RESEARCH PAPERS

A comparison of different phytoplasma DNA extraction methods using competitive PCR

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Summary. The primer pair 16endF-Tmod, specific for the16S/23S rDNA spacer region, amplifies different length fragments when the target DNA belongs to phytoplasmas of the X-clade and AP-clade in compared with phytoplasmas of the AY-clade. The cloned 16S/23S spacer of the tagete witches'-broom phytoplasma (TWB, AY-clade) was used in a competitive PCR assay to quantify the DNA of the apple proliferation phytoplasma (AP, AP-clade) and the clover phyllody phytoplasma (CP, X-clade) present in the nucleic acids extracted from infected periwinkle plants. Thus, four methods, normally used for the extraction of phytoplasma DNA from infected plant tissue, were compared to determine which was the most efficient in recovering phytoplasma nucleic acids. The different methods detected relatively minor differences in the yields obtained. It was concluded that the sampling and detection protocols are more critical than the DNA extraction method as far as sensitivity is concerned.

Key words: 16S rRNA, rDNA, quantitative PCR, *Mollicutes*.

Introduction

Phytoplasmas are the cause of more than 300 plant diseases (McCoy *et al.*, 1989). They affect herbaceous and woody, wild and cultivated plants, and the damage they cause to crops has a noticeable impact on agricultural economics.

These wall-less prokaryotes, previously known as mycoplasma-like organism (MLOs), belong to the class *Mollicutes* and are systemic pathogens, which inhabit the phloem cells of the host plant. The inability to grow them *in vitro* has seriously hampered studies on these organisms. Nevertheless great advances have been made on their differentiation and characterization using molecular analysis.

Techniques based on serology, DNA/DNA hybridization, restriction fragment length polymorphism (RFLP) analysis, and selective amplification using polymerase chain reaction (PCR) are useful tools for the rapid and reliable diagnosis of these microorganisms in host plants. Polyclonal and monoclonal antibodies (Clark *et al.,* 1989), DNA probes (Davis *et al.,* 1988; Bonnet *et al.,* 1990; Kuske *et al.,* 1991), RFLP and PCR analysis (Deng

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and Hiruki, 1990; Schaff *et al.,* 1990; Lee and Davis, 1992) have shed light on their taxonomic position and permitted the identification of different groups of phytoplasmas (Seemüller *et al.,* 1998a).

However the suitability of most of these molecular methods depends closely on the amount of phytoplasma cells or nucleic acids in the extract. Since the concentration of these phloem-inhabiting pathogens is subject to significant variations according to the season (Seemüller *et al.,* 1998b), and is very low especially in woody hosts (Kartte and Seemüller, 1991; Lederer and Seemüller, 1991), the importance of obtaining phytoplasma DNA at a concentration and purity high enough for precise analysis is obvious.

This work compares different, previously described techniques to extract DNA from phytoplasma-infected plant tissue in order to determine the best procedure to satisfy the above-mentioned needs. The comparison of the efficiency of the different DNA extraction methods is based on a quantitative evaluation of the presence of phytoplasma DNA by competitive PCR (Gilland *et al.,* 1990).

The principle of competitive PCR is based on the co-amplification of target DNA with competitor DNA of known concentration but whose amplified fragments have different sizes. During the reaction the two DNA templates compete for the primers and as a consequence the PCR-amplified products, when analyzed with electrophoresis, reveal fluorescence intensities that are proportional to the amount of the template DNA present in the reaction.

Comparing sequences available from public databases, it was found that the length of the 16S-23S rDNA spacer region shows considerable variation between the phytoplasma groups (Palmano and Firrao, 2000). The pair of primers used in this work amplifies fragments of different sizes of the 16S-23S rDNA spacer region for phytoplasmas belonging to different phylogenetic groups, and they were used to determine the amount of template DNA present in the reaction.

