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Co-operational PCR coupled with dot blot hybridization for detection and 16SrX grouping of phytoplasmas

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A protocol based on Co-operational PCR has been successfully applied to the detection of phytoplasmas. A triprimer reaction coupled with hybridization using general and specific probes permitted detection of '*Candidatus* Phytoplasma mali', '*Ca*. Phytoplasma prunorum' and '*Ca*. Phytoplasma pyri', and their identification as members of 16S ribosomal quarantine group X. The sensitivity of this method was at least one hundred times greater than conventional PCR and similar to that achieved by nested PCR and real-time PCR. The method was validated by testing field samples collected from *Malus*, *Prunus* and *Pyrus* spp. and *Olea europaea* and compared with seven phytoplasmas maintained in *Catharanthus roseus*.

Keywords: '*Ca*. Phytoplasma mali', '*Ca*. Phytoplasma prunorum', '*Ca*. Phytoplasma pyri', DNA targets, molecular detection and diagnostics, Nested PCR

Introduction

Phytoplasmas are wall-less prokaryotes associated with plant diseases that are transmitted in nature by phloem-sucking insects (Kirkpatrick, 1992; Agrios, 1997). Many phytoplasmas are included in the A2 list of quarantine organisms of the European and Mediterranean Plant Protection Organization (EPPO). Accurate and rapid methods are necessary to detect quarantine phytoplasmas, especially in woody, symptomless plants prior to vegetative propagation. Ultrastructural, serological and molecular techniques have been used to detect phytoplasmas in plant and insect vector tissues (McCoy, 1979; Cousin & Boudon-Padieu, 2001; Jones, 2002; Baric & Dalla-Via, 2004; Jarausch *et al*., 2004; Torres *et al*., 2005). However, morphological techniques based on electron microscopy have very low sensitivity, are expensive and not suited to large scale screening. Phytoplasmas usually are present in low concentrations, especially in woody hosts, limiting the reliability of serological techniques for detection. Although PCR is considered the most suitable diagnostic technique for phytoplasmas (Marzachì *et al*., 1998), it is very often necessary to use a nested PCR in two steps in order to obtain acceptable levels of sensitivity (Gundersen & Lee, 1996). The introduction of a second round of amplification vastly increases the risks of contamination in routine analysis (Roberts, 1996; Olmos *et al*., 1999). In addition, restriction fragment length polymorphism (RFLP) or sequence analyses of the PCR products are necessary to confirm the molecular identification of phytoplasmas (The IRPCM Phytoplasma/Spiroplasma Working Team, 2004). Real-time PCR was recently used for phytoplasma detection. The absence of post-PCR manipulations that could cause carryover contamination gives this method an advantage over nested PCR. However, to date, real-time assays are limited to '*Candidatus* Phytoplasma mali' (Baric and Dalla-Via, 2004; Jarausch *et al*., 2004) and 16SrX group (Torres *et al*., 2005).

A new method called Co-operational PCR (Co-PCR) has been described for sensitive detection of plant viruses and bacteria (Olmos *et al*., 2002; Caruso *et al*., 2003). This technique, carried out in a single reaction, minimizes contamination risks and has a level of sensitivity similar to nested PCR and real-time PCR. In addition, it can be coupled with dot blot hybridization, making it possible to characterize the nucleotide sequence.

The aim of this work was to validate Co-PCR coupled with hybridization as a method for detecting all phytoplasmas, and characterization of fruit tree quarantine phytoplasma organisms in large numbers of samples.

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Materials and methods

Phytoplasma sources and plant material

Table 1 shows the samples used as phytoplasma sources, listed by reference number and their host plants. Seven phytoplasma strains [chrysanthemum yellows (CHRY), (16SrI-A); green valley X disease (GVX), (16SrIII-A); elm yellows (EY), (16SrV-A); ash yellows (ASHY), (16SrVII-A); apple proliferation (AT), (16SrX-A); pear decline (PD), (16SrX-C) and Molière disease (MOL), (16SrXII-A)], belonging to different 16S ribosomal groups according to Lee *et al*. (1998) and maintained in periwinkle, were used as positive controls. Six samples from symptomless *Malus domestica, Prunus domestica* and *P. salicina* plants from a commercial nursery were used as healthy controls. Twenty eight field samples with suspected phytoplasma disease symptoms from *M. domestica*, *P. domestica*, *P. salicina*, *P. armeniaca*, *P. cerasifera* and *Pyrus communis*, previously characterized by Torres *et al*. (2004), were included. In addition *Olea europaea, Prunus avium* and *P. salicina* samples from adult trees showing unusual symptoms that could be associated with phytoplasma were also used.

