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Real-time PCR for simultaneous and quantitative detection of quarantine phytoplasmas from apple proliferation (16SrX) group

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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 '*Ca*. Phytoplasma pyri', '*Ca*. P. prunorum' and '*Ca*. P. mali', quarantine members of apple proliferation (16SrX) group. The selected primers amplify specifically a target of 217-bp fragment from the 16Sr gene region of the 16SrX group and not from any other tested phytoplasma groups. An artificial template consisting in a plasmid clone of a 1785-bp DNA fragment of the 16S rRNA gene, 16S/23S rDNA spacer region, tRNA-Ile and partial 23S rRNA gene of a '*Ca*. P. prunorum' isolate, was used to establish a calibration curve to evaluate the number of amplified targets per sample. The sensitivity of the technique was similar to nested-PCR (10 copies of the amplified target per μl). The estimated concentration of phytoplasmas in infected pear, plum and apricot trees ranged from 9.7×10^3 to 3.0×10^5 phytoplasmas per gram of tissue. The method offers the possibility to detect simultaneously, in a single reaction, all quarantine phytoplasmas affecting fruit trees hosts in Europe.

Keywords: Ca. Phytoplasma pyri; Ca. P. prunorum; Ca. P.mali; SYBR green

1. Introduction

Phytoplasmas are bacteria without cell walls that live in phloem elements of infected plants and that are naturally transmitted by sap-sucking insect vectors. Symptoms of infected plants include stunting, shoot proliferation, yellowish foliage and lack of vigour of the infected plants. Since phytoplasmas cannot be cultured in vitro in cell-free media, early taxonomic system was based on symptomatology, host plants and on the specificity of insect vectors. Phylogenetic analysis of 16S rRNA formed the basis for two tentative of taxonomic systems including 14 phylogenetic groups [1] or 20 major groups [2]. The IRPCM Phytoplasma/Spiroplasma Working Team - Phytoplasma taxonomy group [3] proposed to accommodate phytoplasmas within the genus '*Candidatus* Phytoplasma' gen. nov. and provided its formal description and also summarised its species composition. The apple proliferation (16SrX) group includes quarantine species responsible for major economic losses in Europe, such as '*Ca*. Phytoplasma pyri' (associated with pear decline and peach yellow leafroll), '*Ca*. P. prunorum' (associated with European stone fruit yellows) and '*Ca*. P. mali' (associated with apple proliferation).

Conventional detection of phytoplasmas is based on PCR using 16S rRNA general or group specific primers [4,5], being necessary in many cases a nested PCR in two steps [6] due to low titres of phytoplasmas in plants and in vectors. However, the introduction of a second round of amplification and the simultaneous manipulation of the previously amplified products, vastly increases the risks of cross-contamination in routine analysis [7,8]. Real-time PCR assay measures the accumulation of amplified products during the reaction through the determination of the fluorescence signal. This method has many advantages over the conventional PCR in terms of accuracy, dynamic range, high-throughput capacity, and absence of post-PCR manipulations that prevents carryover contamination [9–11]. The application of this method to plant pathogens

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determination is still limited but considerably is increasing in last years. In the case of phytoplasmas real-time PCR has been up to now only applied for detection [12] and for quantification [13] of '*Ca* P. mali'.

In this paper we have developed a real-time PCR conjugated with the SYBR® Green I dye for sensitive and simultaneous quantitative-detection of all phytoplasmas from 16SrX affecting pear, apple and stone fruit species. The purpose of this work is to provide a system to quantify the level of infection and to improve the routine detection in a large number of samples of 16SrX group phytoplasmas with economic relevance.

2. Materials and methods

2.1. Phytoplasma sources

The phytoplasmas analysed included 2 isolates maintained in *Catharanthus roseus* (periwinkle) and 14 isolates from field samples previously characterised by RFLP and/or sequencing analysis of rDNA [14] (Table 1). The isolates belonged to four different phylogenetic groups [15]: (1) 16SrI (aster yellows), (2) 16SrV (elm yellows), (3) 16SrX (apple proliferation) and (4) 16SrXII (stolbur).

