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# Evaluation of susceptibility of plum-trees to '*Candidatus* Phytoplasma prunorum' using real-time PCR

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## Abstract

One of the ways to control '*Candidatus* Phytoplasma prunorum' infection is to find varieties of *Prunus* that are resistant or tolerant to the phytoplasma. Different real-time procedures have been recently described to quantify the number of phytoplasmas, thus, the main objective of this work was to evaluate some of these methods and apply them to estimate the concentration of the pathogen in field samples. The DNA extraction with PGB/CTAB and the amplification with two diverse real time protocols were the methods that reach amplifications at lower cycles and were the chosen methods to perform the study of susceptibility of 5 varieties of *Prunus salicina* and *P.cerasifera*. *P.cerasifera* presents the lowest percentage of infected trees. The higher percentage was obtained in the 'Pioneer' and 'Fortune' varieties despite not significant differences were obtained with the rest of varieties of *P. salicina*. The comparison of the estimated number of phytoplasmas did not reached statistical differences between the five varieties of Japanese plum due to the high intra-varietal variability detected.

**Key words:** phytoplasma, detection, *Prunus* spp., ESFY.

## Introduction

The *Prunus* species show differences in susceptibility to '*Candidatus* Phytoplasma prunorum', with apricot, Japanese plum and peach trees being more susceptible than the *Prunus cerasifera* (myrabolan) and *Prunus domestica* genotypes. One of the ways to minimize the damages caused by this phytoplasma is to obtain tolerant or resistent varieties. The purpose of this work was to evaluate different methods of the real-time PCR to estimate the phytoplasma concentration in *P. cerasifera* and in different varieties of *P. salicina*.

## Materials and methods

### Comparison of detection methods

Previously to realize the trial of phytoplasma susceptibility, two DNA extraction methods and three PCR detection methods were assayed. For this purpose 11 infected and 5 healthy Japanese plums (*Prunus salicina*) were analyzed.

Two methods of DNA extraction were assayed after a first homogenization with PGB grinding buffer: (1) E.Z.N.A. Plant MiniPrep Kit (Omega Bio-Tek) and (2) CTAB extraction (Ahrens and Seemüller, 1992). The real-time detection methods assayed were two based on TaqMan probes (Christensen *et al*., 2004; Hodgetts *et al*., 2009) and one based on SYBR Green chemistry (Torres *et al*., 2005).

### Susceptibility to '*Ca*. P. prunorum'

For this study samples from 5 varieties of Japanese plums (15 trees of each variety) and ten trees of *P. cerasifera* (mirabolan) were taken in June 2010 (table 1).

Samples were analyzed using PGB/CTAB extraction and two amplification methods selected between those previously carried out: Christensen *et al*. (2004) and Torres *et al*. (2005). Statistical analysis was performed using the R statistical framework (2010), release 2.11, loading additionally the ez package.

## Results

### Comparative of detection methods

The samples from 11 infected trees were positives with all the combinations of amplification and extraction methods, and the negative controls were also confirmed with all methods. However, the extractions with PGB/CTAB amplified at lower number of cycles with the three methods of PCR, and the most sensitive detection was obtained performing this extraction and amplifying with Christensen *et al*. (2004) or Torres *et al*. (2005), with significative differences with the rest but not between them (figure 1).

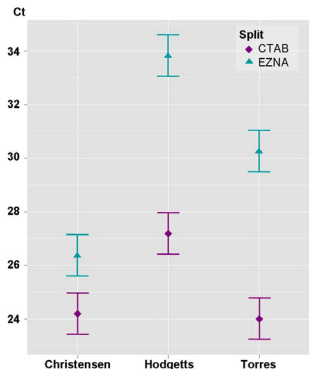


**Figure 1.** Multiple comparison of amplifying and extraction methods. Error bars of each factor level are the Fisher's Least Significant Difference, and plotted in order to facilitate visual post-hoc multiple comparisons.