DNA extraction

To concentrate phytoplasmas, 1 g of fresh tissue (young shoots and leaves) was ground with 8 mL of PGB buffer (Ahrens & Seemüller, 1992). Of this homogenate, 1·5 mL was clarified by centrifugation for 5 min at 2000 *g* and the supernatant was then centrifuged for 20 min at 15 300 *g*. Total DNA extraction from the resulting pellet was performed with E.Z.N.A.® Plant MiniPrep Kit (Omega Bio-tek), as described by Martín & Torres (2001). The extracted DNA was resuspended with 100 μL of sterile Milli-Q water. DNA from olive and Japanese plum samples was also purified with the alternative DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was stored at –20°C until use. Ten-fold serial dilutions of purified DNA from isolates 1 (16SrX-A), 8 (16SrX-B) and 28 (16SrX-C) (Table 1) were prepared in order to compare the sensitivity of different diagnostics methods. Three repetitions of the assays were performed.

PCR-based protocols

Single round and nested PCR were performed using Ready-to-Go PCR Beads (Amersham Biosciences) as described by Torres *et al*. (2004). In addition, single round PCR was also assayed under similar cycling conditions to Co-PCR to measure and compare the sensitivity of each method. PCR primers were P1 (Deng & Hiruki, 1991) and P7 (Schneider *et al*., 1995). Nested PCR primers were R16F2n (Gundersen & Lee, 1996) and R16R2 (Lee *et al*., 1993). The characterization of the different phytoplasma isolates into their specific groups was performed by restriction of amplicons using the endonuclease *TruI*. The procedure was according to Torres *et al*. (2004). Real-time PCR was performed according to Torres *et al*. (2005) using the universal primer P1 and the specific primer R16(X)F1 r.

Co-PCR was performed according to Olmos *et al*. (2002), including modifications described by Caruso *et al*. (2003). The reaction mixture was prepared in a final volume of 25 μL, containing the following reagents: 1 × *Taq* buffer (Invitrogen), 3 mM $MgCl_2$, 0·3 mM of each deoxynucleoside triphosphate (Pharmacia LKB), 0·1 μM of external primer P7, 0·05 μM of internal primers R16F2n and R16R2, 1 U of *Taq* DNA polymerase (Invitrogen) and 5 μL of DNA extract. The amplification consisted of a denaturation phase at 95°C for 3 min followed by 60 cycles at 94°C for 30 s, 50°C for 1 min and 72°C for 2 min, followed by one cycle at 72°C for 10 min, in a MasterCycler Gradient (Eppendorf) thermal cycler. PCR product (1 μL) was then submitted to a colorimetric detection using general and/or 16SrX group probes. To evaluate the reliability and reproducibility of the assayed methods, three repetitions were performed.

Probe design and colorimetric detection

The 3′ digoxigenin-labeled probes were according to Bertolini *et al*. (2001). Sequenced regions of phytoplasma 16S rRNA genes were retrieved from public databases. Sequences were aligned and homologies were identified using Advanced BLAST 2·0 program (Altschul *et al*., 1997). A new universal phytoplasma probe (5′-ATTAAG-TACTCCGCCTGAGTAGTAC-3′) and a new specific probe for the quarantine phytoplasma members of the 16SrX group (5′-CGCAAGGGTATGCTGAGAGAT-3′) were designed from one isolate of apple proliferation phytoplasma (X 68 375).