Materials and methods

Source of phytoplasmas

Nucleic acids were extracted from the midribs of periwinkle plants (*Catharanthus roseus*) main-tained in the greenhouse and infected, by grafting, with the phytoplasma associated with apple proliferation (AP) or the phytoplasma associated with clover phyllody (CP). The plants were obtained as described by Carraro *et al.* (1988, 1991).

DNA extraction

Three series of extractions by four different extraction procedures were performed. Each series of extractions involved the collection of sufficient plant material to perform the DNA extraction using four different methods in parallel.

Method A follows the procedure published by Ahrens and Seemüller (1992) and includes a phytoplasma enrichment step. An amount of 1.5 g of infected plant material was incubated for 10 min in 8 ml of Phytoplasma Grinding Buffer (PGB: 100 mM K_2HPO_4, 30 mM KH_2PO_4, 10% sucrose, 0.15% bovine serum albumin fraction V, 2% polyvinylpyrrolidone-10, 25 mM ascorbic acid) in a mortar maintained on ice, and then finely crushed with a pestle, adding 5 ml more of PGB.

The homogenate was then centrifuged for 5 min at 2,500 *g*. The supernatant of each sample was transferred to clean tubes and centrifuged for 25 min at 18,000 *g*. The pellet was dissolved in 1 ml CTAB buffer (2% CTAB, 100 mM Tris pH 8, 1.4 M NaCl, 20 mM EDTA). After a one-hour incubation at 60°C, the nucleic acids were purified by chloroform-isoamyl alcohol (24:1) extraction and an equal volume of cold isopropanol was added to the drawn aqueous phase, then incubated in ice for 1 h. After centrifugation at 12,000 *g* for 10 min, the pellet was dissolved in 400 µl of TE (10 mM Tris-HCl pH 8, 1 mM EDTA) to which 40 µl of 3 M sodium acetate and 0.9 ml of 95% ethanol were added. After incubation for 2–14 h at -20°C, the mixture was centrifuged for 10 min at 12,000 *g*. Once the supernatant was eliminated and the tubes washed with 80% ethanol, the pellet containing the DNA was dissolved in 100 µl of sterile water.

In method B the samples were initially treated with the same enrichment procedure as above. After incubation in 2% CTAB buffer and two centrifugations of 5 min at 2,500 *g* and 25 min at 18,000 *g*, the pellet was dissolved and treated according to protocol I, supplied with the kit for extraction of NucleoSpin Plant nucleic acids (Macherey-Nagel, Duren, Germany).

The method C follows the procedure described by Daire *et al*. (1997) with some modifications listed below. An amount of 1.5 g of plant material was homogenized in mortars with 7 ml CTAB 3% buffer (3% CTAB, 1 M Tris, 1.4 M NaCl, 20 mM EDTA, pH 8) with 0.2% 2-mercaptoethanol added. One ml of the homogeneous mixture thus obtained was transferred to 2-ml tubes and incubated for 10 min at 65°C. After extraction with an equal volume of chloroform the aqueous layer was precipitated with 1 ml isopropanol. After centrifugation at 12,000 g the pellet was washed with 80% ethanol and dissolved in 100 µl sterile water.

Method D follows the procedure of Prince *et al*. (1993). An amount of 1.5 g of infected plant material was triturated with the addition of liquid nitrogen. Eight ml of PGB were added to the preparation and after a first centrifugation for 20 min at 20,000 g, the pellet was dissolved in 4 ml extraction buffer (100 mM TrisHCl pH 8, 250 mM NaCl, 100 mM EDTA) enriched with 440 µl Proteinase K (0.1 mg ml^{-1}) and 440 µl of 10% N-lauroylsarkosine. The suspension was incubated for 1 h at 55°C and later centrifuged for 10 min at 7,500 g. The pellet was dissolved in 2.5 ml isopropanol, incubated for 30 min at -20°C and centrifuged for 15 min at 7,500 g. The pellet thus obtained was dissolved in 3 ml of TE buffer enriched with 100 mg ml^{-1} of Proteinase K and 5% SDS, and incubated for1 h at 37°C. Later, 525 µl of 5 M NaCl and 420 µl of 10% CTAB buffer (10% CTAB, 0.7 M NaCl) were added and the mixture was incubated for 10 min at 65°C. An extraction was then carried out with phenol:chloroform:isoamyl alcohol (25:24:1) and the resulting aqueous phase was extracted again with chloroform:isoamyl alcohol (24:1). A volume of 2.5 ml isopropanol was added to the aqueous phase and then incubated overnight at 4°C. After centrifugation for 30 min at 15,000 g, the pellet was washed with 1 ml of 70% ethanol, centrifuged for 10 min at 15,000 g and then dissolved in 100 µl sterile water.

