaining the desired results is compliance with relevant technology.

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EFFECT OF SOIL PROCESSING AND FERTILIZATION ON THE OCCURRENCE OF WEEDS IN ALFALFA SEED

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ABSTRACT

The success for growing of alfalfa is being set before its sowing. Important are the choice of area, the method of soil treatment and mineral fertilization to ensure creation of durable and high-yield planting alfalfa.

Major problems in the cultivation of alfalfa are weeds with their high species diversity and high environmental and biological plasticity, which facilitates their rapid dissemination and adaptation.

Researches were conducted in a field experiment for the period of four years to determine the influence of soil processing and fertilization on weeds development in alfalfa crops grown for forage.

The survey results show that at least weeds per square meter were found in versions of loosening without inverting the layers of 12-15 cm, due to good crop garnish and better physical characteristics of soil in the arable layer. The highest is the weeds density in variants with a ploughing depth of 12-15 cm - 10.2%, followed by ploughing of 18 -22 cm and 30 to 35 cm, respectively, 9.24 and 9.90 percent. Lowest is the stage of weeds in the variant with $N_{2,3}$ P_{10} $K_{3,5}$, nitrogen½ in I year, ½ year in III year for the phosphorus and potassium as a stock and non-fertilized control respectively 5.2 and 5.9 percent compared to alfalfa yield, followed by the variants with fractional introduction of phosphorus fertilizer.

Key words: alfalfa, weeds, soil processing, fertilization

In Bulgaria alfalfa is the main grass-fodder crop. It provides the most protein per unit area due to the symbiosis with the nitrogen-fixing bacteria, without requiring high doses of nitrogen fertilization.

The success for growing of alfalfa is being set before its sowing. Important are the choice of area, the method of soil treatment, bringing to gardening state, as the choice of suitable seed to ensure creation of durable and high-yield planting alfalfa. Not a little is the role of good nourishing regimen maintaining through the use of mineral fertilizers.

The creation of high agro-background for growing alfalfa enhances the competition by weeds. Due to their rapid pace of development they grow older and accumulate big biomass, while culture sprout slowly.

Integrated control is best suited to maintaining the purity of crops with alfalfa. Soil processing is the central group in it. The weed vegetation is limited significantly by performing timely processing, at appropriate technological conditions and on the required depth. (Dimitrov etc. 2003, T. Tonev 2000, Albrecht H & Sommer, H., 1998).

The chemical method is one of the highly efficient, fast and easily methods of weed control (Dimitrova, Ts., 2000). The majority of weed species rapidly develop resistance to most of the preparations for a long time (Joene, W. at all.

1991). This imposes the need of new studies to increase the scope of possibilities for weed control and to maintain weed populations below economic harm.

The main problem with weeds is their great diversity of species and high ecological and biological plasticity, which facilitates the rapid dissemination and adaptation.

The aim of the study is to determine the influence of soil processing and fertilization on the development of weeds in crops of alfalfa grown for forage.

MATERIAL AND METHODS:

The experiment was set at the method of the long plots of II experimental field in EFC – Pleven, with slightly leached black earth soil (Haplic Chernozems, loamy) at the area of 6 acres, with a size of the experimental plot of 30 m2 in four iterations with the following agrochemical characteristics:

Table 1. Agrochemical characteristics of slightly leached black earth soil

Sample Nr.	pH (в H_2O)	pH (в KCl)	N/ 1000 g	P_2O_5/ 100 g	K_2O/ 100 g	C_x	Humus %
1	6,95	6,04	31,50	5,19	-	1,03	1,77
2	6,68	5,67	33,95	3,94	-	1,37	2,36
3	6,54	5,55	32,55	3,18	-	1,34	2,31
4	6,31	5,30	35,70	3,16	-	1,28	2,21
5	6,24	5,20	32,55	3,22	-	1,57	2,71

The experiment is made as two-factor and includes:

Factor F – fertilization with the following rates of factoriality:

F_1. Control – Non Fertilized

F_2. N_6 P_{10} K_8 – on the approved technology

F_3. $N_{2,3}$ P_{10} $K_{3,5}$ – the nitrogen ½ in I year, ½ in III year, the phosphorus and the potassium as a stock.

F_4. $N_{2,3}$ P_{10} $K_{3,5}$ - the nitrogen before sowing, the potassium as a stock, the phosphorus 1/3 in 1,2 and 3 years

F_5. $N_{3,5}$ P_8 $K_{5,0}$ – the nitrogen before sowing, the phosphorus and the potassium before the processing

F_6. Amofos – 25,0 kg/da – calculated at the rate of fertilization $N_{2,7}$ P_{12} K_0.

and the factor T – soil processing:

T_1 loosening at the depth 12 – 15 cm

T_2 ploughing at the depth 12 – 15 cm

T_3 ploughing at the depth 22 – 24 cm - control

T_4 ploughing at the depth 18 – 22 cm
T_5 ploughing at the depth 30 – 35 cm

The experiment was set in 2002 and is made for the period of four years. The processing is carried out in October with a tractor Class 1.3 Zetor. In loosening variant the shallow loosening tool is used, so the depth is 12-15 cm, but the soil is only made friable without to overturn the soil layer. In other variants of soil processing the layer turns with a plow, as the control is the proposed in the conventional technology for growing alfalfa for fodder in areas with heavily weeds (Mamarova, Naydenov et al., 1987) at a depth of 22-24 cm with following ploughing of 28-30 cm after 20-25 days. We preferred the ploughing of 22-24 cm, as there was the variant for deeper processing of 30-35 cm. After processing the fertilization with one soil cultivation is performed. Followed strong low temperatures in the winter helped to homogenize the soil as regard to the structure in the spring.

The soil before-sowing processing includes cultivation has taken place and a rolling. Sowing is done in propagation norm 22 kg / ha. The area is rolled before sowing and after sowing the seeds. The prolonged spring drought had the negative impact, which severely affected the germination and further vegetation of plants. The average rainfall in May was 74.5 mm, in June - 12.8 mm, the average maximum monthly temperature for these months respectively was 26.9^0C and 29.4^0C.

Only mechanical method is used for weed control - to determine influence of the factors soil processing and mineral fertilization.

RESULTS AND DISCUSSION

The alfalfa realizes maximum its biological potential only in the absence of the competitive weeds impact. In this aspect the soil processing and fertilization are essential.

The area before processing was mainly with cereal weeds - Johnsongrass (S. halepense). It was accounted the weeds appearance over the biological threshold of harmfulness with a Johnsongrass (S. halepense), Cat greenbriar (S. glauca), common pigweed (A. retroflexus) and horseradish (P. lapatifolia L.)

Table 2. Influence of the soil processing of the alfalfa sowing weeds occurrence

Soil processing cm		Weeds biomass kg/da	Alfalfa yield kg/da	Correlation weed biomass / Alfalfa %
Loosening	12-15	156	2145,4	7,27
Ploughing	12-15	168	1650,4	10,18
Ploughing	22-24	140	2029,0	6,90
Ploughing	18-22	176	1905,0	9,24
Ploughing	30-35	188	1900,0	9,89

The crop weeds occurrence (mainly with annual and perennial weeds cereals) depends on soil processing factor. The lowest is the weeds occurrence (Table 2) in the variant ploughing of 22-24 cm, as the weeds share to yield is 6.90 percent, and the variant with loosening of 12-15 cm is 7.27%. The highest weeds occurrence is established in the variant of ploughing processing of 12-15 cm - 10.2%, followed by ploughing processing of 30 to 35 cm and 18 -22 cm, respectively, 9.90 and 9.24 percent. In the standard depth of processing the main depot with weed seeds is not affected, which resulting in systemic processing at depths over 30 cm is located in the layer 25-30 cm. As the confirmation of this conclusion is the high relative percentage weeds biomass in plowing depth 30-35 cm.

Table 3. Influence of fertilization of the alfalfa sowing weeds occurrence

Fertilization variants	Weeds biomass kg/da	Alfalfa yield kg/da	Correlation weed biomass / Alfalfa %
Control – non fertilizes	128	2151,2	5,94
N_6 P_{10} K_8 – on approved technology	186	2390,7	7,78
$N_{2,3}$ P_{10} $K_{3,5}$, nitrogen ½ in I year, ½ in III year and P and K for a stock.	138	2635,5	5,24
$N_{2,3}$ P_{10} $K_{3,5}$, nitrogen before sowing, K for a stock, P - 1/3 in 1,2and 3 years	160	2534,5	6,31
$N_{3,5}$ P_8 $K_{5,0}$	175	2409,0	7,36
Amofos – 25,0 kg/da – $N_{2,7}$ P_{12} K_0.	166	2422,0	6,89

In shallow ploughing parcels the largest correlation of weeds biomass towards the alfalfa biomass yield achieved due to strong secondary weeds occurrence and weaker alfalfa undergrowth. Reducing evaporation by capillary action is established on shallow loosening parcels in low soil moisture content in the spring of the first and second year with the formation of soil crust under which soil layers remain in loosen condition. These conditions lead to higher by 3.2% and 3.7% soil moisture, as a result the alfalfa forms stronger and well garnished grass, leading to accumulation of more biomass (Table 4).

In the last year of experiment conducting (Table 4) the results obtained confirmed the trend towards reducing the rate of weeds formation in loosening variant without turning the layers of 12-15 cm. In other variants with increasing depth of processing number of weeds is increased reaching its maximum depth in the treatment of 30-35 cm.

In motoring the influence of fertilization factor (Table 3) on the occurrence of weeds in crop the following trend is observed - relatively lowest is the weeds percentage participation in the total biomass in variants with the highest yield

Table 4. Green and dry mass yield of alfalfa, kg / da, seed yield and number of weeds m2- field experience - an average from three sprouts

Fertilization/ Processing variants	Fresh mass кg/da	Dry mass кg/da	Seed yield		Weeds number at m^2
			average recurrence кg	кg/da	
1. F_1T_1	1791	**459**	**2,530**	**16,61**	**32**
F_1T_2	1787	457	2,400	16,08	92
F_1T_3	1544	380	1,556	10,42	68
F_1T_4	1764	413	1,000	6,07	48
F_1T_5	1625	389	1,000	6,07	90
2. F_2T_1	1797	**421**	**1,820**	**12,19**	**36**
F_2T_2	1776	416	1,535	10,28	110
F_2T_3	1849	452	1,855	12,42	36
F_2T_4	1813	421	1,120	7,50	60
F_2T_5	1724	405	0,570	3,820	106
3. F_3T_1	1941	**472**	**2,070**	**13,86**	**30**
F_3T_2	1877	456	1,800	12,06	52
F_3T_3	1932	467	1,570	10,52	68
F_3T_4	1805	416	1,260	8,44	44
F_3T_5	1757	421	1,350	9,05	58
4. F_4T_1	1891	**440**	**2,420**	**16,21**	**50**
F_4T_2	1805	408	1,550	10,39	56
F_4T_3	1696	427	1,580	10,59	40
F_4T_4	1856	441	1,180	7,91	52
F_4T_5	1933	432	2,210	14,81	80
5. F_5T_1	1983	**421**	**1,920**	**12,79**	**44**
F_5T_2	1693	408	1,510	10,12	94
F_5T_3	1659	396	1,660	11,11	60
F_5T_4	1729	407	1,590	10,65	106
F_5T_5	1969	453	2,025	14,03	84
6. F_6T_1	1683	**431**	**1,690**	**11,32**	**54**
F_6T_2	1669	397	1,560	10,45	110
F_6T_3	1541	371	1,640	10,99	60
F_6T_4	1841	443	0,955	6,40	88
F_6T_5	1583	379	1,335	8,94	108

$GD_{5\%}$ =2.18 ; $GD_{1\%}$ = 2.74; $GD_{0,1\%}$=3.90;

obtained (fertilized with $N_{2,3}$ P_{10} $K_{3,5}$, nitrogen ½ in I year, ½ in III year for phosphorus and potassium on stock) and non fertilized control - respectively 5.2 and 5.9 percent compared to the yield of alfalfa. Relatively highest is the weeds

formation in the variant fertilized on the common accepted technology with N_6 P_{10} $K_{8,}$ followed by the variant fertilized with $N_{2,3}$ P_8 $K_{3,5}$, nitrogen before the sowing, phosphorus and potassium on a stock.

Smaller weeds amount was observed in variants with fractional introducing of phosphorus manure as this fertilizer stimulates legume crops and suppresses wheat weeds, which are mainly nitrogen preferring.

CONCLUSIONS

In variant with loosening without inverting the layers of 12-15 cm at least weeds per square meter were ascertained due to good crop garnish and better physical characteristics of soil in the arable layer.

The highest density of weeds formation is in the variant with ploughing depth of 12-15 cm - 10.2%, followed by plowing of 18 -22 cm and 30 to 35 cm, respectively 9.24 and 9.90 percent.

The lowest weeds formation level is in the variant with fertilization $N_{2,3}$ P_{10} $K_{3,5}$, nitrogen ½ in I year, ½ in III year for the phosphorus and potassium in a stock and non fertilized control respectively 5.2 and 5.9 % compared to the yield of alfalfa, followed by options with the introduction of fractional phosphorus fertilizer.

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ENVIRONMENTAL ASPECTS IN APPLICATION OF GYPSUM OF SERO-CLEANING INSTALLATIONS FROM "MARITSA IZTOK 2" THERMO-ELECTRICAL STATION FOR MELIORATION PURPOSES ON DISTURBED AGRICULTURAL LANDS

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Key words: waste gypsum, soil reclamation, melioration of salt-affected soils

ABSTRACT

We have carried out investigations in two directions for application of waste gypsum from SPI (Sulphur-purifying installation of "Maritza-Iztok" thermo-electrical station.

-as environmental suitable substrate for reclamation of disturbed agricultural lands

-for its use as chemical meliorant of salty soils

The industrial development and intensification of production increase significantly the pollution of the environment. The soil contaminates most often. This is due to the large quantities of waste resulting from different industries.

Very often, these waste products are wasted in the area of the factories, hinders their functioning and pollute the environment.

In "Maritsa-Iztok 2" EAD Thermo-Electrical Station 600,000 tons production gypsum are obtained per year from gas-purification which is deposited on fertile agricultural land rather than to be recovered. Sero-Cleaning Installations / SCI / after 7th and 8th - energy units, and since October 2008 and after 1st and 2nd energy units are designed to purify the flue gases of sulfur dioxide and sulfur trioxide to protect the air clean and to prevent its content over the pollution limits for their content. The resulting calcium sulfate (gypsum) is transported and disposed with other solid waste (slag and ashes) in the existing disposal site № 1 of the TES-2 and then in "Staroselets" waste bank. Due to the exhaustion of the capacity of "Staroselets" waste bank at the present the solid waste is deposited in the "United northern waste banks".

The problems highlighted are not only of regional but also of national importance, in view of the affection of one of the most fertile parts of our country. This requires duly seeking technologies and methods to restore disturbed areas, to propose options for safe disposal of gypsum of sero-cleaning installations in the "United northern waste banks" and for other purposes.

Studies were conducted in both directions for ascertaining the possibilities for use of waste gypsum: 1 / its inclusion as a component of an ecological suitable substrate for reclamation of damaged areas in order to reduce the exploitation costs of its safe disposal, and 2. / its use as chemical meliorant of salted soils.

The purpose of the present study is to present the results of the chemical characteristics, one of the most important indicators of restricting the use of

gypsum from Sero-Cleaning Installations of “Maritsa Iztok 2” Thermo-Electrical Station as a component for biological reclamation of damaged land and meliorant for salted soils.

MATERIAL AND METHODS

1/ The geomorphologic reference is made to achieve the goals on the first direction for the soils in the region of Maritsa East Mines. The initial materials /gypsum, cinder-ashes/, mining overburden/ were analyzed for chemical, agrochemical, physical-mechanical and radioactive indicators.

The vegetation experiments were derived with different participation of gypsum (1, 3, 5, 7.5, and 10%) in soil mixtures of ashes and clay to find the most optimal ratios between them. There were tested the opportunities for growth and development of two indicator plants – fodder peas and spring barley. Before making the experience and after crops harvesting there have been reported chemical, agrochemical and physical indicators of soil mixtures and the production of fresh and dry weight is reported. The plant analysis is made to the selected options.

In accordance with the findings and recommendations of the vegetation experiments an experimental section is built on “Staroselets” heap (from Maritsa Iztok mines region), in which substrates are modeled in layers on depth to 50 cm on mixed filling of gypsum of SCI, ashes and overburden. 5 variants were created on the experimental area of 5 acres; control – with pure clay, with gypsum 3% and 5% in a mixture with ashes and clay 15%, and the same ternary mixtures covered with 40-cm layer of humus soil. A variety of types and depth treatments of substrate and mineral fertilization with nitrogen and phosphate fertilizers are made. The barley and the mixture of winter barley and peas were grown in double field rotation.

A morphological description of soil horizons on the depth up to 100 cm is made. The changes in the physical and physico-mechanical properties of newly formed "soils" are monitored. Periodically is made chemical and agrochemical characteristics of soil substrates as the quantity of water-soluble salts, water-soluble sulfates including the ability for leaching the waste to establish the change in their condition in the long term, total sulfur content of humus and heavy metals pH, nitrate and ammonia nitrogen, total and assimilable K and total mobile P is determined (Arinushkina, 1970). The microbiological characteristic is made.

2 /On the second direction of waste production gypsum using there are derived vegetation experiments with 4 types of salted soils with different levels and types of salinization. 5 variants with three levels of gypsum from the SCI were tested. The mineral fertilizer was added to all the variants as a constant background. The test crop - alfalfa is planted. The necessary amount of gypsum /calcium sulphate/ in non-carbonated salted soils is determined by the formula of Kavardzhiev / Penkov, 1985 /

On the basis the positive results obtained from vegetation studies the most appropriate melioration rates for field conditions have been determined. An experimental plot was established on an area of 4 acres on meadow alkaline soil in the experimental base in the village of Belozem, Plovdiv region. The agricultural activities are carried out and different standards of gypsum processing are tested - 2,0 t / dka; 2,5 t / dka; 3,0 t / dka; 4,0 t / dka and 5,0 t / dka. 2 cultures are grown – vetch-barley mixture and wheat. The biological yield of green mass is reported and the agrochemical evaluation of vegetable production is made. Periodically, soil samples were taken from various variants and the characteristic of meliorated salted soils is made to establish the changes in the salinity extent and water-determining physical parameters.

RESULTS AND DISCUSSION

1. /The results of vegetation and field experiments show that the gypsum of SCI in the three-component mixture has a negative impact on the growth, development and yield of crops grown. According to the chemical characteristics the resulting production meets environmental requirements. Heavy metals are not in high concentrations, which could have a detrimental impact on humans and animals when using the plants for food.

Newly created three-component mixtures with gypsum 3% and 5% have a high content of soluble salts - 1.25% and respectively 1.14% in bulk area, which, according to Bulletin 27 of the MAF of 1994, defines them as salted. The values of heavy metals are under the limit values and do not represent an environmental hazard. The environment reaction does not modify /Table 1/. No detectable movement of sulfates in profiles depth under 50cm layer. According to scientific research and practical knowledge, these soils can be attributed to so called gypsum-bearing soils, which are characterized by insufficient favorable agrochemical and physical and mechanical properties. Soil conditions restrict the choice of appropriate crops, which requires a special approach to the organization of crop rotation.

Best results were obtained in anthropogenic "soils" with gypsum 3% and 5% and humus cover. Humus soil layer leads to significant improvement of the nutrient regimen, structural condition and physic-mechanical properties to 50cm depth. It is rich in organic matter and creates a favorable environment for microbiological activity. This reflects favorably on the growth, development and quality of crop production. The obtained grain yield of barley in variants with humus layer exceeds double the yield in variations with humus without coverage. Statistically is proven that in variation with 5% + humus layer the yield obtained is highest - 323 kg / dka. Soil layers below the humus horizon are characterized by physical and chemical properties identical to those without humus coverage. The highest yield of pea-barley mixture’s green mass is obtained also in variants with humus cover - 295-300kg/dka.

Table 1. Water soluble salts and sulfates, humus, pH and heavy metals content in the field experience variants

Depth cm	Water soluble salts, %	Water soluble sulfates mg/kg	Humus %	pH (H_2O)	Cu	Zn	Pb	Cd	Ni	Cr
					mg/kg soil					
1 variant (substrate of pure clay)										
0-25	0,20	473	0,53	7,3	27	29	11	<0.50	9	8
25-50	0,29	464	0,17	7,4	30	31	13	<0.50	10	11
50-75	0,24	650	0,36	7,6	42	39	12	<0.50	11	9
75-110	0,24	699	0,13	7,6	37	39	15	<0.50	12	5
2 variant (substrate of 3% gypsum, 15% ashes and clays)										
0-25	1,25	7064	0,79	7,6	59	39	11	<0.50	25	13
25-50	1,14	6224	0,46	7,6	37	41	12	<0.50	20	11
50-75	0,38	810	0,17	7,6	37	44	14	<0.50	17	7
75-110	0,24	678	0,30	7,8	38	43	14	<0.50	22	7
3 variant (substrate of 5% gypsum, 15% ashes and clays)										
0-25	1,14	7950	0,46	7,6	44	39	12	<0.50	20	15
25-50	0,77	4464	0,56	7,6	40	35	13	<0.50	17	13
50-75	0,23	971	0,52	7,7	38	40	14	<0.50	14	11
75-110	0,16	954	0,26	7,6	28	33	13	<0.50	15	11
4 variant (humus layer covering the substrate of substrate of 3% gypsum, 15% ashes and clays)										
0-40	0,18	354	2,75	7,7	60	48	13	<0.50	16	19
40-65	1,26	5151	1,18	7,6	62	39	14	<0.50	29	19
65-90	0,77	4019	0,95	7,7	41	30	14	<0.50	11	7
90-120	0,57	1164	1,70	7,6	33	29	14	<0.50	12	5
5 variant (humus layer covering the substrate of substrate of 5% gypsum, 15% ashes and clays)										
0-40	0,21	281	2,56	7,7	44	29	9	<0.50	11	9
40-65	1,42	6064	1,47	7,6	39	25	8	<0.50	18	8
65-90	1,03	5021	0,53	7,6	36	23	9	<0.50	13	7
90-120	0,42	1528	1,21	7,7	33	24	8	<0.50	12	7

The agrochemical characteristics of soils in newly formed soils of the beginning of the field experience shows their weak reserves of basic nutrients nitrogen and phosphorus and their good reserves of potassium. In the process of

field research after the fertilization with mineral nitrogen and phosphorus fertilizers a positive change in reserves of substrates with assimilated forms of nutrients is reported.

2. / The investigations conducted for gypsum use as a chemical meliorant show that with introduction of increasing doses of gypsum the exchangeable sodium in meliorated arable layer 0-25 cm significantly reduced at the end of the study. The same trend is observed in its percentage from sorption capacity. In the layer 0-25 cm the values of exchangeable Na before starting experience are 2,57 meq/100g soil, and correspondingly decreased at the end of the experiment to 0,47 - 0,06 meq/100g. In lying below horizon 25-50 cm exchangeable Na reduces from 73,4 meq/100g soil to 8,21 -1,4 meq/100g soil. The data in Table 1 for the degree of alkalescence show clearly that in a standard gypsum processing with a rate 3 t / dka gypsum the soil from deep alkaline is already weak alkaline, and by the land melioration in with and 5 t / dka gypsum we can talk about non-alkaline soils .

Therefore the best ameliorative effect was obtained in standards rates of gypsum processing with 4 and 5 t / ha gypsum of SCI. In all variants the soil reaction in the arable horizon 0-25 cm is slightly alkaline to neutral, in the alkaline B horizon (25-50 cm) it is still high (8,9-9,6) and only in the variants with the addition of 4 and 5 t / da gypsum pH decreased respectively to 7,6 and 7,2. At the end of 3-year experiment the positive ameliorative effect is reached, but to fully improvement the chemical properties of alkaline soils a longer period of time is required to respond to difficult soluble gypsum into the soil in order to replace the exchangeable sodium of the soil absorption complex with calcium.

CONCLUSIONS

The investigations conducted for the evaluation of gypsum of SCI by chemical, agrochemical and physical-mechanical properties show that they do not contain harmful substances which can have negative effects in using as a component of disturbed land reclamation and as a chemical meliorate of salted soils.

The complex studies characterizing the anthropogenic "soils" created by mixed filling of waste from the power production of “Maritsa Iztok 2” TES with mine overburden indicate that the concentration of gypsum from SCI to 3% in the three-component mixture for filling /disposal/ is no ecological risk is growing of agricultural crops. For the implementation of successful biological reclamation the filling of upper insulation layer of clean overburden / homogeneous mineral materials / is necessary, with layers thickness 2m after subsidence over the deposited waste and the spreading of humus soil layer on the lined surface of the upper insulation layer with a thickness of the layer 30-35cm after the subsidence.

On the basis the results obtained from the field studies conducted as the existing environmental legislation documents the technology for reclamation

regulation in mixed filling of ashes and plaster of SCI of “Maritsa Iztok 2” TES with mines overburden is proposed.

The reducing of pH values to neutral area, the exchangeable sodium and the alkaline rate decreased significantly in the studied meliorated salted soils is observed. The highest ameliorative effect of gypsum of SCI for the period of the experiment is occurred in the rate 4t/dka. The received plant production meets the environmental requirements. It does not contain harmful substances and heavy metals in hazardous concentrations and has a high content of essential nutrients.

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THE USE OF DIRECT VIABLE COUNT METHOD FOR QUANTIFICATION OF BACTERIA IN MARINE MICROCOSMS SUPPLEMENTED WITH GASOLINE

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ABSTRACT

The direct viable count (DVC) introduced by Kogure and colab. (1979) is one of the simplest methods to determine which bacterial cells are active and capable of cellular growth and multiplication. This microscopic method of direct enumeration of viable cells is based on incubation of the samples in the presence of an antibiotic (nalidixic acid as inhibitor of cell division, all other metabolic properties remaining active) and nutrients (yeast extract). Viable cells may continue to metabolize nutrients and grow but will not be able to divide, thus becoming more elongated after incubation whereas inactive cells do not elongate during the incubation period. Measurements were performed using two automatic measurement software digital imaging, such as ImageJ to measure cell length and CellC software used for cells quantification, compared with manual counting and measuring. In preliminary experiments we calibrated the original conditions indicated by Kogure, using acridine orange as fluorochromes. These results showed clear increase in cell's length (2-4 times) after 6-8 hours of incubation. This method was used to determine direct viable counts (DVC) in microcosms supplemented with petroleum hydrocarbons (gasoline-0.5% v/w) as compared with the control. The results clearly show that this method is very useful for counting viable bacteria in marine microcosms supplemented with gasoline.

Key-words: direct viable count, fluorochromes, microcosms, gasoline, software digital imaging

INTRODUCTION

Direct viable count has been used as an indicator of the general metabolic activity of bacteria in different media, including in marine environment (Kogure et al., 1979; Barcina et al., 1995; Joux & LeBaron, 1997; 2000; Bakermans & Madsen, 2000; Baudart et al., 2002; Wu et al., 2009) but it has not yet coupled with automated image analysis. In the last decades there is an increase in the development and use of different softwares for automated analysis of the digital images thus obtained (Ishii et al., 1987; Estep & Macintyre, 1989; Walsby, 1996; Congestri et al., 2003; Embleton et al., 2003; Selinummi et al., 2005, 2008; Ardelean et al., 2009a), automated quantification and sizing of single cells by microscopy and image analysis are routinely used to determine microbial biomass (Zeder et al., 2010). The automated approach will not only remove the need for tedious manual analysis work, but also enable biologists to measure cellular features not feasible by the standard manual techniques (Wang et al., 2007; Selinummi, 2008).

The aim of this paper is to use the original direct viable counts method (Kogure et al., 1979) to count cells that are capable of division in the presence of gasoline, and to couple this method with automated image analysis.

MATERIALS AND METHODS

Samples and microcosms were as follows: we collected water samples in volume 300 mL of natural sea water from the Black Sea (0.5m depth; Tomis seaport, 44°10'44''N; 28°39'32''E) in sterile Polyethylene transparent bottles, natural sample- control (M3); control supplemented with petroleum hydrocarbons (gasoline-0.5% v/w) (M2) and iii) control supplemented with petroleum hydrocarbons (gasoline-0.5% v/w) and nutrients (ammonium nitrate 0.005% w/w) (M1). Variables where with respect to: i) nature and concentration of nutrients; ii) temperature of incubation (18ºC- fluorescent light); iii) the use of filtered (Millipore membranes 0.45 µm) seawater, in order to eliminate bacteriovorus microorganisms (Ghita & Ardelean, 2010). At different interval of time, samples were collected in sterile Falcon tubes, fixed with buffered formaline (2% final concentration) and kept at 4°C in darkness without any added fluorochrome. This fixation has been made to be measured other parameters (Ardelean et al., 2009b).

In parallel, for preliminary experiments, one have collected in the same locations mentioned above fresh seawater sample (S1), which was supplemented with yeast extract to 50 mg / L final concentration (S2), or with yeast extract 50 mg / L and nalidixic acid (20mg/L), and incubated 6 hours at 30°C in the dark .

Direct viable counts. The method of direct enumeration of viable cells is based on incubation of natural samples in the presence of an antibiotic (nalidixic acid as specific inhibitor of DNA synthesis, without affecting other metabolic processes) and nutrients (yeast extract). Active cells may continue to metabolize nutrients and thus become more elongated after incubation period. Sterile flasks containing seawater samples (40mL) were kept at a constant temperature (30°C) incubated in dark and continuous stirring. Samples from each microcosms were supplemented with yeast extract (50 mg / L final concentration), nalidixic acid (20 mg / L final concentration) (Kogure et al., 1979) and gasoline (0.5% final concentration). Subsequently samples were harvested each two hours (considering the time T_o, T_1 –after 2 hours, T_2- after 4 hours; T_3- after 6 hours, T_4- after 8 hours). Then samples were stained with acridine orange and visualized by epifluorescence microscopy. Total cell counting and DVC were done tacking into account 15 fields per sample and 120 fields per sample, respectively.

Acridine Orange and Propidiu Iodide direct counts. Aliquots with volume of 5 ml were stained with 10 µl Acridine Orange (0.1mg/mL final concentration) and filtered on Isopore polycarbonate membrane filters (0.2 µm pore size, black) and washed with 10 ml distilled water (Robertson & Button, 1989; Jansson & Prosser, 1997; Kawai et al. 1999; Marie et al., 1999). Staining time was 5 minutes. PI

staining was performed as previously shown (Ardelean et al., 2009 b; Ghita & Ardelean, 2010).

Epifluorescence microscopy. Samples were viewed immediately by epifluorescence microscopy (N-400FL, lamp Hg 100 W, type on the blue filter-450-480 nm) with immersion 100X objective and 10X eyepieces. Cell enumeration was done manually (Fry, 1990; Sherr et al., 2001), 400-600 cells being counted on each filter, and this number of cells was converted to cells/mL following classic equation (Fry, 1990; Sherr et al., 2001), using a calibrated square eye piece (surface 0.01mm^2), as previously shown (Ardelean et al.,2009a; Ghita & Ardelean, 2010).

The automatic cell analysis was performed using the CellC software, the main software used for multiple digital microscope images analysis to count cells (http://www.cs.tut.fi/sgn/csb/cellc/). ImageJ software was applied to digital images of whole cells color-stained bacteria, used to display, edit, analyze, process, save and print 8–bit, 16–bit and 32–bit epifluorescence digital images, many image formats including TIFF, JPEG, BMP, supporting 'stacks'and hyperstacks, a series of images that share a single window; also we used this software for measure the length of cells and pixel value statistics of user-defined selections, creating density histograms and line profile plots, supports standard image processing functions such as contrast manipulation, sharpening, smoothing, edge detection and median filtering. Because the digital images are two-dimensional grids of pixel intensities values with the width and height of the image being defined by the number of pixels in *x* (rows) and *y* (columns) direction, that's way the pixels are the smallest single components of images, holding numeric values – pixel intensities – that range between black and white (ImageJ user guide). Microphotographs used in this study was RGB images, RGB/HSB stacks, and composite images.

CellC software proceeds few important steps: the background is separated from the objects based on the intra-class variance threshold method; noise and specks of staining color in the image can affect the reliability of the analysis, so those was removed. The removal was done applying mathematical morphology operations to the image; then separation of clustered objects was performed (Selinummi, 2008). The CellC software was used for cell enumeration and measurements of cell's properties (size, shape, intensity). We applied the algorithms of CellC software for digital images, because this have three important parts: a MATLAB figure file of the segmented image (this can be exported in any common image file format; a comma separated value (CSV) - file with quantitative data of the cells (was opened in a spreadsheet program Excel for further analysis); a summary CSV-file with the cell count for each of the analyzed images for a quick overview of the analysis process (this file were only saved in the batch processing mode). Fluorescence microscopy digital images were analyzed and the objects has different intensity than the background.

RESULTS AND DISCUSSION

Preliminary experiments showed that the incubation of natural marine samples in the presence of yeast extract and nalidixic acid induces the formation of elongated cells (S3) but the elongation (the increase in length at least 2 times) is not seen nor in control(S1- any addition) neither in yeast extract supplemented sample (S2).

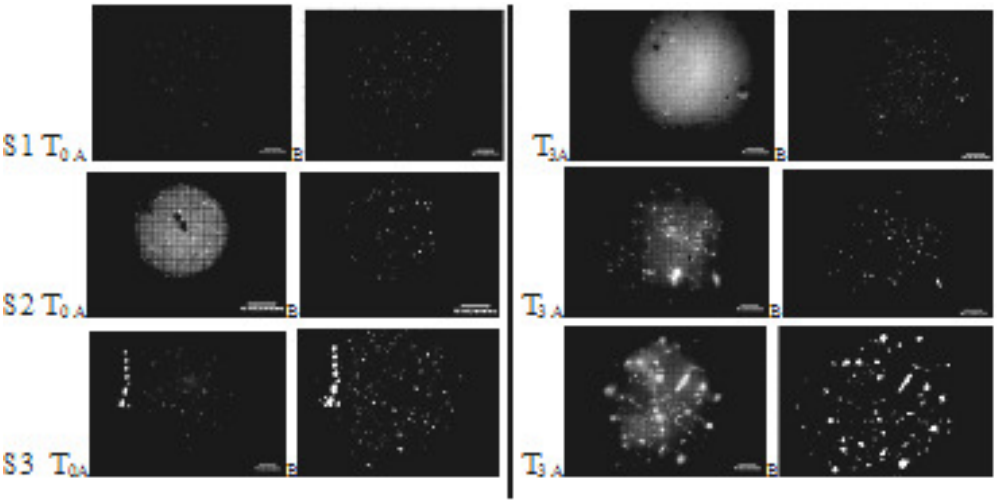

Fig1. In natural marine samples cells elongation occurs only in the presence of both yeast extract and nalidixic acid (S3).A – original digital images analyzed with ImageJ software; B – Panel of original images used to count cells with CellC software.