The amplicons (1 μL) were bound to positively charged nylon membrane (Roche Molecular Biochemicals) with UV light (4 min, 254 nm) for the colorimetric detection. Membranes were submitted to a prehybridization phase in a Roller-Blot HB-3D (Techne) hybridizer. Prehybridization was performed at 50°C for 1 h in a buffer containing 5X SSC, 0·1% (w/v) N-lauroyl-sarcosine, 0·02% (w/v) SDS and 1% Blocking Reagent (Roche), plus 50% formamide for the 16SrX specific probe. This solution was then discarded and hybridization was carried out at 50°C for 2 h using 10 pmol mL^{-1} of the 3′DIG labelled probe. The colorimetric detection was performed using a DIG Nucleic Acid Detection Kit and DIG Wash and Block Buffer set (Roche) according to the manufacturer's instructions. Three repetitions of the assays were performed.

Results

Sensitivity of PCR-based techniques

The sensitivity of Co-PCR coupled with dot blot hybridization was compared with single round, nested PCR and real-time PCR (Table 2). Phytoplasma DNA was only detected in the undiluted purified sample by single round PCR and up to the 1:10 dilution when Co-PCR cycling

Table 1 Host, sample reference number, origin of the phytoplasma isolates and subgroup affiliation according to Lee *et al.* (1998) for PCR, nested PCR and Co-operational PCR amplification results

			PCR results (primers)		Co-PCR (P7/R16F2n/R16R2)	
Sample[a]	Host	16Sr group	PCR (P1/P7)	Nested PCR (R16F2n/R16R2)	General probe	16Sr X probe
PC-1	*Catharanthus roseus*	16SrI-A	+	+	+	-
PC-2	"	16SrX-A	+	+	+	+
PC-3	"	16SrIII-A	+	+	+	-
PC-4	"	16SrVII-A	+	+	+	–
PC-5	"	16SV-A	+	+	+	–
PC-6	"	16SrX-C	+	+	+	+
PC-7	"	16SrXII-A	+	+	+	–
HC-1	*Malus domestica*		–	–	–	–
HC-2	"		–	–	–	–
HC-3	*Prunus salicina*		–	–	–	–
HC-4	"		–	–	–	–
HC-5	*Prunus domestica*		–	–	–	–
HC-6	"		–	–	–	–
1	*Malus domestica*	16SrX-A	+	+	+	+
2	*Prunus armeniaca*	16SrX-B	+	+	+	+
3	"	16SrX-B	–	+	+	+
4	"	16SrX-B	+	+	+	+
5	"	16SrX-B	–	+	+	+
6	"	16SrX-B	–	+	+	+
7	"	16SrX-B	–	+	+	+
8	"	16SrX-B	+	+	+	+
9	"	16SrX-B	–	+	+	+
10	"	16SrX-B	+	+	+	+
11	"	16SrX-B	–	+	+	+
12	"	16SrX-B	–	+	+	+
13	"	16SrX-B	–	+	+	+
14	*Prunus domestica*	16SrX-B	+	+	+	+
15	*Prunus cerasifera*	16SrX-B	+	+	+	+
16	"	16SrX-B	+	+	+	+
17	*Prunus salicina*	16SrX-B	+	+	+	+
18	"	16SrX-B	–	+	+	+
19	"	16SrX-B	+	+	+	+
20	"	16SrX-B	+	+	+	+
21	"	16SrX-B	–	+	+	+
22	"	16SrX-B	–	+	+	+
23	"	16SrX-B	–	+	+	+
24	"	16SrX-B	–	+	+	+
25	*Pyrus communis*	16SrX-C	–	+	+	+
26	"	16SrX-C	+	+	+	+
27	"	16SrX-C	–	+	+	+
28	"	16SrX-C	+	+	+	+
29	*Prunus avium*		–	–	–	–
30	*Olea europaea*		–	–	–	–
31	"		–	–	–	–
32	"		–	–	–	–
33	"		–	–	–	–
34	"		–	–	–	–
35	"		–	–	–	–
36	"		–	–	–	–
37	"		–	–	–	–
38	"		–	–	–	–
39	*Prunus salicina*	16SrX-B	+	+	+/–[b]	+/–[b]
40	"		–	–	–	–
41	"	16SrX-B	–	+	+	+
42	"		–	–	–	–
Total	55		21	37	37	32

[a]PC: Positive control; HC: symptomless plant control.

