PHYTOPLASMAS DETECTION EFFECTIVENESS BASED ON SYMPTOMATOLOGY, DAPI STAINING AND SPECIFIC PCR AT PLUM PLANT MATERIAL OF DIFFERENT CATEGORIES

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ABSTRACT

Phytoplasmas are important pathogenic invaders of fruit trees. Numerous symptoms associated with the destruction of the normal equilibrium of growth regulators are among the most displayed, and because of this, symptomatology only may not serve for accurate diagnosis of phytoplasmatic infection. Microscopic and molecular methods are among the most used in this respect. DAPI staining detection is based on the use of 4', 6-diamidino-2-phenylindole, a fluorescent stain, which lights under UV light, and makes phytoplasmas visible. The PCR based methods of detection address mainly the amplification of ribosomal and non-ribosomal specific DNA from phytoplasmas. Present study compares the results on the detection of phytoplasmatic infection at 300 plum samples, from four collections in Korça, Albania based on symptomatology, DAPI staining and specific PCR. From the symptomatology results of three consecutive years (2014-2016) is clear that symptoms are displayed differently in different periods of time for the same trees; DAPI staining also provides different situation on presence of phytoplasmas in different periods of sampling; Specific PCR proves successful for the amplification of ribosomal DNA fragment from all the analyzed plant material. In conclusion, the three methods complement each-other and might be used to detect phytoplasmas infection, but specific PCR is more accurate, fast and reliable method.

Keywords: Symptomatology, DAPI staining, PCR, phytoplasma, Plum leptonecrosis phytoplasma.

INTRODUCTION

A large number of plant diseases of yellowing types were thought to be caused by viruses, this relying on ways of spreading, symptomatology and the fact that they are transmitted via insects. The first demonstration that agents who cause the yellowing disease can be wall-less prokaryotes (phytoplasmas) was made earlier than 40 years ago (Doi *et al*., 1967).

Plants infected with phytoplasmas demonstrate diverse symptoms. Disturbances in normal equilibrium growth factors lead to virescence/phyllody (development of structures similar to leaves instead of flowers), sterility of flowers, the proliferation of axillary buds forming a display similar to a witches broom, abnormal extension and asthenia (Bertaccini, 2007). Other symptoms that are commonly found are: reduced development of specific parts or the entire plant that can reach up to rickets; deformities or malformations of leaves or flowers, delay in the opening of buds or fruit trees or in bloom; general yellowing of leaves that is a widespread symptoms named after some phytoplasmas and redness leaves during autumn. All these symptoms are often caused by viruses, so their diagnosis, despite the long field experience, necessarily requires laboratory analysis (Susuri and Myrta, 2012).

For the detection of phytoplasmas are used a variety of laboratory techniques. Detection of phytoplasmas now is routinely performed by various techniques based on nucleic acid, polymerase chain reaction (PCR). These techniques developed in the last 20 years are suitable for detecting phytoplasmas in plant material and in vector insects. The success of PCR in the detection of phytoplasmas in plant material depends mainly on the extraction of genetic material of good quality, enriched with phytoplasmatic DNA, which presents many difficulties (Firrao *et al*., 2007). The amount of phytoplasmatic DNA is lower than 1% of the total DNA extracted from plant tissue (Bertaccini, 2007). Different protocols for the extraction of total DNA were used for detecting these pathogens in plants with main purpose the concentration of phytoplasmal DNA. This is generally achieved by including in the extraction protocol a step for the enrichment with phytoplasmas. PCR assays using primers based on cloned DNA fragments, specific to a given phytoplasma, provided sensitive as well as specific means for their detection. In contrast, PCR assays using generic or broad-spectrum primers based on conserved sequences allowed detection of a wide array of phytoplasmas associated with plants and insects (Firrao *et al.*, 2007). However, PCR-based methods are effective, but they have some limitations mainly related to the high cost and level of specialization of personnel in laboratories. For this reason, other techniques including DAPI staining (4',6-diamidino-2-phenylindole), which are quick and inexpensive, are seen as the best solution in many cases. DAPI is a relatively sensitive method but its effectiveness depends on the number of phytoplasmas (Kartte, S., and Seemuller, E. 1991). It has the ability to connect with regions rich in A-T of phytoplasmas making them fluorescent and visible in fluorescence microscope. Phloem cells of infected plants show stronger fluorescence than that of typical of nuclei of other parenchymal cells. Infected tissues generally show bright spots in the sieve tubes of phloem, which are not present in healthy tissue (Arismendi AS et al., 2010). The DAPI staining technique is considered as a fast and accurate method to locate phytoplasmas in various tissues such as roots, leaves, etc (Sinclair et al., 1992, Thomas and Balasundaran 1998, Bricker and Stutz 2005., Arismendi A.S. et al., 2010).
Continued efforts aim at improving diagnostic procedures, development of rapid and more economical methods.

