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A real-time PCR method for detection and quantification of '*Candidatus* Phytoplasma prunorum' in its natural hosts

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Abstract

A real-time PCR assay conjugated with the fluorescent SYBR® Green I dye has been developed for rapid, sensitive and quantitative detection of '*Candidatus* Phytoplasma prunorum' in its natural hosts, in stone fruits such as apricot plants and in the insect vector *Cacopsylla pruni*. The selected primers amplified specifically a fragment 153 bp long from the *rplV* (*rpl22*) gene of '*Ca.* P. prunorum' and not from closely related '*Ca.* P. mali' infecting apple trees and *C. picta* and *C. melanoneura*, and '*Ca.* P. pyri' infecting pear trees. Absolute quantification of '*Ca.* P. prunorum' in plant and insect samples was achieved establishing two standard curves with serial dilutions of the plasmid containing '*Ca.* P. prunorum' *rpl22* target gene.

The estimated concentration of phytoplasmas in infected apricot trees ranged from $1{,}55*10^2$ to $6{,}18*10^3$ genome units (GU) of phytoplasma DNA per nanogram of plant DNA, while in infected *C. pruni* ranged from $1{,}07*10^7$ to $4{,}24*10^7$ per tested insect.

Key words: apple proliferation group, ribosomal protein gene, SYBR® Green I, *Cacopsylla pruni*.

Introduction

'*Candidatus* Phytoplasma prunorum' is the causal agent of European stone fruit yellows, a quarantine phytoplasma disease present mainly in Europe and recently reported also in Turkey (Sertkaya *et al*., 2005). Together with '*Ca.* P. mali' and '*Ca.* P. pyri' belongs to a major phylogenetic group, the apple proliferation (AP) phytoplasma group (16SrX).

Conventional detection of fruit trees phytoplasma is mainly based on nested-PCR using 16S rDNA universal or group specific primers; a multi-step procedure that increases the risk of cross-contamination.

Real-time PCR represents the most recent innovation in phytoplasma detection. Aim of the present study was to develop a real-time PCR assay for detection and quantification of '*Ca.* P. prunorum' in its plant hosts and insect vector *Cacopsylla pruni* (Scopoli).

Materials and methods

Total DNAs were extracted from phytoplasma infected apricot, apple and pear trees or healthy apricots, from periwinkle infected by AP15, AT, PD and LNp phytoplasma reference strains using a CTAB extraction method modified from Doyle and Doyle (1990). Total DNAs were extracted from batches of 5-6 phytoplasma infected vectors, *C. pruni*, *Cacopsylla picta* (Foerster) and *Cacopsylla melanoneura* (Foerster), or healthy *C. pruni* using the Doyle and Doyle (1990) procedure. The presence of '*Ca.* P. prunorum' in plants and insects was assayed by conventional nested-PCR.

'*Ca.* P. prunorum' was quantified by SYBR® Green I real-time PCR as number of '*Ca.* P. prunorum' genome units (GU)/ng of plant DNA and as number of '*Ca.* P. prunorum' genome units (GU)/insect (Marzachì and Bosco, 2005). Ribosomal protein (rp) gene *rplV* (*rpl22*) was chosen as a target for amplification of '*Ca.* P. prunorum' (EF193369). Primer pair rpLNS2f (5'-GTG CTG AAG CTA ATT TAT TG-3')/rpLNS2r2 (5'-CAA TAT GGC TAG TTC TTT TT-3') amplifying a 153 bp fragment, was designed based on variable regions of the *rplV* gene sequence using Primer3 software. The 18S rDNA was chosen as a target for the amplification of plant DNA (Christensen *et al*., 2004).

For quantification of '*Ca.* P. prunorum' in apricot and insect samples two standards curve were established by diluting plasmid pGEM® Easy Vector (Promega, WI, USA) containing rp genes of LNp phytoplasma. Serial dilutions (1: 10) of the plasmid starting from 1 ng/µl to 1 fg/µl were prepared by mixing with 20 ng/µl and 10 ng/µl of total DNA from healthy apricot plant and phytoplasma-free *C. pruni* respectively. To quantify plant DNA a standard curve was prepared with 1 : 10 serial dilutions of total DNA from healthy apricot starting from 50 ng/µl to 5 pg/µl.

Real-time PCR was performed in a DNA Engine Opticon® 2 System (MJ Research). Two µl of each diluted sample and standard (run in triplicates) were added to a mixture containing 0.3 µM each primer, 2.5 mM $MgCl_2$, 200 µM each dNTPs, 1X PCR buffer, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, USA), 0.25X SYBR® Green I (Molecular Probes, Invitrogen, USA) in DMSO. Cycling conditions were as follows: initial denaturation at 95 °C for 11 min; 40 cycles of 15 sec at 94 °C; 15 sec at 54 °C (60 °C for plant DNA); 20 seconds at 72 °C, and a final extension at 72 °C for 8 min. A melting curve analysis (ramp from 65 °C to 95 °C at 0.2 °C/second) was programmed at the end of the cycling reaction to evaluate the purity of the amplification product.

