Detection and identification of phytoplasmas in peach based on woody indexing and molecular methods

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Summary: Symptoms resembling phytoplasma disease have been observed on peach trees in a seed-source plantation of stone fruits in south Hungary quite recently. In this publication we report on the results of woody indexing of symptomatic peach trees on GF 305 indicator in the field and under greenhouse conditions as well as on molecular studies. Phytoplasma infection detected on GF 305 indicators in greenhouse and field indexing was confirmed by PCR. Nested PCR was conducted using universal primer pairs followed by group and subgroup specific primers for the second amplification. RFLP analysis of nested PCR products was performed using Rsal restriction enzyme. Based on the results of molecular studies it can be concluded that phytoplasmas, belonging to the European stone fruit yellows subgroup (16SrnX-B) were identified in peach trees. Further studies on symptomatic peach trees originating from different parts of Hungary are in progress.

Introduction

Phytoplasmas (formerly called mycoplasma-like organisms = MLOs) can cause severe diseases on fruit tree species (Németh 1986). Detection of phytoplasmas has been performed with biological indexing on woody indicators for several decades, but identification of the causal agents became possible only with recent advances in laboratory methods. Molecular techniques such as Polymerase Chain Reaction (PCR) assays using specific primers based on cloned phytoplasma DNA sequences as well as Restriction Fragments Length Polymorphism (RFLP) analyses applying different restriction enzymes for digestion of PCR products (amplified 16S rDNA) and ribosomal gene sequences provided possibility to develop reliable and rapid means for differentiation and classification of phytoplasmas. So nowadays laboratory techniques became available not only for detection, but also for distinction between phytoplasmas, belonging to the same 16S group or subgroup and causing similar symptoms on different host species.

In the previous decades phytoplasma diseases of fruit trees were not widespread in Hungary. Apple proliferation was the first such disease to be detected in the 1960s. Indexing on woody indicators proved that the pathogen can be transmitted. A similar method was applied for the detection of apple rubbery wood in apple cultivars and rootstocks, and the causal agents were described as apple proliferation and rubbery wood viruses (Németh, 1960). Later on, according to literature data, they were classified among mycoplasma diseases (Németh, 1979). In Hungary del Serrone et al. (1998) detected apple proliferation and pear decline phytoplasmas using PCR and RFLP analysis, but the latter could not be confirmed by biological indexing (Németh, 2000, pers. comm.). Until the middle of the '90s causal agents could not be detected in stone fruit trees with symptoms resembling phytoplasma infection in Hungarian orchards, though in the southern parts of Europe, especially
in the Mediterranean area it has been considered a severe disease since a half century.

In France a virus disease was described on apricot in 1957 (Morvan, 1957). Later it was found that its causal agent is amytoplasma (Morvan et al., 1973). All the knowledge obtained until that time was summarised also in France under the name of apricot chlorotic leaf roll mycoplasma (Morvan, 1977). The current name of the phytoplasma is European stone fruit yellows = ESFY (Lorenz et al., 1994). Recently Lee et al. (1998) classified ESFY in subgroup 16SrX-B within their scheme containing 14 groups and 32 subgroups. The syndrome on apricot has already been known in other southern countries like Italy (Goidanich, 1934), Greece (Agiros, 1971) and Spain (Sanchez-Capuchino et al., 1976) for several decades.

The disease causes severe losses in other Prunus species, too, thus in Japanese plum (Prunus salicina) in France (Morvan, 1977), Spain (Sanchez-Capuchino et al., 1976), Greece (Syrigianidis et al., 1976; Rumbos & Basalidis, 1985) and Italy (Glunchedi et al., 1982).

Since the early 30s various symptoms on peach, caused by phytoplasmas presumably, were described under different disease names in the US such as X disease or peach yellow leaf roll (PYLR) etc. (Gilmer & Blodgett, 1976). The earliest record of the disease on peach in Europe is from Switzerland (Bovey, 1956). Significant damages in this fruit species were reported from France, (Bernhard et al., 1977), Greece (Agiros, 1971; Syrigianidis, 1974; Tsakalis & Rumbos, 1980) and Spain (Sanchez-Capuchino et al., 1976), too. Based on molecular studies Kison et al. (1997) demonstrated that the causal agents of ESFY in peach from Germany and PYLR in peach from California are clearly distinguishable phytoplasmas. Paltrimieri et al. (2000) detected, besides ESFY (16SrX-B), phytoplasmas belonging to groups of aster yellows (16SrI-B), apple proliferation (16SrX-A) and stolbur (16Sr XII-A), furthermore, in one tree mixed infection of 16Sr I-B and X-A groups was also found in peach. Navratil et al. (2000) reported on the presence of ESFY and pear decline (16SrX-C) in peach trees.