In the following experiments, nalidixic acid has been added to the three microcosms in order to induce the elongation of those bacterial cells that are active metabolic and able of cellular growth and division. In figure 2 is shown the time evolution of medium cell size in the three microcosms, using Image J and CellC program for measuring cell's length (μm).

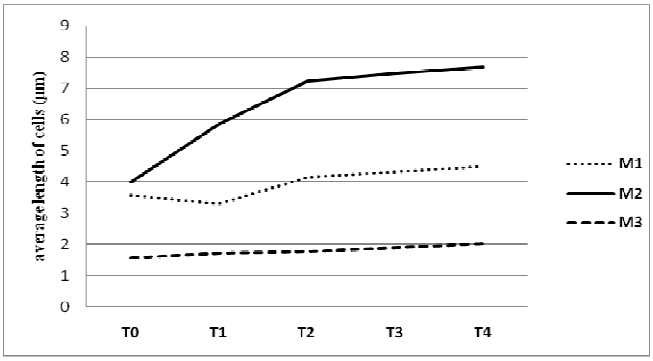

Fig 2. Average length of cells from T_0 to T_4 (during 8 hours of incubation with nalidixic acid and yeast extract) in the three microcosms

As one can see in figure 3 at the start of the incubation period (in the presence of nalidixic acid and yeast extract) there is a difference in the average length of cells in M2 (sea water and gasoline) or M1 (sea water, gasoline and ammonium acetate) as compared with the control (sea water). The average cell length in control (M3) is lower than in M2 or M1, probably because of the lack of extra nutrients. In M3 (control) one can see an almost constant length of cells during incubation (1.79± 0.17) whereas in M2 there was a sudden increase in cell length at time T_2 (4 hours incubation). However, after 8 hours of incubations, the average length of cells in microcosms is about 4µm (M1) and 7 µm (M2), compared with the M3 where the cells were maintained in high proportion in the form of cocci (average length of about 1.7 µm). So far, it is not clear why the cells from M2 reach a greater length (7 µm) as compared with those from M1 (4µm), a result that deserves further attention to understand its signification.

The average cell length was obtained by measuring the length of all cells in the 10-40 microscopic fields, including cells which didn't really increase their length during incubation period.

In order to count the number and to show the increase in cell length of those cells which really doubled (or more) their cellular size during incubation period (8 hours) all the measurements done on each microcosms at each sampling time were ordered in Excel, from the smallest to the greatest cell length. Using the performance of Excel, at this stage of the work was counted only those cells which really increase as compared with the average length at the beginning of the incubation period (fig. 3). The length of these selected cells was taken into account to calculate the average length of only growing cells (see fig. 4).

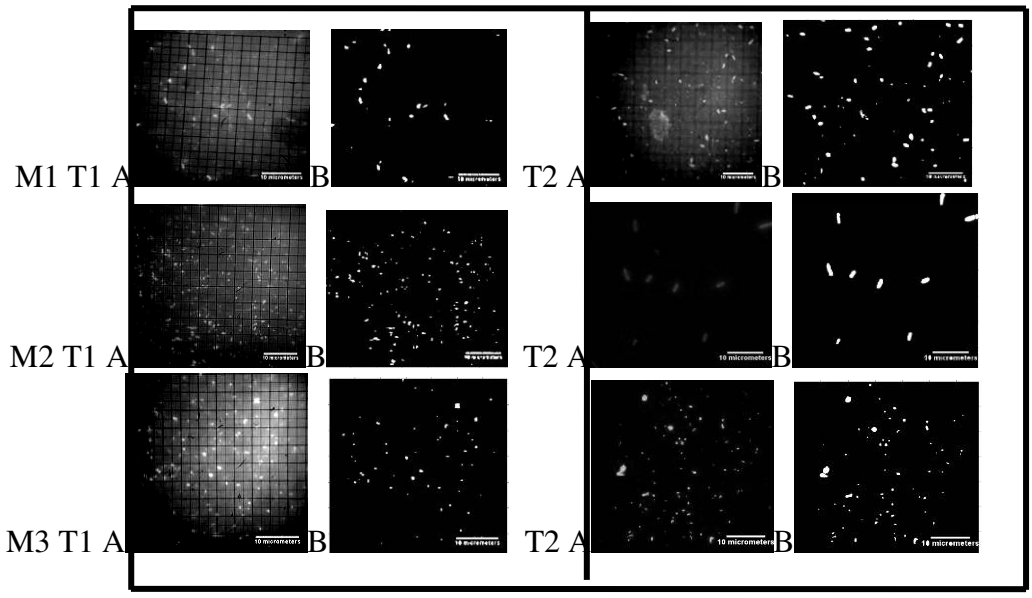

Fig3. Cell elongation after T1 (two hours) and T2 (four hours) from the start of the experiment, cells were stained with acridine orange and viewed with epifluorescence microscopy (x1000); A - original microphotographs; B – total count of bacteria from original microphotographs using CellC software.

In figure 3A there are microphotographs of bacteria from M1, M2, M3 and in figure 3 digital analysis of pannel A using CellC software. We adjust contrast/brightness of digital image with ImageJ software, then analyse measure of graticula and set the calibration bar to determine correctly the length of each bacteria. Image processing methods used guarantee that all images are analyzed using the same criteria, and therefore results between different images are comparable. Images for analysis were done with a Sony DSC-P200, 7.2 megapixels digital camera). Brightness and contrast were adjusted for the first image and kept unchanged throughout the image acquisition procedure.
The images (1600 by 1200 pixels, 256 dpi) were acquired at 50x magnification and stored as 543-KB JPG files. Additional images acquired at 100x magnification were used to verify that measurements of individual cells.
Because CellC software included graphical user interface, and the batch processing mode enables fast and convenient processing of hundreds of cell images, we easilly enumerate bright cells on a dark background (epifluorescence). We also used two

different methods to process the images: one image/image pair at a time; several images pairs sequentially in batch processing mode.

If the background of the image is uneven (because of misaligned lighting), it was preferable to choose this option. The default option in CellC is to present the measured parameters in pixels. By checking this box we defined how many micrometers one pixel corresponds to, and receive all measurement results in micrometers. The correct value of this setting obviously depends on the imaging setup, such as on the camera and the objective, and must be determined outside CellC, using ImageJ to calibrate the scale.

Automatic removal of over/undersized cells were selected, because CellC automatically decides which particles are too small to be considered as real cells. All detected objects that are smaller than 1/10 of the mean size of all objects, were removed. Because the sizes of under/oversized particles were known using "Analyze Measure" option of ImageJ, it was possible to set the thresholds manually by using the text boxes. The unit of size depends on the user defined unit (pixels/μm^2).

In Figure 4 one can see the number of cells in elongation in the three microcosms, all supplemented with yeast extract and nalidixic acid.

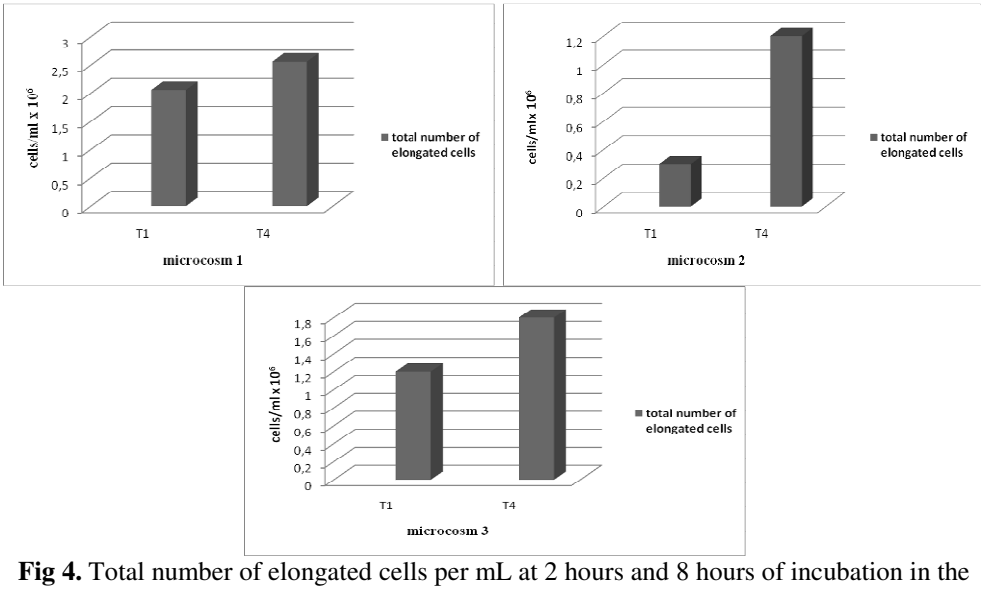

Fig 4. Total number of elongated cells per mL at 2 hours and 8 hours of incubation in the presence of yeast extract and nalidixic acid.

As one can see in figure 4, the highest active cell densities were found in M1 and the lowest in M3 (control) probably because of the extra nutrients in M1 (gasoline and ammonium acetate); however, the fastest increase in the number of active cells was found in M2 ($0.3x10^6$ cells/mL at 2 hours and $1.2x10^6$ cells/mL at 8 hours of incubation).

Taking into account only the cells which increased their length (fig.4) to calculate the average cell length of these cells, the following values have been obtained: 5.82±1.20 for M1; 7.71±0.54 for M2 and 3.15±0.86 for M3. As expected, these values are larger than average values obtained for all cells, both growing and non growing (fig.2).

CONCLUSIONS

In this paper the original direct viable counts method (Kogure et al., 1978) is used to count cells that are capable of division in the presence of gasoline, and for the first time, up to our best knowledge, the microscopic images obtained with this method were treated by special software (Image J and Cell C) for automated image analysis which increases the accuracy of the measurements.

The presented methods including image analysis systems were performed for counting and estimating the length of bacteria, but does not totally exclude the need for double blind validation of both manual microscope analyses and automated image analysis.

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STUDIES ON IMMOBILIZATION OF CELLULASES BY SORPTION AND ENTRAPMENT ON SOLID SUBSTRATES AND PERMSELECTIVE MEMBRANES

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ABSTRACT

Cellulases, due to their capacity to depolymerise cellulose into fermentable sugars are essential components in the production of bioethanol from lignocellulosic materials. Developing of new and efficient techniques of immobilization leads to superior biocatalysts with increased performances.

Immobilization experiments were performed using a commercial cellulase complex and Amberlite polymeric resins XAD-7, XAD-8, chitosan and glutaraldehyde. Determination of cellulolytic activity and protein content were performed spectrofotometrically.

Immobilization yields of 90 percent were obtained when the ionic strength of the enzymatic solution was of 10-25 mM and the concentration of cellulases in the solution was 5%, corresponding to 0.2 g of enzyme per g of adsorbent. Maximum irreversible immobilization yield by reticulation of the enzyme with glutaraldehyde was 75 percent and was obtained using a glutaraldehyde/protein ratio of 2.5/1. In case of chitosan entrapment, experiments showed that the maximum immobilization yield of 60 percent was obtained at 1/0.053 chitosan/glutardehyde ratio and 12.5/1 chitosan/protein ratio.

INTRODUCTION

Cellulases, due to their capacity to depolymerise cellulose into fermentable sugars are essential components for the production of bioethanol from lignocellulosic materials. Developing of new and efficient techniques of immobilization leads to superior biocatalysts with increased performances.

Objectives

The presented study had two main objectives: first the immobilization of cellulases by adsorption on solid substrates combined with reticulation and second, the immobilization of cellulases by entrapment in natural polymer matrices.

MATERIALS AND METHODS

Experiments were performed using a commercial cellulase complex and Amberlite polymeric resins XAD-7, XAD-8, chitosan and glutaraldehyde as immobilization supports.

Determination of cellulolytic activity and protein content were performed spectrofotometrically (λ=540 nm).

RESULTS

Immobilization yields of 90% were obtained when the ionic strength of the enzymatic solution was of 10-25 mM and the concentration of cellulases in the solution was 5%, corresponding to 0.2 g of enzyme per g of adsorbent.

Table 1. Adsorption of cellulases on synthetic supports

Initial concentration of protein (%)	Protein/support ratio	Loading degree*		Adsorption yield (%)	
		XAD-7	XAD-8	XAD-7	XAD-8
1	1 : 10	0,090	0,075	90	92
2	1 : 8	0,100	0,090	85	90
3	1 : 5	0,170	0,165	83	83
4	1 : 4	0,200	0,180	80	84
5	1 : 3,5	0,225	0,200	79	80
5	1 : 2	0,225	0,200	„	„

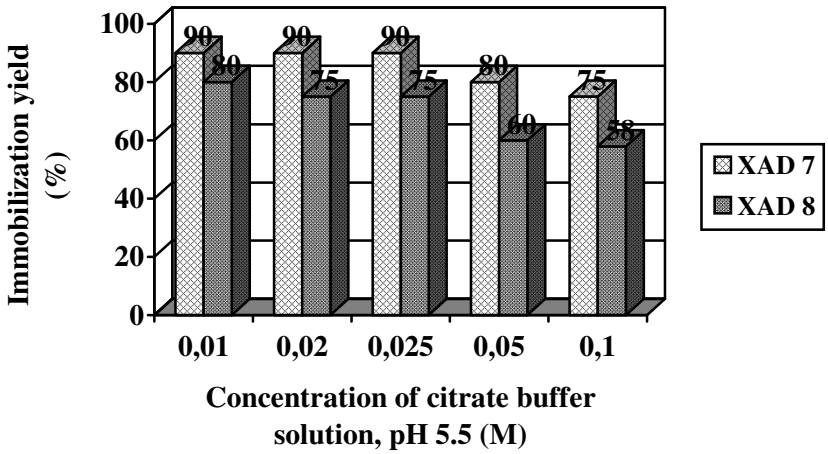

Fig 1. Influence of ionic strength of the buffer solution on immobilization yields

Maximum irreversible immobilization yield by reticulation of the enzyme with glutaraldehyde was 75% and was obtained using a glutaraldehyde/protein ratio of 2.5/1.

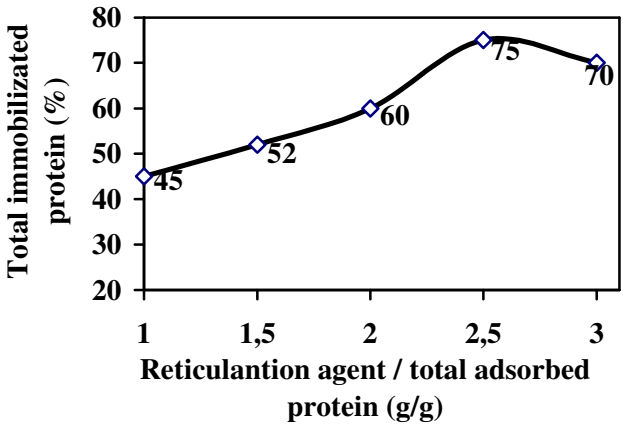

Fig 2. Influence of glutaraldehyde/total adsorbed protein ratio on immobilization yields

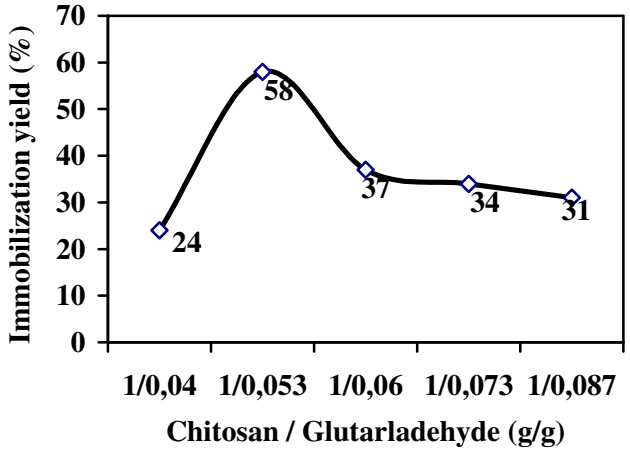

Fig 3. Influence of the chitosan/glutaraldehyde ratio on the immobilization yields

In case of chitosan entrapment, experiments showed that the maximum immobilization yield of 60% was obtained at 1/0.053 chitosan/glutardehyde ratio and 12.5/1 chitosan/protein ratio.

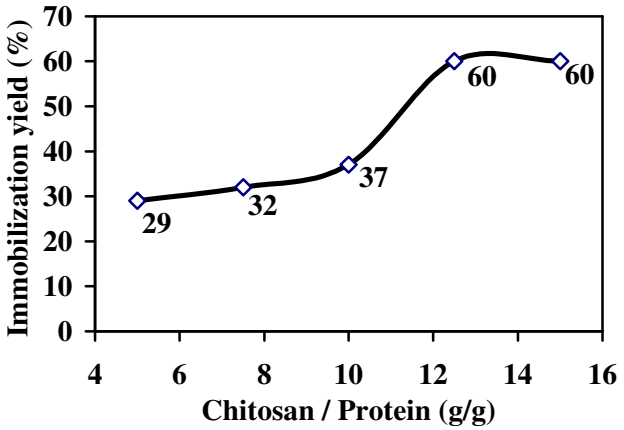

Fig 4. Influence of the chitosan/protein ratio on the immobilization yields

CONCLUSIONS

- Maximum immobilization yields of 90% were obtained by adsorption;
- In case of irreversible immobilization by reticulation with glutaraldehyde, the maximum immobilization yield was 75%
- Maximum immobilization yield by entrapment was 60%
- Experiments pointed out the positive effect of reticulation with glutaraldehyde and the efficiency of cellulases immobilization by adsorption on Amberlite polymeric resins and by chitosan entrapment

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PHYTOREMEDIATION OF LEAD CONTAMINATED SOILS

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Key words: Morus sp., phytoremediation, lead

ABSTRACT

In the last decade, several researches have shown that in soils contaminated with heavy metals may reduce the content of chemical elements through phytoremediation.

Basic and applied research has demonstrated unequivocally that certain plant species have genetic potential to remove, degrade, metabolize, or immobilize a wide range of contaminants.

Hyper-accumulator plants, which have ability to extract high concentrations of metals in the soil in their tissues while retaining metabolic functions, are considered primary candidates for phytoremediation process.

Despite regulatory measures adopted in many countries to limit contamination by Pb in the environment, it continues to be one of the most serious human and environmental hazards. Considering the fact that many Pb pollutants are essential to modern human life, soil contamination with Pb seems to fall in the near future.

The article describes several models of plantation wood species belonging to the genus Morus. The phytoremediation process, metals are extracted from soil by plants of the genus Morus having a high potential in this respect (plant extract heavy metals from soil and remedied biomass's quality of the soil).

INTRODUCTION

Long-term application of fertilizers, sewage, pesticides and commercial waste tailings containing heavy metals, resulted in surface contamination of agricultural soils over the allowable limits, or on small areas or large surfaces. Techniques are currently available physico-chemical ex-situ extraction, usually expensive. Moreover, they destroy the soil structure and leave it biologically inactive.

The term phytoremediation is derived from the Greek word "phyton" - plant and from the Latin word "remediare" - to address and describe the system by which certain plants, interacting with soil organisms, can transform contaminants into harmless forms and sometimes valuable. This method is often used to improve land contaminated with heavy metals and toxic organic compounds.

Extraction of heavy metals accumulation in plants (phyto-extraction) was suggested as a strategy of in-situ remediation, mild to surface soils. Hyper-accumulator plants are able to accumulate exceptionally high concentrations of metals in biomass without symptoms of phyto-toxicity.

However, such hyper-accumulator plants have typically slow growing and produce only small amounts of biomass, leading to a period of years the need for decontamination of polluted sites. As an alternative, trees have been proposed due

to extensive root system, high rates of sweating, rapid growth and large biomass production.

Phyto-remediation efficiency is directly dependent on the system characteristics of plant growth, biomass size, ease of harvest, and tolerance / accumulation of large amounts of heavy metals in roots, stems or fruit.

Literature studies have led to the recognition of plant roots Morus sp. as essential components of ecosystem productivity and stability, being able to synthesize a remarkable diversity of secondary metabolites and to adjust their metabolic activities in response to abiotic stress factors.

An important characteristic of ecosystems is the morus tree biomass production and longevity than their considerable (50-100 years), compared with annual or biennial plant ecosystems of spontaneous and cultivated flora present in the same biotype.

Perennial nature of the morus tree in time lead to the formation of an environment and ecosystem in which plants are influencing each other.

Romania is situated in an area favorable for morus culture; it is cultivated in all areas except the alpine and coniferous forest area.

MATERIALS AND METHODS

Getting morus seedling by cuttings

Working protocol for obtaining the morus bush provides the following steps:

- Branches for cuttings are harvested in February and stratify in moist sand at a temperature of 4-5 ° C.

- Rooting substrate preparation is done on a level ground with good soil structure and fertility, which is run by fall plowing at 30-40 cm depth, preceded by fertilizing with manure in the dose of 20 t / ha. With about 30 days before planting bush are mobilizing soil depth of 20 cm and 1.25 m wide swaths picketing long and 25-50 m; soil mix with manure mobilized at a dose of 30 t / ha and carbonate calcium in quantity of 50 kg / ha. Furrows are covered with white polyethylene film very tight and fixed with earth on the sides, in order to achieve and maintain optimum soil temperature and humidity.

- Planting bush - the bush is best to plant when the temperature of the substrate so prepared is maintained constant at about 20°C for 5-6 consecutive days; in Romania, this period coincides with the beginning of May. Branches are removed before planting the seedlings stratification and cut a length of 15-20 cm (3-4 buds), is covered with paraffin at the top and introduced in solution stimulators (IAA, IBA, NAA). Seedlings are planted only on the bottom and middle branches, 10-15 mm thick to provide nutrients that allow the vegetation and settling. Before planting, foil Drill distance of 25 cm between seedlings, cuttings

are placed through holes in the ground, at a depth of 7-8 cm, leaving the outside of a bud.

In this article, we present three models of planting a slope-planting pattern, planting pattern of rectangular or square shape, pattern of planting in an irregular triangle

1) Model in slope planting

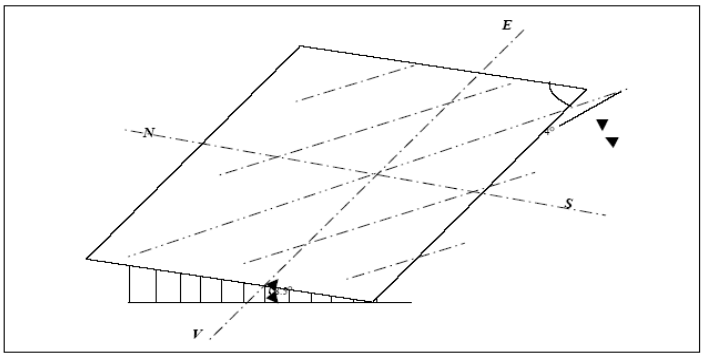

Fig.1: Model in slope planting

It was chosen to illustrate a sloping plateau of 15% which is equivalent to a 8.5° tilt. Directing EV eliminate the difficulties created by the alternation of slope-ramp, but require the car to work with an inclination of 8.5° from the horizontal which induces a significant shift to the south and center of gravity can overturn the machine.

2) Planting pattern of rectangular or square shape

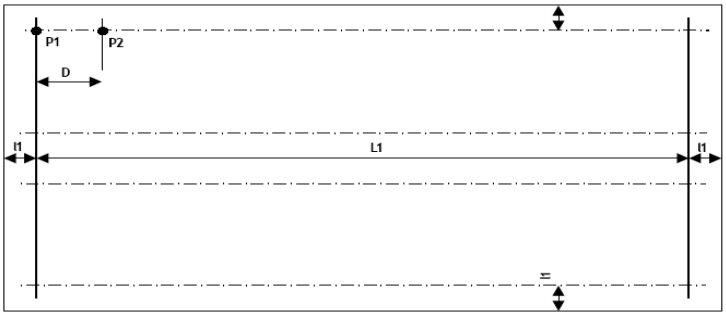

Fig.2: Planting pattern of rectangular or square shape

It sets the size (size valid for all three models), and the land will figure drawing point P1 is the location of the first picket. Land length (L) minus two distances l1 is the length available for the first row of trees.

L-2l1 =L1

Let D imposed distance between trees, and the number of trees per row will be n+1

L1 : D=n

3) Model for planting in an irregular triangle

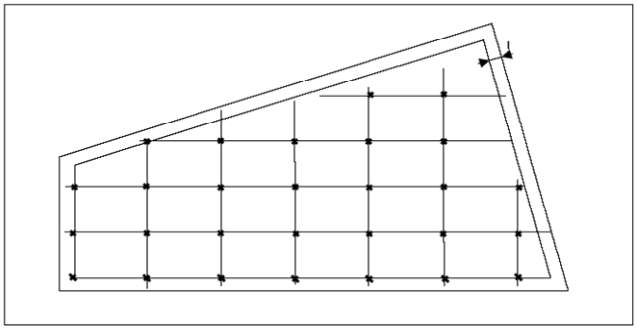

Fig.3: Model for planting in an irregular triangle

Operation refers to the definition of free bandwidth on the perimeter lot and will choose one of the directional long sides of the quadrilateral. First and these will be parallel with that, and as the lines approach the other side of the quadrangle longer possible for them to be shortened to a single tree.

After planting, soil moisture is made to ensure regular watering in foil, when shoots were 10-12 cm long, polyethylene film is removed from the layers.

Fig.4: Planting bush - the dry cuttings

Maintenance work throughout the vegetation-run work of destruction of weeds by manual method for loosening soil and removing weeds and irrigation, track.

Fertilization with chemical fertilizer dose of N-60, P-60, K-60 kg / ha is done after removing the polythene and after 30 days longer administers an amount of nitrogen of 60 kg / ha

Prevention of bacterial (Pseudomonas mori) and brown staining (Mycosphaerella mori) is achieved by combined treatment with Topsin - and Ridomil 0.2% - 0.2% (3 treatments every 10-12 days).

In this system of rooting substrate preparation, it is obtain a maximum rooting of 93 - 95%.

To highlight phyto-remediation testing is done ex situ which involves the use of plant growth substrate morus obtained by scraping soil from areas contaminated with lead.

All test patterns phyto-remediation capacity of Morus species variants include variants uncontaminated and contaminated with lead:

V0 - uncontaminated control consisting of a solid growth substrate land consists of celery, manure and sand in a ratio of 1:1:1, sterilized by autoclaving at 120oC for 60 min and rehydrated with distilled water.

V1-experimental version consists of solid substrate growth area consists of soil contaminated with lead overburden

Throughout the unfolding experiment will provide environmental conditions, temperature, light and moisture in accordance with the requirements of the morus plant.

RESULTS AND DISCUSSION

Lead leaches into the soil to air, water and mud and soil from the surface down through the processes of diffusion, adsorption, leaching and water stripping, or macroorganisms. In soil, microorganisms are soluble, or turn them in suspension in water reaching the plant roots.

As a result of pollution and high load of soil with heavy metals and of enhancing their mobility in soil, plants absorb large amounts of these elements from the soil particles plus deposits with the pollutants existing in air, leaves, buds, etc.

Once inside the ground, lead is very difficult to remove. Metal resist layer of 0-15 cm from the surface, which is strongly linked through processes of adsorption, ion exchange, precipitation and complexation with organic matter absorbed.

Pb reaches the entire cortex and accumulates near the endoderm. To reach the root xylem, lead must go through endoderm and Casparian bands, which is very difficult. Consequently, the highest uptake of metals is performed by the young root, where the Casparian bands are not yet fully developed.

Studies done on plants have shown that the roots have the ability to take significant amounts of Pb and its translocation to other parts of the plant takes place through root. The way in which Pb enters the plant through the leaves depends on their ability to absorb Pb from air sources, capacity, in turn, depends on leaf morphology. Fuzz on the leaves absorb heavy metals in the atmosphere. However, most of the Pb taken up by plants remains in the roots.

The leaves have different abilities in terms of their ability to accumulate Pb, depending on age. Maximum Pb content is found in mature leaves, in young leaves is finding a small amount.

Pb content in different organs of plants tends to decline in the following order: roots> leaves> stem> inflorescence> seeds.

Ultrastructural studies have shown that a variable amount of lead is present especially in the intercellular space, cell wall and vacuoles, while small deposits of lead are present in the endoplasmic reticulum. Cell walls and vacuoles contain, together, about 96% of the amount of Pb absorbed. A small amount of Pb reaching the nucleus, chloroplasts and mitochondria and exert toxic effects on these organelles.

All interactions that occur in the matrix are dependent on soil pH. Soil pH has a significant effect on the mobility of lead and other metals in the soil. Soil pH generally varies from 4.0 to 8.5. In acidic conditions (pH <5.5), metal cations are more mobile, while anions tend to be absorbed on mineral surfaces. Under these conditions, metals are much more available to plant roots.

A major limiting factor for potential phyto-extraction of lead is the low bioavailability of the metal consumption by the plant. To overcome this limitation, you may need to add synthetic chelating chemical contaminated soil to increase the amount of bioavailability lead plant. Using synthetic chelator in phyto-remediation process is not only to increase the consumption of heavy metals by plants by increasing metal bioavailability, but also to increase the availability of micronutrients, which decreases the possibility of deficiencies of nutrients for the plant.

Were tested for lead different chelators EDTA, CDTA (trans-1 ,2-ciclohexilen-dinitrilo-acetic acid), DTPA (dietilentrinitrilo-pentaacetic acid), EGTA (etilbis [oxzetilentrinitrilo]-acetic acid), HEDTA (hydroxyethyl-ethylene-dinitrilo-triacetate acid), citric acid and malic acid.

Adding chelates resulted in high levels of lead in plants. EDTA proved to be most effective. In soils with pH 5, adjusted with EDTA, the plants accumulated a larger amount of Pb around 2000 mg/kg, compared with other treatments.

In order to enable a substantial accumulation of lead in plants (> 5000 mg/kg), the concentration of synthetic chelates (EDTA, DTPA, CDTA) must exceed 1 mmol / kg.

Water is also an important factor in the process of phyto-remediation. Increasing soil water content removes oxygen from the soil pores, favoring the reduction reaction. Strongly reducing conditions are favored precipitation of lead sulphide, shrinking their accessibility and mobility in the soil for plants.

CONCLUSIONS

- As a result of pollution and high load of soil with heavy metals and of enhancing their mobility in soil, plants absorb large amounts of these elements from the soil particles plus deposits with the pollutants existing in air, leaves, buds, etc.

- The leaves have different abilities in terms of their ability to accumulate Pb, depending on age. Maximum Pb content is found in mature leaves, in young leaves is finding a small amount.
- Pb content in different organs of plants tends to decline in the following order: roots> leaves> stem> inflorescence> seeds.
- The phytoremediation of soils contaminated with lead is realized through the use of the phytoremediation potential of woody plants from Morus species.
- To highlight the processes of absorption, accumulation and translocation of lead contaminants there have been developed, in experimental conditions, three models of planting: a slope planting pattern, a planting pattern of rectangular or square shape, a planting pattern in an irregular triangle.
- There have been established the proportions between rows of plantation sizes, as well as distances between the planted trees.
- Throughout the unfolding experiment will provide environmental conditions, temperature, light and moisture in accordance with the requirements of the morus plant.
- All test patterns phyto-remediation capacity of Morus species variants include variants uncontaminated and contaminated with lead.

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XYLANASE PRODUCTION ON GLYCEROL - WHEAT BRAN SUBSTRATE

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The present study aimed the xylanase production by growing the microorganism Bacillus brevis ICCF 500 using glycerol as a co-substrate with wheat bran.

Biodiesel have drawn attention in the last decade as a renewable, biodegradable, and non-toxic fuel. Raw glycerol, byproduct from bio-diesel production process, is used as carbon substrate in several biotechnological applications.Using crude glycerol as a carbon source for fermentation is a promising alternative use for this waste material.

The agricultural wastes, as wheat bran, are energy sources wich can be recovered by enzymatic strategies.

Xylanases have made a remarkable impact in the world of biotechnology because of their applications in the pulp and paper, feed, food and fermentation industries. The most promising application of xylanases is pre-bleaching of kraft pulp.

The experiments were carried on , at laboratory scale, taking into account media optimization in order to obtain the highest enzimatic activity.

Key-words: xylanase, glycerol, wheat bran

INTRODUCTION

Microbial enzymes are more advantageous than enzymes derived from plants or animals because of their great variety of catalytic activities, possible high yields, stability, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations, rapid growth of microorganisms in inexpensive media, more convenient and safer protection methods.

Xylanases (Endo-1,4-Beta-xylanase or XYN, EC 3.2.1.8) are glycosidases which catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in the backbone of complex plant xylan polysaccharides [2].

Plant cell walls are comprised of cellulose, hemicellulose and other polymers that are intertwined.This complex structure acts as a barrier to degradation by a single enzyme.Thus, a cocktail consisting of bi and multifunctional xylanases and xylan debranching enzymes is most desired combination for the efcient utilization of these complex materials.

First reported in 1955, they were originally termed pentosanases, and were recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 when they were assigned the enzyme code EC 3.2.1.8. Their official name is endo-1,4-b-xylanase, but commonly used synonymous terms

include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β –xylanase [2].

Xylanases have been extensively studied and could potentially be employed in the production of hydrolysates from agro-industrial wastes, nutritional improvements of lignocellulosic feeds, processing of food [1]and increasing animal feed digestibility, biobleaching of paper pulp [8]. Microbial xylanases enhanced dough rheological properties as increase in loaf volume that improves its baking performance and so have great importance in cereal industry [5]. Other potential applications include the clarification of fruit juices and wine, the extraction of plant oil, coffee and starch, the production of oligosaccharides and improvement of the nutritional value of feed [4].

Biodiesel have drawn attention in the last decade as a renewable, biodegradable, and non-toxic fuel. Raw glycerol can become an important feedstock when biodiesel is applied on a large commercial scale.With the production of 10 kg of biodiesel from rapeseed oil, 1 kg of glycerol becomes available. Few microorganisms can be used for direct glycerol biovalorization.

MATERIALS AND METHODS

In this study, Taguchi method [6] was applied to test the relative importance of medium components (glycerol, wheat bran, corn steep liquor, ammonium sulfate) in xylanase production by *Bacillus brevis*.

