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OCCURRENCE OF PHYTOPLASMAS OF THE APPLE PROLIFERATION GROUP IN FRUIT TREES IN KYUSTENDIL REGION OF BULGARIA

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Abstract

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Apple, pear and plum trees showing symptoms typical for apple proliferation, pear decline and European stone fruit yellow from Kyustendil region of Bulgaria were analyzed for phytoplasma infection by polymerase chain reaction technology. PCR was performed by the use of specific for Apple proliferation group set (Loewe Phytodiagnostica, Germany) and the protocol recommended by the manufacturers. The results obtained showed the presence of phytoplasmas in 85.0% and 62.0% tested apple and pear trees, respectively and in all tested plum trees. Restriction fragment length polymorphism analysis revealed that apple and pear were infected with apple proliferation and pear decline phytoplasmas, respectively, while European stone fruit yellows was detected in plum trees. Because many trees showed symptoms like those of sampled and analyzed trees, this preliminary assay suggest a high incidence of phytoplasmas from AP group in the surveyed region of the country.

Key words: fruit trees, phytoplasmas, AP, PD, ESFY, detection, PCR, symptoms

Introduction

Apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) are among the most economically important plant diseases that are caused by the phytoplasmas *Candidatus* Phytoplasma mali*',* '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', respectively. Those three agents are closely related to each other, with 98.5-99% homology in the nucleotide sequences of the 16S gene and are therefore combined in the group of apple proliferation (16SrX group) (Lee et al., 1998; Seemüller and Schneider, 2004; IRPCM, 2004; Marcone et al., 2010).

Phytoplasmas are transmitted by insect vectors, mainly by leafhoppers (*Cicadellid*ae) and psyllids (*Psyllidae*) in a persistent-propagative manner (Carraro et al., 1998; White et al., 1998). They are also graft-transmissible agents and the use of infected planting material contributes largely to their spread.

Phytoplasmas cannot be distinguished from each other by morphological or structural characteristics with optical and/or electrono-microscopic methods (CABI, 2009). Their detection is based on use of biological, serological and molecular methods, including indexing on woody indicators, ELISA method with monoclonal antibodies (Loi et al., 2002), hybridization, direct/nested PCR, RFLP and real-time PCR. The highly sensitive PCR technology with universal, as well as group- and pathogen-specific primers direct to both ribosomal and nonribosomal DNA sequences has widely been applied (Jarausch et al., 1994; Lorenz et al., 1995; Smart et al., 1996).

The three phytoplasma from AP group are quarantine pathogens, both in Bulgaria and Europe (EPPO/CABI 1997) and are under official control. In 2005 the Central Laboratory for Plant Quarantine developed a programme for monitoring of quarantine pathogens on fruit trees and vine, including phytoplasmas. In the implementation of the program Bulgarian Agency for Food Safity (BAFS) found the presence of

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the three mentioned above phytoplama diseases in 10 out of 28 surved regions of the country (Etropolska and Laginova, 2012; Avramov et al., 2013).

The present study describes the results obtained in preliminary survey carried out in apple, pear and plum orchards in Kyustendil region of Bulgarian using PCR assay.

Materials and Methods

Plant samples

A survey and collection of plant samples was carried out in the fall (September–November) of 2013 and 2014. All trees were naturally infected and showed symptoms typical for the diseases in AP group. Apple trees from five differently aged (6 to 23 aged) orchards planted with Florina, Granny Smith, Freedom, Prima, Coop10, Golden delicious, Jonafree, Priam cultivars and several old cultivars named White winter kalville, Aport, Cox's orange Renette, Kandile, Cardinal were subject of the study. In addition pear trees from one approximately 22 year's old abandoned orchard and Japanese plum trees (*Prunus salicina*) from one young (4 years old) orchard were included. In total, twenty nine apple samples, eight and five samples from pear and plum, respectively were collected for molecular analyses.

DNA extraction, PCR amplification and RFLP analysis

Total DNA was extracted from leaf midribs and/or stem phloem pre-grounded to a fine powder under liquid nitrogen according to Dellaporta et al., 1983 and Doyle and Doyle, 1990.