In Hungary, ESFY phytoplasma was first described in apricot (Lorenz et al., 1994), confirmed by further studies of Viczian & Sile (1996) and Viczian et al. (1997a, b). ESFY was detected also in almond (Viczian & Sile, 1996), Japanese plum and cherry (Sile, 2000, pers. comm.), and recently in Prunus mahaleb, too (Varga et al., 2000).

In the frame of indexing of peach trees from a seed-source plantation of stone fruit species during assessment on GF 305 indicators both in greenhouse and in the field, phytoplasma specific symptoms were found in 1999 and 2000. Based on these results, in the course of the inspection of the seed-source plantation in August 2000, appearance of symptoms, different phases of the disease and the caused damage were observed. In 2000 molecular investigations were conducted allowing identification of the pathogen. The present paper summarises the results of biological indexing and molecular studies.

Material and methods

Woody indexing

In the frame of the regular official phytosanitary control of seed-source mother trees, symptoms, characteristic for phytoplasma infection, were observed on woody indicator GF 305 peach seedling in the field for the first time in 1999. The tested peach trees are in a seed-source plantation in the southern part of Hungary. The trees are 12 years old. Both in the greenhouse and the field GF 305 indicator was used in 4–5 replicates. Transmissions were done using chip budding (2 chips per plant) in late August of 1998 and 1999. Symptoms appeared on the indicator plants after 4–5 months in the greenhouse and in late June - early July of the next year in the nursery.

Visual observation

Seed-source mother trees, found positive for phytoplasma infection in greenhouse and field indexing in 1998–1999, were visually inspected for presence and severity of symptoms in August 2000. Furthermore, based on symptoms, the entire seed-source plantation was surveyed to determine the distribution of the disease.

Molecular studies

Mother trees providing budwoods for indexing in 1999 died by 2000, thus their molecular studies could not be conducted. Therefore, in the same plantation, samples were taken for the molecular studies from two trees showing phytoplasma symptoms, the field indexing of which was positive in 1999.

Leaf samples were collected twice in 2000: in April and August, from the seed-source mother trees as well as from GF 305 plants in the greenhouse and in the nursery, which were inoculated in late August 1999.

R16F2/R3 nested PCR products of apple proliferation (AP), pear decline (PD), and European stone fruit yellows (ESFY) phytoplasmas, kindly provided by Milan Navratil (Czech Republic), were used as positive controls for RFLP analysis.

DNA extraction

Total DNA from infected and healthy peach leaf material was extracted in Doyle buffer (Doyle & Doyle, 1990).

Primers and PCR

Universal primer pairs R16F1/R16R0 (for the first nested PCR) and R16F3/R16R3 (for the first nested PCR) were used for amplification of phytoplasma 16SrDNA. Second nested PCR was primed by group and subgroup specific primers: R16F2/R3, R16XF/R1, R16IF/R1, R16IF/R1, R16VF/R1, as well as Stolbur and EY specific primers (Lee et al., 1994).

PCR was performed in mixtures of in 50 µl final volume containing 5 µl 10xPCR buffer (100 mM Tris-HCl, pH=8.3,
500 mM KCl, 0.01% gelatin, 1.5 mM MgCl$_2$, 250 µM each dNTP, 20 pmol each primers, 2U Taq polymerase. About 2 ng total DNA was amplified in the first PCR.

PCR products were diluted in 1/50 and 1 µl DNA was used for the second nested PCR.

The following amplification conditions were used: 94 ºC for 3 min, 94 ºC for 1 min (30 sec for nested), 55 ºC for 2 min (30 sec for nested), 72 ºC for 3 min (1 min for nested), 35 cycles and 72 ºC for 5 min. PCR products were analysed in 1.5% ethidium-bromide stained agarose gel.

**RFLP**

R16F$_{2r}$/R$_2$ nested PCR products were digested with RsaI restriction enzyme (NBL Gene Sciences) according to the manufacturer’s instructions, to distinguish the phytoplasmas belonging to different subgroups of the 16SrX group.

**Results and Discussion**

**Woody indexing**

**Field indexing**

First symptoms on GF 305 indicators appeared in July 1999 and 2000, a year after inoculation.

At shooting stage GF 305 plants seemed to be normal. From the middle of June leaves began to turn yellow, then to bright red, leaves developed later became narrow, enrolled towards the midrib, bark of the shoot also turned red, in the middle of July leaf drop began from the bottom (Fig. 1). The infected plants dried and died by September – October (Fig. 2), next year they did not shoot in spring.