Results

Primer pair rpLNS2f/rpLNS2r2 amplified a fragment of the expected size from '*Ca.* P. prunorum' infected apricot trees and *C. pruni*. No amplification was obtained

Table 1. Quantification of '*Ca.* P. prunorum' in relation to plant DNA.

Sample	'*Ca.* P. prunorum' DNA in plant DNA		Plant DNA		'*Ca.* P. prunorum' DNA/ Plant DNA (GU/ng)
	CT (mean ± SD)	Quantity (GU, mean ± SD)	CT (mean ± SD)	Quantity (ng, mean ±SD)	
A1	17.51 ± 0.08	$4.99*10^5 \pm 2.85*10^4$	10.07 ± 0.29	89.47 ± 16.48	$5.58*10^3$
A2	19.64 ± 0.15	$1.18*10^5 \pm 1.21*10^4$	10.34 ± 0.08	73.81 ± 4.09	$1.60*10^3$
A3	23.80 ± 0.28	$7.17*10^3 \pm 1.40*10^3$	11.04 ± 0.02	46.27 ± 0.60	$1.55*10^2$
A4	18.34 ± 0.05	$2.85*10^5 \pm 9.59*10^3$	10.63 ± 0.43	62.57 ± 16.25	$4.55*10^3$
A5	20.83 ± 0.26	$5.33*10^4 \pm 9.59*10^3$	10.22 ± 0.05	80.13 ± 2.68	$6.65*10^2$
A6	17.88 ± 0.21	$3.9*10^5 \pm 5.45*10^4$	10.58 ± 0.11	63.09 ± 4.60	$6.18*10^3$

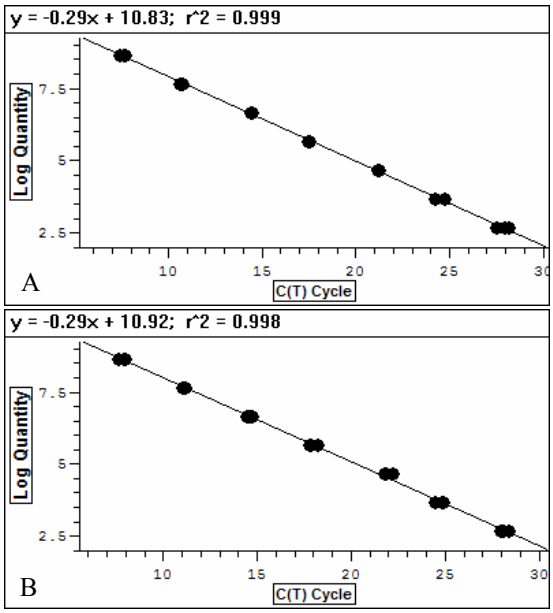

Figure 1. Standard curve of '*Ca.* P. prunorum' diluted in 20 ng/µl of total DNA from healthy apricot A) and in 10 ng/µl of total DNA from healthy *C. pruni* B) obtained by plotting C_T vs log of starting quantity (ng).

from total DNA of phytoplasma infected apple and pear trees, from *C. picta* and *C. melanoneura* and from PD and LNp reference strains. A unique melting peak at 77.06 °C (±0.17) was observed after real-time PCR with DNA from plasmid, infected apricots and *C. pruni*.

Slopes of real-time PCR for quantification of '*Ca.* P. prunorum' in healthy plant and insect DNA were very similar, indicating a PCR efficiency close to 100% (99.9 and 99.8% respectively), irrespective of the type of host DNA background (figure 1). For quantification of infection levels in plants, 18S rDNA served to normalize the data. Slope of real-time PCR for quantification of plant DNA also indicated PCR efficiency close to 100% (99.8%). Infection levels in symptomatic apricot ranged from $1.55*10^2$ to $6.18*10^3$ GU of '*Ca.* P. prunorum'/ng of plant DNA (table 1). Mean CTs for *C. pruni* were in the range from 17.06 ± 0.02 to 18.84 ± 0.11, indicating a phytoplasma titer from $1.07*10^7$ to $4.24*10^7$ per individual (data not shown), assuming that each insect per batch was equally infected.

Discussion

A specific and efficient real-time PCR assay based on *rpl22* gene was developed for detection and relative quantification of '*Ca.* P. prunorum' in infected apricots and *C. pruni*. Real-time PCR represents a fast and sensitive alternative to nested-PCR. SYBR® Green I chemistry is the most economic alternative and the precision of the PCR amplification can be checked by melting curves analysis. An accurate and rapid detection of '*Ca.* P. prunorum' is a major requirement to control the disease and may also contribute to explain in detail the biological and epidemiological properties of the pathogen. Preliminary quantitative results demonstrated a greater variability in the concentration of the pathogen in infected plants than in insect vectors. As future study it will be interesting to quantify '*Ca.* P. prunorum' in *C. pruni* adult reimmingrants and new generation at different stages, testing each insect individually.

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