2.2. DNA extraction

Total DNA was isolated using PGB grinding buffer [16] and E.Z.N.A.® Plant MiniPrep Kit (Omega Bio-tek), without RNase and mercaptoethanol [17]. The DNA extracted was resuspended with 100 μl of Milli-Q water and stored at −20 °C until use.

2.3. PCR and nested PCR

PCR and nested PCR were performed using Ready-to-Go PCR Beads (Amersham Biosciences). PCR was done with the general phytoplasma primers P1/P7 [18,19]. The nested amplification was done with the 16SrX group-specific primers R16(X)F1/R1 [4] using as template 1 μl of the amplicon obtained in direct PCR. Controls, DNA-free, were run for each experiment to check for DNA carryover. Amplified products were detected by 2% agarose gel electrophoresis in TE buffer, stained with ethidium bromide and visualized under UV light.

2.4. Construction of an artificial template

PCR primers P1-P7 were used to amplify a 1785-bp DNA fragment of the 16S rRNA gene, 16S/23S rDNA spacer region, tRNA-Ile and partial 23S rRNA gene of the phytoplasma isolate 2 (Table 1). Amplicons obtained were purified with E.Z.N.A. clean kit (Omega Bio-tek) and cloned using pGEM®_T Easy vector (Promega Corporation). The purified plasmid clon was quantified with 'GenQuant II RNA/DNA Calculator'. To construct standards, the artificial template was diluted at final concentrations of 10^8 to 1 copies of the target 16S rRNA gene per microlitre of sterile water.

2.5. Real-time PCR primers and amplification conditions

The universal primer P1 [18], and the specific primer R16(X)F1r (5′-CATCTCTCAGCATACTTGCGGGTC-3′) were used to amplify a 217-bp DNA fragment located near the 5′ end of the 16S rRNA gene. The R16(X)F1r primer is

Table 1
Origin, classification, 16Sr group affiliation and PCR, nested PCR and real-time PCR amplifications, of the phytoplasma isolates used

Isolate	Host	Disease/candidatus phytoplasma	16Sr group	PCR results		
				PCR	Nested	Real-time
1	Pear cv. Williams	Pear decline/*Ca*. P. pyri	16SrX	+[a]	+	+
2	Japanese plum cv. Fortune	European stone fruit yellows/*Ca*. P. prunorum	16SrX	+	+	+
3	Japanese plum cv. Black diamond	European stone fruit yellows/*Ca*. P. prunorum	16SrX	+	+	+
4	Japanese plum cv. Golden Japan	European stone fruit yellows/*Ca*. P. prunorum	16SrX	+	+	+
5	Apricot cv. Modesto	European stone fruit yellows/*Ca*. P. prunorum	16SrX	−[b]	+	+
6	Apricot cv. Moniqui	European stone fruit yellows/*Ca*. P. prunorum	16SrX	+	+	+
7	Apricot cv. Traver	European stone fruit yellows/*Ca*. P. prunorum	16SrX	+	+	+
8	Apricot cv. Traver	European stone fruit yellows/*Ca*. P. prunorum	16SrX	+	+	+
9	Apple cv. Reineta gris	Apple proliferation/*Ca*. P. mali	16SrX	−	+	+
10	Periwinkle	Pear decline/*Ca*. P. pyri	16SrX	+	+	+
11	Periwinkle	Apple proliferation/*Ca*. P. mali	16SrX	+	+	+
12	Tomato	Aster yellows/*Ca*. P. asteris	16SrI	+	−	−
13	Grapevine	Bois noir/*	16SrXII	+	−	−
14	Grapevine	Bois noir/*	16SrXII	+	−	−
15	Grapevine	Flavescence dorée/*	16SrV	+	−	−
16	Grapevine	Flavescence dorée/*	16SrV	−	−	−

**Candidatus* Phytoplasma not yet described.
[a] Positive amplification.
[b] No amplification.

the complementary and reverse of the R16(X)F1 primer [4] that specifically amplify phytoplasmas belonging to apple proliferation group.