We considered Taguchi methodology as a good positive option for the optimization of biotechnological processes of microbial enzymes production . The experimental design proposed by Taguchi involves using orthogonal arrays to organize the parameters affecting the process and the levels at which they should be varies. Instead of having to test all possible combinations like the factorial design, the Taguchi method tests pairs of combinations [6,3,7]. This allows the collection of the necessary data to determine which factors most affect process performance with a minimum amount of experimentation, thus saving time and resources.

Table 1. Medium components and levels

FACTORS	LEVELS %		
	Level I	Level II	Level III
• A. Glycerol	1	1,5	2
• B. Wheat bran	3	5	7
• C. Corn steep liquor	0,5	1	2
• D. Ammonium sulfate	0,2	0,3	0,4

Selection of medium components and their levels are made on the basis of some preliminary trial experiments conducted in the laboratory.

Four medium components such as glycerol, wheat bran, corn steep liquor and ammonium sulfate are selected for the study. Each of the four components was treated at three levels, as shown in Table 1. Using orthogonal array design the effects of multiple process variables on the performance characteristic can be estimated simultaneously while minimizing the number of test runs. An L_9 (3^4) standard orthogonal array as shown in Table 2 was employed for the present investigation. This array is most suitable to provide the minimum degrees of freedom as 9 [= 1 + 4 x (3–1)] required for the experimental exploration [6].

Tabel 2. L_9 (3^4) Standard orthogonal array

Experiment no.	Factor A	Factor B	Factor C	Factor D
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

Table 3 represents the layout of the experimental design, which has been obtained by assigning the selected factors and their levels to appropriate columns of L_9 orthogonal array. This array has 9 rows and 4 columns and each row represents a trial condition while each column accommodates a specific process parameter.

Table 3. Layout of experimental design (3^4)

Experiment no.	Factor A	Factor B	Factor C	Factor D
1	1	3	0,5	0,2
2	1	5	1	0,3
3	1	7	2	0,4
4	1,5	3	1	0,4
5	1,5	5	2	0,4
6	1,5	7	0,5	0,3
7	2	3	2	0,3
8	2	5	0,5	0,4
9	2	7	1	0,2

The variants in Table 3 are to be completed with the rest of the ingredients in unmodified concentration, as follows:$MgSO_4$ – 0,05%, KH_2PO_4 – 0,1%

In Taguchi technique, the variation of the response is also examined using an appropriately chosen S/N ratio. Broadly speaking, the S/N ratio is the ratio of the mean (signal) to the standard deviation .The standard S/N ratio computing formula for this type of response is [6]:

$$\left(\frac{S}{N}\right)_i = -10\log\left[\frac{1}{n}\sum_{j=1}^{n}\frac{1}{Y_{ij}^2}\right]$$

where 'i' is the number of a trial; 'Yij' is the measured value of quality characteristic for the ith trial and jth experiment; 'n' is the number of repetitions for the experimental combination.

RESULTS AND DISCUSSION

The control of the optimization scheme was made during 3 experiments which proved to be reproducible.

Tabel 4. Results presenting enzymatic activity obtained after 3 experimental assays

Experiment no.	Enzymatic Activity (UI/ml)			Mean	S/N ratio
	Assay 1	Assay 2	Assay 3		
1	72,8	70,1	71,4	71,4	37,099
2	86,3	90,7	91,4	89,4	37,521
3	213,1	206,8	210,7	210,2	46,459
4	81,2	79,4	78,5	79,7	38,042
5	122,5	130,1	128,4	127	42,072
6	180,5	174,5	179,2	178,06	45,017
7	86,3	87,8	88,5	87,5	38,828
8	109,1	126,2	120,4	118,5	41,439
9	170,6	168,5	171,8	170,3	44,635

A

(S/ N) A1 = 40,359 A1 - 123,66

(S/ N) A2 = 41,710 A2 – 128,25

(S/ N) A3= 41,634 A3 – 125,43

Optim level 2

B

(S/ N) B1 = 37,989 B1 – 79,533

(S/ N) B2 = 40,344 B2 – 111,63

(S/ N) B3 = 45,370 B3 – 186,18

Optim level 3

C	
(S/ N) C1 = 41,185	C1 – 122,65
(S/ N) C2 = 40,635	C2 – 113,13
(S/ N) C3 = 42,453	C3 – 141,56
Optim level 3	
D	
(S/ N) D1 = 40,867	D1 – 120,85
(S/ N) D2 = 40,455	D2 – 118,32
(S/ N) D3 = 42,003	D3 – 133,85
Optim level 3	

The average values of S/N ratios of the four control factors at each of the levels are shown above, and from which the levels corresponding to the highest S/N ratio values were chosen for each parameter representing the optimum condition. It is clear that the optimum levels are: A2, B3, C3, D3.

In addition to S/N analysis, main effects of the process parameters on the mean response are also analyzed. The mean response refers to the average value of the quality characteristic for each factor at different levels. Thus the average values of the enzymatic activity for each factor at the three levels have been calculated. The mean response analysis also indicates the same optimum level of the parameters (A2, B3, C3, and D3) as was obtained in S/N ratio analysis.

Optimum recipe: Glycerol 1,5%
Wheat bran 7%
Corn steep liquor 2%
Ammonium sulfate 0,4%
Magnesium sulphate 0,05%
Monopotassium phosphate 0,1%

CONCLUSIONS

Optimization medium components was performed via Taguchi's parameter design method. An L9 orthogonal array was used to accommodate four control factors and each with three levels for the experimental plan.

The factorial plan considered points out the microorganism potential to use the carbon source glycerol 1.5 % and wheat bran 7 % , and that the organic nitrogen source - corn extract - is best used at 2 % ratio; the variants were reproducible for all of the 3 optimizing experiments.

These experiments provided basic information to improve the efficiency of xylanase production and supported the analysis of the main effect of each constituent of the medium.

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PROTECTIVE EFFECTS OF SOME FLAVONOID PIGMENTS ON OXIDATIVE STRESS

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ABSTRACT

Flavonoids are plant pigments that are synthesized from phenylalanine and generally display marvelous colors in the flowering parts of plants.

The flavonoid pigments are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. Flavonoids' antioxidant properties may be explained also by free radicals capture formed in various pathological conditions: anoxia (when the generation of super-oxide radical takes place), inflammations (when super-oxide anions by NADPH-leukocytes' membrane oxidase, hydroxyl radicals and other reactive radicals are produced, which are forming during phagocytosis), lipids auto-oxidation (with hydro-peroxide radicals formation).

Many investigations have correlated the pharmacodynamic properties of some plant extracts with their antioxidant activity and the capacity to defend the organism against oxidative stress.

This study presents the evaluation of antioxidant activity of some vegetal selective extracts obtained from Agrimonia eupatoria L. and Hypericum perforatum **L.**

Antioxidant properties were studied using the chemiluminescence technique and DPPH free radical scavenging assay.

A correlation between antioxidant activity and flavonoidic content was observed for all the selective extracts. The results obtained recommending the selective vegetal extracts from Agrimonia eupatoria L. and Hypericum perforatum ***L.*** *species for applications in pharmaceutical industry, cosmetic industry and food additives.*

Key-words: chemiluminescence, antioxidant activity, flavonoid pigments, oxidative stress.

INTRODUCTION

Flavonoids are plant pigments that are synthesized from phenylalanine and generally display marvelous colors in the flowering parts of plants.

Flavonoids belong to the extensive group of polyphenols and several biological activities, such as anti-inflammatory, anti-ulcer, anti-cancer, antiviral, antibacterial, antispasmodic, neuroprotectant, antiatherosclerotic and anti-thrombotic, have been attributed to these compounds [1].

Oxidative stress and antioxidant defence imbalance have been associated with inflammatory, carcinogenic and coronary diseases. The recognized antioxidant potential of flavonoids could therefore be responsible for their beneficial actions[2]. Reactive oxygen species (ROS) are generated by many redox processes that normally occur in the metabolism of aerobic cells. These species are highly reactive and harmful to the cells. If not eliminated, ROS can damage important

molecules, such as proteins, DNA, and lipids. Cells express several defence mechanisms, including antioxidant enzymes and nonenzymatic compounds, that help prevent the damaging effects of ROS [3,4]. However, these endogenous systems are often in sufficient for complete scavenging of ROS. Their excess has been implicated in the development of chronic diseases, such as cancer, arteriosclerosis and rheumatism [5,6].

Oxidative stress can also play an important role in the development of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases [7,8].

There is an increasing interest in natural antioxidants, namely phenols, present in medicinal and dietary plants, that might help prevent oxidative damage[9-11].

Many investigations have correlated the pharmacodynamic properties of some plant extracts with their antioxidant activity and the capacity to defend the organism against oxidative stress. Among the plants of interest, the species *Agrimonia eupatoria L.* and *Hypericum perforatum* **L.** is also situated, whose chemical composition rich in flavonoids and polyphenols components confers antioxidant and curative properties. [12,13].

For obtaining of the flavonoid pigments were used two vegetal species *Agrimonia eupatoria L.* and *Hypericum perforatum* **L.**

The aerial parts of agrimony (*Agrimonia eupatoria* L., Rosaceae) are used as infusions, decoctions or tinctures (aqueous-alcoholic extracts) in traditional medicine, for their anti-inflammatory, astringent and diuretic properties [14, 15].

Agrimonia eupatoria L. contain aromatic acids, triterpenes, tannins, and flavonoid pigments. The presence of kaempferide, kaempferol and their derivatives, such as kaempferol 3-glucoside, kaempferol 3-rhamnoside and kaempferol 3-rutinoside, have also been reported [16].

Hypericum perforatum, (St. Jons's wort) is a vegetal species, from which the flowering part is used, which contains: naphtodiantrone (hyipericin, pseudohypericin, protohypericin, isohypericin), anthranols (emodol, anthranol), Flavonol glycosides (hyperoside, rutin, isoquercitrin, quercetin 3-glucuronate, quercetin 3-galacturonate, hyperoside-acetyl, rutin-acetyl, kampferol 3-rutinoside, quercetin 3-rhamnoside), flavonoid aglycones (quercetin, kaempferol, biapigenin, amentoflavone) essential oil, proantocianidoli, cinnamic acid, cafeic acid, chlorogenic acid and neochlorogenic acid.

Hypericum perforatum **L.** extracts contain many phenolic compounds, namely flavonoids and phenolic acids, suggesting that they could have important antioxidant properties. Previous reports indicate that these extracts show significant scavenging capacity for the superoxide radical, produced by the xanthine/xanthine oxidase system [17].

The complex pharmacological action, of choleretic, cholagogue, anti-inflammatory, anti- depressive, cicatrizant is attributed to the active principles mentioned.

Hypericum perforatum **L.** has a wide range of medicinal applications, including skin wounds, eczema, burns, diseases of the alimentary tract and psychological disorders. Nowadays, its use in the treatment of mild to moderate depression has become prominent [18 - 20].
In situations of increased free radical generation the reinforcement of endogenous antioxidants via intake of dietary antioxidants may be of particular importance in attenuating the cumulative effects of oxidatively damaged molecules.
Having in view the aspects presented above, the flavonoidic extracts obtained from vegetal sources present a special interest for their antioxidant effect in the human body. The technological studies of the extraction and separation have led to the obtaining of seven flavonoidic fractions from *Agrimonia eupatoria L.* and *Hypericum perforatum* **L.** species.
This study presents the evaluation of antioxidant activity of some flavonoidic vegetal pigments obtained from *Agrimonia eupatoria L.* and *Hypericum perforatum* **L.** Antioxidant properties was studied using the chemiluminescence technique and DPPH free radical scavenging assay.

MATERIALS AND METHODS

Vegetal material: *Agrimonia eupatoria L.* and *Hypericum perforatum.L* from Fitoterapia SA Romania
Reagents: 2,2-diphenyl-1-picrylhydrazyl (Merck) for DPPH free radical scavenging assay,
MgO (Merck) for UV-VIS spectroscopy; Folin-Ciocalteu (***Sigma-Aldrich***).
For chemiluminescence was used the system luminol – H_2O_2 in buffer TRIS-HCl, at pH 8,4;
Solvents: Ethanol p.a., Methanol p.a., Acetone, Acetic acid, Formic acid, Ethyl Acetate, Chloroform (Sigma-Aldrich).
Standards used: rutin, quercetin, apigenin, chlorogenic acid, caffeic acid, gallic acid and kaempferol (Sigma-Aldrich).
Vegetal material: *Agrimonia eupatoria L.* and *Hypericum perforatum.L* from Fitoterapia SA Romania

Antioxidant character evaluation and physical and chemical characterization of the flavonoidic vegetal pigments obtained from *Agrimonia eupatoria L* and *Hypericum perforatum* species was performed using the following methods and equipment:

- Chemiluminescence, CL by chemiluminometer Turner Design TD 20/20 USA;
- Spectrophotometric measurements were performed on UV-VIS spectrophotometer Anthelie (Secomam) controlled by software **Dathelie**
- Spectroscopy UV-VIS on 190-800 nm domain - spectrometer V-570 Jasco;

- Quantitative determination of sylimarin, flavonoids, polyphenols and polyphenol-carboxylic acids by spectral technique [21,22].

RESULTS AND DISCUSSION

The obtaining of the flavonoidic vegetal pigments from Agrimonia eupatoria L. and Hypericum perforatum L. ***species***

The flavonoidic vegetal pigments (**PVA1, PVA2, PVA3** from *Agrimonia eupatoria* L. and **PVH3, PVH4, PVH4, PVH5 -** from *Hypericum perforatum* ***L.***) were obtained by a succession of technological stages consisting in the first stage in the solid-liquid extraction. Following the extraction procedure, the vegetal material used was removed, and the obtained filtrates were processed by vacuum concentration until obtaining a residue which, passed through successive precipitation with polar and nonpolar solvents, centrifugation, filtering and purification. The variation of the operational parameters, respectively the fine degree of the plant, the solvent, used the plant/solvent ratio, extraction time and temperature, resulted in obtaining, non-hygroscopic, fine powdery samples. The samples were characterized by spectral techniques UV-VIS (table 2) by chemiluminescence and DPPH methods[23] (Figure 2); the quantitative determination of the flavonoids, of the polyphenols, of the polyfenolcarboxilic acids and of specific physical-chemical indicators, were made according to FR X (table 1) [21].

Table 1. Physical-chemical characteristics

Samples	Ash %	Humidity %	Colour	Flavonoids, mass % (as rutin)	Polyphenols, mass % (*as gallic acid*)	Polyfenolcarboxilic acids, mass % (*as caffeic acid*)
PVA1	2.19	2.81	Yellow-brown	3.54	4.501	2.573
PVA2	2.54	2.67	Yellow	3.02	3.735	1.987
PVA3	2.31	2.15	Yellow-brown	3.21	2.948	1.712
PVH4	2.58	3.07	Red-brown	3.49	3.917	2.998
PVH5	2.83	3.11	Green-brown	4.38	4.341	3.112
PVH6	2.21	2.64	brown	2.41	3.136	1.821
PVH7	2.79	3.28	Red-brown	3.27	5.786	3.124

UV-VIS characteristics of the samples

The samples in ethanolic solvent have similar electronic spectra which those come from structures with an extended conjugation (table 2 and figure 1).

Table 2.UV-VIS data of the PVA1, PVA2, PVA3, PVH4, PVH5, PVH6, PVH7 **samples in EtOH**

Domain (nm)	PVA1	PVA2	PVA3	PVH4	PVH5	PVH6	PVH7	Identification
200-230	-	-	-	210 (u) 226	228	205 230	-	$\pi \rightarrow \pi^*$ transition
280-290	281	281	281	280	280	281	280	$\pi \rightarrow \pi^*$ transition
315-385	320 (u) 385	340 -	318 -	324 -	321 370	319 373	321	$n \rightarrow \pi^*$ transition
400-480	- - -	410 - -	411 - -	- - -	412 437 474	410 437 -	408	conjugated structures
590-670	607 670	615 669	616 670	-	615 668	617 669	592	aromatic cycle

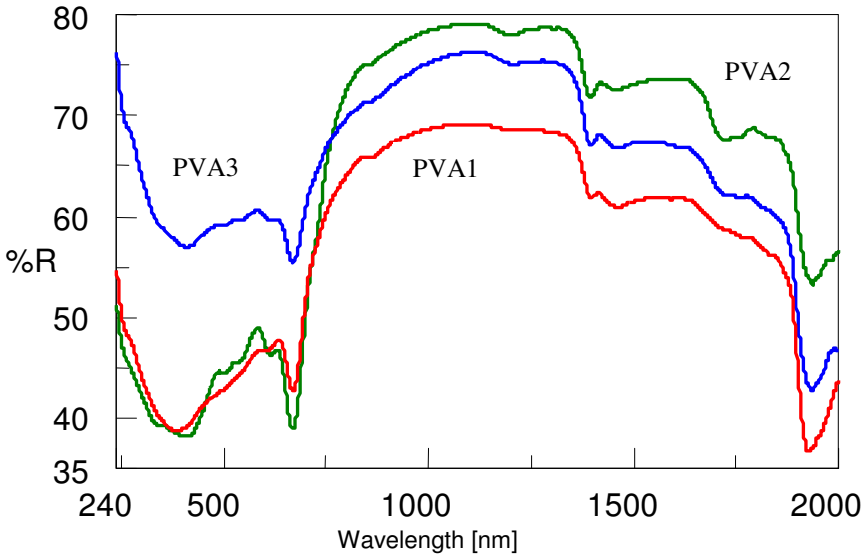

Fig 1. UV- VIS spectra of the samples

The main electronic tranzitions in UV-VIS domain are situated at 300-700 nm indicating carboxylic, hydroxilic and unsaturated conjugated structures specific to quercetine and rutin [24].

Comparing the spectra of the flavonoidic vegetal pigments (PVA1, PVA2, PVA3, PVH4, PVH5, PVH6, PVH7) with the spectra of the standards (rutine and quercetine), we note the presence of common bands, specifics for the flavonoidic structures [25].

Antioxidant activity

DPPH- Free radical-scavenger activity

The free radical-scavenger activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as described in [23, 26] .

The antiradical activity of each fraction was evaluated using a dilutions series, in order to obtain a large spectrum of sample concentrations.

The reduction of DPPH absorption is indicative of the capacity of the flavonoidic vegetal pigments to scavenge free radicals, independently of any enzymatic activity. The extraction methods resulted in different flavonoidic vegetal pigments, with diverse compositions, either in qualitative or quantitative terms (Table 1). All fractions promoted the reduction of DPPH (Figure 2). The best free radical-scavenger activity was reported for the PVH5 and fractions PVA1 and PVH4, respectively (Figure 2). The lowest activity was observed for fraction PVH7. It is interesting to note that all these flavonoidic vegetal pigments possessed high reactivity and had an important flavonoid component (namely fractions PVH5 and PVA1). Fraction PVH7 exhibited the weakest reactivity, significantly different from any other extract, in spite of having a considerable proportion of flavonoids and caffeoylquinic-type acids.

This low reactivity could be related to the presence of other compounds (e.g. sugars, amino acids) that could somehow interfere in the total reactivity of the fraction.

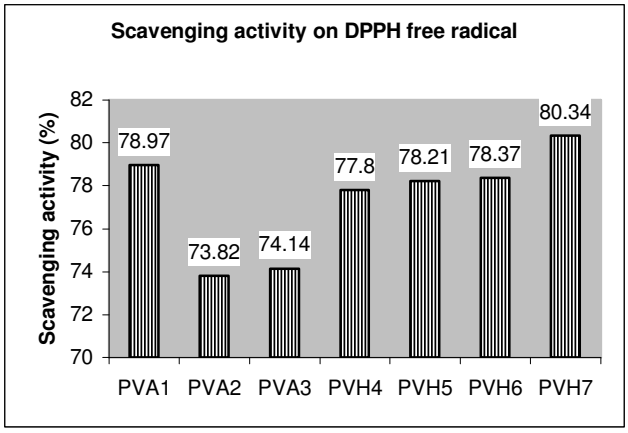

Fig 2. Scavenging activity on DPPH free radical

Chemiluminescence tests

Taking into account the importance and therapeutical role of vegetal antioxidants, the flavonoidic extracts have been tested by chemiluminescence using luminol and hydrogen peroxide at pH = 8.6 in presence of TRIS + HCl, following the procedure described in [25]. The data are reported in table 3.

Table 3. Chemiluminescence characteristics

Sample code	k (s^{-1})	v_i (s^{-1})	AA % [CL]
PVA 1	0.102	120.51	92.40
PVA 2	0.095	291.48	87.10
PVA 3	0.090	104.21	89.70
PVH 4	0.086	145.24	90.15
PVH 5	0.100	118.84	94.02
PVH 6	0.116	183.21	80.01
PVH 7	0.094	291.74	78.91
Quercetine	0.101	128.83	92.80
Kaemferol	0.105	124.80	92.49
Rutine	0.093	291.40	77.40
Apigenin	0.098	289.20	82.62
Chlorogenic acid	0.096	100.00	87.20
Caffeic acid	0.070	318.00	82.90
Galic acid	0.112	166.46	85.70

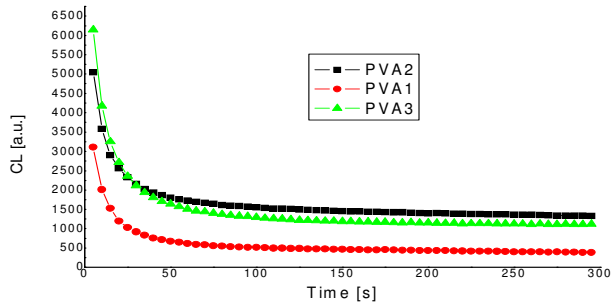

Fig 2. CL evolution in time of the samples PVA1-PVA3

Antioxidant activity and the rate constants of the reaction (table 3) emphasize high protective capacity of the extracted components. The antioxidant activity of the flavonoidic vegetal pigments is attributed to the active principles, especially to the flavonoids content.

The qualitative and quantitative content of the phenolic and flavonoidic constituents in the samples, confirmed by spectral analysis and chemiluminescence determinations, explain the high value of the antioxidant activity, especially for the sample PVH 5 (94.02%) [25].

CONCLUSIONS

The extractive process conducted to the obtaining of seven flavonoidic vegetal pigments from *Agrimonia eupatoria* L. and *Hypericum perforatum* **L**. Spectral investigations (UV-VIS) on the flavonoidic vegetal pigments emphasized the presence of some phenolic and flavonoidic structures, also confirmed by

quantitative determination of the flavonoids, polyphenols and polyphenolcarboxilic acids.

The results of the free radical-scavenger activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay revealed that the samples exhibit high antioxidant activity, being in accordance with the values provided by chemiluminescence *in vitro* tests, and suggesting thus their potential application for prophylaxy and therapy of various free radical related diseases.Experimental data emphasized the structural complexity and antioxidative capacity of the flavonoidic vegetal pigments.

A correlation between antioxidant activity and flavonoidic content was observed for all the flavonoidic vegetal pigments. The results obtained recommending the flavonoidic vegetal pigments from *Agrimonia eupatoria* L. and *Hypericum perforatum* **L.** species for applications in pharmaceutical industry, cosmetic industry and food additives.

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ANTIOXIDANT BIOPRODUCT WITH IMMUNOMODULATORY POTENTIAL

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ABSTRACT

Modulation of cytokine secretion may offer novel approaches in the treatment of a variety of diseases. One strategy in the modulation of cytokine expression may be through the use of herbal medicines.
We analyzed the effects of a selective extract obtained from Rosmarinus officinalis L. which exhibit strong antioxidant activity, on the production of interleukins including IL-1β, IL-2, IL-6, IL-8, IL-10 and TNF-α, in peripheral blood culture.
The results showed increased expression IL-10 (anti-inflammatory interleukin) and inhibition of proinflammatory interleukins production after treatment with the selective extract
This findings suggest that Rosmarinus officinalis and its compounds could be used to reduce inflammatory reactions in certain inflammatory diseases.

Key-words: Rosmarinus officinalis, immunomodulatory, cytokine

INTRODUCTION

Cytokines are the main regulators of the immune response. They induce the balance between inflammatory versus regulatory or antibody mediated reactions. For instance, Th1 cytokines such as IFN-γ and tumor necrosis factor-α (TNF-α) plus TNF-α from monocytes/macrophages, stimulate the function of T cytotoxic cells, natural killer (NK) lymphocytes,and activated macrophages. TNF-α, IL-1β and IL-6 are considered proinflammatory cytokines because they stimulate the synthesis of nitric oxide and acute phase proteins, attract inflammatory cells, and upregulate the synthesis of secondary mediators and proinflammatory cytokines. On the other hand, the anti-inflammatory cytokine, IL-10, derived from Th2 lymphocytes and monocytes, promotes humoral immunity and suppresses monocyte/macrophage and Th1 lymphocyte activation by inhibiting IL-12, TNF-a and IFN-c production [1,2]. Modulating the release of cytokines or inducing them by immunomodulating agents is an attractive mode for treating or help in treating several diseases such as infection, allergy, autoimmune mediated diseases and cancer [3,4].

For instance, patients with viral infections or cancer may be in need of increasing or inducing Th1 or proinflammatory cytokines, which ultimately activate T cytotoxic, NK cells and macrophages to kill virally infected cells and tumor cells, and the

enhancement of the host immune system would increase the ability to combat infection and thus reduce the problems of antibiotic resistance [3,5]. Furthermore, autoimmune diseases can be classified into proinflammatory (Th1) (e.g. rheumatoid arthritis) or antibody (Th2) (e.g. systemic lupus erythematosus) mediated diseases. In the latter cases, immunomodulating the immune cells and shifting the cytokine pattern would help in the treatment of the disease [6].

In order modulation of cytokine secretion may offer novel approaches in the treatment of a variety of diseases. One strategy in the modulation of cytokine expression may be through the use of herbal medicines.

Medicinal plants represent alternative means for the treatment of several chronic diseases, including inflammation. Leaves of *Rosmarinus officinalis* has been reported to possess a variety of bioactivities including antioxidant, antitumor, anti-inflammatory antibacterial and antiviral, possibly due to the presence of flavonoids, phenols and terpenoids (carnosic acid, carnosol, rosmanol, caffeic acid, rosmarinic acid and ursolic acid [7,8].

The purpose of this study is to characterize potential immunomodulatory bioactivities of *Rosmarinus officinalis.*

We analyzed the effects of a selective extract obtained from *Rosmarinus officinalis* which exhibited strong antioxidant activity in our preliminary studies, on the production of interleukins including IL-1β, IL-2, IL-6, IL-8, IL-10 and TNF-α, *in vitro*, in mitogen-stimulated peripheral blood culture.

MATERIAL AND METHODS

Plant material. The rosemary samples (*Rosmarinus officinalis* L.) consisted of dried rosemary leaves were obtained from S.C. Fitoterapia S.A., Romania.

Chemicals. All solvents, reagents and standards used were purchased from Sigma-Aldrich.

Kits. To determine the cytokine levels it has been used the following kits: interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), tumor necrosis factor α (TNF-α□) and Human MultiAnalyte Profiling Base Kit A, all from R&D Systems.

Extraction of plant material. The selective extract (RZ) was obtained by a succession of technological stages consisting in the first stage in the solid-liquid extraction in a Soxlet installation. Following the extraction procedure, the vegetal material was removed and the obtained filtrates were processed by vacuum concentration until obtaining a residue which was passed through successive precipitation with polar and non-polar solvents, centrifugation, filtering at low pressure and purification.

Chemical analysis of the extracts. The samples were characterized by spectral techniques (IR, UV-VIS-NIR) and chemiluminescence. The quantitative determination of the flavonoids, polyphenols, polyphenolcarboxilic acids and the

evaluations of the specific physical-chemical indicators were done according to the FR X and the European Pharmacopeea [9-11].

Subjects

Fourteen healthy volunteers (7 males and 7 females) with a mean age of 30.2 (±4.1) years old enrolled and signed an informed consent prior to their participation in the study. None of the volunteers have taken any medication for at least a week, did any exercise, or ate before the blood sample was drawn. All blood samples were drawn in the morning between 8 and 9 a.m.

Human whole blood culture

Blood samples were drawn from fourteen healthy volunteers into sterilized sodium heparin tubes and processed within 45 min. The blood as diluted with 1:10 with RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 lg/ml streptomycin, without exogenous serum. To each well of the 6-well culture plates, 2.0 ml of the diluted blood was added. To study cytokine modulation, 0.001, 0.01, 0.1, 1, 10 or 100 ug of RZ/ml was added to each well, incubated for 24 h, followed by adding a mixture of Phytohemeagglutinin (PHA)+Lipopolysaccharide (LPS) in 40 µl volume to give a final concentrations of 5 and 1 µg/ml for PHA and LPS, respectively, and incubated in 5% CO2 at 37ºC for another 24 h. At the end of incubation, blood was collected from wells into sterilized tubes and each well was washed with 0.5 ml of RPMI to ensure removal of all well content. The supernatants were aliquoted and stored in sterilized tubes at -30ºC until assayed.

Cytokine analysis

Cytokine level from supernatants was determined with preconfigured kits using the Luminex xMAP technology

Statistical analysis

The results were expressed as the means concentrationof each cytokine (±SD). Statistical comparisons between dosage groups were done using the paired t-test and a one-way ANOVA, with $\mathbf{P} < 0.01$ indicating significance.

RESULTS AND DISCUSSION

Stimulating or modulating the release of cytokines by immunomodulators or immunostimulating agents is an attractive mode for treating several diseases. Of these agents, we found that the selective extract (table 1) obtained from *Rosmarinus officinalis* (RZ) can induce and modulate cytokines in dose-dependent manner. RZ was found to reduce significantly ($p < 0.001$) IL-1β, TNF-α, and IL-6 production from whole blood culture in a concentration dependent manner (fig. 1-3).

Table 1. Physical-chemical characteristics of the sample

Sample	RZ
Ash %	2.19
Humidity %	4.05
Flavonoids, mass % (*as rutin*)	2.94
Polyphenols, mass % (*as gallic acid*)	3.82
Polyfenolcarboxilic acids, mass % (*as caffeic acid*)	2.58
Hydroxicinnamic derivates, % (*as rosmarinic acid)*	1.32

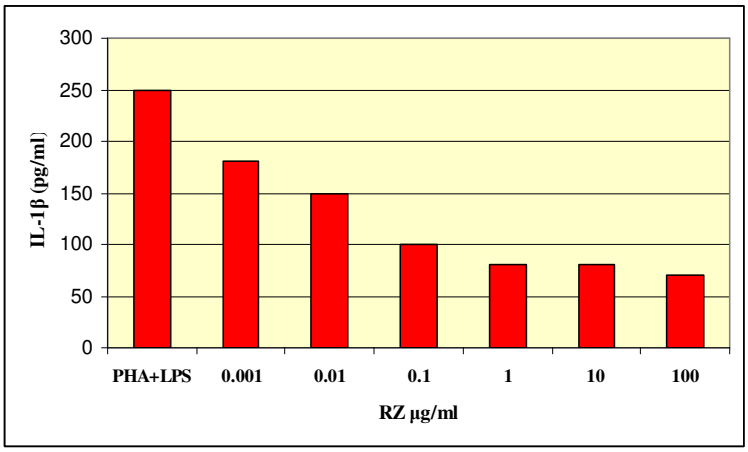

Fig.1. The effect of RZ on modulating IL-1β production after PHA+LPS stimulation

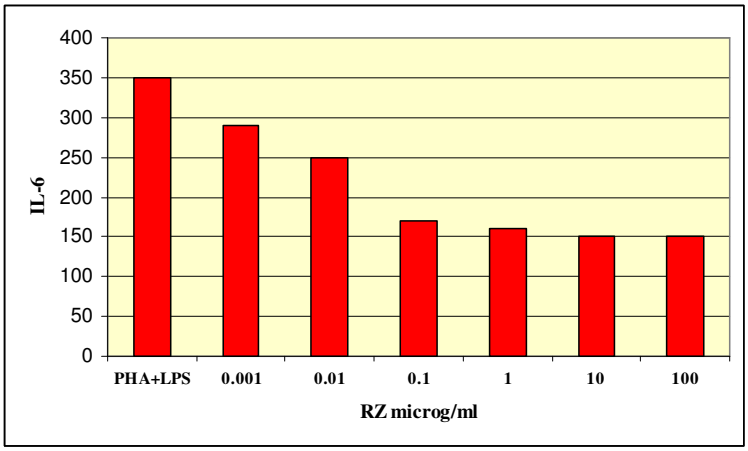

Fig.2. The effect of RZ on modulating IL-6 production after PHA+LPS stimulation

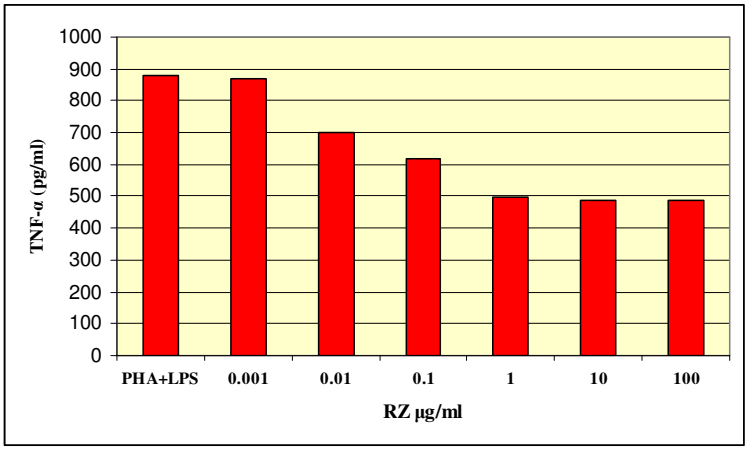

Fig.3. The effect of RZ on modulating TNF-α production after PHA+LPS stimulation

Twenty-four hour exposure at different concentrations of RZ increased significantly ($p < 0.001$) the production of IL-10, from PHA+LPS-stimulated whole blood (fig.4). No significant changes in the production of IL-2, IL-4 and IL-8 were seen.

These results indicate that RZ inhibits proinflammatory cytokines production and increase IL-10 level in a concentration dependent manner.

The differential effect of RZ on cytokines opens several ideas for applications. Those effects should be studied further in animal models to check the efficacy of such cytokine inhibition and/or induction.