METHODOLOGY

Plant material: Plant material was sampled from three plantations in the district of Korca (Dvoran, Zemblak, Cangonj), Albania. According to sampling procedure described by Rekab *et al*., (2010), 10 from 100 trees were selected and sampled as follows: 5 samples were taken for each of the four categories of materials (roots, trunk, stem, leaves). A total of 600 samples were collected from 30 trees of plum cultivars *Tropojane* and *Iliria*.
Field observation: was conducted randomly according to predefined schemes (schemes X, Y, Z).

DNA extraction: DNA extraction was made based on the sample enrichment protocol for phytoplasmas, using the MLO buffer described by Kirkpatrick *et al*., (1987) with some minor modifications. The usage of MLO aimed to enrich the sample with phytoplasmas making possible the extraction of their DNA and eliminating as far as possible the DNA of the plant. The second buffer that was used was CTAB by (Doyle and Doyle., 1990). DNA was extracted from four different categories of plum tissues, roots, trunk, stalks, and leaves.

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.

The selection of primers and the amplification process: four primer pairs were used to amplify the ribosomal sequence, according to Schneider and Semuller, 1993. PCR mixture had a volume of 40 µl containing 2 µl of template DNA, 2 µl of each primer, 0,4 µl the four dNTPs, 0,2 units of GoldStar polymerase, and 1x buffer. It was cycled 35 times at the following conditions (Sakai *et al*., 1988).

The cycling protocol for amplification using these primers fU5/rU3; fO1/rO1; fCPD/rCPD, is as follows			
Initial denaturation	95°C	4’	
Denaturation	95°C	30”	35 cycles
Annealing	51°C	75”	
Elongation	72°C	90”	
Final elongation	72°C	5’	
4°C		Hold, ∞ Infinity	

The cycling protocol for amplification using these primers fCAP/rCAP, is as follows			
Initial denaturation	95°C	4’	
Denaturation	95°C	30”	35 cycles
Annealing	56°C	75”	
Elongation	72°C	90”	
Final elongation	72°C	5’	
4°C		Hold, ∞ Infinity	

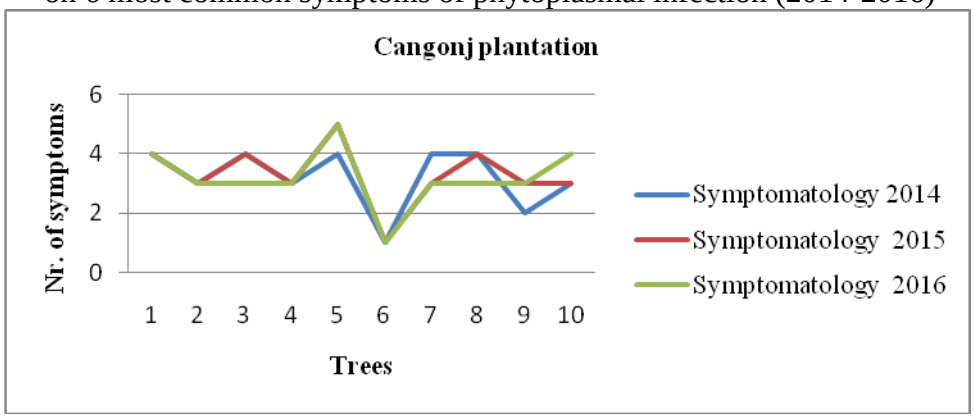
Gel Electrophoresis: 1.5% agarose gel in TAE was used to analyze products multiplied by PCR.