Real-time PCR was performed in 96-well Optical Reaction plates using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems) and data were analyzed using the software version SDS 1.9.1 and Dissociation Curves 1.0. Twenty-five microlitre reactions were pipetted in each well, containing 3 μl of DNA template (1 μl in case of artificial template), 0.2 μM of each primer, 12.5 μl of the SYBR® Green PCR Master Mix (Applied Biosystems) and 8.5 μl of sterile Milli-Q water (10.5 μl in case of calibration standards). Controls, DNA-free, were run for each experiment. Amplification conditions were: (1) incubation step at 95 °C for 10 min; (2) DNA amplification for 35 or 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. A melting curve temperature profile was obtained by 95 °C for 1 min, 60 °C for 1 min and heating to 95 °C in 20 min. Product identity was confirmed by electrophoresis in a 2% agarose gel. The threshold cycles (Ct) were calculated by plotting normalised fluorescence (ΔRn) in relation to cycle number.

Aliquots of some DNA templates were used to repeat the assays in a LigthCycler™ (Roche Molecular Biochemicals). Twenty microlitre reactions were pippetted in each capilar, containing 2 μl template, 1 μM of each primer, 2 μl of the SYBR® Green PCR Master Mix (Roche), 2.4 μl of $MgCl_2$ and 11.6 μl of sterile water provided by the manufacturer. After a preincubation stage of 95 °C for 30 s, amplifications were performed for 40 cycles of 95 °C for 10 s, 60 °C for 5 s and 72 °C for 10 s. After amplification, a melting curve was generated by holding the reaction at 65 °C for 15 s, and then heating to 95 °C with a ramp rate of 0.1 °C per s. To give melting temperature for each sample, the fluorescence signal was plotted against temperature [20].

2.6. Calibration curve

The standards dilutions were used to establish a calibration curve by plotting the Ct, obtained by a 35 cycles Real-time PCR with P1-R16(X)F1r primers, versus the $\log_{10}$ of the copy number of the target 16S rRNA gen ($\log_{10}$ (copy number)). Each calibration standard was tested in triplicate or quadruplicate in two different runs. Two main statistical analyses were performed. The reproducibility of the calibration curve was investigated modelling the two runs simultaneously with a one way analysis of covariance, with the group factor as the run number, the covariate as $\log_{10}$ (copy number), and the dependent variable as Ct. The calibration curve was computed as a linear regression model of $\log_{10}$(copy number) as independent variable for each separate run. All the statistical tests has been computed using the statistical package SPSS 11.5.1 (SPSS Inc.).

2.7. Evaluation of the phytoplasma concentration in plant samples

Phytoplasma isolates (Table 1) from fruit trees showing typical symptoms of phytoplasma diseases were tested by triplicate in order to quantify the number of phytoplasma targets present in the analysed tissue.

Dilutions of artificial template and target isolates were amplified, by 35 cycles of real-time PCR with P1-R16(X) F1r primers, during the same reaction. The estimation of the sample copy number values was performed computing the estimates of linear regression coefficients and the 95% confidence interval of the individual predictions. The copy number of amplified target derived from the calibration curve.

To estimate the number of phytoplasmas (Nph) per gram of tissue, the estimated copy number was multiplied by 100 and divided by 2 (Nph = copy number × 50). The factor 100 is related to the analysis of 3 μl out of 300 μl obtained, after the DNA extraction procedure, from 1 g of plant tissue. The factor 2 takes into account that each phytoplasma has two copies of the gene encoding the 16S rRNA [21,22].

3. Results

3.1. Confirmation of primer specificity

A unique melting peak at 81.5 °C (±0.3) was observed after the real-time PCR with DNA from all phytoplasma isolates tested, belonging to the group 16SrX. The melting curve of '*Ca*. P. pyri', '*Ca*. P. prunorum' and '*Ca*. P. mali' is shown in Fig. 1A (isolates 1, 2 and 9 in Table 1). A melting peak at 76.7 °C was observed with phytoplasmas belonging to other groups and DNA-free control when 40 cycles were run. Fig. 1A shows the melting curve for flavescence dorée phytoplasma (isolate 15 in Table 1) and the DNA-free control. Primer set P1-R16(X)F1r generated a single band, after gel electrophoresis analysis, of the expected size of 217 bp for all isolates tested from 16SrX group (Fig. 1B). Amplification product of negative isolates and DNA-free controls was not observed in electrophoresis gel. All DNA-free controls and DNA from other phytoplasma groups, with a melting peak clearly different, yielded a cycle threshold major to 36 whereas all phytoplasma DNA belonging to apple proliferation group showed a cycle threshold minor to 36 (Fig. 1C).

