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

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Abstract

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

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

INTRODUCTION

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

Meanwhile, molecular techniques based on PCR amplification of pathogenic genome

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

MATERIALS AND METHODS

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

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

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

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

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

Figure 1. Symptomatic plants in Turan plantation.

Table 1. The number of samples collected for DNA analysis

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

RESULTS AND DISCUSSIONS

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

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

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

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

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

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

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

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

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

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

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

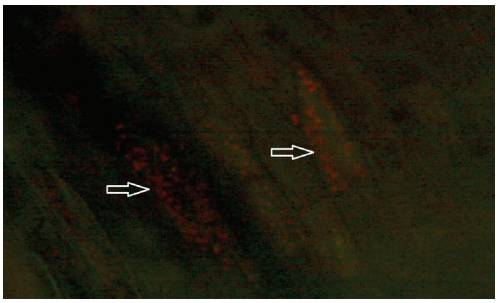

Figure 2. Fluorescent phytoplasma-like bodies in the phloem of leaf main nervure tissue.

CONCLUSIONS

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

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

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

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

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