DAPI staining: leaves 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). Transversal sections from the fixed samples were prepared, and placed from sterile distilled water to 5% glutaraldehyde solution. Fixed samples were transferred to fresh 0.1M phosphate buffer (pH 6.9). The last stage included the Thermo Fisher Scientific DAPI staining 1×working solution, which makes phytoplasmas visible. Preparations were observed with fluorescent microscope at10x, 40x and 100x.

RESULTS AND DISCUSSION

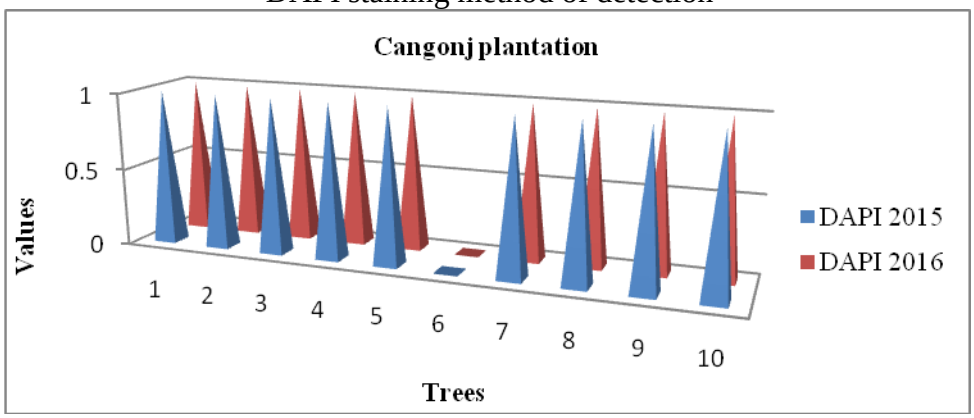
The yearly evaluation of phytosanitary status were conducted for the three plantations of plums from 2014-2016. In the following charts are described results based on symptomatology, and two-year situation according to the microscopic technique DAPI. A matrix was prepared for each sample, where presence/non-presence of viral infection was marked as 1/0.

Picture 1. Graphic presentation of the phytosanitary station of collection of Cangonj based on 6 most common symptoms of phytoplasmal infection (2014-2016)



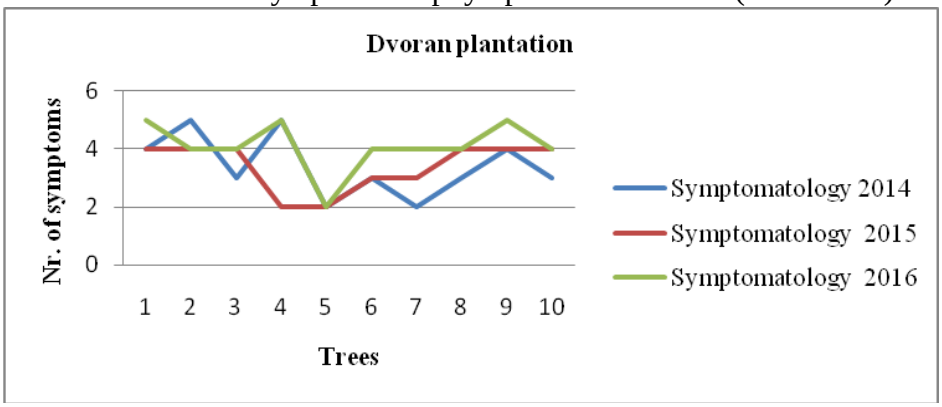
As seen from the graph, the number of different symptoms, in different trees, in different years has fluctuated. An exception from the general rule observed, plums numbered as tree 1, 2, 4, 6 have shown the same number of symptoms throughout the three year period of sampling. The number of symptoms in trees from the collection of Cangonj ranges from 1 to 5.