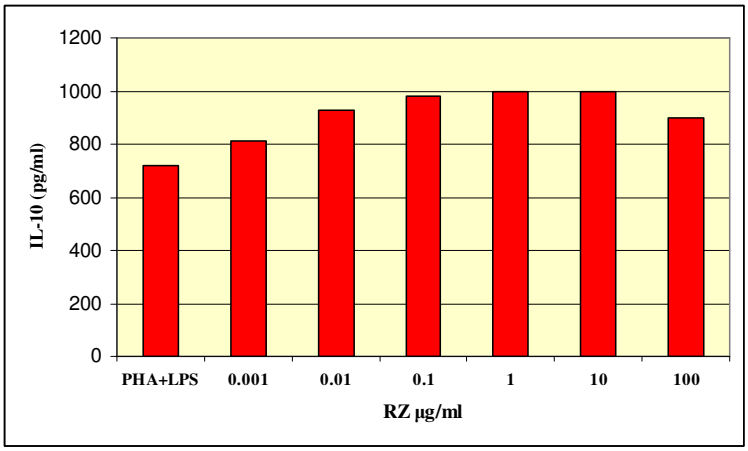

Fig. 4. The effect of RZ on modulating IL-10 production after PHA+LPS stimulation

Based on all the above observations, we suggest a possible immunomodulatory role for **Rosmarinus officinalis** as it relates to inflammation.

CONCLUSIONS

The selective extract obtained from *Rosmarinus officinalis* L.(RZ) modulates cytokines production by PHA+LPS stimulated whole blood.

The results showed increased expression IL-10 (anti-inflammatory interleukin) and inhibition of proinflammatory interleukins (IL-1β, TNF-α, and IL-6) production after treatment with the selective extract, in a concentration dependent manner.

This findings suggest that *Rosmarinus officinalis* and its compounds could be used to reduce inflammatory reactions in certain inflammatory diseases.

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THE ENVIRONMENTAL IMPACT OF VITICULTURE: INFLUENCE OF THE BIOFERTILIZER ON PRUNING AND WINE WASTE

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ABSTRACT

The global wine and table grape industry, with annual sales exceeding US$100 billion, generates large quantities of cane pruning waste each year. This pruning is usually composted or burned as waste, often with a net cost incurred to the winery. The 'end-use' grapevine waste can be degraded by micro-organisms, soil organisms or enzymes. In the European Waste Catalogue (EU, 2001) grapevine waste is indexed as plant waste and as a part of biowaste. Since grapevine waste is subject to the aerobic treatment process, it is compost. Grapevine waste is subject to an anaerobic treatment process or treated by other methods, including mixtures with other materials like grape mark before/after treatment.

The aim of this paper is to investigate quality and quantity of the compost types made of pruning waste, grape mark, and the mixture of both. Preparation of microbiological fertilizers consists of the Bacillus and Pseudomonas straining and their placement, at an adequate amount, in all assemblage. Results indicate that the microbiological biomass causes the improvement of natural degradation.

Key words: pruning vinegrape, pomace, biowaste, microbiological fertilizers *Bacillus*, *Pseudomonas*

INTRODUCTION

Since agricultural secondary products / waste are barely used, highly-valuable substances, which separate during the natural or industrial procedures and which may be re-implemented in the production or as a supplement in human or animal nutrition, are thus ignored. Efficient extraction and commercial implementation of these components may help carry out sustainability of the given production facilities, in this case vinyards and wine cellars (Das and Singh, 2004). So, for example, the total wine and table grapes production, with the basic value exceeding 100 billion dollars, guarantees a large quantity of pruned vine every year. However, the pruned mass is frequently burned, thus increasing expenses in the vineyard. In avereage, there is about a ton (USEPE, 1995) of the pruned vine mass while nearly 7,500,000 hectares are used for the world grape production each year (PricesSTAT-FAOSTAT). Grapes are fruit crops grown widely in many areas of the world and 71% of the fresh grapes produced are used for wine production. During wine making, large quantities of wastes such as grape marc (the residue after pressing for white wines or vinification for red wines) and stalks are produced as by-products. The placing of the enormous amount of waste grape marc generated all over the world is an increasing problem. By processing 100 kilograms

of grape-vine approximately 20-25 kilograms of grape marc is produced. For example, in Europe alone 112 million tons of grapes were processed by the wine industry in 1998, and an estimated 13% (14.5 million tons) of this amount corresponded to the by-product after pressing, consisting mainly of skins and seeds (Radovanović *et al.,* 2009).

Grapes are rich in proanthocyanidins, and the researches are directed toward two aims: elaboration of the production procedure of wine enriched with proanthocyanidins and elaboration of the procedure for industrial extraction from the solid vine parts and the repeated return to the grape production procedure as well as in order to implement them in pharmaceutical, cosmetic and food industry (Kovač *et al*., 1994). Seeds make the most interesting potential raw material for industrial extraction of the mentioned physiologically active components. Pinor gris and Pinot noir have the highest content of catechols and proanthocyanidins in 1 kg of stalks, marc and seeds (29,132 mg/kg and 25,153 mg/kg), while the mentioned content is considerably lower in Pinot blanc and Riesling italico (13,142mg/kg; 7,950 mg/kg) (Kovač *et al*., 1992).

Pruned vine contains a considerable amount of the components jointly called stilbenes while the most prominent substance extraceted from grapes is trans-resveratrol (3,5,4'-trihydroxystilbene). This substance inhibits or delays vine-affecting diseases (Baur and Sinclair, 2006), prevents cardiovascular diseases (Bradamante *et al.*, 2004), cancer (Jang *et al.,* 1997) and increases stress resistance and prolongues human life (Baur, *et al.,* 2006; Valenzano *et al.,* 2006). Trans-resveratrol is the first in the stilbene series; in vinegrape, it synthetizes with stilbene synthase enzyme as the result reaction to occurrence of a disease or stress/freezing. Stilbenes which occur as trans-resveratrols and trans-ε-viniferins are known as anti-phytopathogen reagents when it comes to occurrence of downy mildew (*Plasmopora viticola;* Dercks and Creasy, 1989; Hoos et al., 1990, Dai et al., 1995, Pezet et al., 2004), grey rot (*Botrytis cinera*; Langcake, 1981, Hoos and Blaich, 1990, Adrian et al., 1997), *Phoma medicaginis* (Hipsking and Paiva, 2000), *Rhizopus stolonifer (*Sarig *et al*., 1997) and in the wide range of microbes and fungi which make presence during the post-harvest and grape processing period (Urena *et el.,* 2003, Jimenez *et al.,* 2005). Resveratrol is known for a secondary metabolite with fungicide activity. As a result of its external application to treatment of harvested grapes, their microbiological flora depleats and fruits last longer while their nutritive value remains unaffected. When compared to the resveratrol-untreated grapes, it adopts characteristics of the natural pesticide which diminishes the possibility of infection of harvested fruit (Jimenez *et al.*, 2005).

Potential pruned vine utilization is very high, from compost to anti-phytopathogenic treatment extract, which contributes to a better grape production and processing sustainability. When decomposed, lignin, the major component of composite carbohydrates in agro-industrial waste, produces polycyclic aromatic

hydrocarbon components such as benzopyrenes, catechols, hydroquinones, phenanthrenes and naphthalenes if decomposed by heat. All these components can inhibit DNA synthesis caused by a carcinogen tumor of liver, lungs, throat and cervix in people and animals. According to Rayne (2007), there is 1 mg/g of dry substance (1mg/g dw), that is 7,500 t on the worldwide level or 825 mg per capita worldwide.

From the surface of 30,000 ha in Serbia, estimated as productive (www.stat.gov.rs), nearly 30,000 tons of pruned vine/dry waste and about 74,690 t of wet waste are produced after grapes have been processed into wine, and that 62% marc, 14% lees, 12 % stalk and 12% waterwaste. Pomace or grape marc is made during the processing procedure, and it is mainly deposited in the open space. Grape marc can be used as fodder, especially during the winter when there are no pastures. A high price of labour and transportation expenses frequently limit utilization of direct advantages of this bioproduct (Sánchez *et al*., 2002). Plant material residue is recognized as biowaste (Europen Waste Catalogue, EU, 2001). The best possible effect is attainable through its repeated intake in the soil, since that is the way to achieve optimum ballance among plant/vine growing, soil structure, and the supply of water, nutritions, oxygen and biomass of microorganisms. Soil of a good structure increases the number and diversity of organisms, decreases development of harmful units and promotes decomposing processes from organic substances to minerals (Raičević *et al*., 2003). A stable organic mass is the leading factor for improvement of the soil structure and fertility (White, 2003, Sivčev *et al*., 2010). To make soil fertile would be the basis of organic system while organic substance supply might be the most important factor for the soil fertility preservation. Therefore, bio-diversity of microorganisms, plant nutrients, sustainable water capacity, aggregate supply and land erosion control depend upon quality and quantity of the organic substance in the soil (Kandeler *et al.,* 2005).

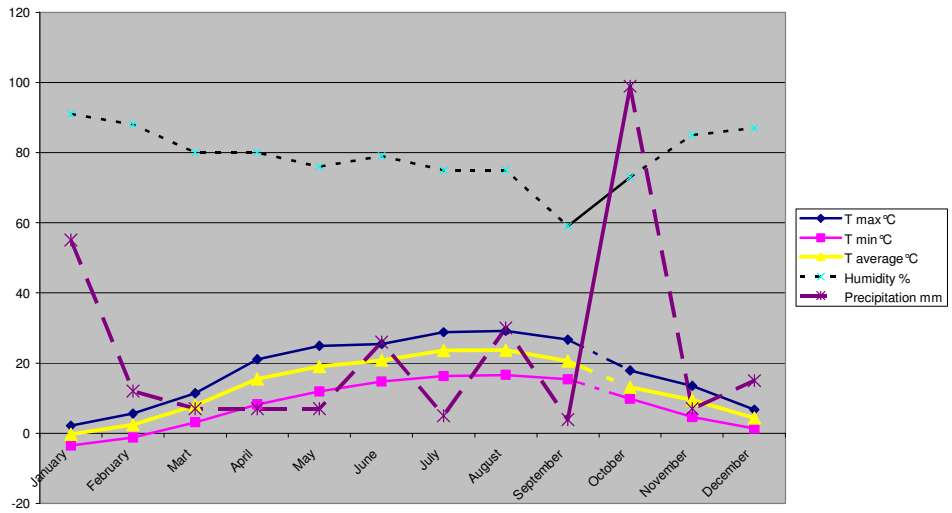
The present paper work is to investigate quality of the biowaste: pruned and chopped up vine, marc (pomace) after the grape processing and the mixture composed of the proportional amount of pruned vine and marc within natural conditions, including the activity of two groups of microorganisms *Bacillus* and *Pseudomonas.*

MATERIAL AND METODS

The experiment has been carried out near the vineyard, in three seedbeds of the size 10 x 2 x 0.2 m. Ripe pruned vine was put in one seedbed, marc in another (grape marc) deposited in the heap from the 2008 harvest, while mixed chopped up ripe vine and marc were, at the same proportion, put in the last seedbed. Each seedbed was provided with the mixture of microorganisms *Bacillus* (10^6/ml) and

Pseudomonas(10^6/ml). The seedbeds were set in March 2009, within completely natural conditions. The mixture would be stirred from time to time to provide aerobic conditions for decomposition.

Fig. 1 Mean air temperature and sum of precipitation in the year 2009



ESE »Radmilovac»: Altitude 130 m, Latitude 44°45´N, Longitude 20°35´E

There is a climatological station on the Faculty of Agriculture's Experimental School Estate «Radmilovac», near which the experiment has been set. Since the data for September and October are missing, data from the first-class meteorology station, 5 km away from the vinyard, have been used instead. Data on average monthly air temperature values, relative air humidity and precipitations are presented in Fig. 1. Seedbed samples were taken in Autumn 2009, Spring 2010, Autumn 2010, and then the analysis of microbiological presence was carried out. Microbiological characterization of samples was carried out through the use of nutrient medium 0.1xTSA (for total microflora) and Fyodorov`s medium for determination of *Azotobacter sp*. The samples were prepared by the dilution method. All experiments were performed in triplicate. The total microflora incubation period was 5 days at 30°C and 2 days at 30°C for *Azotobacter sp*. The population densities were recorded as colony forming units (cfu) g^{-1} of dry samples. In autumn 2010, the following characteristics were determined: pH value, the content of calcium, humus, the total content of ammonium and nitrate forms of nitrogen, the content of available phosporous and potassium as well as the proportion of carbon and nitrogen (C/N), all by means of the standard methods (Jakovljevic *et al*., 1998).

RESULTS AND DISCUSSION

The wine industry uses large amounts of chemical fertilizers and organic substances (Kammerer *et al*., 2005; Arvanitoyannis *et al*., 2006). Prevention of possible vineyard crop diseases is a good way to present composting as the most suitable process for the organic waste reuse in the wine industry in vinayard crops (Ruggieri *et al*., 2009).

Tab. 1 Total bacterial number (CFU $x10^6$) in different types of waste

Type of waste	Autumn 2009	Spring 2010	Autumn 2010	Average
Comminute pruning wood	3.2	5.8	4.23	4.41
Grape marc	6.13	31.6	4.88	14.20
Mixture 1:1 pruning wood and grape marc	10	75.33	5.96	30.43
Average	6.44	37.58	5.02	-

The experiment results indicate different presence of the total number of bacteria, depending on the transformation process duration. Bacteria were the most numerous in Spring 2010, which indicates that the most intensive transformation processes took place during that time (Tab. 1). Bacteria were the least numerous within the comminute pruning wood type of waste, while they were the most numerous within the pruning wood and grape marc mixture.

There were no considerable fluctuations in the number of *Azotobacteras* among different treatments in Autumn 2009 and 2010, while the fluctuations were obvious in Spring 2010 (Tab. 2). *Azotobacteras* were the least numerous within the comminute pruning wood type, while they were the most numerous within the 1:1 proportion of pruning wood and grape marc mixture. Bacteria were more numerous within the 1:1 proportion of pruning wood and grape marc mixture due to the larger quantity of available carbon and higher aeration. There was a gradual drop in pH value, which was mildly neutral. The carbonate content was low.

There were no considerable fluctuations in the total nitrogen content among different treatments, while there was the highest presence of ammonium nitrogen and available phosporous within the 1:1 proportion of pruning wood and grape marc mixture. The highest content of nitrate nitrogen and available potassium was recorded withinn the grape marc type.

As for the C/N proportion values, there were no considerable differences among the treatments, but the most important ones were recorded within the comminute pruning wood type. When organic matter passes through successive cycles of decomposition, the C/N ratio becomes smaller (White, 2003). The previous statement has been confirmed by our results: the C/N ratio of decomposers in well

drained soils with pH~7 is close to 10. It includes the timely biodegradation with maintenance and continuous creation of high quality humus.

Tab. 2 Number of *Azotobacter sp*. (CFU x10^3) in different types of waste

Type of waste	Autumn 2009	Spring 2010	Autumn 2010	Average
Comminute pruning wood	**57.3**	**18.3**	**38.88**	**38.16**
Grape marc	**66.5**	**56.2**	**47.51**	**56.7**
Mixture 1:1 pruning wood and grape marc	**62.9**	**75.3**	**42.81**	**85.4**
	62,2	**49,9**	**43**	

Composting helps manage large quantities of organic wastes in a sustainable manner. It is one of the technologies of integrated waste management strategies, used to recycle organic materials into a useful product (Giglotti *et al.,* 2005)

Tab. 3 Chemical characteristics of different types of waste

Type of waste	pH		$CaCO_3$ (%)	Humus (%)	Total N (%)	NH_4 mg/kg	NO_3 mg/kg	P_2O_5 mg/100g	K_2O mg/100g	C/N
	H_2O	KCl								
Comminute pruning wood	7.11	6.26	0.4	2.71	0.164	126	7	13.8	20	9.6
Grape marc	7.18	6.43	0.1	2.37	0.157	147	10.5	17	28.7	8.8
Mixture 1:1 pruning wood and grape marc	7.51	6.93	0.8	2.12	0.147	161	7	21.4	25.6	8.3

Sustainable organic mass is the leading factor for improvement of the soil structure and fertility (Sivčev *et al*., 2006). The previous statement is confirmed by the content of humus, since its values get close to the vineyard optimum value in all three treatments. Generally, our results are in accordance with the ones acquired by Biale (2000). He has taken into account several results and showed the average vegetative yield and grape yield value obtained in Europe and Australia. The compost microorganisms decompose organic matter while their activity and enzymes, created during these processes, contribute to easier absorption of nutritious elements by vine and other plants.

CONCLUSION

It takes 16 months under the natural conditions to get comminute pruning wood and grape marc tarnsformed into compost. It is characterized by high nutrient absorption capacity with nutrient availability. The highest compost/biofertilizer microbiological activity is recorded in Spring, within the medium precipitation conditions in all three treatments. Chemical analysis of the compost has indicated the coherent relation of the examined components. Differences among the treatments/biofertilizers are not statistically relevant, and all three treatments have become important for the living environment preservation and conclusion of the grape and wine production cycle. The results indicate the possibility to obtain the products from viticulture applicable in agriculture.

Thus, it is possible to contribute to the global awareness of the municipal waste composting and to obtain different compost products from the Balkans agriculture.

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VANILLIN RELEASE FROM AGAR MICROCAPSULES

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ABSTRACT

Encapsulation has found numerous applications in food and pharmaceutical industries because offers several advantages: slows down the degradation processes and/or prevents degradation of bioactive compounds. Flavors can be encapsulated for a sustained release in different food formulations.
The aim of this paper is to present experimental data concerning vanillin release from agar capsules formulated as matrix type delivery system. Because vanillin can adhere to the surface of agar capsules, the surface vanillin and also the encapsulated vanillin was determined for different agar microcapsules.

Keywords: microcapsules, vanillin, agar, flavor

INTRODUCTION

Microencapsulation is a technology that has solved many problems that limit the use of ingredients and food additives. For example, encapsulation increases the stability of the products and can provide control release of the ingredients under prestablished conditions. Microencapsulation has also found widespread use in the food and beverage industry. Flavor microencapsulation entraps tiny volumes of the flavoring substance in micro and nano particles of different materials having a huge impact on the flavor profile of the final product they are used in. Despite the wide range of encapsulated products that have been developed, microencapsulation is still far from being fully developed in the food industry [Anal and Singh 2007, Krishnan et al. 2005, Gouin 2004, Rosemberg and Young 1993]. It is a very active research area, in which there is a constant stream of new, improved techniques and consequently patent applications.

The techniques used for microencapsulation are spray drying, spray cooling/chilling, freeze-drying, spinning disk and centrifugal coextrusion, extrusion, fluidized bed technology, coacervation, liposome entrapment, co-crystallization and microencapsulation processes based on supercritical fluids [Krasaekoopt et al. 2003]. Entrapment of actives in an amorphous matrix is one of the most used techniques of encapsulation.

Numerous materials, especially biopolymers, are available for food application, for example: starch, chitosan, agar, calcium alginate. Agar, for exemple, is a gelatinous substance derived from red algae. Agar consists of a mixture of agarose and agaropectin. Agar is insoluble in cold water but dissolves to give random coils in boiling water. Because it forms hydrogels it is ideal as matrix for food actives. Vanillin is the major component of natural vanilla, which is one of the most widely used and important flavoring materials worldwide. Vanillin displays also antioxidant and

antimicrobial properties and hence has the potential for use as a food preservative (Walton et al. 2003).

The aim of this paper is to present the possibility of vanillin encapsulation in agar microcapsules. Because a burst effect was observed in a typical release experiment, the attention was focused on the amount of vanillin which is really entrapped and the amount which is called surface vanillin.

MATERIALS AND METHODS

Materials

Agar (AG) was purchased from Sigma Chemical Co. and vanillin was obtained from Fluka.

Preparation of encapsulated vanillin

The agar microcapsules were obtained starting from an agar solution, prepared by dissolving agar in hot water under mechanical stirring. In the agar solution was poured another solution of synthetic vanillin of known concentration. The mixture was extruded through a syringe needle in the form of droplets, which were solidified by cooling in sunflower oil under vigorous stirring for ten minutes. Their diameters were measured using an Olympus microscope BX 51 (Olympus). In order to examine how the agar concentration in granules affects the release rate, different granules were obtained by varying the amount of agar used.

Vanillin analysis

The vanillin content was analyzed in the liquid phase using an UV-VIS spectrophotometer (Cintra 6 GBS-Scientific-Australia) at fixed wavelength (λ = 229 nm).

Analysis of surface vanillin and entrapped vanillin

To analyze the surface vanillin, 0.2 g of microcapsules were washed with alcoholic solution (96% v/v alcohol) for five minutes. Then, the capsules were filtrated and the solution was analyzed for vanillin content. The microcapsules which were washed with alcohol, to determine surface vanillin, were contacted under vigorously stirring with a new quantity of alcoholic solution and after 1 hour the solution was filtered and the vanillin content was determined.

RESULTS AND DISCUSSIONS

An optical micrograph of an agar microcapsule is presented in figure 1. The resultant agar particles have a particle size ranging between 300-500 μm. A typical plot for vanillin release from agar microcapsules is presented in figure 2. The microcapsules were obtained starting from 3% w/v agar solution and 2.5 g/L synthetic vanillin dissolved in ethanol (96%) by extrusion method, already presented. From this experiment one can observe a burst effect for the first 5-10 minutes from the beginning of the experiment. This burst effect can be explained

only considering that an amount of vanillin is surface vanillin. Encapsulated vanillin represents vanillin inside the microcapsules and surface vanillin represents the vanillin adhered to the surface of the microcapsule. This surface vanillin can be recovered by washing agar beds with absolute alcohol since vanillin is soluble in absolute alcohol.

To elucidate this aspect the following experiments were done to determine surface vanillin (SV) and entrapped vanillin (EV) in agar microcapsules containing different amount of agar. In the first experiment were used microcapsules obtained starting from agar solution 1.5 % w/v and solution of synthetic vanillin 2.5 g/L. 0.2 g of microcapsules were weighted and then contacted under vigorously stirring with alcoholic solution (96%). To avoid the non-uniformity of the samples, five samples from the same microcapsules were tested.

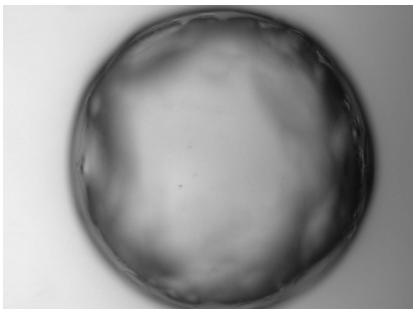

Fig 1. Optical microscopy image of an agar vanillin microcapsule (x10)

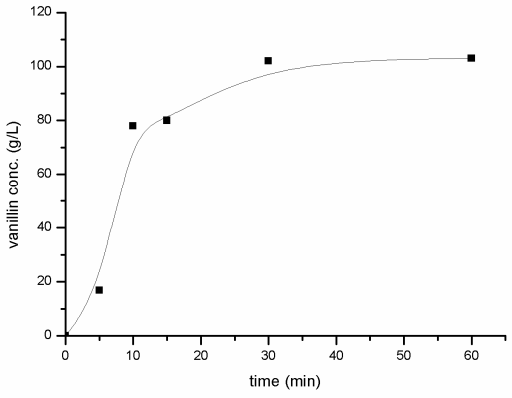

Fig 2. Typical release data of vanillin from agar microcapsules in ethanol (96%).

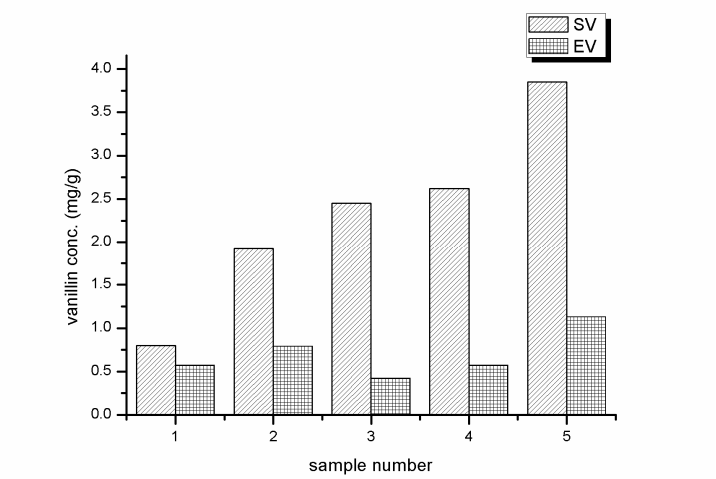

Fig 3. Surface vanillin (SV) vs entrapped vanillin (EV) for agar beds obtained from agar 1.5% aqueous solution.

In figure 3 are presented the results obtained for surface vanillin and entrapped vanillin for agar microparticles obtained starting from an aqueous solution of 1.5% agar. One can observe that the surface vanillin is in a greater amount than entrapped vanillin for all the samples tested. The average of surface vanillin concentration, expressed as mg/g wet microcapsules, is 75% and only 25% is the entrapped vanillin, which explains very well the burst effect which is observed in a typical release experiment. The next experiment was performed starting from a solution of 3% agar and using the same concentration of synthetic vanillin (2.5 g/L) in aqueous solution of ethanol (96%). In this case, from figure 4, one can observe that the entrapped vanillin is increasing and for some samples is greater than surface vanillin which is desired for a sustained release. The average of surface vanillin concentration, expressed as mg/g wet microcapsules, is 50.5 % and 49.5 % is the entrapped vanillin. In this case, even the burst effect is also observed, the amount of entrapped vanillin is greater and this assure a longer period for vanillin release in surrounding medium.

CONCLUSIONS

Control release is in the most cases the aim of encapsulation of flavors and others substances. To have a constant release rate is necessary to have entrapped a significant amount of active ingredient. If the flavor is in the greater quantity disposed on the particles surface it will determine a burst effect which is not desired.

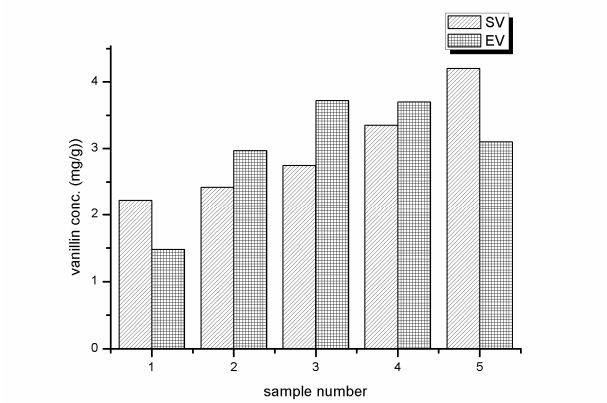

Fig 4. Surface vanillin (SV) versus entrapped vanillin (EV) for agar beds obtained from agar 3% aqueous solution.

In this case, the capsules formulation must assure that the greatest quantity of flavor is entrapped substance. The experimental study presented here pointed out that, using agar as encapsulation medium, vanillin can be used as model flavor. The microcapsules were matrix type and in the case of 1.5% agar solution used to obtain capsules the greatest quantity of vanillin is adherent on the surface. This leads to the burst effect in the first 10 minutes of a release experiment. In the case in which the initial agar solution is more concentrated (3%) the burst release diminishes and the percent of entrapped vanillin is greater. This proves that the microcapsules formulation can influence the control release. Better results are expected using reservoir type capsules, in which the core of active substance is surrounded by the a coating agent.

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EFFECT OF AUXINS ON *IN VITRO* RHIZOGENESIS AND SUBSEQUENT *EX VITRO* ACCLIMATIZATION OF *ARNICA MONTANA*

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ABSTRACT

Micropropagation requires not only stable shoot multiplication, but also successful rooting of plantlets. The effect of auxins on in vitro rooting of A. montana plants was studied. For root induction, half-strength Murashige and Skoog (1962) media (MS) with different types of auxins: NAA (α-naphthalene acetic acid), IAA (indole-3-acetic acid) and IBA (indole butyric acid) at concentrations 0; 0.5; 1 and 2 mg/l were used. The best rooting efficiency was achieved on medium supplemented with 0.5 mg/l IBA (100%). The results revealed that MS medium containing this auxin gave the best results for all parameters. A maximum number of roots/plant (5.0) with mean length (1.5 cm) was obtained after four weeks of culture. The effect of three mixture substrates during acclimatization stage was also studied. Best hardening response (90%) was obtained in peat: perlite: coco (2:1:1 v/v/v) after six week of transplantation in ex vitro conditions. The micro cloned plants with average length from 1.5 - 2.0 cm and average length of roots 1.0 - 1.5 cm were most suitable for acclimatization. The survival rate was 85 %. These hardened plants were subsequently shifted in glass house. A simple method in this study has facilitated the successful transfer of plants from in vitro to ex vitro conditions.

Key words: *Arnica montana*, auxin, root formation, ex vitro acclimatization, hardening response.

INTRODUCTION

Arnica montana L. (Asteraceae) is a valuable medicinal plant species, spread in various regions of Europe (Delabays and Mange, 1991). The flower heads of *A. montana* contain sesquiterpene lactones, exerting cardiotonic and cardiotoxic effects, flavonoids of antimicrobial and antirheumatic effects and essential oils in the roots and rhizomes with antiseptic effects (Nichterlan, 1995; Ganzera et al., 2008). The constant reduction of *A. montana* populations requires urgent endeavor to protect it in order to renovate and enlarge the species area. *A. montana* is law protected in many countries. Development of biotechnological methods such as micropropagation would allow solution of the problem. *In vitro* technique was estimated in order to achieve mass production of shootlets in a short period. The success of the techniques of multiplication and rooting of *A. montana* plants could be influenced by various factors, plant growth regulators and physical conditions being the most important ones (Daniel and Bomme, 1991; Conchou et al., 1992; Malarz et al., 1993; Le, 1998, 2000; Zapartan and Daliu, 2001; Butiuc-Keul and Deliu, 2001). Many of the studies make only brief mention of the rooting of the shoots produced *in vitro*. For rooting stage, the media triggering root formation in *A. montana* are often modified through addition of plant growth regulators. Conchou et al.. (1992) recommended to use NAA (0.1 mg) and Le (1998, 2000) IBA (1.23 µM) into ½ MS. Basic MS medium

containing pumpkin extracts (1 ml/l) and IBA (1 mg/l) was quite efficient to induce root formation and high growth rates (Butiuc-Keul *et al.,* 2002). Malarz et al. (1993) and Weremczuk-Jeżyna and Wysokińska (2000) obtained rooting of shoots in MS medium without addition of growth regulators. The rooted plantlets were then transferred to the greenhouse or to the field where they continued to grow up with a survival capacity varying from 50 to 95% depending of the used rooting media (Zapartan and Deliu, 2001; Butiuc-Keul and Deliu, 2001).

The objective of this study was to develop an efficient method for high frequency root induction and root growth, followed by identification of *ex vitro* conditions.

MATERIALS AND METHODS

Plant material: The micropropagated shoots of *A. montana* (seeds from the Carpathian Mountains, Ukraine) were used as plant materials for *in vitro* rooting and *ex vitro* acclimatization. For root initiation, newly developed *in vitro* shoots (1.5 - 2.0 cm) were excised individually from proliferating cultures and transferred to rooting media.

In vitro rooting of microshoots: Half-strength Murashige and Skoog (1962) (MS) medium containing vitamins, 2% sucrose and solidified with 0.6% agar was used. Indole-3-butyric acid (IBA), α-Naphthalene acetic acid (NAA) and Indole-3-acetic acid (IAA) were the different auxin sources. Several types of rooting media were tested, MS0 medium without auxin (control) and MS media supplemented with IBA, NAA or IAA. The effect of different concentrations of auxins (0.5; 1.0 and 2.0 mg/l) on rooting was studied (Table 1). The plants were cultured vertically in the glass tube (150 x 20 mm), containing 8 ml of rooting medium.Two shoots per culture tube were inoculated and each treatment involved 40 plants.The treatments were repeated two times. Data as percentage of root formation, number of roots per plant and root length were recorded after four weeks of culture. The data were statistically analyzed using Sigma Stat computer package (Sigma Stat 3.1, Systat Software, San Jose, California, USA).

Ex vitro acclimatization of plants: After *in vitro* rooting, the plantlets with newly formed roots were taken out carefully from the culture vessels and washed with tap water to remove agar sticking to the roots. The plantlets were transferred to small plastic pots (6 cm diameter) containing different peat mixtures under *in vivo* conditions for acclimatization. Three types of mixtures were used: Mix1 – peat : coco (2:1 v/v); Mix2 – peat : perlite (2:1 v/v) or Mix3 – peat : perlite: coco (2:1:1 v/v/v). To maintain high humidity, the pots were covered with transparent polyethylene. After two weeks, polythene covers were removed in order to acclimatize the plants to *ex vitro* conditions. Twenty plants were used for each treatment. In our second experiment, the plants were classified according to shoot hight and root length before acclimatization as short (1.0-1.5 cm and 0.5-1.0 cm, respectively), medium (1.5–2.0

cm and 1.0-1.5 cm, respectively), tall (2.0–2.5 cm and 1.5-2.0 cm, respectively). The plants were placed into peat : perlite : coco (2:1:1 v/v/v). The percentage of surviving plants was determined after four weeks. The plants were maintained in the growth room at 24±1 °C and 90% relative humidity. The potted plants were transferred in glasshouse for further acclimatization after six weeks and were planted into garden soil conditions.

Culture conditions: The pH of rooting media was adjusted to 5.6 using 0.1N NaOH or 0.1N HCl before gelling with agar. The media were autoclaved at 121 °C for 20 min for sterilization. All cultures were incubated under controlled temperature (22±2 °C) and 16 h photoperiod with light intensity of 40 µM $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

RESULTS AND DISCUSSIONS

In vitro rooting of A. montana plants: Induction of rooting is an important step for *in vitro* plant propagation. In our previous study, the multiplication of *A. montana* with BAP (1 mg/l) and IAA (0.1 mg/l) yielded normal plants suitable for *in vitro* root formation and *ex vitro* acclimatization stages (Petrova et al., 2005), but these stages were poorly studied. For *in vitro* root initiation, the microshoots derived from multiplication stage were separated and transferred to half strength MS hormone-free media or supplemented with three different concentrations (0.5; 1.0 and 2.0 mg/l) of the auxins IBA, NAA or IAA (Table 1). Root initiation began after 10-12 days of culture. Maximum growth of roots occured in the fourth week of culture. The half-strength MS medium without auxin showed low root induction and the lowest number of roots (1.2) compared to all tested auxins. In contrast, root induction was observed when multiple shoots were cultured on media with different concentrations of auxins. Presence of 2% sucrose in ½ MS with either auxin was found to be suitable for *in vitro* rooting.