Different aliquots, containing not more than 100–150 ng/µl DNA were used in PCR amplifications, performed in 50 µl reaction mixture of AP Phytoplasma group PCR set (Loewe Phytodiagnostica, Germany) and the protocol recommended by the manufacturer's. This AP PCR set allows the detection of Apple Proliferation (AP), Pear Decline (PD), European Stone Fruit Yellows (ESFY) and Peach Yellow Leaf Roll (PYLR). The mixture was subjected to 35 cycles at the following conditions: 20s denaturation at 93°C, 60 s annealing at 55°C and 60 s extension at 72°C. Positive and negative controls from the PCR set were included in each PCR amplification. In addition DNA extracted from infected with AP, PD and ESFY periwinkle (*Catharanthum roseus* L.) leaves (kindly provided by d-r J. Avramov, BAFS) were used as positive controls, too. Five µl of PCR products were analyzed by electrophoresis in 1.5% horizontal agarose gel in TAE buffer in the presence of GelRed™ dye (stock solution ×10 000, Biotium). DNA bands were visualized using UV transilluminator (EC 3 Imaging System).

For RFLP analyses five µl of PCR products were digested separately with *Rsa*I restriction endonuclease, following the manufacturer's instruction (Fermentas). 2.5 µl of the digests were used to resolve the restriction fragments in 1.5% horizontal agarose gel in TAE buffer. After electrophoresis, The DNA was visualized with GelRed™ dye as described above.

Results and Discussion

Symptoms

The main symptoms of AP disease on apple described by Nemeth (1986), as 'witches' brooms, enlarged stipules, small size fruits with poor taste and redding of the leaves were observed in all five surveyed orchards and cultivars. The premature development of axillary buds of trees, which give rise to secondary shoots/shoot proliferation (witches' brooming) were clearly visible in January – February, before the vegetation season. The 'witches' brooms formation directly from the trunk or central branches was much more common, compared to that of the apices branches. The other characteristic symptom, as abnormal enlarged stipules mostly on the wiches'brooms branches was observed, too. The fruits of the infected trees in almost all cultivars were dwarf and reduced in size, compared to those of the healthy trees of the same cultivars. Single large flowers appeared in late summer – early fall on some trees of Granny Smith and Prima cultivars. On the base of the observed symptom all samples for further molecular analyses were collected from the trees with the described symptoms.

On pear trees from the only one surveyed and sampled orchard, leter development in spring and total reduced growth was noted. Leaves were few, smaller and light green with uprolled margins. The growth of shoots was also very weak (a few cm). The most characteristic symptom, however, was reddening of the leaves and their premature fall in early autumn. All samples for molecular analyses came up from the trees showing the described symptoms, which are typical for PD.

The symptoms described for ESFY (Carraro et al., 1992; Jarausch et al., 2000a; 2000b; Carraro and Osler, 2003) were observed on the trees of sampled for analysis Japanese plum trees. They consisted of early bud break, followed by leaf reddening and rolling with smaller size and sparse foliage.

Phytoplasma detection and identification

Twenty two out of twenty five tested samples from apple (Figure 1A), five out of eight pear samples and all tested plum samples (Figure 1B) reacted positive for AP phytoplasma group in PCR analyses. PCR products of the expected size (1050 bp) of the above mentioned samples and the positive controls were observed. No amplification products were

