A Real-Time PCR Assay for the Detection of European Stone Fruit Yellows Phytoplasma (ESFYP) in Plant Propagation Material

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Abstract

A severe outbreak of European Stone Fruit Yellows (ESFY) has been reported recently in apricot (*Prunus armeniaca*) orchards located in the province of Trento (Italy), where partial or total tree dieback caused major economic losses to growers. In order to prevent the disease spreading, the presence of ESFYP-vector, the psyllid *Cacopsylla pruni,* was monitored together with wild reservoirs of the phytoplasma. Five experimental orchards were planted using ESFYP-free material (cv. Bergeron and Goldrich grafted on "Wavit" or "Myrobolan 29C") to perform epidemiological studies. A multiplex real-time PCR procedure (TaqMan) was set up using two primers/probe combinations for simultaneous detection of ESFYP and host DNA, in order to avoid false negatives due to PCR inhibition. Real-time PCR assays were performed on: propagation material, groups of *C. pruni* (2 insects per group) and wild individuals of several *Prunus* spp. collected in areas close to the experimental orchards and individual samples from apricots showing ESFYP-like symptoms. The results obtained indicate that the primers/probe combination used in the real-time PCR procedure allows reliable and specific detection of ESFYP. The pathogen was detected in 93% of the apricot trees showing ESFYP-symptoms and in ~ 33% of the insect groups and in several wild species collected in different locations. No phytoplasmas were found in healthy plants or in propagation material. This result suggests that new infection of trees is presumably due to ESFYP transmission by vectors rather than by contamination of propagation material. Further research is in progress to check the presence of ESFYP-sources in wild plants close to experimental orchards and to monitor pathogen's dissemination.

INTRODUCTION

The potential epidemic threat posed by ESFYP in stone fruit orchards is confirmed by the annual increasing number of infected trees. Symptoms of ESFY have been reported for a long time in French and Italian orchards, but the spreading of severe outbreaks highlights the importance of this disease as a danger for the new cultivars of apricot (*Prunus armeniaca*) (Thebaud et al., 2004). ESFY progression was also noted in apricot orchards located in the province of Trento (Italy), where partial or total tree dieback caused major economic losses to growers.

Our objective was to prevent the disease spreading either by planting ESFYP-free material (cv. Bergeron and Goldrich grafted on "Wavit" or "Myrobolan 29C") in five experimental orchards and by monitoring the presence of ESFYP-vector, the psyllid *Cacopsylla pruni,* as well as wild reservoirs of the phytoplasma.

Recently, a real-time PCR assay was developed for the detection of apple

proliferation phytoplasma (APP), combining high sensitivity and specificity to suitability for high throughput testing (Baric and Dalla-Via, 2004); a new approach for ESFYP diagnosis in plant material and in vectors using a highly sensitive real-time assay, useful for large-scale analyses is described in this paper.

MATERIALS AND METHODS

A real-time PCR procedure (TaqMan) for the detection of ESFYP in stone fruit, apple and pear trees (10 each) naturally infected by different phytoplasmas (ESFYP, APP, and Pear Decline (PDP), respectively) was set up. Pathogen and host DNA was amplified simultaneously in a multiplex assay to distinguish between uninfected plant material and false negatives due to PCR inhibition (Baric and Dalla-Via, 2004). Total DNA was extracted with a phytoplasma enrichment procedure (Marzachì et al., 1999). All the reagents used in real-time PCR were from Applied Biosystem (AB) with the exception of the primers, obtained from Invitrogen Life Technologies (Table 1). A multiplex reaction was carried out in AB PRISM 7000 SDS; 4 µl of template DNA were added to 16 µl of real-time PCR mix containing 1 X TaqMan Buffer A, 5.5 mM $MgCl_2$ solution, 0.5 U AmpliTaq Gold DNA Polymerase, 0.8 mM dNTPs, 750 nM of qAP-16S primers, 150 nM of qMd-cpLeu primers, 160 nM of qESFY-16S probe and 80 nM of qMd-cpLeu probe. PCR conditions were: 10 min at 94°C, followed by 40 cycles of 15 s at 94°C and 1 min at 60°C (Baric and Dalla-Via, 2004).

All samples were also tested with conventional PCR and PCR-ELISA as previously described (Poggi Pollini et al., 2004).

After validating of multiplex real-time PCR efficiency, the same procedures were applied to 68 groups of *C. pruni* (2 insects/group), collected in areas close to the experimental orchards and to phloem tissue of:

- roots from 123 individual plants before planting (3 roots each sample), representing 15,3% of the propagation material;
- shoots from 80 individual plants in autumn, representing another 10% of the propagation material;
- shoots from apricots with off-season growth (17), chlorotic leaf roll (26), total dieback (13), asymptomatic (20) and healthy (10);
- shoots from wild individuals of *P. cerasifera, P. domestica, P. laurocerasus, P. mahaleb* and *P. spinosa* (blackthorn) (166), collected far from the experimental fields and usually asymptomatic, but 2 *P. domestica* showing partial dieback and 11 *P. mahaleb* with off-season growth.

RESULTS

The results obtained showed that the probe allowed specific and exclusive detection of ESFY phytoplasma: amplification was never obtained in reaction employing APP and PDP as a template (Table 2). The application of the three mentioned diagnostic methods to test the same samples showed that the real-time PCR assay used has the same detection sensitivity of PCR-ELISA.