1. Effect of Indole Butyric Acid (IBA) on rooting of A. montana: Data presented (Table 1) clearly show the effect of auxin IBA on the *in vitro* rooting of *A. montana* plants. Maximum rooting (100%) was observed on the ½ MS medium supplemented with all tested concentrations of IBA. The best value of root number per shoot (5.0) was obtained on ½ MS medium with 0.5 mg/l IBA (Table 1, Figure 1a). The mean number of roots per shoot decreased with increasing IBA concentration. The roots were greenish, unbranched and normal in appearance. The root lengths ranged from 1.5 to 2.4 cm depending on IBA concentration. Of the various auxins supplemented, IBA is more effective in promoting root induction than NAA or IAA. Le (2000) reported induction of adventitious roots on ½ MS medium containing IBA (1.23 µM). Butiuc-Keul et al. (2002) obtained highest rooting on basic MS medium supplemented with IBA (1 mg/l) and pumpkin extracts (1 ml/l). In contrast, Weremczuk-Jeżyna and Wysokińska (2000) achieved rooting of shoots in MS medium without the addition of the auxin.

Table1 Effect of different types of auxins in ½ MS medium on rhizogenesis of *A. Montana*

Type of auxin	Concentration of auxins, mg/l	Rooting plants, %	Number of roots per plant (x ± SE)	*Root length, cm (x ± SE)*
Control	0	50	1.2±0.30	*1.3±0.18*
IBA	0.5	100	5.0±0.16	*1.5±0.11*
	1	100	3.7±0.38	*1.8±0.14*
	2	100	3.5±0.31	*2.4±0.38*
NAA	0.5	100	4.1±0.28	*1.8±0.15*
	1	90	3.8±0.27	*2.0±0.22*
	2	90	2.2±0.34	*2.4±0.42*
IAA	0.5	85	2.5±0.41	*1.0±0.15*
	1	80	2.1±0.44	*1.3±0.34*
	2	*70*	*1.9±0.37*	*1.8±0.37*

2. Effect of Naphthalene Acetic Acid (NAA) on rooting of A. montana: The data related to the effect of the auxins on rooting of *A. montana* showed that rooting percentage is high (100%) when the shoots were cultured on ½ MS supplemented with 0.5 mg/l NAA. The average number of roots per plant was 4.1 and average root length was 1.8 cm (Figure 1b). When 1mg/l or 2 mg/l NAA were used, 90% rooted plants with average number of roots 3.8 and 2.2, respectively, were recorded (Figure 1b). Root number was affected also by NAA concentration. Increasing NAA concentration decreased gradually number of roots. On the other hand, root length increased with increasing NAA concentration. Root length ranged from 1.8 to 2.4 cm. After treatment with NAA no significant differences in shoot growth were observed. During four weeks of culture, roots were light green and thicker. Butiuc-Keul and Deliu (2001) found that MS medium containing NAA (5.3 µM), 2iP (5.0 µM) and maize extract (1.0 ml/l) resulted in best root induction.

3. Effect of Iindole-3-Acetic Acid (IAA) on rooting of A. montana: The effect of IAA on *in vitro* root formation of *A. montana* were estimated as root percentage, root number and length (Table 1). Of the tested IAA concentrations, the best treatment for *in vitro* rooting was ½ MS with 0.5 mg/l IAA yielding the highest values of root formation (85%) with highest number of roots per shoot (2.5) (Figure 1c). The results show that the addition of IAA (1 and 2 mg/l) led to lower percentage of rooting (80% and 70%, respectively) compared with the culture medium containing 0.5 mg/l IAA. The root length ranged from 1.0 to 1.8 cm. IAA at high concentration (2 mg/l) resulted in the highest root length (1.8 cm). The *in vitro* grown shoots cultured in all media containing IAA gave thin roots, which teared easily from basis of plant when were transffered in adaptation conditions.

Acclimatization of plants under ex vitro conditions: The effect of different mixture substrates on the survival rate of *A. montana* plants during *ex vitro* acclimatization was examined (Table 2).

Table 2. Effect of different mixture substrates on survival rate of *A. montana* plants during acclimatization

Mix	Different peat mixture	Number of transplanted plants	*Survival percentage of plantlets*
M1	Peat: Perlite (2:1 v/v)	30	*70*
M2	Peat: Coco (2:1 v/v)	20	*65*
M3	*Peat: Perlite: Coco (2:1:1 v/v/v)*	*20*	*90*

It was observed that the tested mixtures had significantly beneficial effect on survival rate and growth of the plants. The data in Table 2 show that the highest percentage of plant survival was obtained after transplantation to peat, perlite and coco mixture (2:1:1 v/v/v). During adaptaion, plants with the greatest number of leaves were observed when M3 mixture was used, where the survival percentage was 90% (Figure 1d, 1e and 1f). The acclimatized plants were of proper growth and development, when M1 mixture of peat and perlite (2:1) was used. In order to maximize the survival of *in vitro* derived plants, it is routine practice to acclimatize them under high levels of relative humidity. In the present study *in vitro* grown plants were acclimatized in humid environment. In the first and second weeks of transplantation the plants were covered with a transparent polythene for providing high humidity and sufficient light. When new leaves developed the polythene was removed. The high humidity and moderate temperature enhanced the initial survival of potted grown plants. A method adopted in this study has facilitated the successful transfer of plants from *in vitro* to *ex vitro* conditions. The rate of successfully acclimated rooted plants was influenced by both shoot and root size. At the time of rooting, shoot height on ½ MS media supplemented with studied auxins ranged between 0.5 and 2.5 cm and root length varied between 0.5 and 2.0 cm for four weeks. The rooted plants of 1.5-2.0 cm height and root length with 1.0 -1.5 cm gave 85% successful survival (Table 3). These plants were more suitable for adaptation. Only 46.1% of the plants with 2.0-2.5 cm height and 1.5-2.0 cm root length survived. Acclimatizated plants were successfully transferred in garden soil conditions. The first growing season is critical for *in vitro* obtained plants and they grew slowly. About 60% of the plants could tolerate and survive under soil conditions. The plants grow normally after overcoming the stress of change in growing conditions, and the second year of development they start to flowering (Fig. 1g).

Table 3. Acclimatization of *A. montana* in depending from size of plants and roots.

Glassified groups of plants	Heigth of plants, cm	Length of roots, cm	Number of transplanted plants	*Survival of plantlets, %*
G1 - Short	1.0-1.5	0.5-1.0	30	*66.7*
G2 - Medium	1.5-2.0	1.0-1.5	40	*85*
G3 - Tall	*2.0-2.5*	*1.5-2.0*	*26*	*46.1*

Note: Peat: perlite: coco (2:1:1)

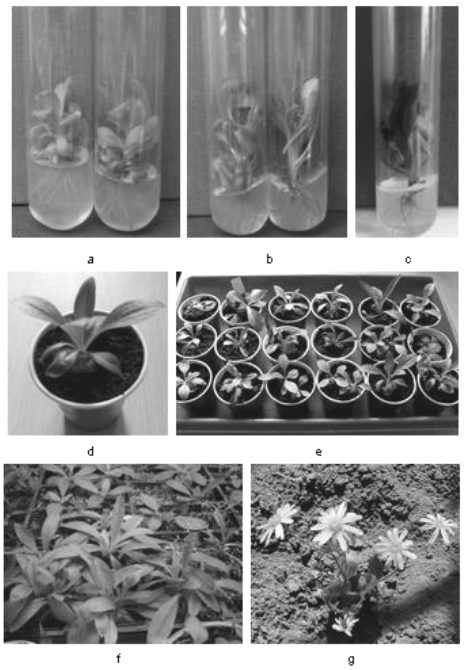

Fig 1. *In vitro* and *in vivo* cultivation of A. montana: a) *In vitro* rooted plants at 0.5 mg/l IBA; b) *In vitro* rooted plants at 0.5 mg/l NAA; c) *In vitro* rooted plants at 0.5 mg/l IAA; d), e) and f) *Ex vitro* acclimatized plants; g) *A. montana* during flowering stage.

CONCLUSION

IBA was found to be the best auxin for rizogenesis of *A. montana*, followed by NAA and IAA. The optimum rooting efficiency for shoots (100%) as well as the best root number per shoot (5.0) were obtained on ½ MS medium supplemented with 0.5 mg/l IBA. Relatively low concentration of IBA promoted both the frequency of shoots forming roots and the number of roots/shoot. Adding NAA to the nutrient medium yielded the longest roots and shoots. The data suggest that different mixture substrates had favourable effect on the survival of plants during acclimatization. The

in vitro grown plants of 1.5-2.0 cm height and 1.0 -1.5 cm root length were preferable for *ex vitro* acclimatization. The developed method in this study has facilitated the successful transfer of 90% of plants from *in vitro* to *ex vitro* conditions.

ACKNOWLEDGMENTS

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SECTION V: FOOD SAFETY

SENSORY ANALYSIS FOR ORGANIC FOOD PRODUCTS

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ABSTRACT

Food nutritional quality and safety levels vary widely around the world. One of the major challenges is reaching these goals, for the near future. The present study covers two principal aspects of research on organic food: the safety of organic foods and the differences between the nutrition profile of conventionally and organically produced foods. In the last decades systematic research has been developed in the field of sensory analysis on food quality control and assessment. Sensory, physical, chemical, physicochemical and biological methods are used to determine food quality. Sensory qualities such as taste, odour and colour, consistent with a particular emotional tone determine food consumption behaviour in consumers. They represent the first contact with the product and they are determinants of food choice. Thus the current study presents the results of sensory analyses conducted on both organic and conventional food products in order to present the role played by all these determinants in the choice for organic food on the Romanian market.

Keywords: organic food, food quality, sensory analysis

INTRODUCTION

Interest in organically produced food is increasing throughout the world in response to concerns about intensive agricultural practices and their potential effect on human health as well as on the environment**.**

Sensory food science has evolved from the need for scientifically sound and systematic sensory evaluation of foods. Capitalising on its multidisciplinary nature, it has to accommodate many research traditions and interests. In the past 15-20 years the field has made substantial progress in developing new methods and approaches and in advancing our understanding of consumer responses to food products.

The importance of sensory food science is based on the relevance of consumer perceptions to the acceptance and commercial success of foods and on the significance of food for human well-being and health. In food companies, sensory food science can be of great value to both tactical and strategic research goals.

The consumer gives priority to sensory qualities when making food choices. Food products that offer pleasant sensations are kept in consumption because only by thinking of their sensory qualities, the consumer gets an appetite. This abovementioned attitude is explained by the fact that the first consumer contact

with food is of sensorial nature. The consumer evaluates the food product according to flavour, taste, colour, consistency, thus becoming determinants in the food choice.

After five years of research, developed in six European countries (Germany, Austria, Belgium, France, Netherlands, Switzerland), CEY-Bert Institute in Geneva presented a series of data, called "new food trends", where the importance consumers give to sensory qualities is highlighted. The studies developed established that sensory qualities, especially olfactory and taste, have a great influence on consumer appetite and food consumption.

Sensory analysis is a scientific method that involves an array of tests and techniques, statistics and orientations to present sensorial results (Banu, 2007; Bordei, 2004, 2007). Sensory analysis implies a multidisciplinary approach i.e. neurophysiology, physiology, psychology, statistics, sensorial evaluation and market studies for researching the mechanism for sensorial perceptions, the effect of physiological differences on perception, the effect of stimuli concentration and composition on perception, and the effect of sensory and non-sensory qualities of products on consumer preferences.

The hedonic approach to sensory analysis implies studying consumer acceptability of a food product by assessing pleasure caused by tasting or consuming that particular product (Lawless, H., Heymann, H. 1999).

The analytic approach to sensory analysis includes techniques for measuring sensory qualities of different food products. This approach may be useful to trace a product's sensory qualities in time, in order to compare different products to verify their conformity to specifications (Bulancea & Iordachescu, 2006; Machado, 2005; Majou & Touraille, 2001; Meilgaard, 2006; Stone & Sidel, 2004; Szabo, 2005). Thus for the current study, the analytic approach is chosen because the health benefits of consuming organic compared to conventional foods are unclear.

MATERIALS AND METHODS

The standard *STAS 12656-88: Food products. Sensory analysis. Methods with scales.*was chosen to differentiate between sensory qualities of conventional versus organic products.

The 16 evaluators were selected based on a questionnaire. The recruiting criteria were sensorial testing skills, eating habits and health problems. Out of the 16 evaluators, ten persons were experienced evaluators in sensory analysis. Eight samples were codified and prepared in the same way, with the quantity and in similar plates for all evaluators.

Each evaluator filled a questionnaire, by scoring each sensorial attribute assessed in the study. Moreover, evaluators chose the preferred sample on a second questionnaire, in order to differentiate between the organic and conventional samples.

Depending on the product analysed, the exterior appearance, the colour on the outside and in section were examined. The consistency was analysed on the outside and in section with the tactile and visual analysers, and through mastication. The smell was evaluated with the olfactory analyser, by simple inhalation, repeated inhalation or by more profound inhalation, on samples with more faint smells. The taste was analysed with the tasting analyser, by tasting the product samples.
These methods allow obtaining the full sensory description of a sample, the identification of hidden ingredients and the determination of the most important product sensory characteristics.
Each evaluator scores every sensory characteristic individually P_i. The average score (P_m) represents the arithmetic mean of the evaluation results of a characteristic point by a group of evaluators. Importance factor (f_i) is the factor that indicates how each organoleptic feature weights in the quality of a product. The sum of all importance factors is always equal to 1. Conversion factor (f_t) is the factor by which we pass from the 5-point scale to the 20-point scale to determine the organoleptic quality of the product. Conversion factor is always equal to 4. Weighting factor (f_p) is the factor resulting from the multiplication of the importance factor with the conversion factor ($f_p = f_i \times f_t$). The weighting average score (P_{mp}) is the result of multiplying the average score of a feature, given by a group of evaluators, with the importance factor and the conversion factor or directly with the weighting factor. The average total score (P_{mt}) is the sum of the weighted average scores in all organoleptic characteristics.

RESULTS AND DISCUSSIONS

Sensory analysis of organic and conventional dairy products

The sensory analysis results for sheep telemea cheese

For the dairy products category, the sheep telemea cheese has been chosen for sensorial examination, procured from SC ASI NATURE Ltd company in Sibiu, a certified producer of organic and traditional products.
The samples were coded as follows: XUM – conventional sheep telemea cheese, and WIP – organic sheep telemea cheese.

Table 1.1 Total average score for conventional sheep telemea cheese

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,800	0,8	0,20	4	3,8400
2	Section appearance	5,000	0,8	0,20	4	3,7872
3	Colour	4,400	0,8	0,20	4	3,5200
4	Consistency	4,534	0,8	0,20	4	3,6272
5	Taste	4,467	0,6	0,15	4	2,6802
6	Smell	4,600	0,6	0,15	4	2,7600
Total average score						**20,2146**

Table 1.2 Total average score for organic sheep telemea cheese

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,867	0,8	0,20	4	3,8936
2	Section appearance	5,000	0,8	0,20	4	3,8400
3	Colour	4,667	0,8	0,20	4	3,7336
4	Consistency	4,334	0,8	0,20	4	3,4672
5	Taste	4,534	0,6	0,15	4	2,7204
6	Smell	4,467	0,6	0,15	4	2,6802
Total average score						**20,3350**

After analysing the total average scores (see Tables 1.1 and 1.2), there can be observed that the two cheese samples are perceived as very similar and of a very good quality (see Figure 1.1). The organic sample has a slightly higher score (20,34 compared to 20,21) as it was appreciated more to some extent on section appearance (3,84 compared to 3,79), colour (3,73 compared to 3,52) and taste (2,72 compared to 2,68).

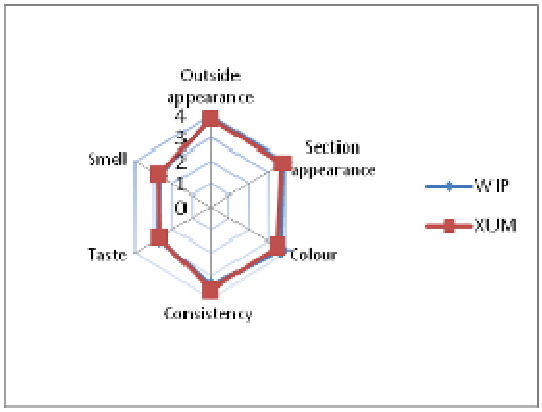


Fig. 1.1. Organic (WIP) versus conventional (XUM) telemea cheese on sensory qualities

From consumer preference point of view, 60% of the evaluators chose the organic sheep telemea cheese (see Figure 1.2).

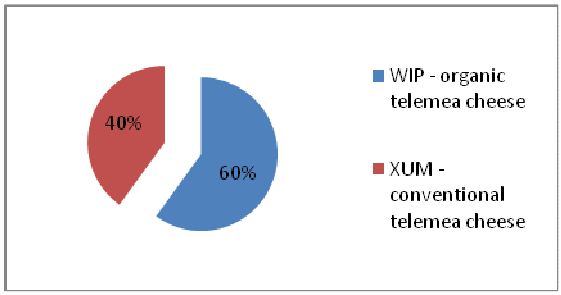


Fig. 1.2. Organic (WIP) and conventional (XUM) telemea cheese on consumer preference

Sensory analysis for organic versus conventional vegetables

Vegetables Research and Development Company - SCDL Bacau, has provided the study with three species of vegetables, considered representative, tomatoes, cucumbers and peppers, both organic and conventional. SCDL Bacau is registered with the Ministry of Agriculture and Rural Development on the list of organic farming producers since 2008. Tomatoes were chosen from the Venezia variety, peppers from the Vedrana variety and cucumbers from the Mirabelle variety.

Vegetables were coded to carry through sensory analysis, as follows:

- MFQ – conventional tomatoes and HAY – organic tomatoes;
- DRA – conventional peppers and EAI – organic peppers;
- KBV – conventional cucumbers and QAF – organic cucumbers.

Table 1.3 Total average score for conventional tomatoes (MFQ)

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,500	0,8	0,20	4	3,6000
2	Section appearance	3,000	0,4	0,10	4	1,1252
3	Colour	3,313	0,8	0,20	4	2,6504
4	Consistency	4,250	0,8	0,20	4	3,4000
5	Taste	3,125	0,6	0,15	4	1,8750
6	Smell	3,625	0,6	0,15	4	2,1750
Total average score						**11,2256**

Sensory analysis results for tomatoes

After analysing the total average scores (see Tables 1.3 and 1.4), there can be noted that the two samples of tomatoes were appreciated as similar (11,23 for conventional tomatoes and 12,64 for organic tomatoes). Nonetheless, the total quality of both products was weak, according to the total average score of only 11-12 out of 20.

Table 1.4 Total average score for organic tomatoes (HAY)

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,625	0,8	0,20	4	3,7000
2	Section appearance	3,000	0,4	0,10	4	1,2252
3	Colour	4,000	0,8	0,20	4	3,2000
4	Consistency	4,313	0,8	0,20	4	3,4504
5	Taste	3,813	0,6	0,15	4	2,2878
6	Smell	4,125	0,6	0,15	4	2,4750
Total average score						**12,6384**

The sample of organic tomatoes scored higher (see Figure 1.3) on colour (3,2 compared to 2,65), taste (2,29 compared to 2,65) and smell (2,48 compared to 2,18).

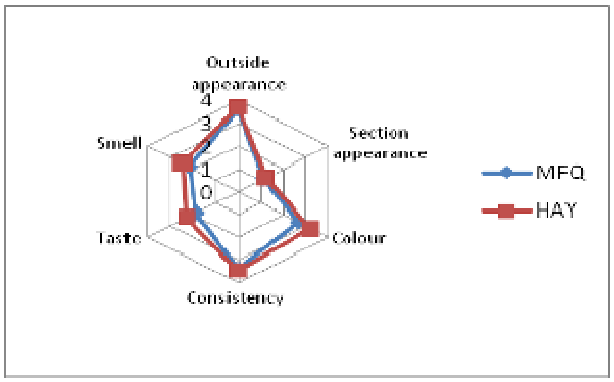


Fig. 1.3. Organic (HAY) versus conventional (MFQ) tomatoes on sensory qualities

From a consumer preference perspective (Figure 1.4), 81% of evaluators preferred organic tomatoes against conventional tomatoes.

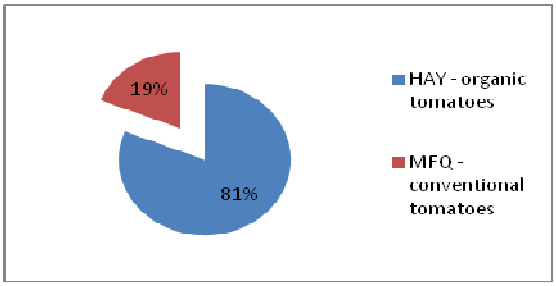


Fig. 1.4. Organic (HAY) versus conventional (MFQ) tomatoes on consumer preference

Sensory analysis results for peppers

After analysing the total average scores for conventional versus organic peppers (see Tables 1.5 and 1.6), there can be observed that the two samples were similarly scored, with rather close total average scores (18,55 for conventional peppers and 17,70 for organic peppers). The total quality for peppers under analysis is possible to consider good, having scored a 18 points of 20.

Table 1.5 Total average score for conventional peppers (DRA)

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,563	0,8	0,20	4	3,6504
2	Consistency	4,750	0,8	0,20	4	3,8000
3	Colour	4,813	0,8	0,20	4	3,8504
4	Taste	4,188	0,8	0,20	4	3,3504
5	Smell	4,875	0,8	0,20	4	3,9000
Total average score						**18,5512**

The samples received the same score for outside appearance, thus there is no significant difference on this characteristic (see Figure 1.5). The conventional cucumbers got a slightly higher score on consistency (3,8 compared to 3,45 for

organic sample), colour (3,85 compared to 3,65), taste (3,35 compared to 3,4) and smell (3,9 compared to 3,55).

Table 1.6 Total average score for organic peppers (EAI)

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,563	0,8	0,20	4	3,6504
2	Consistency	4,313	0,8	0,20	4	3,4504
3	Colour	4,563	0,8	0,20	4	3,6504
4	Taste	4,250	0,8	0,20	4	3,4000
5	Smell	4,438	0,8	0,20	4	3,5504
Total average score						**17,7016**

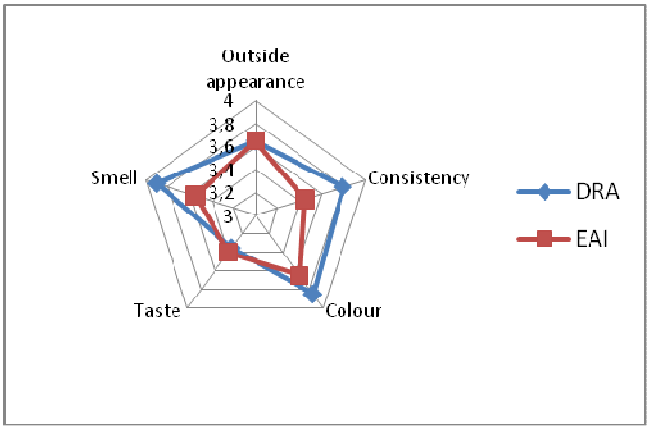
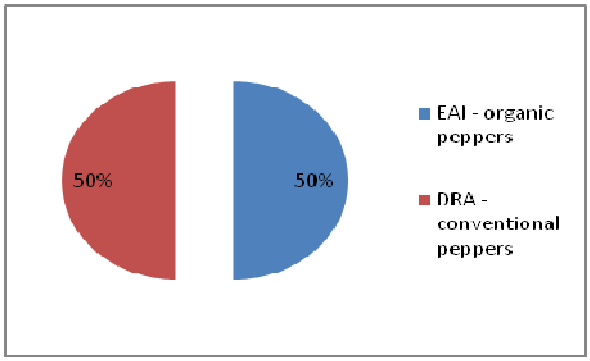


Fig. 1.5. Organic (EAI) versus conventional (DRA) peppers on sensory qualities

Fig. 1.6. Organic (EAI) versus conventional (DRA) peppers on consumer preference



When related to consumer preference (see Figure 1.6), the choices were split equally between the two variants of peppers. Thus these results are inconclusive.

Sensory analysis results for cucumbers

After analysing the total average scores (see Tables 1.7 and 1.8), there can be noticed that the organic cucumbers were evaluated similarly as the conventional cucumbers (17,64 for conventional cucumbers and 18,75 for organic cucumbers). The total quality of the cucumbers analysed can be considered good, with 18 points out of 20.

Table 1.7. Total average score for conventional cucumbers (KBV)

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,313	0,8	0,20	4	3,4504
2	Section appearance	5,000	0,4	0,10	4	1,8252
3	Colour	4,750	0,8	0,20	4	3,8000
4	Consistency	4,563	0,8	0,20	4	3,6504
5	Taste	3,500	0,6	0,15	4	2,1000
6	Smell	4,688	0,6	0,15	4	2,8128
Total average score						**17,6388**

The organic cucumber samples (see Figure 1.7) received highest scores for almost all organoleptic characteristics, with only a small difference in smell where they received a slightly lower score (2,7 compared to 2,8 for conventional cucumbers). More important differences can be observed on outside appearance (3,85 compared to 3,45), section appearance (1,93 compared to 1,83), and taste (2,78 compared to 2,1).

Table 1.8 Total average score for organic cucumbers (QAF)

no	Organoleptic characteristics	P_m	$f_p=f_i*f_t$	f_i	f_t	P_{mp}
1	Outside appearance	4,813	0,8	0,20	4	3,8504
2	Section appearance	5,000	0,4	0,10	4	1,9252
3	Colour	4,750	0,8	0,20	4	3,8000
4	Consistency	4,625	0,8	0,20	4	3,7000
5	Taste	4,625	0,6	0,15	4	2,7750
6	Smell	4,500	0,6	0,15	4	2,7000
Total average score						**18,7506**

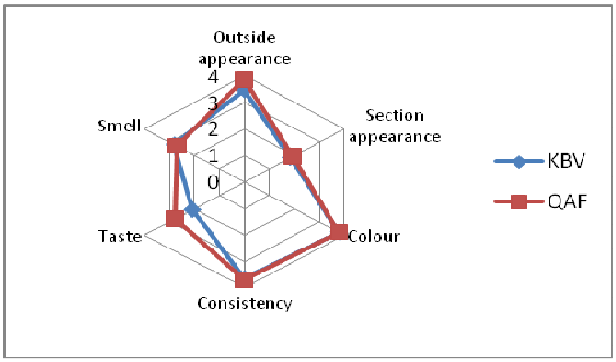


Fig. 1.7. Organic (QAF) versus conventional (KBV) cucumbers on sensory qualities

As preferred choice, evaluators chose in a proportion of 100% the organic cucumbers.

CONCLUSIONS

After conducting the sensory analysis on sheep telemea cheese and the three vegetables (tomatoes, peppers and cucumbers), with both organic and conventional samples, the following can be concluded:

- from the point of view of outside appearance, section appearance, smell and taste, both organic and conventional sheep telemea cheese samples scored very close to each other;
- the organic cheese variant scored slightly better on colour, while the conventional variant scored rather more on consistency, but from consumer preference perspective, the organic sheep telemea cheese received 60% of the evaluators' votes as preferred choice;
- the samples of organic and conventional tomatoes got similar scores on outside appearance, section appearance and consistency, while organic tomatoes scored considerably better on smell, taste and colour;
- scoring 12 points out of 20, the samples of tomatoes were assessed as having a poor quality in both organic and conventional variants;
- 81% of the evaluators opted for organic tomatoes;
- conventional peppers scored well on smell, consistency and colour compared to organic peppers, while the pepper samples were similar on outside appearance and taste;
- 50% of the evaluators chose organic peppers and 50% for conventional peppers, thus results are inconclusive for these samples;
- organic cucumbers received a better score on taste and outside appearance, while both cucumber variants were assessed similarly on taste, colour, consistency and section appearance;

• organic cucumbers were preferred in a proportion of 100%.

As a final conclusion, it can be inferred that organic food products are preferred from sensorial point of view (at least when talking about sheep telemea cheese, tomatoes and cucumbers), showing the organic food products' potential on the market from the perspective of sensory analysis.

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CONTROL OF MICROBIAL GROWTH AND ENZYMATIC BROWNING OF FRESH APPLE SLICES BY ESSENTIAL OILS SPRAYING

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ABSTRACT

The major obstacle of purchasing ready-to-eat fresh-cut fruits is their short shelf life, leading to quick spoilage and decomposition of the product and undesirable look or negative palatability. The purpose of this work was to study the shelf life of minimally processed apples, inoculated with S. cerevisiae, using E-2-hexenal for spraying. The results have showed that the antimicrobial effect depends on the essential oil application method (respectively slow release, spraying without or in presence of electrostatic field. The most effective method to apply the essential oil over the minimally processed apples was demonstrated to be electrostatic field spraying, which led to a reduction of 3-4 log units for the total plate count. Using E-2-hexenal for prolonging fresh apple slices shelf life has proved being an effective way to replace chemical additives. Electrostatic field improved the process effectiveness and that's why the dosage of essential oil applied is not so high to change the flavor and the intrinsic aroma compounds of the product.

Keywords: fresh apple slices, essential oils, electrostatic field spraying, shelf-life, color changes.

INTRODUCTION

The production of fresh-cut fruits is increasingly becoming an important task as consumers are more aware of the importance of healthy eating habits, and have less time for food preparation. Fresh-cut apple production has increased dramatically in recent years and further growth can be anticipated. Minimal processing operations like peeling, coring and cutting damage the tissue integrity of these products, resulting in cellular decompartimentalisation of enzymes and substrates and leading to various forms of biochemical deterioration such as browning, off-flavors and texture breakdown. Degradation also favors the growth of microorganisms due to the release of nutrients. [5]

The color of minimally processed food products is considered to be an important quality indicator. Undesirable color changes have been found to be one of the major problems in preserving cut fruits; they are due to oxidative reactions of phenolic compounds by polyphenoloxidase (PPO). Some alternative treatments to control browning have been developed, for example ascorbic acid has long been applied in combination with organic acids and calcium salts to prevent enzymatic browning and maintain fruit firmness [5]. The inhibitory effect of ascorbic acid is due to the reduction of the o-quinones to phenolic substrates generated by the action of the PPO enzymes [6].

Using essential oils for extending the shelf life of the minimally processed apples have proved being an effective way to replace chemical additives, provided

that the dosage of essential oil applied are not too high in order to change the flavor and the smell of the product. Different studies relieved that hexanal, (*E*)-2-hexenal, and hexyl acetate had a significant inhibitory effect against pathogen microorganisms frequently isolated from raw materials (*E. coli*, *S. enteritidis*, and *L. monocytogenes*) when inoculated in both model and real systems. In this last condition, these compounds, at the levels used (150, 150, and 20 ppm for hexanal, hexyl acetate, and (*E*)-2-hexenal, respectively), displayed a bactericide effect on *L. monocytogenes* and they exhibited significant extensions of lag phase of *E. coli* and *S. enteritidis* inoculated at levels of 10^4–10^5 CFU/g [2].

The main objective of this work was to study the shelf life of minimally processed apples, inoculated with *S. cerevisiae*, using E-2-hexenal by different application method, respectively slow release, spraying without or in presence of electrostatic field.

MATERIALS AND METHODS

SAMPLES PREPARATION

The work was carried out with commercial maturity apple fruits of the *Golden Delicious* variety. The apples were peeled; the seminal house and stems were removed. The apples were cut into slices and immersed for 10 minutes in 1% ascorbic acid in order to prevent the enzymatic browning. After the treatment with *trans*-2-hexenal, Golden Delicious apples sliced were packed in a big plastic container (1 200 g apples with 200 µl *trans*-2-hexenal /25 l container capacities).

ESSENTIAL OIL APLICATION METHODS

The essential oils application methods on the apple slices studied in this paper were slow release, using Whatman filter paper discs (Ø = 6 mm), spraying without or in presence of electrostatic field.

An electrohydrodynamic sprayer was used to spray, with different concentrations of *trans*-2-hexenal (dilution 1/10 in ethylic alcohol), the apple samples, wound-inoculated or non-inoculated with *S. cerevisiae*. The principle of this atomizer is based on the separately enter into the nozzle of the airflow which disintegrates liquid into droplets. High speed airflow passing through the annular area assures that the droplets are swept past the electrode and propelled outward from the orifice of the nozzle. When the particle oils pass through atomization zone, they get high moves charged, either corona charging or induction charging. The charge of the droplets increase with the high voltage applied and with the pressure of the air used, but this charge has a limit, which is function of electrical discharge in air.

METHODS USED FOR QUALITY ASSESSMENT

Physico-chemical indicators, such as pH, color, dry matter content, aw or titratable acidity and microbiological indicators, such as total aerobic count on Nutrient Agar and number of yeasts and moulds on Malt Extract Agar were

determined in order to establish the shelf life of apple slices treated with *trans*-2-hexenal.

COLOR MEASUREMENT

A HunterLab MiniScan XE Plus Spectrocolorimeter was used for measuring the remission (reflectance/transmittance ratio) for the full spectrum between 400 and 700 nm as well as at one wavelength. The tristimulus values of color CIE 1976 $L^* a^* b^*$, in terms of hue, saturation and brightness were computed from remission spectra. The L*a*b* color system is one of the uniform color spaces recommended by CIE in 1976 as a way of more closely representing perceived color and color differences. In this system, L* is the lightness factor; a* and b* are the chromaticity co-ordinates:

• L* (lightness) axis – 0 is black; 100 is white.

• a* (red-green) axis – positive values are red; negative values are green; 0 is neutral.

• b* (yellow-blue) axis – positive values are yellow; negative values are blue; 0 is neutral.

The degree of browning was analysed in terms of changes in L^* and a^* values [5].

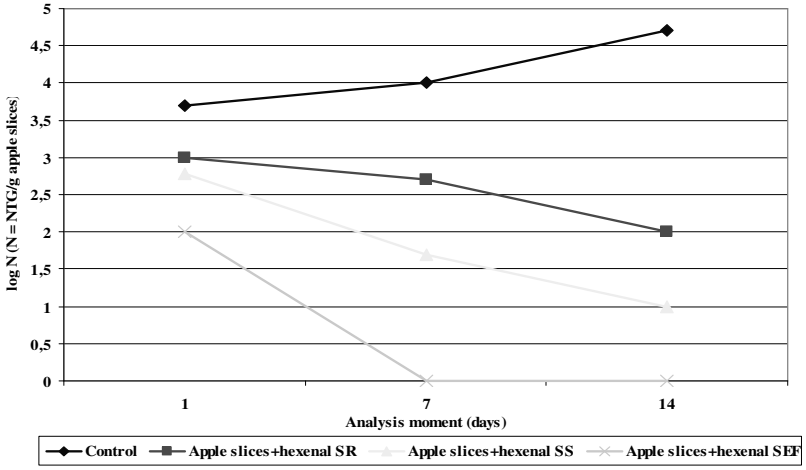
RESULTS AND DISCUSSIONS

The effects of *trans*-2-hexenal treatments by different application methods on the microbial population and on color retention of fresh sliced apples were evaluated.