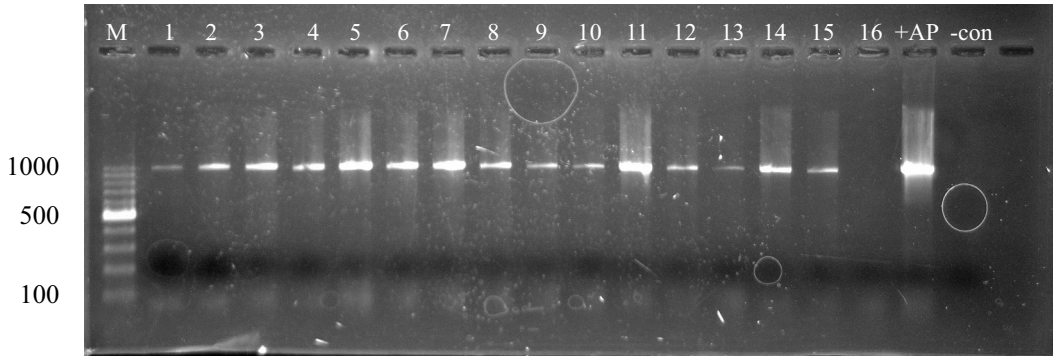
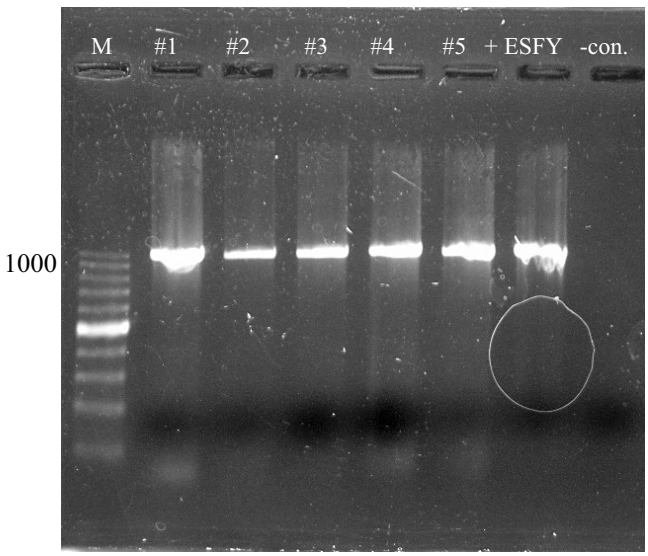


Fig. 1. PCR amplification with AP phytoplasma group PCR set (Loewe, Germany)
A/ M: 100 bp Ladder (O'GeneRuler); 1–16 DNA target from different apple cultivars, as Coop 10, Freedom, Prima, White winter kalville, Aport, Cardinal, Cox's orange Renette, Kandile, Priam and Jonafree. Positive control: AP (periwinkle); negative control: PCR set



B/ M: 100 bp Ladder; 1–5 DNA target from different plum trees. Positive control: ESFY (periwinkle); negative control: PCR set

obtained with three apple samples (in a sample of Florina, Golden delicious and Jonafree cultivars), in three pear samples and the negative controls.

Nevertheless, all 25 and 8 tested samples from apple and pear, respectively had the disease symptoms, 22 apples (88%) and five pear (62%) samples were positive for phytoplasma infection. This could be due both to the absence of the disease, but most likely to the poor quality of the DNA targets. It should be noted also that the association of phytoplasmas only with symptom expression is not sufficient to determine health status of the inspected trees having in mind that other pathogens, like viruses, fungi or bacteria might affect.

Since all tested samples from Granny Smith, Freedom, Prima and several samples from Golden delicious were positive for AP phytoplasma group those cultivars could be accepted as susceptible also in Bulgaria, as already reported in other European countries (Osler et al., 2001; EPPO, 1997; Kartte and Seeműller, 1991).

Following digestion of all amplified PCR products with *Rsa*I restriction endonuclease apple and pear samples were not digested, whereas the corresponding fragments from plum showed a restriction profile with the presence of two bands with a size of approximately 400 and 300 bp (Figure 2). According to Seeműller and Schneider, (2004) ESFY agent can be differentiated from the AP and PD phytoplasma by *Rsa*I digestion, as well as with *Bsa*I since both AP and PD do not contain the restriction sites of these enzymes.

The existence of AP in different orchards from Bulgaria, based on symptomatology has been noted a long time ago by Trifonov (1965; 1967; 1969 and 1975). More recently through the use of molecular diagnostic methods several more phytoplasma diseases on stone and pome fruit species, as pear decline, European stone fruit yellows and apple proliferation were identified, too (Topchiiska et al., 2000; Topchiiska and Sakalieva, 2001, 2002).

