Evaluation of susceptibility of pear and plum varieties and rootstocks to Ca. *P. pyri* and Ca. *P. prunorum* using Real-Time PCR

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Abstract

Real-time PCR was used to quantify phytoplasma concentration in fifty inoculated trees from five *Prunus* rootstocks and in forty-eight symptomatic pear and Japanese plum trees from orchards. Seasonal fluctuation of Ca. *P. prunorum* in different *Prunus* rootstocks, over three years, showed that the highest percentage detected by nested-PCR was in the 'Garnem' rootstock on nearly all sampling dates. Intra-varietal differences were also observed. Phytoplasma titer could be estimated by real time PCR in some trees of the rootstocks 'Garnem', 'Barrier', 'GF-677' and 'Marianna', and ranged from $4.7x10^5$ to $3.18x10^9$ phytoplasmas per gram of tissue. Quantification by real-time PCR was not possible in the 'Cadaman' trees analyzed, probably due to a lower phytoplasma titer in this variety. Samples from infected trees from commercial plots had different phytoplasma concentration and detection percentage depending on the variety, both being lower in 'Fortune' and '606' Japanese plum and in 'Blanquilla' pear trees.

Keywords: Candidatus *Phytoplasma pyri*, Candidatus *Phytoplasma prunorum*, real time PCR, detection

Introduction

There are no direct measures of control for diseases caused by phytoplasmas. One way to avoid the damage they produce is to have resistant or tolerant plant material.

The low expression of symptoms with diseases caused by phytoplasmas is often due to the lack of re-infection and therefore to a low concentration of phytoplasma population. This is associated with the difficulty of detecting the phytoplasma in tolerant varieties, even though they may be infected. For the *Prunus* species there are major differences in susceptibility to European stone fruit yellows (ESFY), caused by Candidatus *Phytoplasma prunorum*, with apricot, Japanese plum and peach trees being more susceptible than the *Prunus cerasifera* (Myrabolan) and *Prunus domestica* genotypes.

Many of the peach rootstocks (*Prunus persicae)* and apricot rootstocks (*P. armeniaca*) are extremely sensitive. The rootstocks Marianna GF 8-1 (*P. cerasifera* x *P. munsoniana*) and GF-677 (*P. amygdalus* x *P. persicae)* are highly sensitive. Slightly less sensitive are Myrabolan (*P. cerasifera*) and the hybrid Ishtara (Giunchedi et al., 1982; Desvignes & Cornaggia, 1982). Differences in detection and presence of symptoms have been observed in different varieties of pear infected by Ca. *P. pyri* (Garcia-Chapa et al., 2003a).

The purpose of this work was to apply real-time PCR to quantify phytoplasma concentration in plum and pear trees previously infected by Ca. *P. prunorum* and Ca. *P. pyri* respectively.

Material and methods

Fifty trees of five different rootstocks were inoculated with Ca. *Phytoplasma prunorum*, in February 2006 at the IRTA greenhouse in Cabrils (Spain), to study its seasonal fluctuation over a period of three years. The rootstocks used were 'Garnem' and 'GF 677' (*P. persica* x *Prunus amygdalus*), 'Barrier' and 'Cadaman' (*Prunus persica* x *Prunus davidiana*) and 'Marianna' (*Prunus cerasifera* x *Prunus munsoniana*).

Trees showing symptoms were also selected from orchards of pear and Japanese plum: twenty-four pear trees, cvs 'Blanquilla' and 'Bartlett', and twenty-four plum trees, cvs 'Pioneer', 'Fortune', '606', 'Golden Plum' and 'TC Sun'.

DNA was extracted from fresh material using PGB grinding buffer (Ahrens and Seemüller, 1992) to concentrate phytoplasmas, and E.Z.N.A. Plant MiniPrep Kit (Omega Bio-Tek) following the manufacturer's instructions. The DNA extracted was resuspended in 100µl of elution buffer and stored at -20ºC until use.

For phytoplasma detection, nested-PCR and real-time PCR amplification were used with phytoplasma ribosomal primers. Nested-PCR amplification was using P1 (Deng and Hiruki, 1991) and P7 (Schneider et al., 1995) primers for the first step. The second step was performed with 16Sr-X group specific primers, fO1/ rO1 (Lorenz et al., 1995), amplifying a fragment of about 1050-bp in length. The first amplification, using 5-10ng of DNA, was in a total volume

of 20µl containing the following mixture: 0.250 µM of each universal primer, 250µM dNTPs, 1 unit $100\mu l^{-1}$ Taq DNA polymerase (Promega, Madison, USA) and 1X Taq buffer (Promega, Madison, USA). Two µl of 1:50 dilution of the first amplification product were used for the second step, in a mixture containing the same components, but a different specific primer concentration (0.375µM each). Real-time PCR amplification was according to Torres et al., (2005) using SYBR Green PCR Master Mix (Applied Biosystems) and the universal primer P1 and the specific primer R16(X)F1r. A melting curve was obtained after amplification.

Results

By nested-PCR, Ca. *P. prunorum* phytoplasma was detected at the first sampling date in a high percentage of inoculated trees and in all plum rootstocks except in 'Cadaman', decreasing in subsequent analysis. The highest percentage detected was in the 'Garnem' rootstock on nearly all sampling dates. The highest percentage detected in 'Barrier' and 'Cadaman' were in spring (Figure 1). The detection varied, with the detection in some trees remaining unchanged on the different sampling dates, while that in other trees fluctuated (data not shown). In 'GF-677' and 'Marianna', phytoplasmas were detected in most trees over the three-year period, but in one tree of each rootstock, despite the initial infection, no phytoplasmas were detected from January 2008.