Microbiological analysis results showed that the most efficient method of essential oil application on apple slices surface was electrostatic field spraying, which inhibited all microbial populations tested.

Essential oil application in slow release system or by spraying without electrostatic field leads to a significant reduction of microbial load for both total aerobic count and number of yeasts and moulds.

Initial number of total aerobic count was reduced, for electrostatic field spraying samples, with 1,5 log unit compared to the control, respectively with 4 log units after 7 days of storage at refrigeration temperature (fig. 1). Also the yeasts and mould number was initially reduced with 1 log, respectively with 3 log units after 7 days of storage at refrigeration temperature (fig. 2).



SR- slow release, SS - spraying, SEF- electrostatic field spraying

Fig. 1. Variation of total aerobic count for fresh-sliced apple treated with *trans*-2-hexenal through different methods of applications and storied at refrigeration temperatures

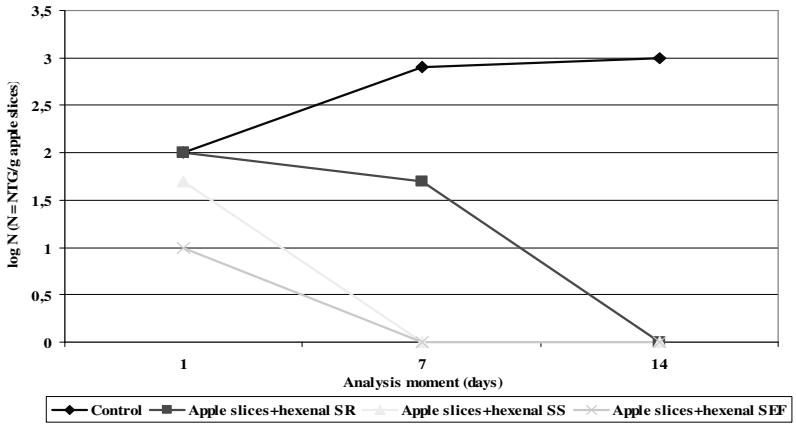
Trans-2-hexenal treatments of apple slices determined a significant extension of shelf life also when *S. cerevisiae* was inoculated at levels of 10^5 colony-forming units/g. Moreover, *trans*-2-hexenal led to a yeast selection favoring species having a reduced spoilage potential due to their prevalent respiratory activity.

The results of microbiological analysis showed that the presence of *trans*-2-hexenal sprayed in electrostatic field and storage at 4 °C totally inhibited mesophilic bacteria and considerably prolonged the lag phase of psychotropic bacteria (fig. 2).

After 14 days at 4°C following essential oil treatment no significant differences in *trans*-2-hexenal content or in sensory traits were detected by a trained panel between untreated and treated "Golden Delicious" apples. *Trans*-2-hexenal, which is a natural molecule characterizing apple aroma, did not affect fruit appearance, color, aw, firmness, dry matter content or titratable acidity.

The presence of these natural compound molecules, *trans*-2-hexenal, in the storage atmospheres considerably prolonged the lag phases of the inoculated yeast and reduced the growth potential of naturally occurring bacteria. Moreover, the addition of low levels of the *trans*-2-hexenal increased the color stability of the products up to 14 days.

Addition of *trans*-2-hexenal to minimally processed apple was also very effective in preventing browning reactions for at least 14 days at 4 °C. No significant changes in hue angle values were observed in samples treated with *trans*-2-hexenal, even after 14 days of storage at 4 °C (table 1).



SR- slow release, SS - spraying, SEF- electrostatic field spraying

Fig. 2. Variation of yeasts and moulds number for fresh-sliced apple treated with *trans*-2-hexenal through different methods of applications and storied at refrigeration temperatures

Tissue browning was evaluated by decreases in lightness (*L**) and increments in *a** values. The brightness indexes L*, describing apple darkness, represents freshness of product and shows intensity of fruit color; most of the Golden Delicious samples range from 47,82 to 56,94.

Table 1. L*, a*, b*, C* and h numeric values for fresh-sliced apple samples inoculated with *Saccharomyces cerevisiae* and treated with *trans*-2-hexenal through different applications methods

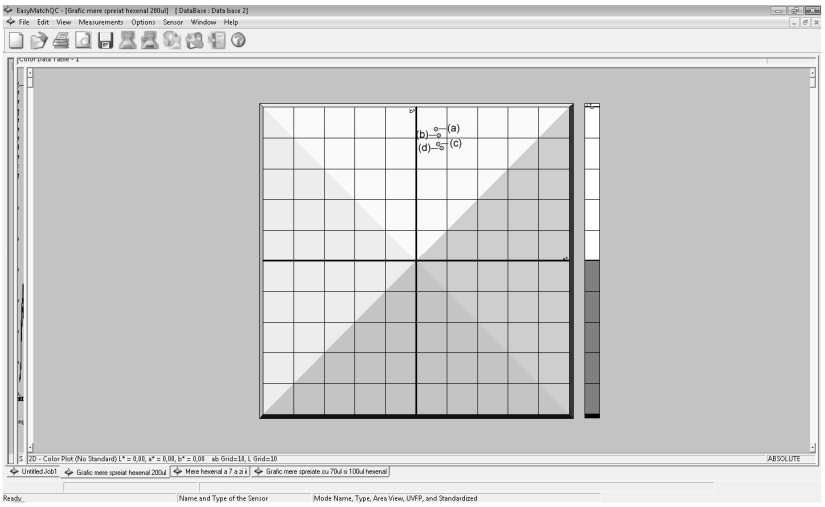
Nr. crt.	The analysis moment (days)	SAMPLE	L*	a*	b*	L*	C*	h
1.\	**Initial**	**C**	55.1	4.30	43.95	55.1	43.23	83.92
2.	After 3 days	C	55.3	4.69	43.57	55.3	43.82	83.86
		SR	51.66	7.59	36.71	51.66	37.49	78.31
		SS	55.51	5.51	41.77	55.51	42.13	82.49
		SEF	48.22	8.85	39.98	48.22	40.95	77.52
3.	After 5 days	C	56.77	7.53	37.83	56.77	38.58	78.75
		SR	50.4	8.39	37.4	50.4	38.33	77.36
		SS	53.29	6.31	35.01	53.29	35.57	79.79
		SEF	47.82	8.76	39.35	47.82	40.32	77.45
4.	After 7 days	C	56.94	6.59	42.97	56.94	43.47	81.28
		SR	56.37	7.54	40.81	56.37	41.5	79.53
		SS	53.24	7.29	38.02	53.24	38.71	79.14
		SEF	50.21	8.44	36.71	50.21	37.67	77.05

Note: SR- slow release, SS - spraying, SEF- spraying in presence of electrostatic field

The darkness of the apple increases each day. The change of this parameter as a result of storage or shelf-life depends on storage conditions or on the treatment applied. Keeping apple for a long time under refrigeration storage conditions involves further darkening and a large differentiation of brightness. Low value of L* for electrostatic field sprayed samples, during 14 days after essential oil treatment, indicates a dark minimally fruit sample.

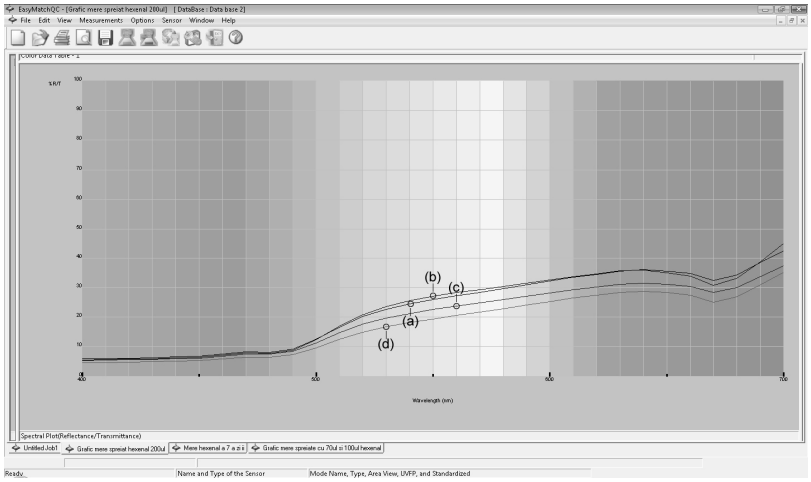
Graphic representation of remission values measured at different wavelengths in the range from 400 to 700 nm is expressed as a percentage (figure 4). This data were collect using a Universal Software V4.01 MiniScan^{TM} XE Plus Program and Hunter Lab MiniScan^{TM} XE Plus spectrocolorimeter.

Enzymatic browning occurring in apple processing should never reach an absorbance difference value higher than 0.15.



a – control; b – apple slices sprayed with *trans*-2-hexenal; c – apple slices sprayed in electrostatic field with *trans*-2-hexenal; d – apple slices treated with *trans*-2-hexenal by slow release

Fig. 3. Graphic representation for L*, a*, b* values for fresh-sliced apple samples inoculated with *Saccharomyces cerevisiae* and treated with *trans*-2-hexenal through different applications methods



a – control; b – apple slices sprayed with *trans*-2-hexenal; c – apple slices sprayed in electrostatic field with *trans*-2-hexenal; d – apple slices treated with *trans*-2-hexenal by slow release

Fig. 4. Graphic representation of remission values (%) function of lenghtwave for fresh-sliced apple samples inoculated with *Saccharomyces cerevisiae* and treated with *trans*-2-hexenal, measured at different lenghtwave, between 400-700 nm

Similar shapes and characteristic features of spectrum were obtained for all samples. The spectra present a blunt peak between 400-500 nm, related to the content of carotenoid pigments and an additional chlorophyll pigments peak at 670 nm, typical for Golden Delicious varieties.

CONCLUSIONS

The results presented showed that electrostatic field spraying of essential oils can be used to retain fresh-like characteristics, preserve sensory attributes and assure a reasonable shelf-life of apple slices.

The difference between electrical field spraying and simple spraying consisting of the amount of essential oil that reaches the product; in case of electrical field spraying a big quantity of substance reaches the product because the charged droplets go along the field lines to the product, so for the same effect a smaller amount will be applied. The essential oil quantity necessary for preservation of apple slices is very small and for a good spraying is necessary a good dilution. The distribution of the essential oil is more uniform on the surface of the product in the case of electrostatic spraying.

Estimation of fruit quality based on the L*a*b* system describing coordinates of color could be useful in connection with marketing, for monitoring consumer preferences and assessing the products after storage and during shelf-life. This system, if properly integrated into a marketing plan, could improve the appearance of fruit, making consumers more aware of true quality factors.

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DETERMINATION OF BIOCHEMICAL MARKER OF CAROTENE TYPE IN HARVESTED GREEN FODDER FROM BUCEGI AREA

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Key words: green fodder, carotene, electronic nose, sensory fingerprint

ABSTRACT

In this paper they were studied five samples of green fodder from the Bucegi area. There were collected from different pastures, samples D, C, A and T were collected from the sub-alpine pasture within the experimental field, and sample E was collected near the experimental field. It was followed the influence of methods for improving of sub-alpine pastures on a biochemical marker carotene type. Each sample of green fodder was analyzed from physico-chemical point of view and using a spectrophotometer AAnalyst 400, by flame atomic absorption at the ppm level, it was determined the metal content (Cu, Fe and Zn) for each sample. Also there were achieved experiments in order to obtain sensory fingerprint using e-Nose FOX 4000.

1. INTRODUCTION

Accordingly to Law no. 150/2004 concerning food safety and animal food, the word „traceability" is defined as „the possibility of identification and following, during all production, manufacturing and distribution stages of the animal food, of an animal for food production and of a substance to be included or which could included in a food product or in the food for animals"(Law no. 150/2004).

In this paper, we are looking just a part of the food product flow which can be obtained in summer grazing conditions on sub-alpine pastures, more precise the influence of different pasture increasing methods on the content of green fodder in β-carotene and metals.

The pastures of Bucegi area of a total ammount of about 3,200 ha, out of which 340 ha on lower level (1,600-1,800 m), 1,610 ha on sub-alpine level (1,800-2,200m) and 1,160 ha on alpine level (2,200-2,500m) are representatives for the mountain area of the Romanian Carpathians. Unfortunately, due to non-rational grazing, decades and even centuries, the grass with valuable fodder species was continously degrading, being now dominated by *Nardus strict* (Maruşca *et al.*, 2003).

The carotenoids may be considered potential bio-markers in nutritional and sensorial characterisation of milk product and for milk traceability as raw material. Despite the fact of existing a large range of carotenoids in plants, in fodder there are maximmum 10, the most important being, as quantity, the β-carotene and lutein. The sensitivity of β-carotene during rumination vary a lot dependeing of

feeding source. The bovines are achieving the highest concentration of carotenoids, mainly β-carotene, most probably due to the low efficiency of vitamine A synthetisation in enterocytes (Noziere *et al.*, 2006).

Due to their properties strongly anti-oxidante, some carotenoids have an anti-cancer potential and others help to the protection against cardiovacular and ocular diseases. The animal body restriction of the food with these pigments may lead to specific colour loosing (Internet, 2009). The biological content, the maturity study and the cow fodder conservation way are factors to influence the milk composition of fatty acids, vitamines and carotenoids (Lucas et al.,2006). Taking into account the fact that carotenoid molecules have a long chain of bonded double liaisons, those react very easy with the acids, the basis, the oxygen and the light, a reason for having a special care when low caretenoids content products are analysed (Schoefs, 2002).

2. EXPERIMENTAL SET-UP AND METHODS

2.1 EXPERIMENTAL SET-UP

For these experiments we have analyzed samples of green fodder from the Bucegi Blana area. This area is located at 1800 m altitude, on a low slop terrain. Primary vegetation was dominated by juniper (*Pinus mugo*) and glade of spruce (*Picea abies*), after their clearing it has installed a herbaceous vegetation dominated by *Festuca nigrescens*, *F. ovina* and *Agrostis rupestris*. After a period these grasses were invaded also by not valuable *Nardus stricta* species.

The samples A, C, D and T were collected from a improved subalpine pasture, situated in the Research Base Blana Bucegi , and the sample E has been collected from the area near of the experimental field. Each plot D, C, A has an area of 0.75 ha with a stocking rate of three cows that graze around 85 days during the summer period. The medium samples within A, C, D and T experimental fields were collected using three metal cages of 2 square meters for each plot. The table below shows the characteristics of the five types sub-alpine pasture where the studied samples have been collected.

2.2 METHODS

UV-VIS spectrophotometry**.** Carotene concentration of green fodder has been determined with UV-VIS 550 spectrophotometer. On each plot have been harvested medium samples of green fodder. The samples have been prepared for analysis to realise an optimal extraction. The carotene extraction has been realised with organic solvents (acetone, n-hexane) and β-carotene separation of the others carotenoids and coloured substances has been done by coloumn chromatography using a filling of activated aluminium oxyde and anhydrous sodium sulphate. The colouring intensity of β-carotene extract (BC) in hexane has been measured with spectrophotomer at 450 nm wave lenght, having as referance the n-hexane.

Tabel 1. Characteristics of experimental plots

Specification	Plot A	Plot C	Plot D	Plot T	Plot E
Type of pasture	natural pasture fertilized	natural pastures, limed, chemical fertilised, padocked	sown pasture,limed, chemical fertilised, padocked	natural pastures from the experimental field	natural pastures near the experimental field
Establishing works	Chemical fertilisation $N_{200}P_{50}K_{50}$ in 2000 year	Chemical fertilisation $N_{50}P_{50}K_{50}$ + liming at 2/3 A_h in 1995 year	Chemical fertilisation $N_{50}P_{50}K_{50}$ + liming at 2/3 A_h + reseeding of grassland in 1995 year	-	-
Maintenance works	- Chemical fertilisation $N_{150}P_{50}K_{50}$ in 2001 year - $N_{100}P_{50}K_{50}$ in 2002 year - $N_{150}P_{50}K_{50}$ in 2010 year	- Chemical fertilisation $N_{150}P_{50}K_{50}$ during 1996, 1997, 1998 - organic manure by padocking, 1 cow / 6 m^2 / 5 nights +100 kg /ha P_2O_5 during 2003 and 2009	- Chemical fertilisation $N_{150}P_{50}K_{50}$ during 1996, 1997, 1998 - organic manure by padocking, 1 cow / 6 m^2 / 5 nights +100 kg /ha P_2O_5 during 2002 and 2008	A rational utilisation has been applied during the 30 years.	-

The calibration curve has been done between 0 - 0.5 µg β-carotene/ml. The β-carotene content, measured in mg at 100 g of product has been computed with formula:

$$\beta\text{-carotene} = C \cdot V/10 \cdot m \quad [mg/100g]$$

where: C- concentration of β-carotene, red calibration curve, [ml]
V- extract volume of β-carotene in hexane, [ml]
m- sample weight, [g]

The methods sensitivity is of 0.2 µg β-carotene at 1 ml of extract

Atomic Absorption Spectrometry**.** The sample preparation has been done by mineralisation at MWS-2 microwave oven, with a mixture of nitric acide and perhydrol. For determination of Cu, Fe, Zn an AAnalyst 400 Perkin Elmer atomic absorbtion spectrophotometer with flame has been used (with air-acetylene) with corection of absorbtion (lamp D2).

The recommended wave lenght for determined elements are:

Cu = 324,8 nm;Fe = 248,3 nm; Zn = 213,9 nm;

The absorbtion of each element has been measured in standard recommended conditions (wave lenght, slot optimal distance (width, height), noise, specific concentration, corected specific concentration, estimated liniar area).

The concentration of analyte regarding the absorbtion has been determined, for each element, with the sampling curve:

absorbtion = f (concentration), done with the respective sampling solutions

Physico-chemical properties have been achieved by following standard methods:

- Moisture SR ISO 6496/2001
- Ash SR ISO 5984/1996
- Protein SR EN ISO 5983-2/2006
- Fat SR ISO 6492/2001
- Fiber SR EN 6865/2002

3. RESULTS AND DISCUSSIONS

Determination of beta carotene. In the below figure, the beta carotene concetrations are presented after analysing the studied green fodder.

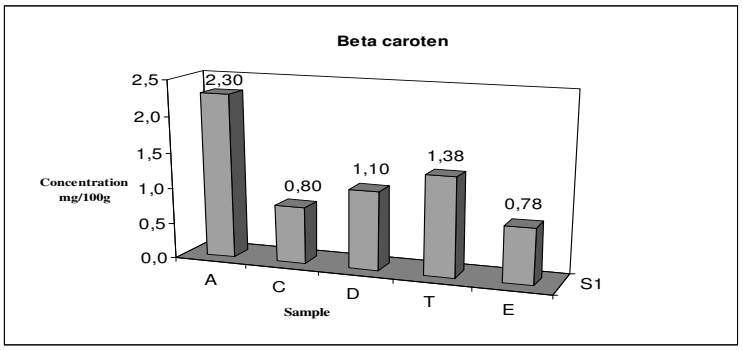

Fig 1. Concentration in β-carotene of the studied samples

Concentration of β-carotene at green fodder samples varied between 0.78 and 2.3 mg BC/100g. The minimmal values have been recorded in the case of sample E from a natural pasture closed to experimental area (0.78 mg BC/100g), respective sample C from an ammended natural meadow, fertilised and padocked (0.80 mg BC/100g).

The maximmal value has been recorded at sample A from natural meadow fertilised with chemical fertilisers (2.30 mg BC /100g).

The other considered samples (samples D and T) have closed values of β-carotene. 1.10 mg BC/100g has been recorded at sample D from a seeded and ammended meadow, fertilised and padocked. At sample T of the experimental area has been recorded a β-carotene concentration of 1.38 mg BC/100g.

Metal content (Cu, Fe, Zn). After metal content determination of each green fodder, the results are shown below:

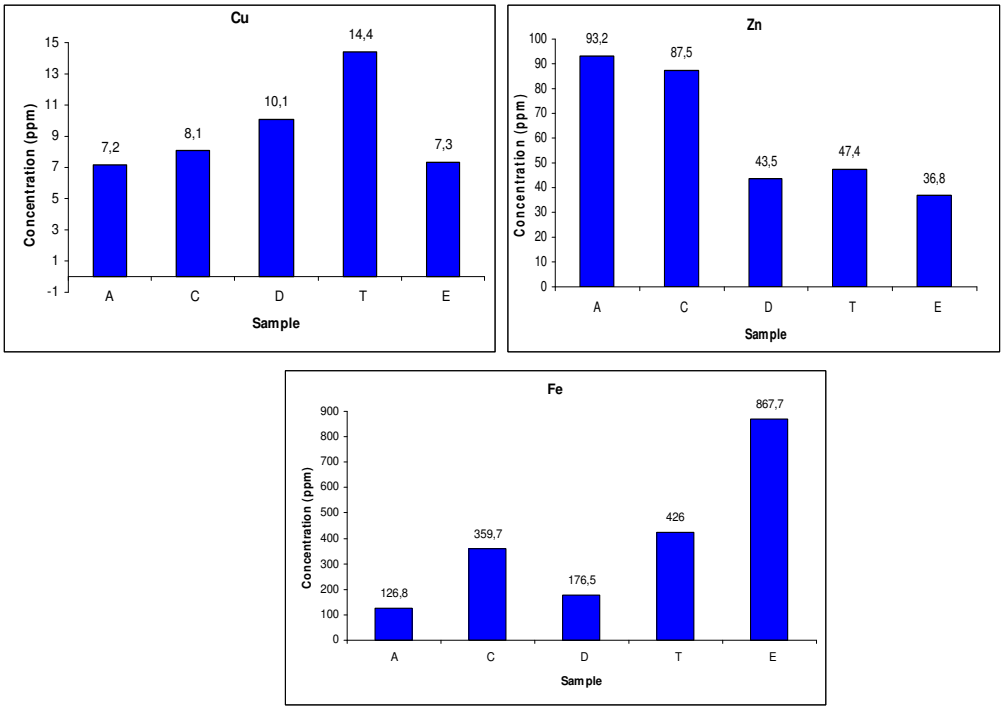

Fig 2. Metal content of green fodder

The metal content of fodders varies different as follows:

- Cu between 7.2 and 14.4 ppm
- Zn between 36.8 and 93.2 ppm
- Fe between 126.8 and 867.7 ppm

In the case of copper, the minimmal value has been recorded at sample A from a natural meadow fertilised with chemical fertilisers and the maximmal value has been recorded in the case of sample T from experimental area on a natural pasture.

The samples A and C have been recorded the highest content of Zn - 93.2 ppm and 87.5 ppm respectively. The iron (Fe), an element with major value in nutrition, has been recorded a big variation in the case of studied fodder samples, the biggest in the case of sample E (867.7 ppm).

Physico chemical caracterization. The studied green fodder samples has been physico-chemical analysed and the results are shown below:

Tabel 2. Physico-chemical characterization of samples

Sample	MOISTURE (%)	ASH (%) SU	PROTEIN (%) SU	FAT (%) SU	CRUDE FIBER (%) SU
A	57.30	**4.08**	12.40	0.44	32.07
C	65.93	2.73	10.59	0.44	34.91
D	63.61	2.49	10.95	**0.77**	**37.67**
T	67.80	2.07	12.22	0.26	36.31
E	62.19	2.81	**13.62**	0.55	28.97

The analysed fodder humidity varied between 57.30 and 67.80%. The studied fodder have a high protein content, which varies between 10.59 and 13.62 (the maximmal value has been recorded in the case of the sample from outside experimental area and the minimmal value has been recorded in the case of the sample from a natural meadow, ammended, fertilised and padocked). The analysed green fodder samples have a low fat content between 0.26 and 0.77% su. The analysed fodder content in fibers varied between 28.97 and 37.67% su.

Sensory fingerprint. The electronic nose (e-nose) FOX 4000 (Alpha MOS, France) is equipped with an automatic headspace sampler HS100 and with 18 metallic oxide sensors. Green fodder analyzed were homogenized**.** Due to specific reactions to various kind of molecules, e-nose sensors produce signals for specific odor compounds. Several experiments were conducted to obtain an optimum method.The analysis was performed using the same conditions at all the samples:

- ✓ Flow rate: 150 ml/min
- ✓ Sample quantity in the vial: 0.5 g
- ✓ Headspace incubation time: 180 s
- ✓ Headspace incubation temperature: 60 ^{0}C
- ✓ Syringe temperature: 65 ^{0}C

From each sample 0.5 g was taken and placed in a glass vial. Headspace was generated by heating and shaking vials containing homogenate sample.Using the syringe from the autosampler, a portion of the headspace of each sample was injected into the FOX and measured. Each sample was analyzed for three times. From each sample was obtain a sensor responses like in figure 4.

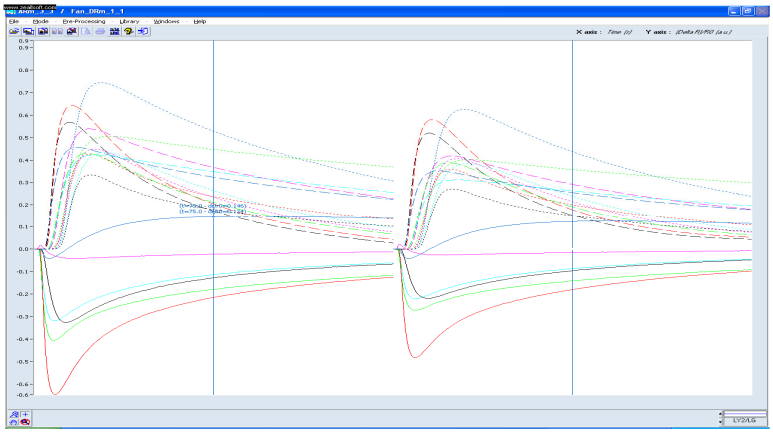

Fig 3. Sensors responses for sample A (left) and D (right)

The information collected during the analysis was used to build up a data base. They were then processed using discrimation analysis.The plots shows that using PCA (Principal Component Analysis) the system is able to differentiate the samples of green fodder collected from different pastures.The obtained index of discrimination was 74%.

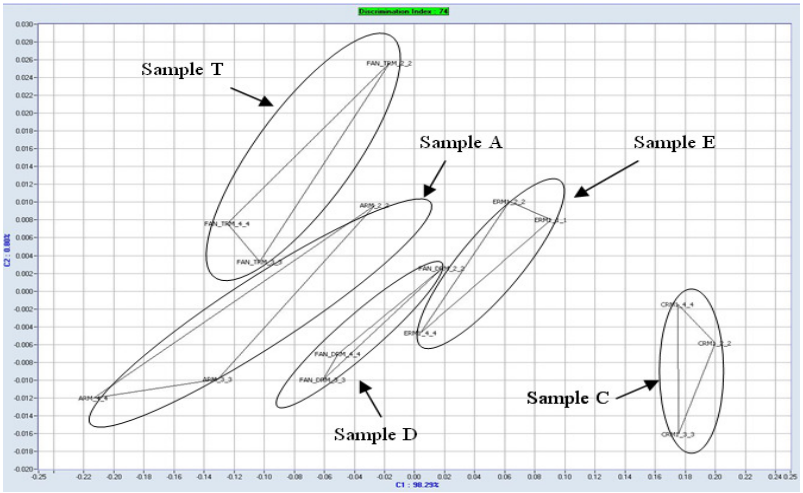

Fig 4. PCA discrimination of green fodder

4. CONCLUSIONS

1. The paper shows the partial results of the researches done to identify a biochemical marker of carotene type in fodder and then in milk, to improve the traceability on the total food chain.
2. In the study, samples of green fodder from Blana Bucegi area have been considered (on different plots). The influence of different methods of improving the meadow (concerning the content of green fodder in beta-carotene and metals) has been followed.
3. The recorded content in beta-carotene varied between 0.78-2.30 mg/ 100g and the maximmal value has been recorded at the sample from a fertilised natural pasture in 2000, 2001, 2002 and 2010.
4. Experiments have been done to obtain a sensorial fingerprint using the e-nose, and by processing the achieved data, a discrimination between fodder samples has also been done (using PCA).
5. The researches will continue to follow the β-carotene biochemical marker traceability in milk.

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AN OVERVIEW OF THE METHODS USED TO ESTABLISH THE RELATIONSHIP BETWEEN FEEDSTUFF AND COMPOSITION OF REGIONAL DAIRY FOODS

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ABSTRACT

In this review are presented different techniques and methods for separation and chromatographic analysis of some feedstuffs and regional diary products in order to establish a relationship between them. For the identification of the structures of different compounds the mass spectrometry method is most widely used.

The majority of sampling procedures for determination of biomarkers from plants are based on the introduction of the extract in the GC or HPLC columns.

Among the most important biomarkers we refer to carotenoids, terpenes, flavonoids, which are important factors determining the composition and flavor of dairy products that are linked to the diet of the animal producing the milk and influenced also by the seasonal changes.

Keywords: chromatographic methods, feedstuff, dairy products, biomarkers

The relationships between the origin of dairy products and the type of pasture was intensively highlighted by using popular and well-known analytical methods such as gas/liquid chromatography, isotope ratio mass spectrometry, olfactometry or chemical analysis [1, 2, 3].

Among the analytical methods used for volatile compound identification, important to establish the relationship between feedstuff and composition of regional dairy foods, we mention:

- headspace technique linked to a GC/MSD/FID system (gas chromatography / mass spectrometry / flame ionization detector) [4]
- steam distillation of milk and plant extraction (extractions made with diethyl ether) and analyzed with GC/MSD/FID) [5]
- high –performance liquid chromatography (HPLC)
- Fourier transform infrared spectroscopy (FTIR) may be a good supplement to mass spectra detect terpenes (it hepls to distinguish the isomers [6)

Solid-phase microextraction (SPME) coupled to gas chromatography–mass spectrometry (GC–MS) is a method which has as result identification of a great number of volatile compounds and flavour compounds of the dairy products

(cheese, yoghurt). This method gives useful information for establish the shelf-life of dairy products, being a rapid, simple and reproducible method.
This method is simply and efficient for terpenes analysis.

Gas chromatography coupled with mass spectrometry (GC-MS) is often used as an analytical method for analyzing the flavour of foods, but needs sample preparation.

Using dynamic head space-gas chromatography coupled to mass spectrometry, it was found that milk collected in highland regions were richer in sesquiterpenes than those collected in lowland regions [**Error! Bookmark not defined.**].

In the case of liquid chromatographic techniques, the stationary phase consists of a finely powdered solid adsorbant packed into a thin metal column and the mobile phase consists of an eluting solvent forced through the column by a high pressure pump. The mixture to be analyzed is injected into the column and monitored by a detector.

Terpene content and profile in milk and dairy products are influenced by feed and especially by grazed herbage. This relationship could be used to discriminate milk or cheese originating from grazing or not grazing system and to trace the geographical origin of these products, or production site.

Viallon et al. [7] have used firstly dynamic head space to extract monoterpenes and sesquiterpenes in milk fat, and then gas chromatography to separate these compounds collected from different cows fed successively with forage containing high and low amounts of terpenes. Their researchers have shown, on the one hand, that a modification of the plant species composition of forages strongly influences the proportions and quantities of monoterpenes and sesquiterpenes in milk fat and, on the other hand, that a rapid transfer of these compounds takes place.

An array of methods can be used for carotenoid analysis. The choice is usually guided by the type of information needed. Generally, the pigment must be extracted before analysis, sometimes from a complex matrix. Therefore, efficient extraction and analytical protocols are requested.

Chromatographic methods are the traditional methods to separate and quantify the carotenoids, which, structurally, are polyisoprenoid compounds.
A first step is the sample extraction, a lot of solvents being widely used, such as acetone [8,9], tetrahydrofuran [10], *n*-hexane [11], pentane [12], ethanol [13], methanol [14], chloroform [15], and also, mixtures of solvents, such, dichlorometan : methanol (6:1, v/v) [16], acetone : petroleum ether (50:50, v/v) [17], tetrahydrofuran : methanol (1:1, v/v) [18], [19], *n*-hexane : toluene (5:4, v/v) [20], *n*-hexane : acetone (6:4, v/v) [21], 2-propanol : dichlorometan (2 :1, v/v) [22], *n*-hexane : ethyl acetate (85 : 15, v/v) [23].

After the sample extraction, the second step in the protocol of carotenoid determination is the alkaline saponification, which is carried out in a KOH solution: aqueous, ethanolic or methanolic solution and the concentration may vary from 10 to 60% [24]. The conditions under which the saponification is carried out may be at ambient temperature overnight or under heating which reduces the time of reaction [25]. After the saponification, the carotenoids are extracted with diethyl ether or hexane, and then the extract is washed several times with water until all the KOH is removed. In the case of dairy products, the saponification is required because of the high fat content (~45%) and carotenoids are contained in the lipid matrix [26].

The saponification procedure is usually used before the chromatographic analysis, for removal of substances, like lipids and chlorophylls, which could interfere with the chromatographic detection.

Generally, the carotenoids analysis is carried out by HPLC. Acetonitrile, methanol and their mixtures are the majority constituents used in the analysis of carotenoids. To optimize the separation of some carotenoids (for example geometrical isomers), the mobile phase is often modified by the addition of small amounts of other organic solvents [27]. Methanol has been recommended in several reports [28] because it provides better recoveries than acetonitrile or acetonitrile-based solvents. Antioxidants, such as ascorbic acid or BHT are often added to mobile phases to stabilize them.

For the purpose of separation of either different carotenoids or *cis /trans* isomers of a special carotenoid, a polymeric C_{30} phase is necessary. This is more effective for the separation of non-polar geometric isomers than the C_{18} phases [29]. An important factor that should be taken into account to achieve a satisfactory separation of carotenoids is the column temperature. Several authors [30] have reported that changes in ambient temperature cause significant changes in the chromatographic response of carotenoids; therefore, it is important to work at constant temperature.

An important analytical tool for the identification of carotenoids is the liquid chromatography-mass spectrometry technique (LC-MS), since it provides information about the structure and in addition, it is a very sensitive technique.
The LC-MS methods developed for carotenoid analysis include mainly atmospheric pressure ionization interfaces (APCI) [**Error! Bookmark not defined.**] or electrospray ionization interfaces (EIS) [31].