### Susceptibility to '*Ca*. P. prunorum'

The phytoplasma concentration in all the samples analysed in this study was estimated by both selected methods. Results showed that in month of June the percentage of phytoplasma detection in *P. cerasifera* was lower than in *P. salicina* (table 1). Although in September a high percentage of positives were obtained (results not shown) neither of the trees of *P. cerasifera* showed symptoms. Almost all negatives of *P. salicina* are related with asymptomatic trees, 16 out of 23.

The percentage of phytoplasma detection in Japanese plum ranged from 46% to 73% (table 1). The higher percentage of infected trees was obtained in the 'Pioneer' and 'Fortune' varieties and the minor in the '606' variety, despite not significant differences were obtained.

The comparison of the phytoplama concentration gave not significative differences between the five varieties of Japanese plum due to the high intra-varietal variability (figure 2). The same statistical result was obtained by both methods. However the estimation of the phytoplasma concentration was lower when SYBR green method was applied (data not shown).

**Table 1.** Proportion of infected trees, showed as positives/total (percentage), detected in *P. cerasifera* (myrabolan) and 5 varieties of *P. salicina*.

| Varieties | Christensen | Torres |
|---|---|---|
| Pioneer | 10/15 (66.7%) | 11/15 (77.3%) |
| Autumn G. | 9/15 (60.0%) | 9/15 (60.0%) |
| Fortune | 11/15 (77.3%) | 10/15 (66.7%) |
| Anne Gold | 10/15 (66.7%) | 10/15 (66.7%) |
| 606 | 8/15 (53.0%) | 7/15 (46.7%) |
| Mirabolan | 1/10 (10.0%) | 1/10 (10.0%) |

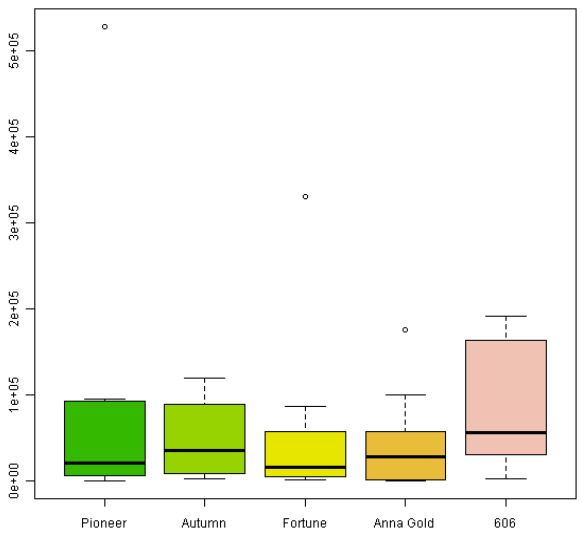


**Figure 2.** Multiple boxplot of phytoplasma's concentration. Results obtained with Christensen *et al*. (2004) method, in positive trees of five varieties of *Prunus salicina*, are expressed as number of copies of the gene 16S RNA per µl of extract template.

## Discussion

All the methods analyzed are suitable for the detection of '*Ca*. P. prunorum'. Some combinations seem to be more sensitive, amplifying the same number of phytoplasmas at lower number of cycles. The SYBR Green method offers an efficient detection at a lower price and could be a reliable option for screening. The estimation of the phytoplasma concentration could vary depending on the real-time applied, in a future work possible matrix effects and the efficiency of the reaction should be analyzed in order to explain these differences.

*P. cerasifera* presents a low percentage of infected trees and not presence of symptoms. The late detection of the phytoplama in these species indicated a late colonization of the phytoplasma in the tree. The mirabolan shows tolerance to the disease but not resistance.

The susceptibility of the varieties of Japanese plum is similar, but the range of phytoplasma concentration estimated in trees of the same variety is wide. More work is to be done in order to evaluate the severity of symptoms and the phytoplasma concentration.

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