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

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Abstract

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

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

INTRODUCTION

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

Table 1. Oligonucleotide primers used for phytoplasma detection.

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

RESULTS AND DISCUSSIONS

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

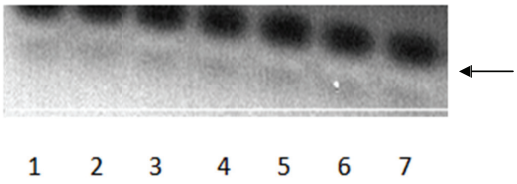

Figure 1. Examples of the results obtained with ribosomal primer pairs fCPD/rCPD, in amplifying template DNA extracted from apple trees.((From left to right: 1, 2. Leaves from Korca plantation; 3. Root from Korca plantation; 4, 5. Trunk from Korca plantation; 6, 7. Stalk from Korca plantation.

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

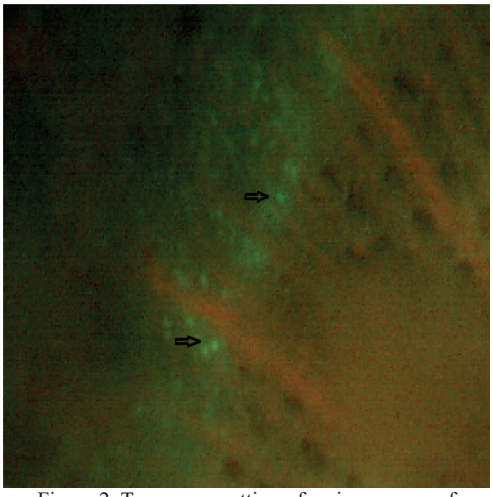

Figure 2. Transverse cutting of main nervures of symptomatic apple leaves, DAPI staining.

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

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

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

Figure 3. Symptomatic tree in the collection of Turan.

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

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

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

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

CONCLUSIONS

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

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

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