At the end of the extraction cycles, three samples of AP- and three samples of CP-phytoplasma DNA were obtained for each of the four procedures utilized, resulting in a total of 24 samples to be quantified.

Competitive template source and quantitative PCR

In order to obtain the competitive DNA template, the plasmid DNA which carries the 16S/23S rDNA spacer region of the TWB-phytoplasma, was extracted from the pPTWB clone, obtained as described by Pressacco and Firrao (1999).

For this purpose, a High Pure Plasmid Isolation Kit (Roche Diagnostics, Mannheim, Germany) was used and the plasmid was digested with the *Eco*RI restriction enzyme (Roche Diagnostics) and with RNAase, and purified using phenol and chloroform, to be finally quantified by spectrophotometrically measuring the absorbance at 260 nm wavelength. Five dilution series, each comprising five dilutions: 10, 4, 2, 1.3 and 1 x 10^n ng, where n varies from 1 to –3, were carried out from the initial concentration of 100 ng µl^{-1} of plasmid DNA. Then, 1 µl of DNA extracted from the phytoplasma-infected plants was co-amplified with 1 µl of the serial dilutions of competitor DNA of known concentration, using specific primers for the phytoplasma 16S/23S rDNA spacer region. The 16endF primer (5'-GTCTAAGGTAGGGTCGA-3') identified in the 3' terminal region of the 16S rDNA region, and the Tmod primer, which is a modification of the Tint primer (Smart *et al.,* 1996) suggested by Palmano and Firrao (2000), were used. The amplification conditions were: denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 40 s, extension at 72°C for 40 s, followed by a final 10 min incubation at 72°C. The PCR products were fractionated using 4% agarose gel electrophoresis and visualized using an UV lighting after staining with ethidium bromide.

Results

PCR amplification of the 16S/23S spacer region is a valuable tool for detection of phytoplasma strain diversity, and the pair of primers used in this work amplified fragments of 221 bp for TWB-phytoplasma and 194 bp for AP- and CP-phytoplasmas (Fig. 1, 2, 3 - lanes TWB, AP, CP). The cloned spacer region of TWB-phytoplasma was used as a competitive template (competitor DNA) for the quantification of AP- and CP-phytoplasma DNA (target DNA), extracted from infected periwinkle plants with the four different extraction methodologies.

Fig.1 shows the products of co-amplification of the competitor DNA, when diluted in the range of 10^{-2} to 10^{-3} ng, with 1 µl of the target DNA from the first extraction series. The co-amplified products had similar fluorescence intensities when com-

pared to a 10 pg concentration of competitor DNA (Fig. 1, lanes 1, 6, 11, 16) for AP-phytoplasma with all methods; and 10, 4, 4, and 10 pg for CP-phytoplasma (Fig. 1, lanes 21, 27, 32, 36) with methods A, B, C and D, respectively.

The amplification products of AP- and CP-phytoplasma DNA from the second series of extractions (Fig. 2) was equal to the concentration of the amplified product of competitor DNA respectively at 10 and 2 pg with method A (Fig. 2, lanes 1, 23); at 4 and 1 pg with method B (Fig. 2, lanes 7 and

30); at 10 and 1.3 pg with method C (Fig. 2, lanes 11 and 34); finally at 10 and 1.3 pg with method D (Fig. 2, lanes 16 and 39).