[b]No consistent results in the three repetitions.

Table 2 Sensitivities of the different PCR assays tested using tenfold serial dilutions of purified DNA from phytoplasma-isolates 1 (16SrX-A), 8 (16SrX-B) and 28 (16SrX-C)

	Isolate number		
PCR assay	1	8	28
Conventional PCR (P1/P7)	Undiluted	Undiluted	Undiluted
PCR (P1/P7) with Co-PCR conditions	1/10[a]	1/10	1/10
Nested (R16F2n/R16R2)	$1/10^3$	$1/10^3$	$1/10^3$
Real-time PCR (P1/R16(X)F1r)	$1/10^3$	$1/10^3$	$1/10^3$
Co-PCR (P7/R16F2n/R16R2)	$1/10^3$	$1/10^3$	$1/10^3$

[a]The fraction indicates the highest dilution at which a positive amplification was detected. Gel stained with ethidium bromide for PCR and nested-PCR, colour precipitation on membrane for Co-PCR and fluorescence signal for real-time PCR.

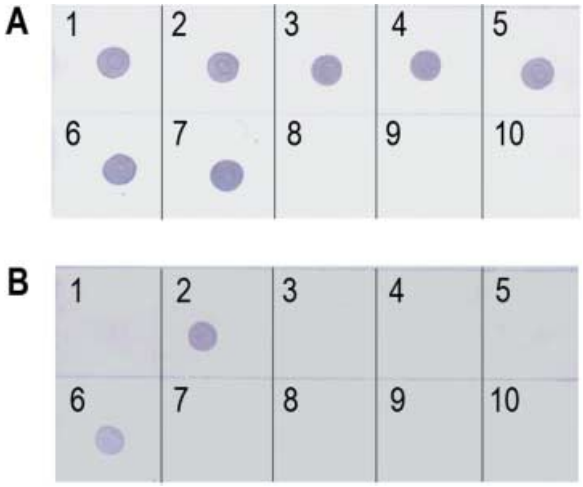

Figure 1 Colorimetric detection of Co-PCR products. A, hybridization with the general probe; B, hybridization with the 16Sr X group probe. Numbers indicate 16Sr phytoplasma groups: 1, 16Sr I-A; 2, 16Sr X-A; 3, 16Sr III-A; 4, 16Sr VII-A; 5, 16Sr V-A; 6, 16Sr X-C; 7, 16Sr XII-A. 8 and 9, symptomless controls from *Prunus salicina*. 10, PCR cocktail control.

conditions were used in single round PCR. However, nested PCR, Co-PCR and real-time PCR detected up to 1:1000 dilution. Consistent and coincidental results were obtained after three repetitions of the assays. The sensitivity of nested PCR, Co-PCR and real-time PCR was 100 to 1000 times higher than single round PCR. No amplifications were obtained from healthy plant and water controls.

Specificity of PCR-based techniques

All phytoplasma isolates used as positive controls were successfully detected by single round, nested and Co-PCR. Amplicons of the expected size were obtained by conventional PCR with P1/P7 primers (1784 bp) and nested PCR with R16F2n/R16R2 primers (1241 bp). Restriction analysis confirmed the identification of the isolates AT (16SrX-A) and PD (16SrX-C) as belonging to the 16SrX group (not shown). In the case of Co-PCR, specific hybridization and colour precipitation were observed for all phytoplasma isolates when the general probe was used (Fig. 1a). In addition, use of the16SrX group probe permitted the specific detection of the two isolates belonging to this group. No hybridization was observed when isolates from other groups were tested (Fig. 1b) or from symptomless plant material.

Validation of Co-PCR coupled with dot blot hybridization

Using single round PCR, 14 out of 42 field samples were phytoplasma positive. Using nested PCR and Co-PCR with the general probe, 30 out of 42 samples were phytoplasma positive. *Olea europaea* and *Prunus avium* samples from trees with unusual symptoms collected in springtime (29 to 38 in Table 1) were negative. Sample 41 from one *P. salicina* with unusual symptoms was positive only by nested PCR and Co-PCR. In sample 39, bands of expected size were obtained for all PCR based methods. However, the specific hybridization with general and 16SrX probes demonstrated that the amplified nucleotide sequence was from a phytoplasma isolate in only two out of the three repetitions. Restriction analysis of the positive samples obtained by nested PCR, and hybridization of Co-PCR products with the16SrX specific probe, indicated that all isolates belonged to the 16SrX group (Table 1).