Picture 2. Phytosanitary status of the collection of Cangonj during 2015-2016, according to DAPI staining method of detection



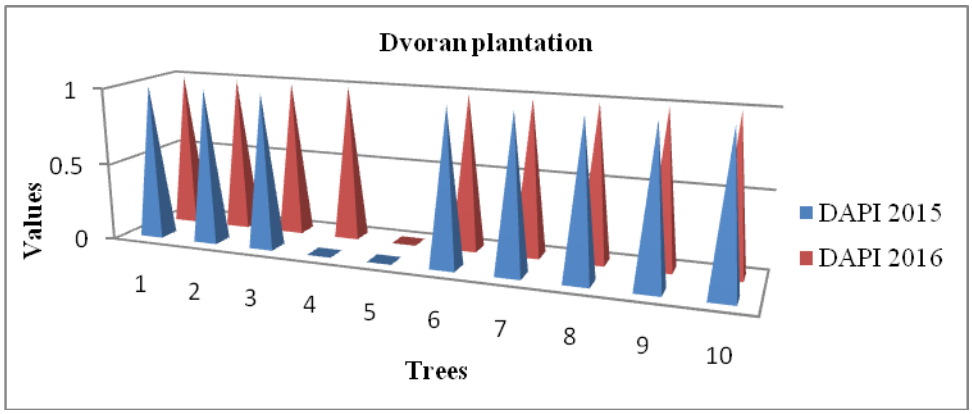
As seen from Picture no 2, the situation of the infection seems to not have changed from year to year. All trees are infected, except samples taken from tree number 6. If compared the results of evaluation based on symptoms with those from DAPI staining, the tree number 6 displays less symptoms than the rest of the trees.

Picture 3. Graphic presentation of the phytosanitary station of collection of Dvoran based on 6 most common symptoms of phytoplasmal infection (2014-2016)



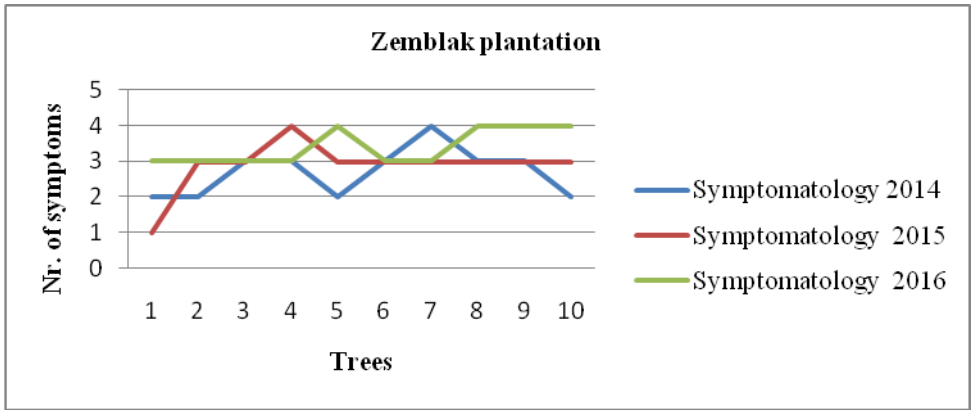
As seen from the chart, most of the trees have different number of symptoms displayed in different years. Tree number 5 is an exception, from this rule. The number of symptoms of infection observed at tree samples from the collection of Dvoran varies from 2 to 5.

Picture 4. Phytosanitary status of the collection of Dvoran during 2015-2016, according to DAPI staining method of detection



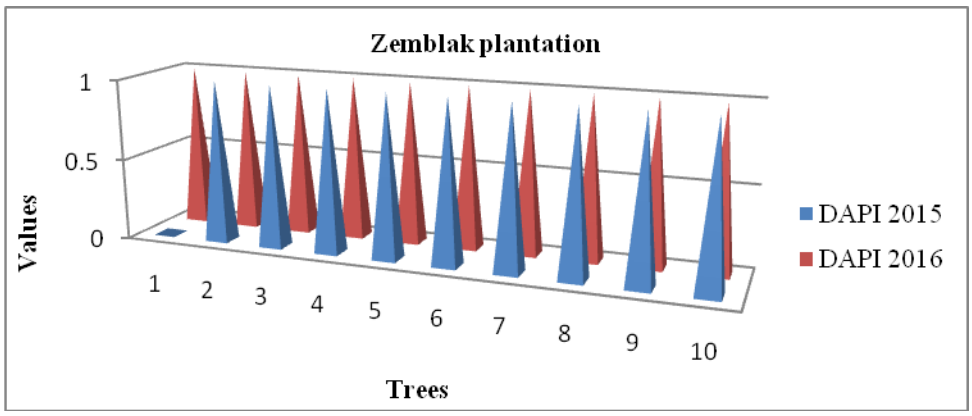
According to picture 4 in 2015, 8 out of 10 trees were infected. In 2016 it is noted that only 1 out of 10 trees is infected. Tree number 4 displayed different number of symptoms from year to year. This fact might be explained by two hypotheses. The first possibility is that tree number 4 has not been infected in 2015, or the lack of symptoms might be a result of non-systemic spread, which is a characteristic of infections caused by phytoplasmas.