In the third series of extractions, the amplified fragments of AP-phytoplasma had similar fluorescence intensities compared to that of the competitor DNA when their concentration was about 13 pg for method A (Fig. 3, lane 4), 10 pg for method B (Fig. 3, lane 10), 13 pg for method C (Fig. 3, lane 14) and 13 to 20 pg for method D (Fig. 3, lanes 18, 19). The CP-phytoplasma DNA concen-
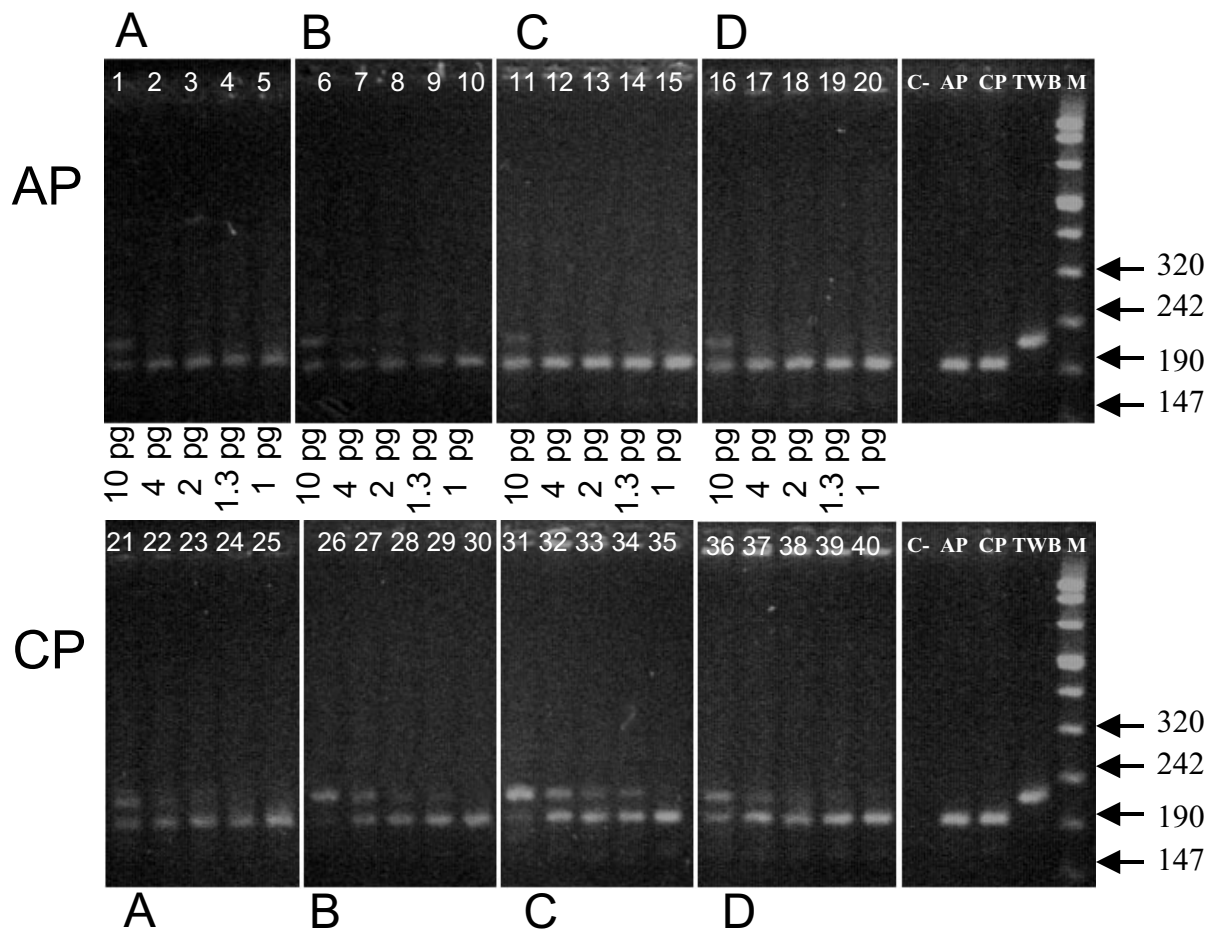