Similar results were obtained using the LigthCycler™. All phytoplasma DNA belonging to apple proliferation group showed a melting peak around 84.2 °C. The DNA obtained from phytoplasmas belonging to others groups and DNA-free controls gave a melting peak at 79.9 °C (data not shown).

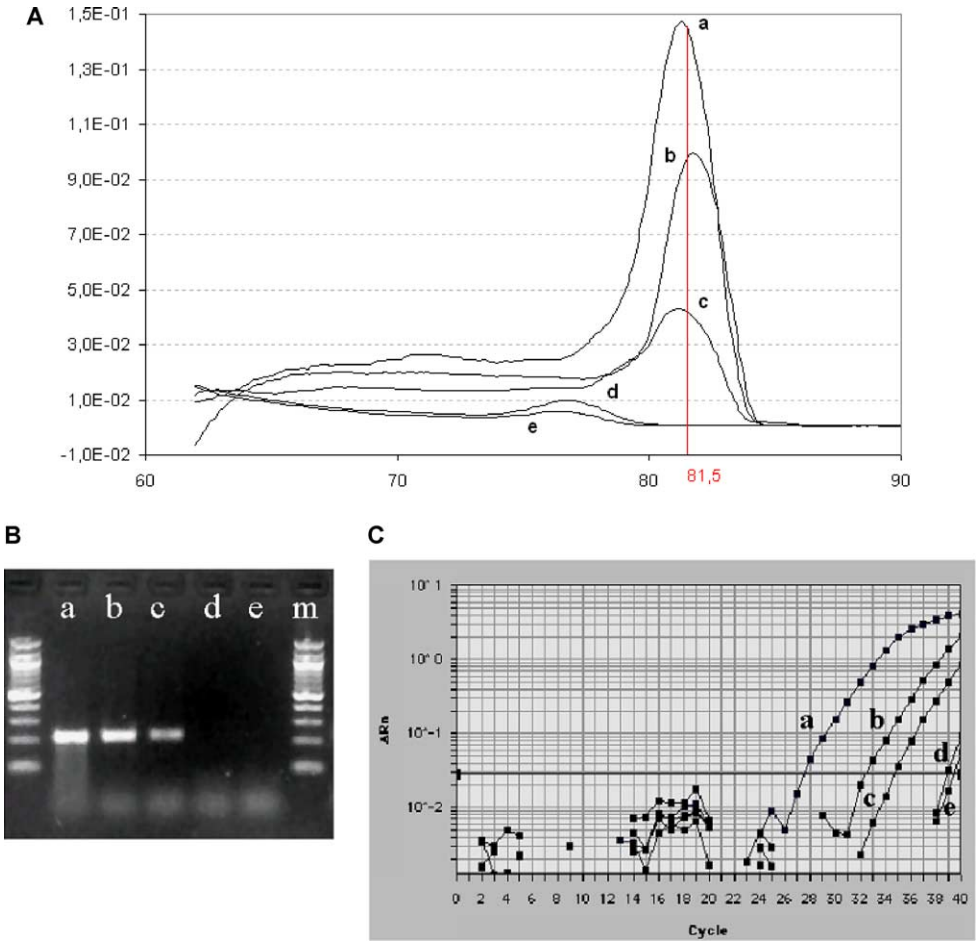

Fig. 1. Specificity of the real-time PCR for detection of phytoplasmas from apple proliferation (16SrX) group. (A) Melting curves. (B) Gel analysis. (C) Amplification plot of normalised fluorescence (ΔRn). Isolates (a) '*Ca*. P. pyri', (b) '*Ca*. P. prunorum', (c) '*Ca*. P. mali', (d) Flavescence dorée phytoplasma, (e) control DNA-free. Melting show a single peak at 81.5 (±0.3) for isolates (a), (b) and (c) and primer-dimer accumulation (76.7 °C) for isolate (d) and DNA-free control (e). The band of the expected size (217 bp) is showed in isolate (a), (b) and (c) (M = 100 bp molecular ladder).