The high sensitivity and selectivity of mass spectrometry (MS) facilitates the identification and structural analysis of small quantities of carotenoids that are typically obtained from samples such as plants, animals, or different products.

Flavonoids are an important group of secondary metabolites, which are synthesized by plants; structurally, they are phenolic compounds with two aromatic rings bonded by a pyran ring.

Reversed-phase liquid chromatography (RPLC) of flavonoids over a C18 derivatised silica stationary phase is most commonly used [32]

For the separation of flavonoids, the chromatographic conditions of HPLC method include the using of a C_{18} column with reversed-phase; a UV-Vis detector and a binary system solvent containing acidified water and a polar organic solvent (methanol, acetonitrile).

Mass spectrometry is one of the physical-chemical methods applied to determine the structure of organic compounds. The MS characteristic is based on using different physical principles, both for sample ionization and for ions separation, ions that are generated according to m/z ratio (m – mass, z – charge).

Among the methods of sample pretreatment for GC–MS analysis, headspace SPME offers certain advantages. Firstly, it is a sample non-destructive method.

Secondly, it concentrates volatile compounds allowing their detection even at trace level and so requires only small amount of sample. Thirdly, non-volatile compounds such as fats, waxes and polysaccharides, are not extracted by such a method, thus avoiding the fastidious sample pre-treatment required for classical GC–MS analysis.

Terpenes are a group of lipophilic aliphatic compounds originating from the secondary metabolism of plants [33].

Terpenes are volatile compounds synthesized and stored in plant vegetative organs [34]. These compounds can be transferred from the forages eaten by the animals into dairy products and used as a marker to distinguish milk and cheese originated from animals in grazing systems from those produced from indoor-fed animals or to trace the geographical origin or production site of the milk [**Error! Bookmark not defined.**].

Mono- and sesquiterpenes are highly volatile compounds, diterpenes exhibit low volatility, triterpenes very low volatility and polysaccharides are not volatile.

Mono- and sesquiterpenes can be easily trapped by headspace SPME, which implies the terpenes volatilization from the sample in a closed space, followed by the analysis of the constituents in this gaseous phase.

The GC method is the most efficient chromatographic techniques for the separation of terpenes. The capillary columns with dimethyl polysiloxane (nepolar phase) and Carbowax 20M (polar phase) are used. Sometimes, when the selectivity of one column is not sufficient, especially when the mixture is very complex, there have been used coupled techniques, such as GC-GC and HPLC-GC, which give a better resolution. Both of them allow the using of techniques with different abilities of separation, combining chiral and achiral or polar and nepolar columns.

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CASE STUDY – METHODS AND FILM PACKAGING EQUIPMENTS APPLIED AT SC RODAX IMPEX TO ENSURE THE TRACEABILITY OF THE PRODUCTS IN THE FOOD CHAIN

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[1]*SC RODAX IMPEX SRL*

ABSTRACT

In the food chain, traceability means the ability to trace and follow a food, feed, food producing animal or substance through all stages of production and distribution.

The basic characteristics of traceability systems, i.e. identification, information and the links between, are common in all systems independent of the type of product, production and control system that are served.

Stages of production and distribution means any stage including import and including the primary production of food, up to its sale or supply to the final consumer and, where relevant to food safety, the production, manufacture and distribution of feed.

The insurance of the quality of the foods has in view the whole food chain, from the designing of the product to the purchasing of the raw materials to the whole production environment, as well as being focused on the production. This solicits a systematic approach and a quality system which is introduced, maintained and administrated, in conformity with the quality management; for reaching maximum performance in ensuring the quality of the foods the first step is represented by the management of the raw materials. The quality systems used by the enterprises in the food industry sector are based on the requirements of the ISO 9000 family standards, as well as other standards specific to the food sector elaborated at international, national or regional level.

To ensure a traceability system in an organization must be realized the process traceability, the product traceability, the documents traceability.

Both products and processes may form key components (known technically as core entities) in a traceability system with information stored in relation to each.

The traceability of products is based on the ability to identify them uniquely at any point in the supply chain.

In the food packaging domain it is very important the application of the traceability system, this being representative for the functions of the packaging.

The case study is describing the modified atmosphere packaging method and the afferent equipment realized by RODAX.

Keywords: food safety, packaging method, packaging equipment, traceability

1. INTRODUCTION

Food traceability systems are record keeping procedures, or tracing systems, that record the path of a food product or an ingredient in a food product from its initial supplier through all processing stages until it reaches the end consumer.

A traceability system allows the food industry to:

- promptly locate and remove unsafe products in case of a recall;
- protect brand reputation — keeping precise records allows companies to quickly identify and recall only unsafe products, reducing the scope of a recall, demonstrating good corporate citizenship and a high level of

concern for public health, therefore limiting negative media exposure and perhaps even turning it positive;

- ❖ minimize the size of a recall and reduce the cost incurred in recovering or disposing of products in the marketplace;
- ❖ diagnose problems in production and determine liability where relevant — traceability can help resolve process problems and determine third-party responsibility if records show that an ingredient supplier or co-packer was the source of the recalled ingredient. Although the manufacturer of the final product is still responsible for the recall, complete records tracing ingredients to their sources may allow seeking indemnification from responsible third parties.

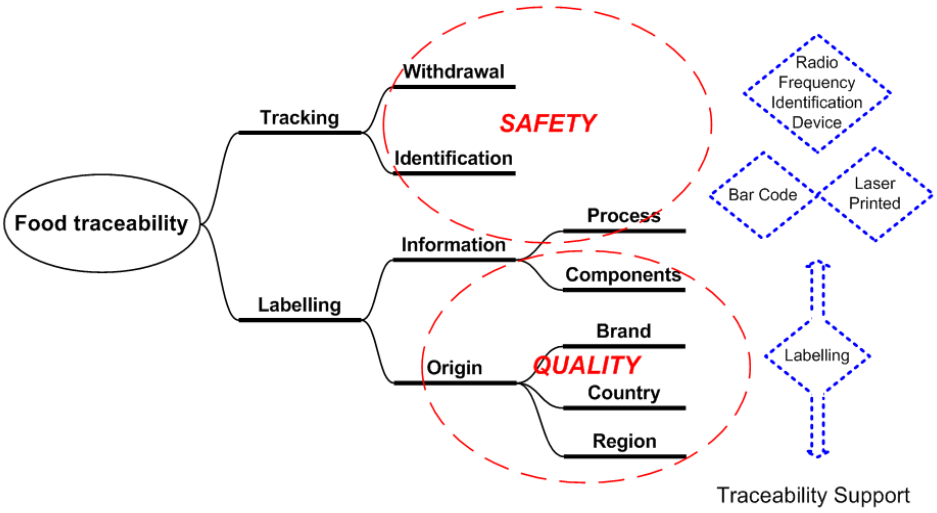


Fig.1 Consumers associations with food traceability issued from focus groups

Due to a number of factors, food safety and quality have been issues of growing importance in the last few years. As a result a number of information technology supported tracking and tracing and quality assurance systems have been developed. Most of the existing solutions focus on a certain production chain or a part of a chain. That kind of setting can currently be handled. However, changes in supply chain configurations and interconnections between chains lead to the transformation of the linear structure into a highly dynamic food sector network. To achieve appropriate transparency across the whole food sector, different tracking and tracing systems have to be interconnected. There is currently no satisfying solution available that is up to this task. One of the objectives of the Transparent Food project is thus to create a blueprint proposal for a European

Backbone Solution that provides basic and simple functionalities to enable integration of different systems.

2. TYPES OF TRACEABILITY SYSTEMS

Traceability systems can be manual or computer based. Small companies manufacturing limited numbers of products with simple formulas, short shelf-lives and fewer customers may find paper-based, manual systems adequate. Large companies may find computerized systems more reliable and efficient. Computerized systems can help:

- speed data and product handling
- reduce errors
- reduce paper waste
- track product movements precisely

Several steps can be followed to establish a tracing system in the food industry:

- **Receipt of raw materials, ingredients or packaging materials** — record information in a logbook, check product specifications and assign a lot number.
- **Use of raw materials, ingredients and packaging material for production** — when products are used in processing, link them to the manufacturing process by logging information such as units used, recipes, work order numbers, dates and times.
- **Packaging finished product** — include a code that allows products to be grouped and links to the information recorded by the tracing system.
- **Shipping finished products to clients or customers** — products are grouped on pallets or in boxes and given separate identification codes for use in dispatch and subsequent handling in the supply chain.

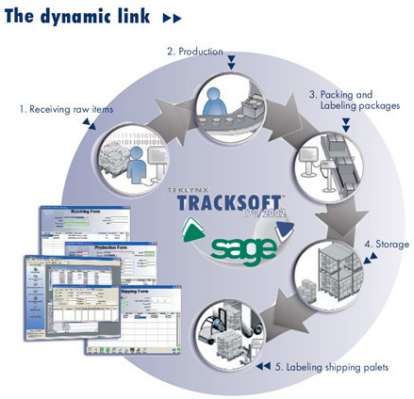


Fig. 2 Example of software used in traceability chain

3. FOOD PACKAGING METHODS

Packaging is an integral part of the processing and preservation of foods and can influence the products quality and shelf-life. It can influence physical and chemical changes, including migration of chemicals into foods. So, in designing the packaging equipments and materials it is very important to analyze and take in consideration the functions of the packaging.

The development of the package and packaging techniques are related to the permanent changes regarding the consumer behavior and the role of the package in the modern supplying chain. In the last decades the energy crisis, the ecological awareness and the demand for healthy and fresh foods has created a need for technologies that allows distribution of fresh products. To reach this aim there are needed packaging methods that realize modified atmosphere in the interior of the package.

In packaging fresh products, especially meat products it is important to preserve its freshness a long period of time and considering their specific properties there were been discovered specific methods as the followings:

- ❖ Vacuum packaging;
- ❖ Modified atmospheric packaging;
- ❖ Intelligent packaging;
- ❖ Aseptic packaging.

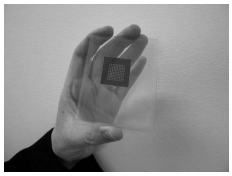
Also, the packaging materials have to accomplish some requirements:

- ❖ Not to damage the consumer health;
- ❖ Not to modify the composition of the aliment;
- ❖ To preserve the organoleptic qualities of the products;
- ❖ To present a commercial aspect.

Examples of intelligent packaging are as following:

Packaging using **printed relative humidity indicators**

These would ideally be incorporated inside the packaging, but visible from the outside through a clear window. Color and text changes indicate changes in humidity.



Packaging using **temperature logger**

Could only detect abuse events –

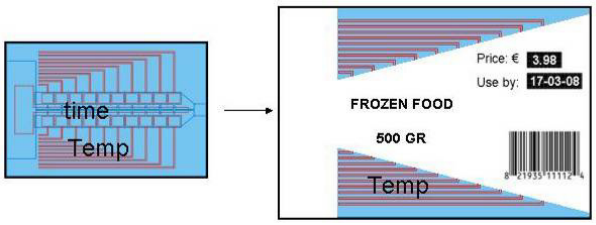
• Ideal temperature less than -18ºC at 5 to 10 minutes time intervals.

3 levels:

• Below ideal

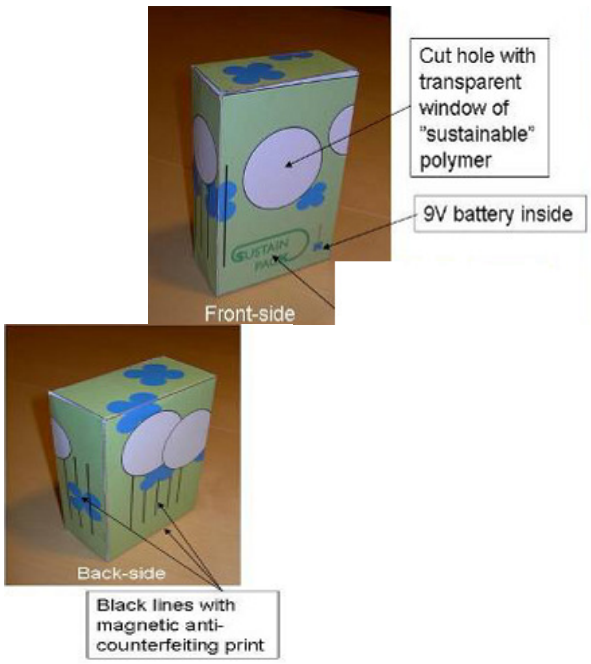
• Abuse above -18ºC (24 hours – 1 time interval)

• Abuse above -2ºC (1 to 24 hours)



85 X 55 MM (CREDIT CARD SIZE) OR SMALLER

Packaging using biodegradable polymer window and sustainable communicative technology



4. FOOD PACKAGING EQUIPMENTS

In order to achieve modified atmosphere in the interior of the package, various techniques have been developed such as: vacuum packaging, modified atmosphere packaging, use of oxygen scavenger or ethylene or gas producers, using ethylene vapor emitters.

Modified atmosphere packaging (MAP) is increasingly used with minimally processed fresh products. Equilibrium Modified Atmosphere Packaging (EMAP) of Fresh Product is also increasingly used to extend shelf-life of fresh product.

Increased MAP and EMAP usage coupled with negative environmental views associated with non-degradable synthetic packaging materials creates a societal and SMEs need for biodegradable films.

Technical characteristics:

Electric supply: 220V/50Hz Sealing length: max. 340mm
Productivity :80-100 bags/h

The gases mainly used are the following:

CO2 – antimicrobial effect;

O2 – a reduced oxygen concentration in the head-space of a package below 1-2 %, even to 0.2 % by replacing oxygen with nitrogen and /or carbon dioxide;

- a high oxygen concentration, even 80 % or above is used in order to inhibit microbial growth through oxygen shock;

N2 – inert gas (equilibrium of atmospheric pressure).

The method of packaging developed by the described equipment ensures an extended shelf-life allowing the products to survive in the distribution period to the stores as well as during its presentation to the consumers.

APLICATIONS: the packaging equipment it is used for the following products: fresh cut food products (especially meat and meat products, fresh cut poultry, fish) which needs packaging in thermo-sealing and thermo-shrinking plastic bags.

The beneficiaries of these equipments are economic units which process the meat and the meat products.

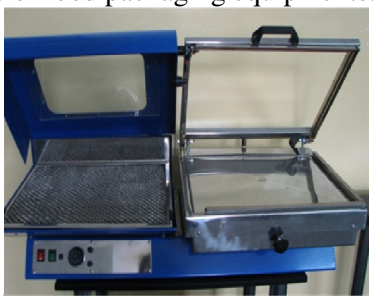
ADVANTAGES of using the MAP equipment are:

- the extension of the food products self life;
- the good storage of the products;
- the emphasize of the advertising characteristic of the package (the packages may be printed and labeled);

- the exposing of the entire range of available products on small stands;
- the integral presentation of the products.

RODAX designed and manufactured more types of food packaging equipments:

Technical characteristics:
Electrical supply: 220V/50Hz
Power: 600W
Packaging rolls: max. width 500mm



Technical characteristics:
Sealing arm: 500x400mm
Sealing temperature : 0°C-300°C
The height of the package: max. 260mm

Electrical supply: 220V/50Hz

Technical characteristics:
Electrical supply: 220V/50Hz
Cycle time: 10 sec.
Packaging rolls: max. width 300mm

Technical characteristics:
Package dimension: 360x390x100mm
Max. sealing length: 400mm
Vacuumation : 1bar
Water temp.: 80°C-120°C
Cycle time: max. 30sec.
Electrical supply: 380V/50Hz

5. CONCLUSIONS

The introduction of improved food traceability systems has aimed to restore consumer confidence in food safety and quality, in part by being able to provide consumers with more information about the origins of foods and food ingredients.

Little is known about consumer attitudes and beliefs associated with traceability, nor their preferences for information provision. Given the heterogeneity of European consumers, it is not surprising that they have different perceptions and expectations regarding and understanding the concept of 'traceability'. Consumers may also perceive traceability differently for different product types.

The benefits for consumer associate with traceability are in terms of health, quality, safety and control, of which the latter was associated with trust and confidence.

Importantly, both quality and safety were shown to be related to traceability in the consumers' minds with quality implying safety. The results show that traceability may contribute to improving consumer confidence.

Traceability lets you identify:

- All the stages involved in the fabrication of a product;
- The origin of the ingredients and components;
- The ingredient and the raw material suppliers;
- The places where the products and ingredients are warehoused;
- The equipment used in the production or manipulation of the products
- The production, distribution and sales batch numbers.

In short, traceability lets you improve the quality, the service and the global efficiency of your company.

It is clear that traceability comes at a cost, but the costs of not having it, or having inefficient systems in place may be severe both for governments, consumers, individual companies and the food industry as a whole.

Traceability systems need to be checked to determine if they meet the following objectives:

- provide traceability forwards and backwards
- include all raw materials, ingredients and packaging materials
- give a response in an appropriate time
- provide simple and readable traceability information to the consumer
- in some cases, provide a feedback

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COMBINED SYSTEM, PEF AND UV, FOR THE INACTIVATION OF PATHOGENIC MICROORGANISMS IN LIQUID FOOD

POPA M., TUDORACHE A., BRINDUSE E., CRAMARIUC R., KONTEK A., MITELUT A., FOTESCU L., CRAMARIUC B., GEICU M., NISIPARU L.

ABSTRACT

The PEF treatment is a non thermal food preservation technology, based on the use of pulsed electric fields, to inactivate pathogens in food and to control the pathogen content of the treated products. This technology is highly valued for its ability to prolong the shelf life of the product treated without use of heat and also to keep the value of sensory and nutritional qualities of the product and the microbiological control of food. PEF technology has no limitations related to the spread of lethal microorganisms in the treated product, for example, in heat treatment where this phenomenon can take place by conduction or convection. The electric field has a density, which enables a rapid and uniform inactivation of lethal microorganisms throughout the product volume.

UV irradiation has been used for decades to disinfect water and is an effective method for microbial decontamination of surfaces and food packaging.

The Food and Drug Administration (2000) approved the UV irradiation as an independent method for preserving fruit juices, unless turbulences occur in the process. This condition is not required if the UV exposure is not the only decontamination factor.

This paper gives the PEF and UV treatment, or a combination of PEF and UV inactivation of microbial liquid food.

Experiments were performed using the micro-organisms: yeast, lactic acid bacteria and acetic bacteria in the systems: PEF alone, UV alone, UV + PEF and PEF + UV combined.

The result of these experiments was that it can get a total inactivation of microorganisms in the combined UV + PEF, leading to the possibility of increasing the quality of liquid foods in relation to thermal pasteurization.

Key words: Pulsed Electric Field (PEF); UV radiation; microbial inactivation; liquid food.

1. Introduction

Recently the market demand of fresh or minimum processed products has rapidly increased. Currently, heat processing is the most used method for inactivating microorganisms that allows increased product life on the shelf. However, this process can adversely affect the nutritional and sensory qualities of food [5]. In this context, recent years have been paid attention to non-thermal technologies (ie PEF, UV), For the preservation of beverages, thanks to their potential on inactivation of pathogenic microorganisms. In addition, they can help minimize the loss of quality in terms of taste, color and nutritional value [2].

The PEF treatment is a non thermal food preservation technology, based on the use of pulsed electric fields, to inactivate pathogens in food and to control the pathogen content of the treated products. This technology is highly valued for its ability to prolong the shelf life of the product treated without use of heat and also to

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UV irradiation has been used for decades to disinfect water and is an effective method for microbial decontamination of surfaces and food packaging.

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Experiments were performed using the micro-organisms: yeast, lactic acid bacteria and acetic bacteria in the systems: PEF alone, UV alone, UV + PEF and PEF + UV combined.

2. Materials and methods

2.1. Treated materials

Experiments were performed on suspensions of microorganisms in distilled water sterilized by autoclaving for 30 min. at a pressure of 1 bar.

Microorganisms for testing were taken from the collection of microorganisms IC-DVV Calugareasca Valley, microorganisms present in wines: they used the yeast species Saccharomyces cerevisiae, strain PN III-11, the lactic bacteria Oenococcus oenos strain 149 and acetic bacteria Acetobacter aceti, strain 11. After a program of successive replications, average liquid suspensions were obtained, from which, after some dillutions, inoculation titers were made.

Highlighting active microorganisms was performed using microbiological control by membrane filter of 0.45 μ, followed by incubation environments specific to each type of microorganism:

- for yeast, an agarose-based yeast extract, peptone and glucose to which was added chloramphenicol
- for lactic bacteria, an agarose-based yeast extract, meat extract, peptone, sodium acetate, magnesium sulfate, glucose, to which was added actidiona
- for acetic bacteria, an agarose-based yeast extract, to which were added actidiona and bromphenol blue.

Experiments were conducted focused on the following aspects:

1. Influence of the duration of UV exposure over the inactivation of microorganisms;
2. Influence of the duration of PEF exposure over the inactivation of microorganisms;
3. The effect of the UV and PEF treatments, alone and combined, over the inactivation of microorganisms;

2.2. UV radiation processing

Exposure to UV radiation was performed in a UV water sterilizer, shown in fig. 1.

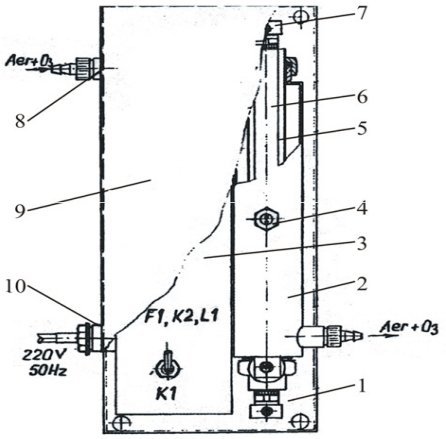

Fig. 1. Machine for purifying water by UV radiation: 1- basic underlying; 2-chamber body; 3-power unit; 4-UV detector; 5-quartz tube; 6-UV lamp; 7-lamp holder; 8-gas pipe link; 9-lid; 10-power supply connection

Overview of the machine is shown in fig. 2.

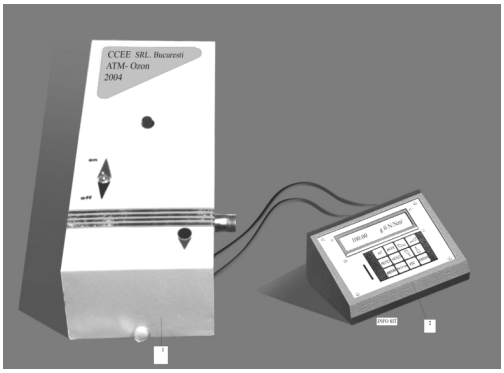

Fig. 2. Overview of the machine: 1-transducer; 2-microcontroller

The sterillizer features are:

- Work fluid – filtered water
- Water flow – 0.1-0.3 l-min
- Maximum pressure in the irradiation chamber – 1 bar
- Wave length of the UV radiation – 254 nm
- UV lamp power – 8 W
- Power supply – 220 V; 50Hz
- Dimensions – 260 x 160 x 82 mm

Material exposure to UV radiation was performed in 750ml batch of treated products, referred to in section 2.1. The device allows a continuous operation. The treated materials were displayed at different times, shown as results. After exposure the materials were transferred into sterile bottles and passed for examination or treatment with high voltage pulses (PEF). Treatments were performed in the following order: UV + PEF or PEF + UV. The results are presented in section 3.

2.3. Processing using pulsed electric fields (PEF)

High voltage pulse processing was carried out in a PEF treatment chamber, cylinder, shown in Figures 3, 4 and 5.

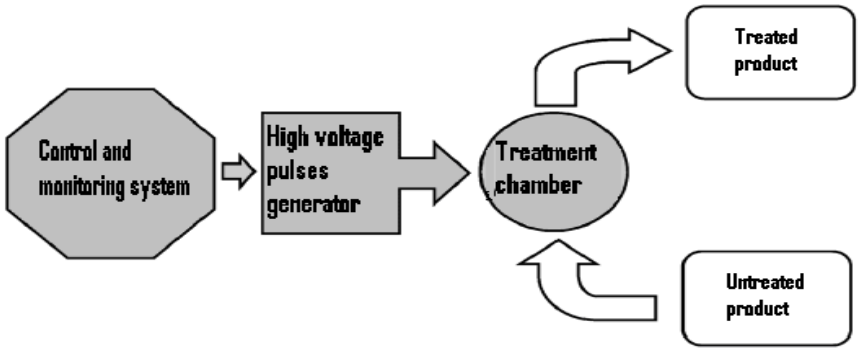

Fig. 3. PEF technology application scheme

UV treated materials were placed in the PEF treatment chamber. The PEF cylindrical treatment chamber has a capacity of 750 ml and the measurements given in fig. 4.

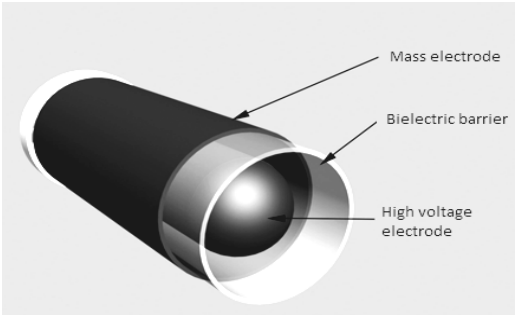

Fig. 4. Cylindrical treatment chamber

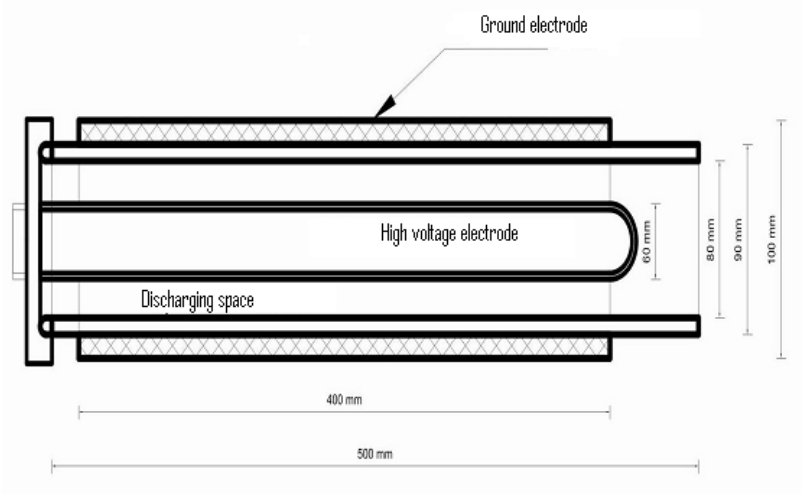

Fig. 5. Cylindrical treatment chamber. Longitudinal section.

High voltage pulses used had the following characteristics: maximum pulse voltage: 30 kV; pulse front duration: 100 ns, pulse length: 1 microsecond.

The variable duration of treatment is referred to the results. Before and after each test PEF system was cleaned and sterilized.

3. Results

In the tables and graphs below are presented the experimental results.

UV treatment resulted in a reduction of just 36% - 62% of microorganisms, depending on the type of organism and duration of exposure. Inactivation rate was higher for bacteria. For yeast was recorded a mortality of 55% in terms of application of UV treatment for 5 min. Under the same conditions, lactic acid bacteria were inactivated at a rate of 59% and acetic bacteria in 62%. Increased duration to 10 min UV of treatment did not significantly alter the inactivation rate of microorganisms (Table 3.1, Fig. 6).

Table 3.1. The influence of UV treatment over microorganisms viability

Type of microorganism		V1 – 2 min.	V2 – 5 min.	V3 – 10 min.
Yeast	Viability (%)	47	45	47
	Lethality (%)	53	55	53
Lactic Acid Bacteria	Viability (%)	64	41	44
	Lethality (%)	36	59	56
Acetic Bacteria	Viability (%)	54	38	38
	Lethality (%)	46	62	62

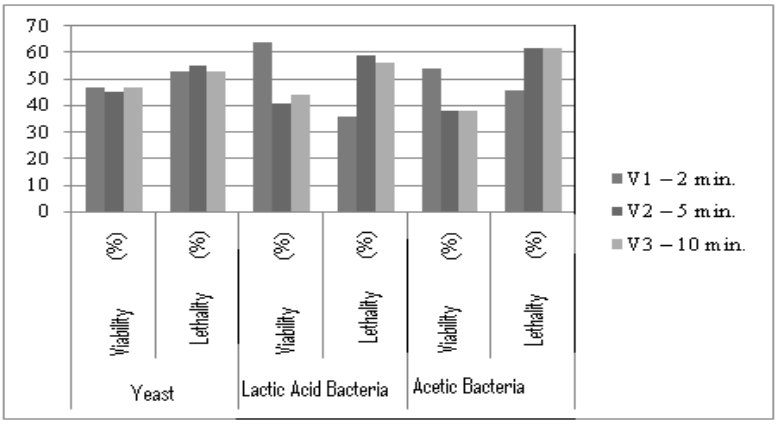

Fig. 6. Influence of UV duration on the inactivation rate of microorganisms

Simple PEF treatment reduced microbial load of the suspension, the reduction rate may vary depending on the type of organism and treatment duration (Fig. 7).

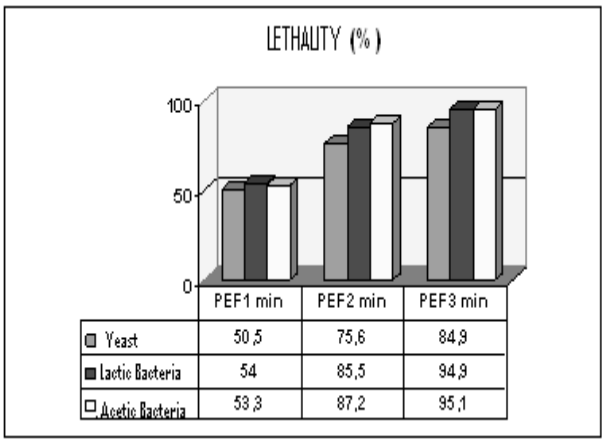

Fig. 7. Influence of the duration of the pulsed electric field (PEF) over the inactivation of microorganisms

Between the microbial load (expressed in CFU / ml) of the groups of organisms (yeast, lactic acid bacteria and acetic bacteria), and the PEF treatment duration were established 1st degree relations.Only regression equation parameters are different (Table 3.2).

UV + PEF treatments combined (Table 3.3, Fig. 8) and PEF + UV (Table 3.4, fig. 9) have been very effective in causing inactivation of nearly all microorganisms.

Table 3.2. Correlation between microbial loads of groups of microorganisms and PEF treatment duration

Type of microorganism	***Statistical characteristics***	
Yeast	The form of the regression line	y = 17,181x + 35,951
	Regression coefficient	0,9338
Lactic acid bacteria	The form of the regression line	y = 20,471x + 37,198
	Regression coefficient	0,9115
Acetic bacteria	The form of the regresion line	y = 21,382x + 35,417
	Regression coefficient	0,8829

Inactivation rate was greater in PEF + UV treatment for both lactic acid bacteria and for the acetic bacteria. The yeasts were more resistant to treatment, registering a 96% rate of mortality in both treatment conditions (Table 3.3, fig. 8).

Table 3.3. The influence of UV + PEF treatment on the viability of microorganisms

Type of microorganism		UV 5 min	PEF 2 min	UV+PEF (5min + 2min)
Yeasts	Viability (%)	48,5	22	4,5
	Lethality (%)	51,5	78	95,5
Lactic acid bacteria	Viability (%)	39	9,1	2,8
	Lethality (%)	61	90,9	97,2
Acetic bacteria	Viability (%)	37,58	6,05	2,15
	Lethality (%)	62,4	93,9	97,9

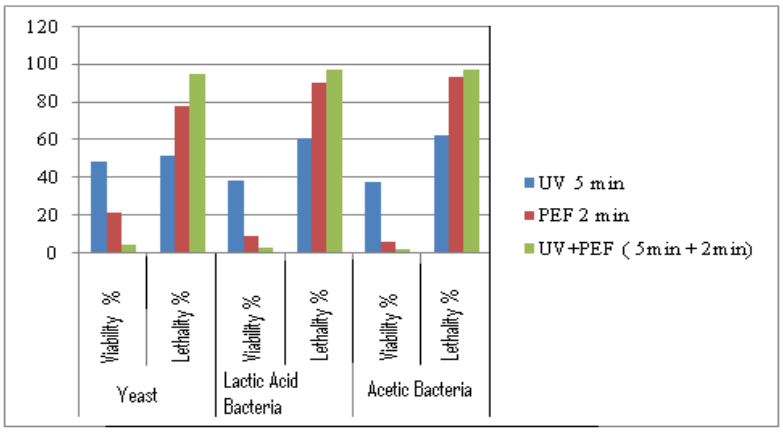

Fig. 8. The influence of UV + PEF treatment on the viability of microorganisms

Table 3.4. The influence of PEF + UV treatment on the viability of microorganisms

Type of microorganism		PEF 1 min	PEF 2 min	PEF 3 min	PEF + UV(2min+5min)
Yeasts	Viability (%)	49,5	24,4	15,1	3,9
	Lethality (%)	50,5	75,6	84,9	96,1
Lactic acid bacteria	Viability (%)	46	14,5	5,1	0,4
	Lethality (%)	54	85,5	94,9	99,6
Acetic bacteria	Viability (%)	47,7	12,8	4,9	1,7
	Lethality (%)	52,3	87,2	95,1	98,3

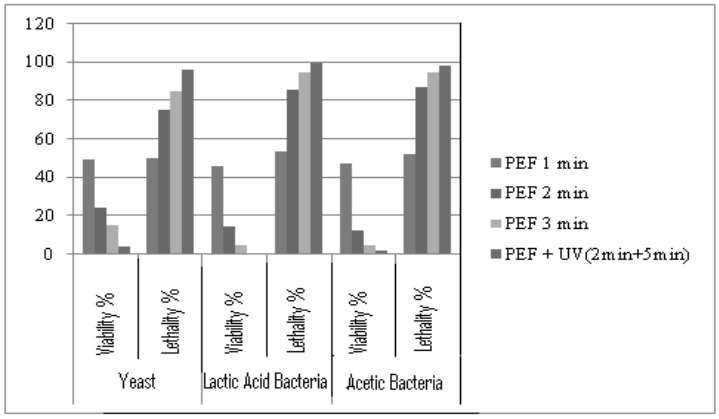

Fig. 9. The influence of PEF + UV treatment on the viability of microorganisms

4. Discussions

The literature stated that it is difficult to inactivate microorganismsusing only PEF technology for natural contaminated liquid products. For this reason it was reported the possibility of obtaining higher levels of microbial inactivation through PEF treatment combined with other effects, such as: heat, pressure or use antimicrobials [1-4].