Fig. 1. Gel electrophoresis of the competitive PCR amplification products obtained with the first series of the DNA extractions. The DNA, obtained with the four extraction techniques from plants infected with AP-phytoplasma (top: method A, lanes 1–5; B, lanes 6–10; C, lanes 11–15; D, lanes 16–20) and CP-phytoplasma (bottom: method A, lanes 1–5; B, lanes 6–10; C, lanes 11–15; D, lanes 16–20) was amplified in the presence of serial dilutions of competitor DNA. The dilutions of competitor DNA are shown and expressed as pg. Lane C-: negative control. Lanes AP, CP, TWB: positive control for AP, CP and TWB phytoplasma DNA. Lane M: molecular size Marker VIII (Roche diagnostic, Mannheim, Germany).
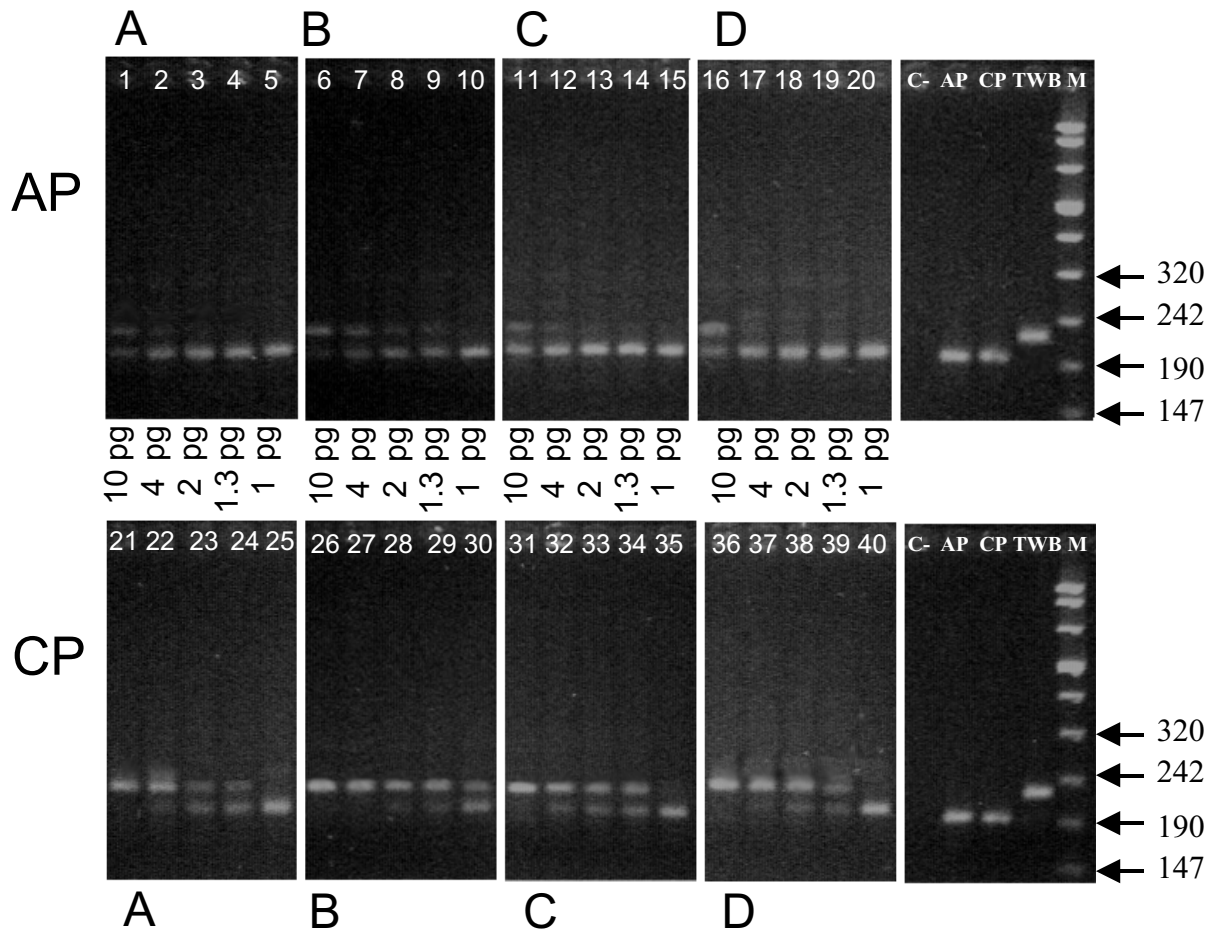
Fig. 2. Gel electrophoresis of the competitive PCR amplification products obtained with the second series of the DNA extractions. The DNA obtained with the four extraction techniques from plants infected with AP-phytoplasma (top: method A, lanes 1–5; B, lanes 6–10; C, lanes 11–15; D, lanes 16–20) and CP-phytoplasma (bottom: method A, lanes 1–5; B, lanes 6–10; C, lanes 11–15; D, lanes 16–20) was amplified in the presence of serial dilutions of competitor DNA. The dilutions of the competitor DNA are shown and expressed as pg. Lane C-: negative control. Lanes AP, CP, TWB: positive control for AP, CP and TWB phytoplasma DNA. Lane M: molecular size Marker VIII (Roche diagnostic, Mannheim, Germany).