3.2. Real-time PCR sensitivity

The same calibration standards, controls and field samples isolates were analysed by real-time PCR (40 cycles reaction), direct PCR (using phytoplasma general primers) and nested PCR (using 16SrX group-specific primers) in order to compare the sensitivity. Samples infected with 16SrX group phytoplasmas (isolates 1–11, Table 1) yielded agree positive reaction when amplified by nested-PCR and real-time PCR, whereas direct PCR was not able to detect isolate 5 and 9. Field samples infected with phytoplasma species belonging to other groups (isolates 13–16, Table 1) yielded negative results with both specific methods, nested-PCR and real-time PCR. Calibration standard of only one copy number could not be amplified by all assayed methods (Table 2). Calibration standards equal or up to 10 copy number where amplified showing products of expected size (1100 bp) when tested by nested PCR. The same samples yielded significant fluorescence increase when amplified by real-time PCR. Direct PCR only amplified standards with more than 100 copy number (Table 2).

Table 2
PCR amplification results of the standards (1to10^8 copy number of target DNA) used to compare the sensitivity of conventional PCR, nested-PCR and real-time PCR

Copy number	Amplification			Quantitative assay (35 cycles)	
	PCR P1/P7	Nested 16Sr(X)	Real-time 40 cycles	Ct[a] average	St[b] deviation
1	−[c]	−	−	−	
10	−	+[d]	+	−	
10^2	+	+	+	32.89	0.08
10^3	+	+	+	28.93	0.57
10^4	+	+	+	24.29	0.10
10^5	+	+	+	20.09	0.31
10^6	+	+	+	16.69	0.31
10^7	+	+	+	12.82	0.22
10^8	+	+	+	10.11	0.12

[a] Threshold cycles (Ct) values obtained by Real-time PCR of the standards used for the quantitative assay and the determination of the phytoplasma concentration in field samples.
[b] Ct values standard deviation.
[c] No amplification.
[d] Positive amplification.

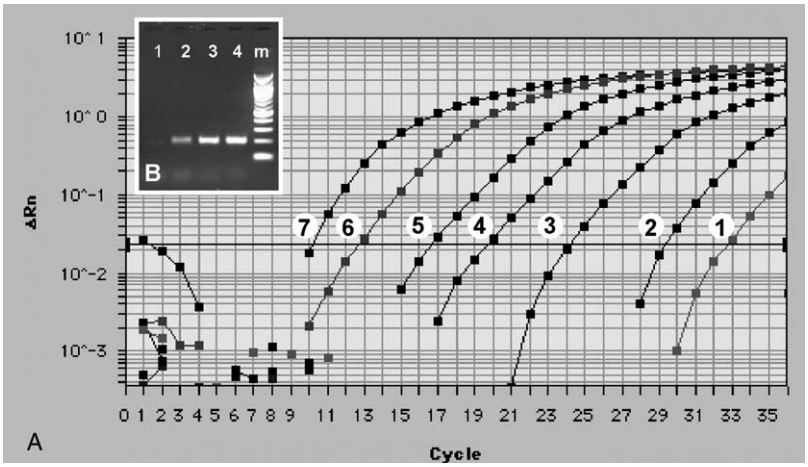

Fig. 2. Sensitivity of the quantitative real-time PCR (35 cycles). (A) Amplification plot of normalised fluorescence (ΔRn) for the standards (10^2–10^8copy number of target DNA) used in the calibration curve. (B) Gel analyses of some calibration standards is showed (1–4; m: 100 bp molecular ladder). Standards: (1) 10^2, (2) 10^3, (3) 10^4, (4) 10^5, (5) 10^6, (6) 10^7, (7) 10^8 copy number of target DNA.

3.3. Calibration curve for quantitative assay

All standards of partial 16S rRNA gene were run in a 35 cycles real-time PCR to obtain only specific amplification products according to the results of primer specificity. The Ct values increased while the amount of copy number of partial 16S rRNA gene decreased (Table 2 and Fig. 2). These PCR reactions where checked by gel electrophoresis analysis and only a single band of the expected size (217 bp) was visible for copy numbers equal or up to 10^2 (Fig. 2).