The data of real-time PCR assays performed on field samples are reported in Table 3: ESFYP was detected in 93% of the symptomatic apricot trees, in some wild *Prunus* and in ~ 33% of the insect groups.

No phytoplasmas were found in asymptomatic and healthy apricots as well as in the propagation material tested, representing 25% of the new plants.

DISCUSSION

The potential epidemic threat posed by ESFYP in stone fruit orchards in Europe, justifies efforts to develop diagnostic methods which combine high sensitivity and reliability in large–scale tests. A real-time PCR assay was developed to detect ESFYP; the advantages of this method compared to conventional diagnostic procedures (PCR, PCR-ELISA) are its suitability for routine testing of numerous samples and the possibility to distinguish between uninfected plant material and false negatives caused by inhibitors.

The results obtained indicated that:

- ESFYP is widespread in most of the apricot orchards located in the province of Trento, with a high incidence of mortality. Latent infections on apparently healthy apricot trees (especially cv. Luizet) have been recently reported in Valais (Western Switzerland) elsewhere (Genini and Ramel, 2004). Our results, however, confirmed a strict association between symptoms — especially off-season growth and dieback — and the presence of ESFY phytoplasma;
- the high proportion of blackthorn hedges resulted infected by ESFYP (18%) (Table 3) suggests that in our conditions, this species could provide an efficient ESFYP source for *C. pruni*; previous reports suggested that an epidemiological cycle of ESFY can be achieved in blackthorn even in the absence of *Prunus* orchards (Yvon et al., 2004);
- it has already been established that several wild *Prunus* species represent good host for ESFYP, as well as for the insect vector. In contrast *P. mahaleb* is considered to be highly resistant to ESFYP-infection and a poor host for *C. pruni* (Carraro et al., 2004)**.** Our results showed, however, that also this species is a natural host of ESFYP and that the infected plants show clearly phytoplasma–like symptoms such as off-season growth. Moreover transmission trials by grafting seedlings of "G.F. 305" with ESFYP-infected *P. mahaleb* plants shown that ESFYP can also be transmitted from *P. mahaleb* in low percentages (Poggi Pollini et al., 2005);
- real-time PCR assays allowed detection of individuals of *C. pruni*, recorded in every prospected areas, infected by ESFYP, the most captured on *P. cerasifera* and *P. spinosa* hedges, but also directly on apricot trees.

The annual progression of ESFY in the apricot orchards located in the province of Trento suggests the presence of a vector together with an important source of ESFYP inoculum in the proximity and/or the introduction of diseased planting material. The real-time PCR results obtained, testing 25% of propagation material, could suggest that in our conditions new infection of trees is presumably due to the transmission of ESFYP by the vectors rather than to the contamination of propagation material, as indicated in epidemiological studies performed in Western Switzerland (Ramel and Gugerli, 2004); however, all necessary steps have to be taken to guarantee the initial sanitary status of the material to reduce the impact of this disease on production.

Further research is in progress to perform a spatio-temporal analysis of disease spread and to extensively search for presence of ESFYP-sources in wild plants close to experimental orchards.

Our goal is also to confirm the possible role played by wild *P. mahaleb*, extremely common in this fruit growing areas, in the epidemiology of the disease.

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Tables

Table 1. Primer and probe sequences for simultaneous detection of the ESFY phytoplasma and the host chloroplast gene for tRNA leucine.

Name	Specifity	Sequence 5'-3'
qAP-16S-F	Phytoplasma belonging to AP group (16SrX)	CGA ACG GGT GAG TAA CAC GTA A
qAP-16S-R	Phytoplasma belonging to AP group (16SrX)	CCA GTC TTA GCA GTC GTT TCC A
qESFY-16S	ESFY phytoplasma	**FAM**-TAA CCT GCC TCT CAG GCG
qMd-cpLeu-F	Gen. *Malus, Prunus, Pyrus*	CCT TCA TCC TTT CTG AAG TTTCG
qMd-cpLeu-R	Gen. *Malus, Prunus, Pyrus*	AAC AAA TGG AGT TGG CTG CAT
qMd-cpLeu	Gen. *Malus, Prunus, Pyrus*	**VIC**-TGG AAG GAT TCC TTT ACT AAC

Table 2. Detection of ESFYP by direct PCR, PCR-ELISA and real-time PCR.

Samples	PCR (fOI/rOI) (positive/tested)	PCR-ELISA (positive/tested)	Real-time PCR (positive/tested)
Apple trees with APP	8/10	0/10	0/10
Apricot and peach trees with ESFYP	9/10	10/10	10/10
Pear trees with PDP	9/10	0/10	0/10
Healthy pome – stone fruit trees	0/15	0/15	0/15

Table 3. Detection of ESFYP in plants.

Species	Symptoms observed	Nb. infected/tested plants
P. armeniaca	Off-season growth	17/17
	Chlorotic leaf roll	23/26
	Total dieback	12/13
	No symptoms*	0/233
P. cerasifera	No symptoms	0/10
P. domestica	No symptoms (8) Partial dieback (2)	3/10
P. laurocerasus	No symptoms	0/10
P. mahaleb	No symptoms (66) Off-season growth (11)	11/77
P. spinosa	No symptoms	11/59

* including: 20 asymptomatic plants collected in the orchards, 203 samples from propagation material and 10 healthy apricots.