tration reached those of the competitor at 40 pg with methods A, B and C (Fig. 3, lanes 22, 27, 32); at 40 to 100 pg with method D (Fig. 3, lanes 36, 37).

In each co-amplification reaction, when the target DNA fragment reached the intensity of the competitor DNA, the two DNA fragments should have the same molar concentration. Since the phytoplasma genome size is about 200-500 times the plasmid vector size, the quantities of phytoplasma DNA obtained with the different extraction methods could be estimated, as shown in Table 1 which

also shows other parameters such as the necessary execution times, amount of work involved in the procedures and dangerous reagents which have to be used and might affect the operator's safety. These parameters are useful for a more complete comparative evaluation of the different methodologies and for determining the method of choice.

Discussion

The purpose of the study was to quantify the phytoplasma DNA extracted from infected plants,
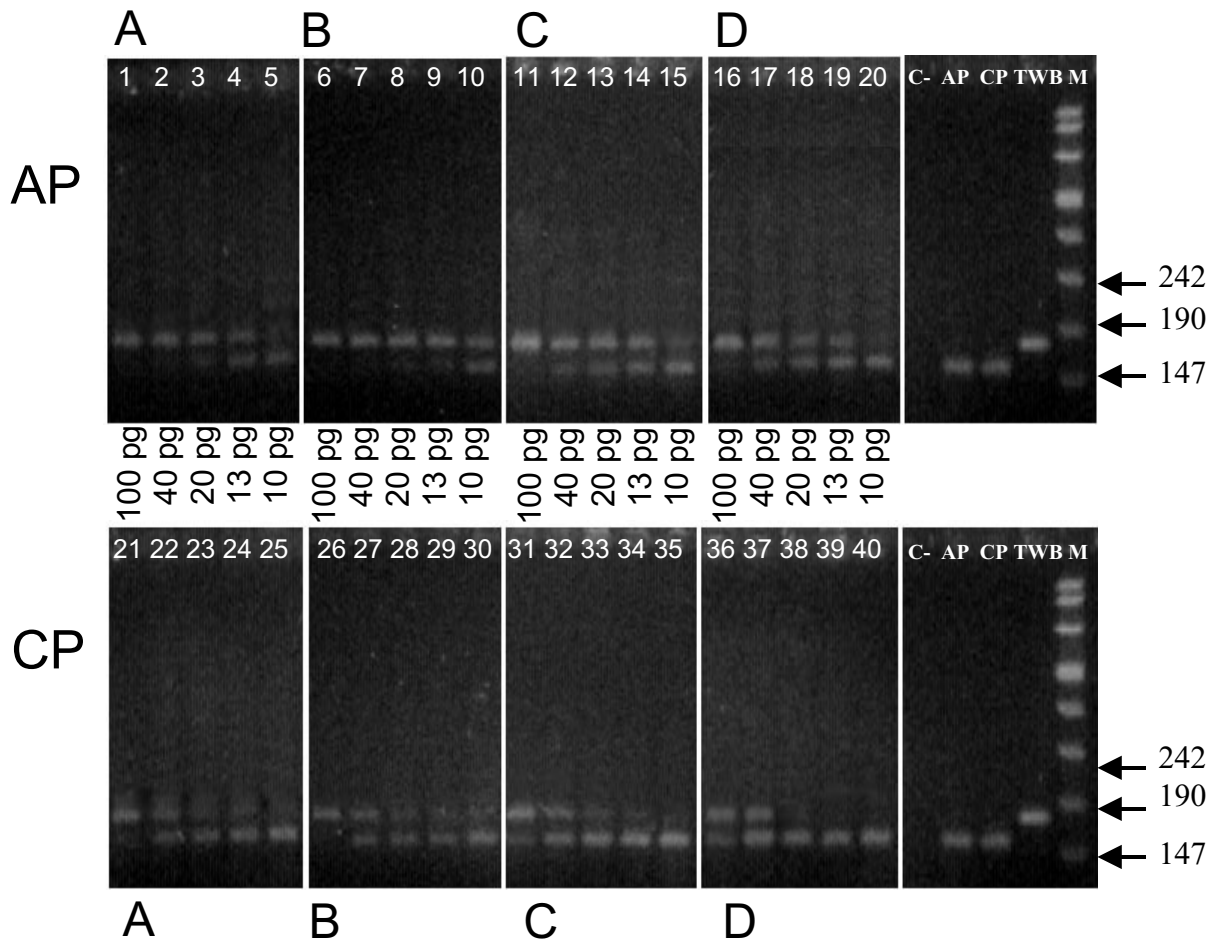
Fig. 3. Gel electrophoresis of the competitive PCR amplification products obtained with the third series of the DNA extractions. The DNA obtained with the four extraction techniques from plants infected with AP-phytoplasma (top: method A, lanes 1–5; B, lanes 6–10; C, lanes 11–15; D, lanes 16–20) and CP-phytoplasma (bottom: method A, lanes 1–5; B, lanes 6–10; C, lanes 11–15; D, lanes 16–20) was amplified in the presence of serial dilutions of competitor DNA. The dilutions of the competitor DNA are shown and expressed as pg. Lane C-: negative control. Lanes AP, CP, TWB: positive control for AP, CP and TWB phytoplasma DNA. Lane M: molecular size Marker VIII (Roche diagnostic, Mannheim, Germany).

using four different methods. Three series of extractions were performed for each method, each series using the same source of plant material for all methods. The first round of extractions was performed using one plant infected with AP- phytoplasma and one infected with CP-phytoplasma. The second series was performed using the same two plants to evaluate the repeatability of the data at different sampling times. The third series was performed using plant tissue from a different set of periwinkle plants, also infected with AP- and CP-phytoplasmas, to evaluate variations between sample sources. The concentration values of AP-phytoplasma DNA obtained in the three extraction series were similar, while the CP-phytoplasma DNA values presented larger differences. A decrease in the concentration of phytoplasma DNA between the first and the second extraction was noted although the material was from the same plants. This can be partly explained by the short

Table 1. Summary of the four methods evaluated in this work. Each column corresponds to one of the DNA extraction methods, designated A, B, C, and D. In the first and second row are shown the estimated amounts of AP- and CP-phytoplasma DNA obtained in the three series of extraction for each method and expressed as ng. In the other rows are shown some parameters which characterise the four extraction techniques: execution time, difficulty and use of dangerous reagents.