Concurrent results were obtained by nested PCR and Co-PCR coupled with colorimetric detection using the general probe, as well as restriction analysis of amplicons with Co-PCR coupled with the specific 16SrX probe.

Discussion

The PCR methodology described in this paper has been compared to the conventional PCR method (Lee *et al*., 1993; Gundersen & Lee, 1996; Smart *et al*., 1996; Seemüller *et al*., 1998) and validated. The method was successfully applied to a large number of field samples from naturally infected plants. The sensitivity and specificity afforded by Co-PCR coupled with colorimetric detection agreed with results obtained by nested PCR. Since Co-PCR only requires one amplification step, the risk of contamination is reduced. In addition, the coupled dot blot hybridization facilitated routine detection and avoided the use of mutagenic ethidium bromide as previously described by Bertolini *et al*. (2001, 2003a).

The first attempt to use Co-PCR, carried out using the procedure described by Olmos *et al*. (2002), included the four primers (P1/P7/R16F2n/R16R2) used in the nested PCR. Co-operational amplification generated two specific bands 1784 bp and 1241 bp in size. However, the largest amplicon was sometimes also detected in symptomless plant samples. Sequencing of those fragments and their alignment analysis using the similarity search tool advanced BLAST 2·0 and NCBI's integrated databases, revealed cross-reactions and nucleotide sequences of several 16S rRNA saprophytic bacteria, i.e. *Acetobacter* spp. or

Roseomonas spp. These problems of specificity with 16S rRNA primers were previously reported by other authors (Skrzeczkowski *et al*., 2001; Boccardo *et al*., 2002). Testing different magnesium chloride concentrations as well as increasing annealing temperature did not improve specificity. For this reason the P1 primer was excluded from the assay, dramatically increasing the specificity and maintaining the excellent level of sensitivity.

The sensitivity afforded by Co-PCR coupled with dot blot hybridization was similar to that obtained by real-time PCR using SYBR green (Torres *et al*., 2005). However, Co-PCR has the advantage of detecting all known phytoplasmas with the possibility of identifying those belonging to 16SrX group. The protocols for real-time PCR described by Baric & Dalla-Via (2004) and by Jarausch *et al*. (2004) are only able to detect '*Ca*. Phytoplasma mali' and that described by Torres *et al*. (2005) only diagnosed 16SrX phytoplasma group.

The routine use of the selected 16S rRNA primers revealed specificity drawbacks after gel visualization that were solved with the probe hybridization. Nevertheless, it is convenient to use these primers that are able to recognise all phytoplasmas. Results of the three analyses of sample number 39 (Table 1) were not coincidental. Although a band of the expected size was obtained in each repetition, in one case no hybridization occurred with the general probe. Sequencing of this fragment again revealed a cross-reaction with saprophytic bacteria. Detection could therefore fail when the target of interest is in small quantities compared with the PCR-interfering bacteria.

The application of the specific probe to the apple proliferation group (16SrX) ('*Ca*. Phytoplasma mali', '*Ca*. Phytoplasma prunorum' and '*Ca*. Phytoplasma pyri'), which are considered quarantine organisms, simplifies the characterization step that takes place with detection. The identification of quarantine phytoplasmas in mixed infections is of importance due to the frequency of this event in woody plants or in insects (Boccardo *et al*., 2002).

The developed Co-PCR assay constitutes a high throughput detection method to simultaneously detect and characterize members of the 16SrX phytoplasma group that are subjected to quarantine regulations. The method also provides a high degree of accuracy, avoiding false positives that can be associated with nested PCR (Olmos *et al*., 1999; Bertolini *et al*., 2003b). The relative simplicity of the method and its high sensitivity make it very appropriate for large scale use in routine processing of the numerous samples necessary in quarantine, sanitary or eradication programs. This method could also be very useful in laboratories where real-time PCR equipment is not available.

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