In paper [5] was mentioned the use of the combined system UV + PEF for the pasteurization of fresh apple juice. The results obtained in the combined UV + PEF were higher, concerning the microbial inactivation with only the application of PEF technology. Separate contribution of UV radiation is small and it has been used only as part of the Hurdle strategy. In this paper we used the combined system UV + PEF for the inactivation of the next microorganisms: yeast, lactic acid bacteria, acetic bacteria, found in different concentrations in distilled water, those microorganisms that occur in the processing of grapes. The present research seeks a new technology based on the combined system UV+PEF, for the pasteurization of grape wine and microbiological stability. The results confirmed the superiority

of the PEF + UV system over the PEF technology alone. The UV radiation treatment is used only combined with PEF, with the Hurdle strategy.

CONCLUSIONS

- Non-thermal PEF and UV treatments, simple or combined, are future treatments in oenology, being able to replace thermal treatments, which have negative effects over the products quality.
- The microorganisms in wine have different resistance to the action of the pulsed electric field, yeast and lactic acid bacteria being the most affected, and the acetic bacteria the most resistant. As a result of the diversity of species in each group of microorganisms, different resistant strains may appear, but the results confirm the fact that larger microorganisms are less affected by the PEF than the smaller ones.
- Simple UV treatment have determined a reduction of 36% - 62% of microorganisms, depending on the type of microorganism and exposure duration, inactivation rate being higher for bacteria.
- Combined treatments improve the performance of the system, being more efficient. These treatments can be applied in oenology for the inactivation of specific wine microorganisms. Technological steps in which they can be applied depend on the intended purpose.

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SECTION VI: MISCELLANEOUS

COMPUTERIZED SYSTEM FOR CONTROLLING PACKAGE ENCASING IN THE FOOD INDUSTRY

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ABSTRACT

The computerized system for controlling package encasing in the food industry is an integrated system of computing and control shall be realized which shall be used for a total control of the encasing of the canned product. Thus, there shall be measured the encasing parameters and the dimensional and shape parameters of the packages subject to the sterilization process to find out defects that may lead in time to the alteration of the content.

Keywords: computerized system, encasing of the canned product, sterilization

1. INTRODUCTION

The increase in the national economy competitiveness in producing high quality products in compliance with the international standards and especially, the alignment of quality at technical performances thereof to the requirements imposed by the European Union is one of the main targets.

According to the data furnished by the Sanitary Veterinary National Authority for Food Safety (ANSVSA), more than 500 units of the food industry could be closed, unless they are modernized and restructured, so that to comply with the European regulations.

The immediate consequences of such situation could be summarized as follows:

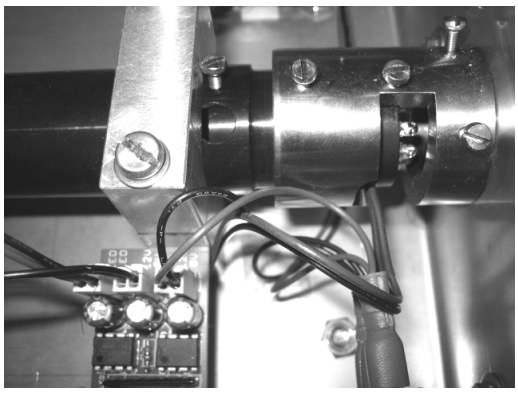
- A serious risk people's food safety;
- Weak presence of the Romanian canned products on the European market though Romania has a high agricultural potential (vegetables, fruits), and the Danube Delta represents a huge piscicultural capacity (canned fish);
- Immediate shut-off of all trade agents not complying with the quality regulations of the European Union;
- Cost price increase of the products, if, due to market requisites, control equipment for the food industry packages are to be imported;
- neglecting the scientific capacity of the Romanian researchers and specialists.

The computerized system for controlling package encasing in the food industry, dimensionally verify the packages, according to the European Union regulations,

furnishing information on certain parameters that may influence the quality of the canned product and decides if it has any potential risk for consumer's food safety.
The geometrical parameters of the package are extremely important, as the quality of the encasing of such food stuff depends on them. The encasing is one of the most important stages in the technology for canned product manufacturing, being decisive for the conservations interval, and implicitly food safety of such product.
Considering the long life canned products should have (2-3 years) and the fact that the defects (content alteration) are not immediately detectable, as they appear after a long time and being extremely dangerous for consumer's health, a special attention should be granted the control of canned stuff packages.

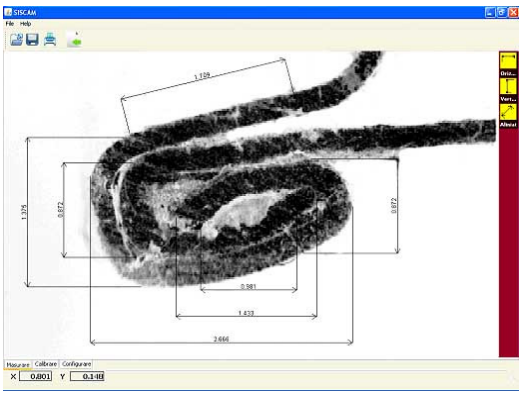
2. SCIENTIFIC AND TECHNICAL DESCRIPTION

From the point of view of the technical and constructive complexity, it was design an integrated system of computing and control shall be realized which shall be used for a total control of the encasing of the canned product. Thus, there shall be measured the encasing parameters and the dimensional, subject to the sterilization process to find out defects that may lead in time to the alteration of the content.
From a constructive point of view, the computerized system for the control of package encasing in the food industry has the following functional structure:
● *the equipment for checking package encasing.* It is an integrated system of optical-electronic control, containing a high resolution video camera and a telecentric lighting system.



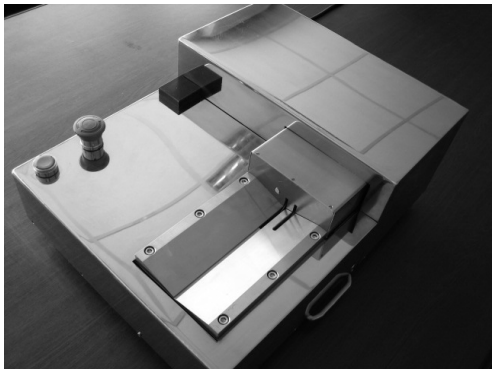
The camera takes over the real image of the encasing (welt) of the controlled package and sends it to the main computer, which display the image on a LCD screen.

The computer measures the encasing parameters and calculates the *real union*.

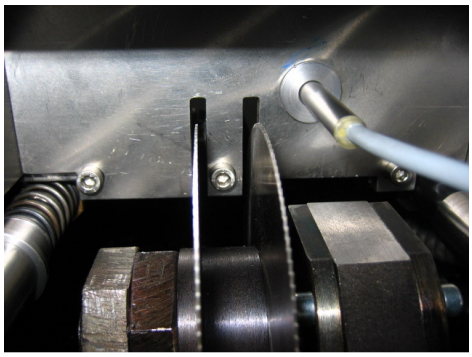
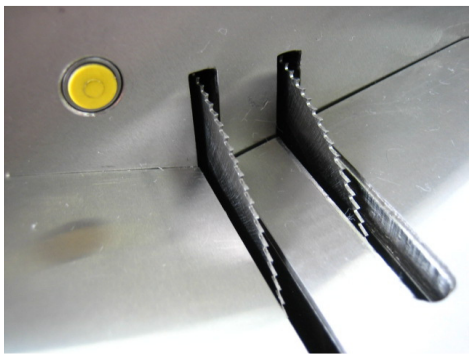


The real union represents the synthesis of all composing elements of the encasing, is expressed in percentages of total union and consists in the welt portion where sealing effectively takes place, by gripping the rubber seal between the hook of the body and that of the tap. A welt is correctly formed when the real union has a value above 50%. Below the mentioned value, the welt may be suspected of leakage. The results of the measurements are displayed on the electronic block and are sent to the main computer.

● the *cutting device* carries out the cutting of the tin with the view to measuring it, being acted by a mono-phase motor with an electronic command block.




The cutting system is made up of two parallel milling discs, with the width of 0.5 mm that, by a system of manual advance cuts the falt section that is to be measured. The revolution of the mill may vary between 400rpm and 800 rpm.

The cutting device is provided with safety systems meant to prevent possible accidents.
In the cutting areas is placed a proximity sensor that validates the presence of the box, and prevents the accidental start of the mill.
Stopping the cutting process is carried out automatically, with a stroke limiter, the operator no longer being required to push another button. The device is also provided with an emergency switch, mushroom-like, that at a simple fast push interrupts the cutting process instantaneously. The device no longer sets in motion until the switch is no longer connected.
● *the main computer* correlated the measurements directly on the package, to those obtained after encasing and identifies the elements that could cause a defect. The

measurements bulletin is typed by the editing system of the computer to the complete data of the measurements, including the lot, change, date and time. All these operations are made with no intervention of the human factor (operator). There is a possibility of printing the real image of the welt for a complex analysis of the material and sealing after the encasing process. All measured date shall be stored for monitoring the entire process in time. Thus, there shall be statistically monitored the evolution of such elements in time and the appropriate measures can be taken as soon as there may be found out deviations from the interval considered normal. The values thus obtained may furnish accurate indices as to the causes having generated an inappropriate encasing.

3. PERFORMANCE AND QUALITY PARAMETERS

The level of performance and quality parameters of the suggested solution can be found in the *technical characteristics* aimed at by the new product:
- The resolution of the measuring system for the linear quotas: 0,01mm;
- Resolution of the system for the measuring of shape and surface deviations: 0,01mm;
- Accuracy of measurement: ± 0,01mm;
- Optical enlargement on the LCD screen: 30X;
- Parameters measured at encasing: welt thickness and height, tap depth, body hook, tap hook, outer reinforcement and inner indentation (in line with the body union), as well as encasing percentage;
- parameters measured for packages: diameter and height on the encasing quota, deviation from surface smoothness of the surface on which encasing is made, circularity (ovality) deviation of the surface on which encasing takes place;
- encasing parameters of the taps: outer diameter, tap height, edge height, area „elongated metal";
- working field: this equipment may control:
  - metallic canned products (STAS 1687/81, SR ISO 3004/92, SR ISO 10653/96): ø73mm and ø99mm;
  - taps for metallic tins with dimensions: ø73mm and ø99mm.
  - metallic taps for steel (Twist-Off, PT, etc.) and aluminum (Omnia, Keller, etc.) recipients. The most used of them are Omnia with the following variants P (pasteurization) and S (sterilization) with the dimensions: ø56mm, ø68mm and ø83mm.

Due to the high flexibility provided by the equipment, it may be used for checking an important sort of packages for the food industry. There should be underlined the fact that when designing the equipment, there shall be considered the possibility of permanently adjusting the measuring accuracy and field to the demands of the

future beneficiaries. There shall also be considered the *future evolution of the field*, by the occurrence of new typo-dimensions of packages.

4. DEGREE OF NOVELTY AND COMPLEXITY

The idea of this project proposal occurred because of the many requests which were received from certain trade agents of the food industry.
They are interested in acquiring some systems measuring the encasing, but also the dimensional and shape deviations of the packages that may influence on their encasing. Accurate control systems were requested where the *operator's influence be manifest as little as possible* to avoid any possibility of occurring objective or subjective errors. For this reason, this project proposes the creation of a computer-assisted system, to carry out the control and monitoring of the technological process for the encasing of packages and to automatically issue the measurement bulletin with all the identification data on it, with no intervention of the operator.
Project contribution to the settlement of certain high complexity problems, in the area of industrial applications, is that ***it settles the problem of encasing control for packages used in the food industry***. This issue is very important as canned products should resist long time (2-3 years), and the consequences of a package defect (content alteration) *are not immediately detectable*; occurring after a long time and may become extremely dangerous for consumer's health.
By mathematical models and dedicated measuring software to be worked out, the project directly contributes to the *development of new S/T field,* determining its *research character at the border of scientific and technical border*. From this point of view, we should mention: the use of dimensional measurements, and computer-assisted deviations in industrial applications and transfer of the decisional factor of the control operation, from man to computer.
The degree of novelty and originality of this project is represented by *the creation for the first time* of a computerized system for the control of package encasing to be integrated in the manufacturing technology of trade agents producing packages for the food industry and meat, fish, vegetable and fruit canned products, in order to comply with the quality demands set by the European Union.
Another objective sustaining the high degree of ***novelty and originality on international level*** of the project is represented by *the working out of dedicated, flexible software*, so that the measuring diagram may contain as many data as required by the user of the equipment. This original software shall be designed in a modular (reconfigurable) way as reusable elements. The hardware and software components shall be the basis for the subsequent development, in order to refit the systems and to adjust them to the new types of packages to appear in the future. For this reason, we may say that the project has a high degree of novelty and originality, falling within the *major tendencies* manifested on international level.

5. CONCLUSIONS

The implementation of "Computerized system for controlling package encasing in the food industry", shall have a significant technical, economic, scientific, and social impact.

The technical-economic impact consists in:

- the creation of a new, salable product demanded by the market;
- the creation of a new product and the implementation of modern technologies for control in the production processes;
- increase of consumer's food safety;
- economic growth in regional plan, including in the developed areas, but with a significant potential for the food industry;
- increase of the turnover and profit by the significant increase of the productivity, quality and viability;
- increase of economic competitiveness by economic relaunching of the field-related trade agents;

The scientific impact shall be evidenced by:

- increase of the personal capacity of research - development;
- training young researchers with experience in complex research-development projects;
- scientific knowledge obtainable after the completion of the project tat shall be easily usable in other projects;
- developing a system of acquisitions and data processing of high velocity;
- use of highly accurate mechanical elements;
- development of algorithms for the control of products and processes in the food industry.

The social impact of implementing the project in the mechanical processing fields shall be significant as well:

- boosting the regional economic growth by providing trade units with modern equipment;
- increase of the canned stuff production with a positive impact on the raw material producers;
- providing an important data basis for the Romanian technical education;
- establishing better labor and life conditions by reducing the risk of occurrence of accidents at the working place;
- reducing the level of noise in the canned stuff production units.

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AGRI-FOOD KNOWLEDGE MANAGEMENT PLATFORM

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*** Romanian Accreditation Association (RENAR)*

Key words: knowledge management, platform, information, competencies, agri-food Research and Development

ABSTRACT

It is conceived a Romanian collaborative platform in the Agri-Food field. Its aim is to increase the organizational competitiveness and knowledge management, information, competencies, expertise and partnerships in the agri-food Research and Development, assembling representative organizations in the field. It will offer the needed interface for the integration of the agri-food R&D into the large European R&D partnerships, projects and programs, thus contributing to an increased competitiveness of the research activity.

INTRODUCTION

Knowledge Management is dealt with today, in the majority of the existing systems, as being based on a structured organization of the Best Practices, knowledge, information, competencies, expertise and partnerships concerning various fields, generically reunited as such under the concept of intangible values.

These static systems have the drawback of creating the intangible values through processes and possibly, through procedures centered on the individual and not on the community and hence of creating a great amount of information stored at the individual's level, very probably redundant, with uncertain utility, relative accessibility and difficult, time consuming extraction.

Furthermore, the lack of a common collaborative platform at the level of the communities involved in joint programmes for the creation of knowledge represents a major hurdle in accomplishing a management of the knowledge, aligned to the strategies of the organization.

The main assumption of this collaborative platform was the importance of knowing the current mechanisms, conditions and aspects of the production of knowledge, notwithstanding those related to the transfer and the utilization of the new knowledge and hence, extending the study of the connection between research and innovation as it is intermediated by the cooperation in R&D activity.

A system for the management of information and knowledge is today a necessity due to the following factors:

- the economic environment is more and more competitive and the innovation rate is increasing

• the knowledge and the information needed to carry out the essential activities of an organization are held by a small number of persons; it is required that these knowledge and information were disseminated within the organization;
• the time allocated to the accumulation of experience and knowledge has diminished under the pressure of the fast adaptation to the requirements of the more and more dynamic environment;
• retirements and the increased mobility of the employees may lead to an important loss of knowledge for the organization.

MATERIALS AND METHODS

The scope of the Agri-Food Knowledge Management Platform (Agri-Food KMP) is oriented on the following directions:

1. development of research from the perspective of institutes and universities;
2. development of the interactions and the cooperation between research institutes, universities and commercial organizations;
3. improving the cooperation in R&D;
4. improving the transfer of knowledge.

Agri-Food KMP will involve the promotion of the knowledge society, which assumes the activation of the technological vectors (e.g. Internet, artificial intelligence, etc.) and of the functional vectors (management of information, knowledge and competencies, innovations, etc.)

The technological development rapidly affects the way information is generally distributed, accessed, controlled and published, and in particular, the scientific information. The Internet and the intranets have more and more important role in the production and distribution of scientific and educational material.

As research and the technical and scientific knowledge is a strong vector of the economic progress and growth, the speed of accessing information and specialized studies is a decisive factor of the quality and of the competitiveness of the research-development-innovation activities.

The Agri-Food KMP aligned to the European research objectives areas, once accomplished and used, can be extended to the research organizations or those working with mostly intellectual personnel (education institutions, etc.). Through the implementation of the collaborative platform, materialized in a system of management of the knowledge and competencies in the Agri-food R&D, the valuation of the forecasted results is observed through:

1. building a structure of information and knowledge in electronic format, on classes of various pertinent and relevant areas, needed for the various activities in the organization;
2. establishing the domains of interest, function of which the classification and the searching of the documentation in the virtual library are done;

3. identification, analysis and consolidation of various information and documentation sources and of the optimal solutions for feeding this virtual library, for each domain.
4. deciding on the domain responsibles and of their role in the process of building and managing this virtual collaborative platform.
5. creation of a motivation mechanism, contributing to the stimulation of personnel, collaborators and partners involvement in the feeding of this virtual platform.
6. establishing the solutions of integration and collaboration with other virtual libraries of current or future R&D partnering institutions.

RESULTS AND DISCUSSIONS

One of the main results of the Agri-Food KMP consist of stimulating the participation in international research projects, the platform will offer a comprehensive framework for the management of intangible values and an instrument for protecting the competitive and specific knowledge, with a maximization of the partnerships contributions and results.

Other results concerning the improvement of the performance of the intangible values are:

1. capture and dissemination of the researchers' expertise;
2. conservation of the knowledge base, within the context of experts leaving the institutes/organizations;
3. stimulating the collaboration and value contribution of companies;
4. improvement of the knowledge management process;
5. a better administration of the intangible resources of the organization and monitoring their contribution to the fulfillment of the strategic objectives of the organization.

Synthetically, the correlation between these results and their utility is presented in *Picture 1*:

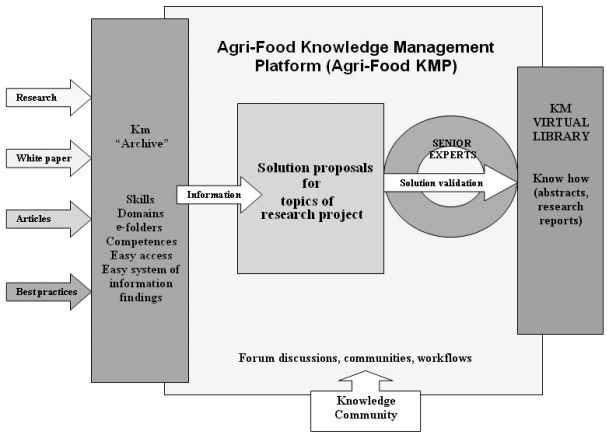

Fig 1. Leveraging the Results

The Agri-Food KMP will be extended, at significantly decreased costs, to organizations with similar profiles (R&D, education institutions), through roll-out mechanisms, considering that the activities and the processes are relatively similar, which leads to a diminishing of the analysis and modeling phases.
The optimization of the web page relevance on the Internet search engines, using SEO (Search Engine Optimization) techniques, will bring about increased visibility notoriety of the platform and of its results.
This research knowledge management system may be successfully implemented afterwards, in various economic domains and sectors: education (similar to the virtual libraries of the great educational centers in Europe and not only –see the OLIS system of Oxford University); health system, economy, etc.

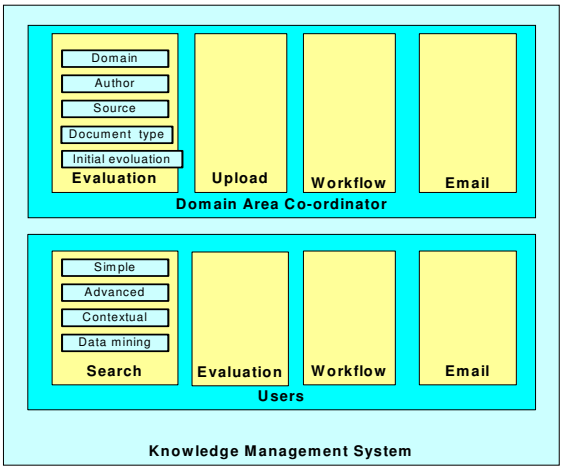

Fig. 2. Using the Results

The Agri-Food KMP may generate the perspective of partnerships at European level in view of joint participation to the specific European research programmes. The system will have two major categories of users (Pic. 2 – Using the Results):

1. **Domain Responsibles –** the only users having system loading authorizations. These will be personalities with authority in their field of expertise, who will have the mission of evaluating the documents to be loaded in the virtual library. They will establish the document attributes (domain, specialty, author, source, document type – handbook, scientific paper, work, etc.), will give the document an initial mark (between 1 and 10) and will load the document (or will give such orders to an administrator). They will be able to send documents on workflows or to send information to various users who subscribed to the respective domain.
2. **Users** – the beneficiaries of the system. They will access complex documents search facilities (simple, multi-criteria, contextual searches), will be able to rank the visualized documents – preferable only in their domain of expertise (otherwise, together with the initial mark, the documents will accumulate during

time multiple rankings from different qualified persons, which will lead to an "average" lacking subjectivism, an important attribute among the search criteria). Also, function of their belonging to various communities, they will be able to send documents on workflows and be able to send documents and information via email. Other technical categories of users: administrators and configurators.
The users of the virtual library will have the possibility to subscribe to various domains organized in communities. The community membership status will allow the user to receive information on the new developments in the domain, as well as on the most relevant works (function on the ranking of a document). As a matter of fact, this ranking will be an important search criterion.

CONCLUSIONS

In conclusion, information and knowledge, as sources of the progress and the sustainability of organizations, tend to become, in the absence of a proper management system, sources of the problems confronting the organizations. Therefore, information and knowledge management is the zone where the organization can get substantial savings, significant developments and long term competitive advantage. The absence of an information management system has a negative effect on the organization competitiveness, affecting its long term development.
The creation of a contextual collaborative platform to manage the intangible values solves the frequent situations when too much information "*kills*" information, since the accessibility and easy extraction of the created and stored intangible values are generally hindered or limited by the absence of a filtering based on a contextual values system, which the users can use to find not only all the information concerning a certain problem but, especially, to find only that information which best solves their problem, under a given context.
An example would be: if, around a technological procedure, we address a classical knowledge management system, we will come up with an extremely important amount of documents. These will contain an impressive volume of information, uncorrelated with the contexts under which they were generated, used or retrieved. This further brings about an inefficient utilization and, most frequently, of an *ad-hoc* nature, of the accumulated knowledge.
The collaborative, integrated and secured platform for the increase of the organizational competitiveness and management of knowledge, information, competencies, expertise and partnerships in the agrifood R&D, assembling representative organizations in the field will be an interactive, intuitive, flexible, easy to use working instrument for all members of the communities involved in the processes of creating and contextually managing the intangible values in the agrifood R&D.

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AN APPLICATION OF STOCHASTIC APPROXIMATION TO A PROBLEM OF VITICULTURE

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ABSTRACT

In this paper, we discussed about an application of stochastic approximation to a problem of viticulture. We presented some fundamental concepts and then, we considered a simple model of competitive antagonism to determine the optimal quantities of two factors: Atonik and Ronilan, so the main shoots reach a well-balanced growth obviously influencing on the grape yield. We solve this problem applaying the stochastic approximation technique and we discuss its comparison with a Newton-Raphson method.

Keywords*: stochastic approximation, random variable, distribution function, regression function.*

1. INTRODUCTION

Multidimensional techniques of stochastic approximation have become very popular due to their practical utility and simplicity.There are experimental situations where the stochastic approximation may be applied, but those might be solved also by using statistical methods or techniques of numerical analysis. In the construction of systems there are many problems which often force one to be satisfied with a design that is less than optimum and the effects of certain factors may not be known and so forth. Under these conditions, it is often desired, once a reasonable system has been built, to attempt to optimize its performance by experimenting with the values of its adjustable parameters.

We presented in [5] some advantages of the stochastic approximation when compared to other methods: first of all, it consists in the fact that it is not necessary to know x value (that value which the experiment depends on) at the initial moment, but only its value at the final moment, that one being easily measured in practice. Moreover, it is not necessary to know the form of the regression function, or to estimate the unknown parameters; therefore, the stochastic approximation is a non-parametric technique that often generates a stochastic process, which is not a Markov process.

2. MATERIAL AND METHOD

The following genuine data come from the results of several analyses, which the Research & Development Institute for Viticulture and Enology, Valea Calugareasca made available for us.

For a better rooting and a successful joining of the grafting partners used in the grapevine grafting (an European grapevine susceptible to phylloxera was used as a scion; an American grapevine resistant to phylloxera was used as a rootstock), Atonik was used as a growth regulator. This product stimulates plant growth and fruit development in case of all the crops by activating the cytoplasm flow, by a

quicker translocation of the raw sap and assimilates, ensuring a faster flow of the minerals and leading to an increase of the chlorophyll content. It also stimulates plant-rooting, seed and pollen germination, shoot growth and proliferation, flower fertility and fruit development; it stimulates the multiplication of micro-organisms in the soil, accelerates the decomposition of the organic matter, indirectly improving soil fertility. Atonik is different from the hormonal stimulators, avoiding the tissue over-saturation in water.

Immediately after using Atonik, the fungicide Ronilan 50 DE is used for controlling grapevine diseases. The former is used for growth stimulation, the latter for destroying different fungus-caused diseases (downy mildew, powdery mildew, grey rot).

We are interested by to determine the optimal quantities of two factors: Atonik and Ronilan, so the main shoots reach a well-balanced growth obviously influencing on the grape yield. We try to solve this problem, applying the multivariate approximation methods. For this, we use the papers indicated in references with [2] and [6].

A general problem of two dimensional stochastic approximation can be formulated mathematically as it follows:

We consider an experiment and two families of random variables

$$\left\{Y_i\left(x\right)\right\}, \quad i=\overline{1,2}, \tag{1}$$

with corresponding distribution functions:

$$F_i\left(Y\left(x\right)\right), \quad i=\overline{1,2}, \tag{2}$$

the regression functions:

$$M_i\left(x\right)=\int_{-\infty}^{+\infty} Y dF_i\left(Y\left(x\right)\right), \quad i=\overline{1,2}, \tag{3}$$

and the variances:

$$\sigma_i^2\left(x\right)=E\left(Y-M_i\left(x\right)\right)^2, \quad i=\overline{1,2} \tag{4}$$

We suppose there exists:

$$\theta_s=\left[\theta_1,\theta_2\right], \tag{5}$$

uniquely determined, by $\theta_1+\theta_2=\theta_s$ and $M_1\left(\theta_1\right)=M_2\left(\theta_2\right)$, with the means $M_1\left(x\right)$ and $M_2\left(x\right)$ are strictly monotone increasing, continuous and the intersection of their ranges is an interval of positive length.

Sequential sets of two experiments are to be performed in the following manner.

To initial levels, we consider a vector

$$X_1=\left[X_{11},X_{21}\right], \tag{6}$$

arbitrarily chosen and succeding levels for each set of experiments are defined by:

$$X_{n+1} = X_n + a_n\left[\overline{Y_n} - Y_n\right], \quad a_n > 0, \sum_1^\infty a_n = \infty, \sum_1^\infty a_n^2 < \infty, \tag{7}$$

where:

$$X_n = \left[X_{1n}, X_{2n}\right],\ Y_n = \left[Y_{1n}, Y_{2n}\right] \text{and} \overline{Y_n} = \left[\overline{Y_n}, \overline{Y_n}\right], \quad \overline{Y_n} = \frac{1}{2}\left(Y_{1n} + Y_{2n}\right). \tag{8}$$

We assume that both experiments in each set are performed independently and we suppose for all x and $i = 1,2$, there exist V, A and B, positive real numbers, such that:

$$\sigma_i^2\left(x\right) < V < \infty, \tag{9}$$

$$\left|M_i\left(x\right)\right| < A\left|x\right| + B < \infty. \tag{10}$$

this means that the averages and the variances are real numbers.
There is a theorem which ensures that under the above-mentioned conditions:

$$P\left[\lim_{n\to\infty} E\left\|X_n - \theta_s\right\|^2 = 0\right] = 1. \tag{11}$$

We remind the most important aspects in a procedure of stochastic approximation. First of all, one will be interested in the convergence and mode of convergence of the sequence generated by the method to the desired solution of the equation. Next, one would like to know the asymptotic distribution of the sequence. Finally, one will be interested to know an optimum stopping rule for a given situation.

As a conclusion of what was said above, we mention that when a character variable depends on two factors, each of whom enforcement response is a random variable with mean, dispersion, distribution function and the corresponding regression and checking certain conditions, the approximation method ensures that an iterative sequence of values assigned to variables which depends on the experimental nature is convergent and the limit is that value of the variable for which we obtain optimal response.

An alternative to stochastic approximation is Newton-Raphson method. This is a iterative technique of numerical analysis, which is frequently used in problems. Let M be a function $M : \left[a,b\right] \to \left[a,b\right]$. We are interested in solving the following equation:

$$M\left(x\right) = \alpha, \tag{12}$$

where the functional form of M is not known, but it is known that M is increasing and continuous. We construct a sequence $\left\{X_n\right\}_{n\geq 1}$ by recursive relation:

$$X_{n+1} = X_n - a^{-1}\left[M\left(X_n\right) - \alpha\right], \quad a \neq 0, \tag{13}$$

with $X_1 \in [a,b]$ arbitrarily chosen. Then, under suitable conditions, $X_n \xrightarrow[n\to\infty]{} \theta$ such that $M\left(\theta\right) = \alpha$.

By Newton-Raphson method, we consider iterative relation:

$$X_{n+1} = X_n - a_n^{-1}\left[M\left(X_n\right) - \alpha\right], \tag{14}$$

where:

$$a_n = \begin{cases} a_1, & M'\left(X_n\right) < a_1 \\ M'\left(X_n\right), & a_1 \le M'\left(X_n\right) \le a_2, \\ a_2, & M\left(X_n\right) > a_2 \end{cases} \tag{15}$$

a_1 and a_2 are constants such that $0 < a_1 < a_2 < \infty$ .Though this method involves the computation of the derivative of *M* at each stage of iteration, the eventual rate of convergence is improved.

The problem of existence of the solution and its uniqueness is solved by Ostrowsky [4]; this implies a result for the problem under consideration.

3. RESULTS AND DISCUSSION

Several grapevines were taken into study and each 5 days the growing process of the main shoots was registered after applying only Atonik in case of some plants and only Ronilan in case of the others.

We applied the previous theory and the following results were obtained:

Table 1- The observational data for 5 experiments

Atonik dosage (mg / l)	Ronilan dosage (mg / l)	Treated by Atonik (growth -cm)	Treated by Ronilan (growth -cm)	$\overline{Y_n}$
$X_{11} = 5$	$X_{12} = 4,3$	$Y_{11} = 5,2$	$Y_{12} = 5,4$	$\overline{Y_1} = 5,3$
$X_{21} = 5,3$	$X_{22} = 4,5$	$Y_{21} = 5,8$	$Y_{22} = 6,2$	$\overline{Y_2} = 6$
$X_{31} = 5,6$	$X_{32} = 4,7$	$Y_{31} = 7,3$	$Y_{31} = 7,5$	$\overline{Y_3} = 7,4$
$X_{31} = 5,7$	$X_{32} = 4,8$	$Y_{31} = 7,5$	$Y_{31} = 7,5$	$\overline{Y_3} = 7,5$
$X_{31} = 5,8$	$X_{32} = 4,9$	$Y_{31} = 7,6$	$Y_{31} = 7,8$	$\overline{Y_3} = 7,7$

Using the Newton-Raphson method, we determine the optimal quantities of both substances (Atonik and Ronilan) so that the main shoots reach a well-balanced growth obviously influencing on the grape yield.

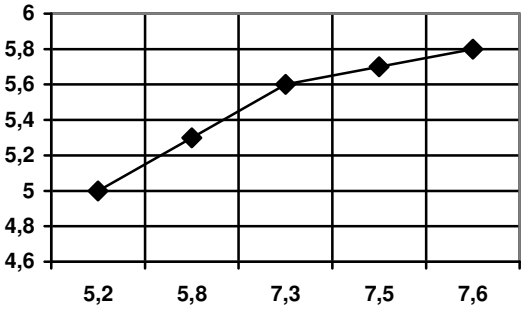

Fig.1 The growth (cm) of plants treated by Atonik

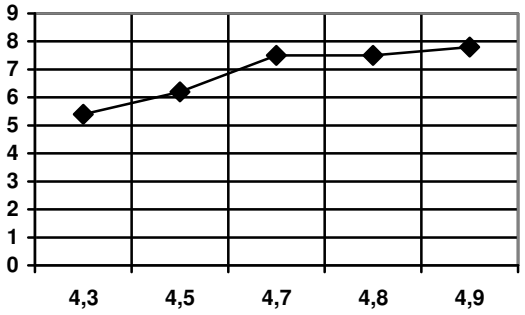

Fig.2 The growth (cm) of plants treated by Ronilan

4. CONCLUSIONS

With the use of these methods, the algoritm to determine optimal amounts of both substances (Atonik and Ronilan) becomes more effective, but also more complex. It is desirable to have robust algorithms, wich are not overly sensitive to unusually random values. Many problems have constraints in the sense that the vector-valued iterate must be confined to some given bounded set. Robbins and Monro recognized that taking an excessive number of observations at each of the $\{X_n\}_{n\geq 1}$ and modeling the algorithm on (13) was inefficient, since X_n is only an intermediary in the calculation and the response $Y(X_n)$ is only of interest in so far

as it leads us in the right direction. More ideas and results are given by Kushner and G.G. Yin in their book [2].

We intend in future, work to find a solution of the problem previously analyzed, by applying a nonlinear model of resource allocation in Biotechnology, specifically to determine an optimum vine growth, establishing the contributions of the factors contributing to the development of plant.

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