The one-way covariance analysis of calibration standard data obtained in two different runs (Table 3) showed a significant interaction (p-value < 0.001) between run an the covariate $\log_{10}$ (copy number), therefore the two regression lines had different shapes. Table 3 showed also significance on run factor (p-value < 0.001) therefore the regression lines had different intercepts. Both runs showed a high linear dependence between the two variables, with correlation coefficients of 0.998 and 0.991. The later analysis confirmed the linearity between 10^7 and 10^2 (Fig. 3) of the Ct versus the logarithm of the copy number of the target gene. In the $\log_{10}$ (copy number) scale, the replicates showed a small variability and a very narrow individual confidence interval.

3.4. Real-time PCR quantification of phytoplasmas in plant samples

Total DNA extracted from seven trees infected with 16SrX group phytoplasmas yielded a significant fluorescence increase when amplified by 35 cycles real-time PCR (Table 4). The average of the three samples replicates yielded copy number values that ranged from 194 to 6046. The number of phytoplasmas per gram of pear leaves ribs resulted in 7.7×10^4 and the range of estimated phytoplasma number varies from 9.7×10^3 to 3.0×10^5 in Japanese plum and apricot buds (Table 4).

4. Discussion

Various types of detection chemistries can be used for monitoring real-time PCR products. The double stranded DNA-binding dye SYBR® green I, selected for this study, is an economic alternative based on the monitoring of the fluorescence intensity after each PCR cycle. This dye binds in the minor groove of double-stranded DNA in a sequence-independent way. Using such a generic dye, different PCR amplification products can be accurately distinguished by the generation of so-called DNA melting curves and first-derivative melting peaks [23].

Table 3
ANOVA table. P-values for the test of shape homogeneity and intercept homogeneity are shown

Source	Sum squares	df[a]	Mean squares	F	p-value
Run	19.565	1	19.565	43.916	0.000
Log_{10}(copy number)	1815.698	1	1815.698	4075.492	0.000
Run * $\log_{10}$(copy number)	9.958	1	9.958	22.352	0.000
Error	17.821	40	0.446		

[a] df = degrees of freedom.

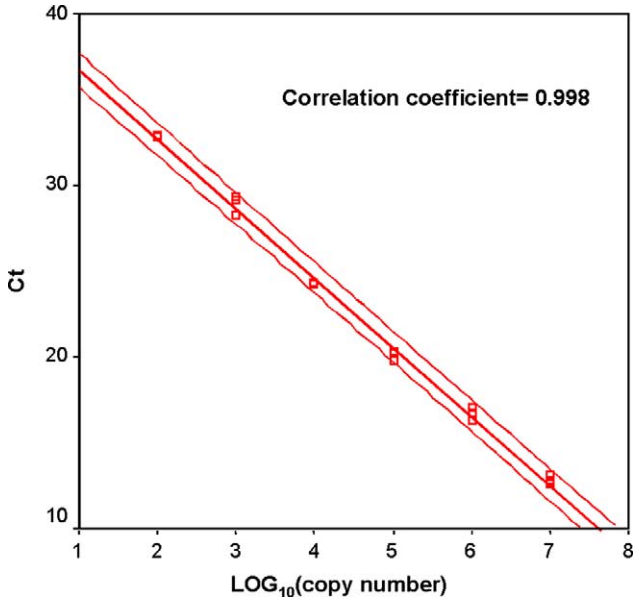

Fig. 3. Calibration curve of the second run. Correlation of the threshold cycle (Ct) to the logarithm of the copy number of partial 16S rRNA gene.