Picture 5. Graphic presentation of the phytosanitary station of collection of Zëmblak based on 5 most common symptoms of phytoplasmal infection (2014-2016)



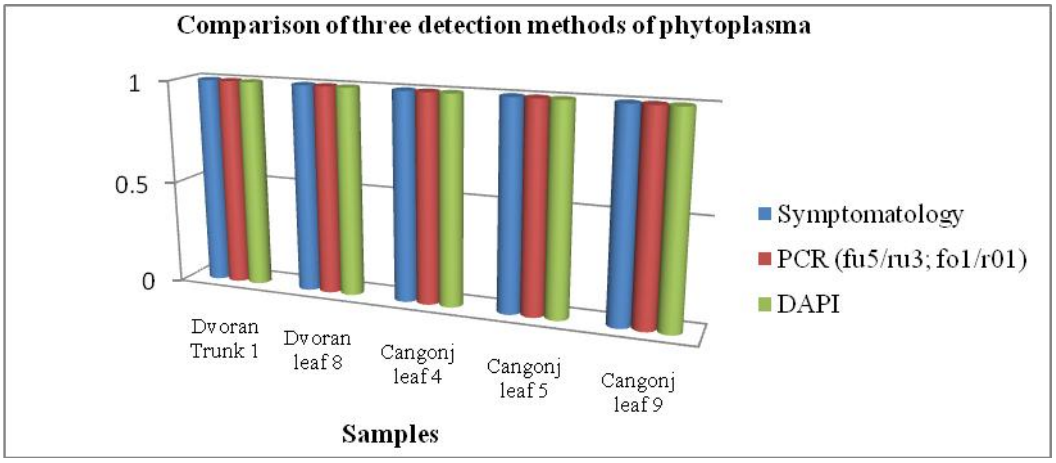
As seen from the graph, the number of symptoms in different trees varies from year to year. An exception are trees number 3 and 6. The number of symptoms in the collection of Zemblak ranges from 1 to 4. The comparison of the number of symptoms in all three plantations shows that Cangonj plantation has the highest number.

Picture 6. Phytosanitary status of the collection of Zëmblak during 2015-2016, according to DAPI staining method of detection



Only 1 out of 10 trees resulted non-infected in both years of observation. Tree no 1, which was not infected in 2015, was infected after one year. There may be two ways of explaining this fact. Either tree number 1 was infected in 2016 by transmitters from neighbor plants, or the result comes due to the non-systemic spread of infection. If we compare the results of DAPI staining for all three collections it is noticed that at Zemblak the level of infection is higher.

Picture 7. Graphical presentation of the effectiveness of three methods for phytoplasmal infection (PCR, DAPI, symptomatology)



Five samples of plums from Dvoran and Cangonj collections were analyzed for presence of phytoplasmas with the three methods. Results show that 5 out of 5 are infected, based on the three methods. The PCR result is considered as the most reliable and fast, however, we faced difficulties in obtaining DNA of proper quality to be used for PCR amplification of phytoplasmal gene fragments from plant tissues other than leaves. The five DNA samples (picture 7) represent mixtures of DNA extracted from tress named respectively, from leaves mainly.

CONCLUSIONS

- The symptoms and DAPI staining method of detection results depend on season of sampling, and on the sampling position at each tree;
- While symptomatology can provide information on presence of disease in advanced phases, DAPI can help identify earlier stages of infection. However, in order to be successful a certain concentration of phytoplasmas is required.
- PCR based diagnostic is strongly dependent on the quality of the DNA, extracted from plant tissues and enriched for the phytoplasmas DNA content, but is not dependent on the amount of template DNA.
- In conclusion, we recommend the simultaneous use of the three methods, which complement each-other assuring this way a more reliable result.

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