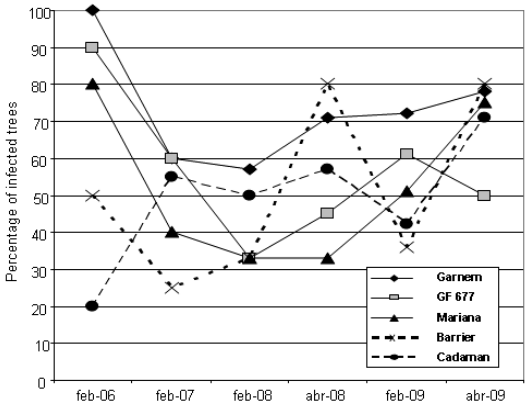

Fig. 1. Evolution of *Ca.* Phytoplasma prunorum detected in fifty inoculated rootstocks, by nested PCR (% of positive trees), over a period of 3 years.

The estimation of phytoplasma titer by real-time PCR, carried out in 2009 in some trees of the rootstocks 'Garnem', 'Barrier', 'GF-677' and 'Marianna', ranged from $4.7x10^5$ to $3.18x10^9$ phytoplasmas per gram of tissue (Table 1). Those trees with high phytoplasma concentration coincide with those that also tested positive in nested PCR analysis on all sampling dates after 2007 (data not shown), and that showed the typical symptoms of off-season growth.

Tab. 1 Percentage of inoculated rootstocks infected with Ca. *Phytoplasma prunorum*, by real-time PCR and average of the estimated number of phytoplasmas.

Rootstock	Cultivar	Real-time PCR detection (%)	Number of phytoplasmas/gr.
P. persica x P. amygdalus	Garnem	33	$2{,}054x10^8$
P. persica x P. davidiana	Barrier	20	$1{,}591x10^7$
P. persica x P. davidiana	Cadaman	0	-
P. persica x P. amygdalus	GF 677	13	$3{,}180x10^9$
P. cerasifera x P. munsoniana	Marianna	13	$4{,}752x10^5$

Although phytoplasmas were detected by nested PCR in 71% of the 'Cadaman' rootstock trees analyzed (Figure 1), quantification by real-time PCR was not possible, probably due to a lower phytoplasma titer in this variety (Table 1). The most susceptible rootstock was 'Garnem', where Ca. *P. prunorum* was detected in a high percentage of trees on all sampling dates, with quantification possible in 33% (Table 1).

The samples of infected plum trees from commercial plots had different phytoplasma concentration depending on the variety (Table 2). The variety 'Pioneer' had the highest phytoplasma concentration. The percentage detected and the estimation of phytoplasma concentration. The percentage detected and the estimation of phytoplasma concentration were lower in 'Fortune' and '606', however, these data were not related in this case with a lower expression of symptoms (Table 2).

Tab. 2 Detection of Ca. *Phytoplasma prunorum* by nested PCR and real-time PCR in symptomatic plums of a commercial plot.

Species	Cultivar	Nested-PCR detection (%)	Real-time PCR detection (%)	Number of phytoplasmas/gr.
Prunus salicina	Pioneer	100	80	$5{,}288x10^6$
Prunus salicina	Fortune	100	40	$4{,}275x10^3$
Prunus salicina	606	100	40	$3{,}552x10^4$
Prunus salicina	TC Sun	66	66	$5{,}725x10^5$
Prunus salicina	Golden Plum	66	66	$1{,}958x10^6$

Infected trees of *Pyrus communis* cv 'Barlett' had a higher phytoplasma concentration than infected 'Blanquilla' trees. Detection was not possible in 'Blanquilla' by real-time PCR, and was only detected in 17% of the trees by nested PCR, despite all trees being infected (Table 3).

Tab. 3 Detection of Ca. *Phytoplasma pyri* by nested PCR and real-time PCR in infected trees.

Species	Cultivar	Nested-PCR (%)	Real-time PCR (%)	Number of phytoplasmas/gr.
Pyrus communis	Barlett	83	67	$2{,}372x10^6$
Pyrus communis	Blanquilla	17	0	-

Discussion

Results of phytoplasma fluctuation in *Prunus* rootstocks indicate that the most susceptible rootstock is 'Garnem'. This is in agreement with other studies that have shown a higher susceptibility of *P.persica* x *P.amygdalus* hybrids (Giunchedi et al., 1982). Intra-varietal differences were also observed, especially in 'GF-677' and 'Marianna' rootstocks, with some trees apparently more resistant to infection than others. This is in agreement with the variability that has been observed within progenies of *Pyrus* taxa (Seemüller et al., 2009).

Quantification by real-time PCR allowed differences in susceptibility among plum and pear tree varieties and rootstocks to be assessed. The quantification of Ca. *P. pyri* in trees of cv 'Bartlett' and 'Blanquilla' confirmed the results obtained previously using nested-PCR for detection (Garcia-Chapa et al., 2003a). In those varieties where phytoplasma is readily detected, such as cv 'Bartlett' and cv. 'Llimonera', the symptoms are much more evident than in cv 'Blanquilla', where it was difficult to detect phytoplasma and there were fewer symptoms (Garcia-Chapa et al., 2003b). The sensitivity of the Japanese plum to the phytoplasma, cited in several studies was also confirmed with real-time PCR

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