The melting temperature of the amplicons depends mainly on its nucleotide composition [24], thus it is possible to identify the signal obtained from the right product. The specific reaction with P1/R16(X)F1r was confirmed since the characteristic melting peak of the amplification products, at 81.5 °C (ABI Prism 7700) or at 84.2 °C (LigthCycler™), was only obtained when using DNA isolated from '*Ca*. P. pyri', '*Ca*. P. prunorum' and '*Ca*. P. mali'. The amplification artefacts obtained using DNA isolated from stolbur, elm yellows, aster yellows groups and DNA-free controls, melt at lower temperature and in broader peaks (76.7 °C ABI Prism 7700 or 79.9 °C LigthCycler™) in accordance with the specificity of the primers. This lower melting temperature of 76.7 °C is due to primer-dimers formation.[24] The melting curve results were also confirmed by the gel electrophoresis analyses. It is important to note that the negative controls only gave fluorescence when Ct was higher to 36 cycles, consequently adjusting the real-time PCR to 35 cycles allows avoiding these amplicon artefacts. Even although, when using stranded DNA-binding dye SYBR® green I, the positive results should be confirmed through DNA melting curves analyses.

Table 4
Threshold cycles (Ct) values, copy numbers and number of phytoplasmas per gram of tissue of the phytoplasma isolates belonging to apple proliferation group evaluated

Isolate	Ct average	Copy number[a]	Number of phytoplasmas/g
1	27.90	1557	7.78×10^4
3	26.64	3581	1.79×10^5
4	31.07	260	1.30×10^4
5	31.89	194	9.70×10^3
6	28.58	1056	5.28×10^4
7	25.58	6046	3.02×10^5
8	30.31	392	1.96×10^4

[a] Copy number of target gene was calculated with the equation of the calibration line obtained with the second run.

Two previous studies had developed real-time PCR assays to detect [12] and also quantify [13] '*Ca*. P. mali', both works allow the specific detection of this phytoplasma whereas the method described in the present paper allows both, detection and quantification of all quarantine phytoplasmas belonging to 16 SrX group. The specificity of the R16(X) F1r primer for phytoplasmas belonging to apple proliferation group was confirmed since only PCR products were obtained when using DNA isolated from '*Ca*. P. pyri', '*Ca*. P. prunorum' and '*Ca*. P. mali'. The negative results obtained using DNA isolated from stolbur, elm yellows and aster yellows groups are in agreement with the specificity of the primers.

The sensitivity of the real-time PCR is similar to that obtained with nested-PCR, tested with field samples and calibration standards, allowing the detection of 10 copies of the target gene present in the reaction mixture.

The developed real-time PCR provides an estimation of the phytoplasma concentration in the sample, standard curves confirm the linearity of the quantification process between exponential increases of DNA copy numbers (from 10^2 to 10^7) and real-time PCR threshold cycles. The reduced confidence and prediction intervals showed the possibility to get adjusted curves with four standards and two replicates. In the natural scale of copy number, even the small variability observed between replicates, the confidence interval had approximately an order of magnitude length. So it is recommended running at least three replicates of unknown samples to get an appropriate estimation of copy number. In agreement with Rasmussen [25], repeated runs of the same standard curve gave differences in slope and intercept. Thus, the linear regression analysis must be therefore done in each separate run.

The number of phytoplasmas per gram of tissue, estimated considering the copy number derived from the calibration curve, was between the same ranges in three of the species of fruit trees analysed in this work. The concentrations of phytoplasmas in apricot, pear and Japanese plum estimated ranged from 9.7×10^3 to 3.0×10^5 cells per gram of tissue. These estimated values are between those calculated by Berges et al. [26] with a method based on competitive PCR. In that work, apple proliferation phytoplasma concentration ranged mainly from 6.5×10^2 to 1.0×10^8 cells per gram in apple trees tissues. The apple tree analysed (isolate 9) gave a copy number out of the range of linearity of the calibration curve. All cultivars of fruit trees studied were sampled during first year of symptom manifestation, in the same fenological stage and in the same season; however, it is possible that the number of phytoplasmas increases as the disease evolves. The real-time PCR assay developed constitutes a high throughput detection method to detect all destructive

phytoplasmas from apple proliferation group that are subjected to quarantine regulations. It also provides a high security with the positive results due to the specificity of the primers used and avoiding false positive due to post PCR manipulation, allowing the application in a wide sanitary program in nursery selection. Furthermore, the quantitative property of the assay can be useful to monitor phytoplasma kinetics such as the progress of an infection, to estimate the number of copies carried by vectors, or the evaluation of phytoplasma tolerance levels.

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