The symptoms observed on diseased apple, pear and plum trees were similar to that described in Bulgaria by Topchiiska et al. (2000), Topchiiska and Sakalieva (2001, 2002) and from other geographic areas (Goidanich, 1933; Woodbridge et al., 1957; Bovey, 1963; Nemeth, 1986; Malinowski et al., 1996; Carraro et al., 1992; Del Serrone et al., 1998; Seeműller et al., 1998; Vorackova et al., 1998; Jarausch et al., 2000a; 2000b; Carraro and Osler, 2003).

Presently the most widely used method for detection and differentiation of phytoplasma agents is by PCR method,

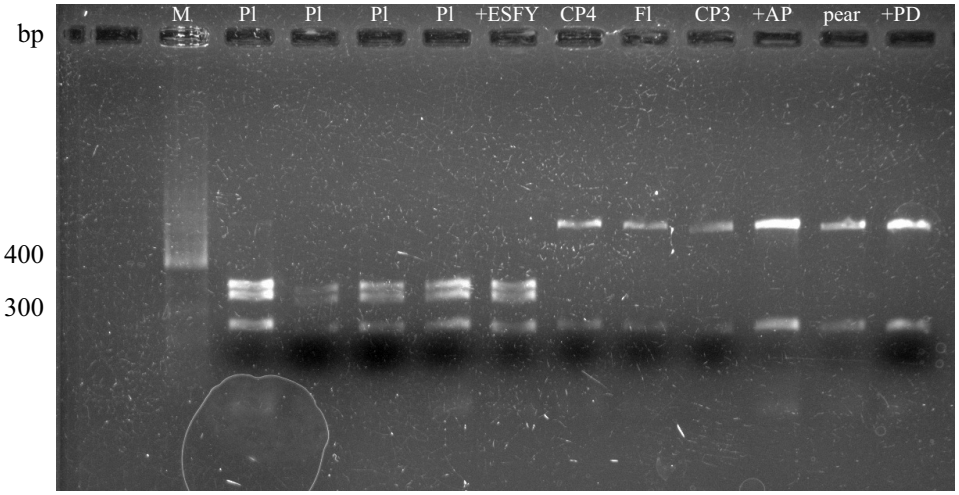


Fig. 2. RsaI restriction analysis of amplified with AP phytoplasma group PCR set products
M: 100 bp Ladder; 2–5 DNA target from different plum trees. Positive control: ESFY (periwinkle); 7–9: DNA target from different apple trees (Coop 10, Florina and Coop 10). Positive control: AP (periwinkle); 11: DNA target from pear; Positive control: PD (periwinkle)

which is highly sensitive and suitable for the detection of the pathogens in different plant tissues and in their insect-vectors. The three phytoplasma in the group, as AP, PD and EFSY are closely genetically related and after the standard PCR with the use of some of phytoplasma-universal primers, nested PCR with group-specific primers, RFLP analyses and/or sequence analyses should be performed for their differentiation.

With few exceptions all DNA samples from apple and pear trees sampled in the region of Kyustendil region of Bulgaria yielded PCR products when amplified with detection set specific for AP phytoplasma group including AP, PD, ESFY and PYLR. In addition, digestions with *Rsa*I restriction endonuclease resulted in a profile typical for ESFY agent. These results clearly showed that tested plum trees were infected with ESFY. ESFY infection on stone fruit species, as apricot, peach and almond was reported for the first time in Bulgaria by Topchiiska et al. (2000) All together the results of our study indicates that AP, PD and ESFY phytoplasmas are more widespread in Bulgaria and occurred in more fruit growing areas of the country known up to now.

Conclusion

The detection of diseases from AP group in 85.0% and 62.0% tested apple and pear trees, respectively and in all tested plum trees, together with the observed many symptomatic trees is indication for a high occurrence of phytoplasmas within and around the surveyed orchards in the region of Kyustendil. To have a clear insight into the distribution and strain composition in the region and in the country, the surveys should be continued. Further establisment of the insect-vectors in the region will help the development of chemical contol measures.

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