**Greenhouse indexing**

In the greenhouse 4–5 months after transmission interveinal reddening was observed (Fig. 3), terminal shoots formed rosettes with narrow leaves followed by wilting, finally the whole plant died (Fig. 4).
Visual observation

In early August 2000, during visual observation of nine seed-source peach mother trees found infected with phytoplasma according to the results of indexing. It was observed that five trees completely died (Fig. 5). Among the living trees, two ones showed partial decline (Fig. 6), while leaf reddening, rolling of the leaves (Fig. 7) and yellowing (Fig. 8), symptoms characteristic for phytoplasma infection also could be found. Symptomatic leaves were drooping as if the tree needed water. Shaking the diseased branches, dropping of the lower leaves already began.

In the survey to determine the distribution of the disease in the seed-source peach plantation, among the trees, not included in field indexing in 1998–1999, there were many plants showing similar symptoms or completely died. It was concluded that about 60% of the trees were infected. According to the observation of the phytosanitary inspector, in 1999 the infection rate was only around 25–30%. First leaf symptoms, characteristic for phytoplasma infection, such as reddening (Fig. 7), yellowing and rolling (Fig. 8),
Molecular studies were conducted in 2000 for confirmation of woody indexing and identification of the pathogen.

Phytoplasmas were detected in all the six symptom-showing samples collected from peach mother trees of the seed-source plantation as well as from GF 305 indicators in the greenhouse and in the field in the nested PCR system using universal primers R16F1/R0 and R16F2/R2 (Fig. 9). ESFY was identified with group specific primer pair R16(X)F1/R1 in a nested PCR in every tested tree (Fig. 10). No phytoplasma specific PCR products were detected using other group or subgroup specific primer pairs R16(D)F1/R1, R16(II)F1/R1, R16(V)F1/R1, with either Stolbur or EY primers (Fig. 10).

RFLP

RFLP analyses confirmed results of group specific nested PCR, when F16R2/R2 nested PCR products were digested by RsaI enzyme. Only phytoplasmas belonging to the 16SrX group, subgroup of European stone fruit yellows (16SrX-B) were present in all the peach samples found infected in nested PCR (Fig. 11).

Based on the results of this study on six symptomatic peach trees we may assume that European stone fruit yellows is very likely to be one of the most common phytoplasmas in Hungarian peach orchards, as also in other European countries. However previous and recent surveys of higher number of trees in Europe demonstrated that several other phytoplasmas can occur in peach as apple proliferation (Lorenz et al. (1994), pear decline (Navratil et al., 2000),

**Figure 8** Yellowing and rolling of leaves on ESFY infected peach seed-source mother trees in August

where shown up in 1998. This observation is supported by the fact that presence of phytoplasma could not be detected before 1998, during the regular official phytosanitary control of the plantation. Rapid spread of the disease is demonstrated by the high incidence of the infected trees. Based on several years' experience of the phytosanitary inspector, in the second half of the vegetation the leafhopper population significantly increased in the plantation after the sprayings against aphids were stopped in July.

**Molecular studies**

**PCR**

**Figure 9** Results of nested PCR for detection of phytoplasmas in peach leaves with universal primer pair R16F1/R0, followed by amplification with primers R16F2/R2 (Lee et al., 1994)

| Samples: | 1-2 phytoplasma infected GF 305 indicator from the greenhouse | 3-4 phytoplasma infected GF 305 indicator from the field | 5-6 phytoplasma infected peach tree from the seed-source plantation | 7 healthy GF 305 indicator | 8 DNA marker | 9 positive control |

**Figure 10** Result of nested PCR of one of the studied seed-source peach mother trees with different group and subgroup specific primers (Lee et al., 1994)

<table>
<thead>
<tr>
<th>Lane</th>
<th>PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>R16(X)</td>
</tr>
<tr>
<td>2</td>
<td>Stolbur</td>
</tr>
<tr>
<td>3</td>
<td>R16(II)</td>
</tr>
<tr>
<td>4</td>
<td>R16(V)</td>
</tr>
<tr>
<td>5</td>
<td>R16(IV)</td>
</tr>
<tr>
<td>6</td>
<td>EY</td>
</tr>
<tr>
<td>7</td>
<td>DNA marker</td>
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**RFLP**

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Figure 11 Results of RFLP analyses of the R16F2/Rg nested PCR products from one of the studied seed-source peach mother trees using Rsal restriction enzyme.

Controls 1–3:
1 Apple proliferation
2 Peach decline
3 European stone fruit yellows

stolbur, aster yellows and related strains, very often in mixed infections (Paltrinieri et al., 2000).

This wide range of phytoplasmas found on peach in other countries, the rapid spread, as well as the great economic importance of the disease inspire us to start a national survey of stone fruit orchards for determining distribution of phytoplasma diseases, planned for the near future. Molecular studies of symptomatic peach trees originating from other parts of the country are already in progress.

References