Parameters evaluated		DNA extracted (ng)			
		A	B	C	D
AP[a]					
extraction	I	2–5	2–5	2–5	2–5
extraction	II	2–5	0.8–2	2–5	2–5
extraction	III	2.6–6.5	2–5	2.6–6.5	3.3–8.25
CP[b]					
extraction	I	2–5	2.6–6.5	2.6–6.5	2–5
extraction	II	0.4–1	0.2–0.5	0.26–0.65	0.26–0.65
extraction	III	8–20	8–20	8–20	14–35
Execution time (h)		8	2	2	36
Execution difficulty		Medium	Easy	Easy	High-medium
Use of hazardous reagents		Chloroform	None	Chloroform 2-mercaptoethanol	Phenol chloroform liquid nitrogen

[a] AP, apple proliferation phytoplasma.
[b] CP, clover phyllody phytoplasma.

lapse of time between the collection of two sizeable samples, which might have stressed the plants as well as limited the choice of plant material. This highlights the importance of proper identification of plant parts for sampling; in this case, the leaves had to show obvious symptoms but without being necrotic or completely yellow. In the third series of extractions, performed on different plants, the concentration of phytoplasma DNA was higher, especially for CP- phytoplasma. Variance in phytoplasma titers between infected plants of the same species has been observed in a previous study (Berges *et al.*, 2000) and may be caused by different stages of the disease, or even by different states of development and age of the plants.

Method B was included to evaluate if the simplicity and speed offered by certain commercial kits for the extraction of nucleic acids, would also work for the extraction of phytoplasma DNA. Although an enrichment phase for phytoplasmas was added, that was not required in the original protocol, this methodology proved to be the less sensitive. In the evaluation of the other three methods, other parameters had to be considered as well as the quantification of the phytoplasma DNA. These were: the speed of analysis, the amount of work involved in the various phases, the number of samples that could be simultaneously processed by a single operator, and the operator safety. Method C responded positively to the need for a quick, uncomplicated procedure which allowed a single operator to perform a large number of samples simultaneously but it slightly lowered the concentration of the phytoplasma DNA recovered, compared to methods A and D. From the point of view of quantity, methods A and D were the most efficient, yielding similar concentrations of phytoplasma DNA. Method A is however preferable to D,

because it is quicker (it can be completed in one working day) and is safer for the operator by limiting the use of toxic substances.

In order to make an accurate diagnosis it is fundamental to have a pure and concentrated quantity of phytoplasma DNA. Phytoplasma DNA concentration showed little variation in the results between methods.

The DNA concentrations obtained with methods A and D are at most 2.5 times higher than the concentrations obtained with methods B and C, when the DNA extraction was performed using material from the same plant. Whether this factor is significant or not, is strictly dependent on the diagnostic procedure adopted. For example, serological methods and DNA/DNA hybridization assays are reasonably sensitive but are unsuitable when the phytoplasma titer is very low (Ahrens and Seemüller, 1992; Lee *et al.*, 1993). Considering the variability of phytoplasma DNA concentration in the host plant (Berges *et al.,* 2000) and especially in woody hosts, where the concentration was reported to be particularly low (Kartte and Seemüller, 1991; Lederer and Seemüller, 1991), the choice of protocol can be crucial to determine success or failure.

The use of PCR for the detection of phytoplasma DNA is likely less limiting in the choice of extraction protocols, given the high sensitivity of this technique (Deng and Hiruki, 1990; Schaff *et al.,* 1990). Moreover, as stated by Berges *et al.* (2000), a gradual extension of amplification could be obtained with the introduction of the nested PCR, which increases sensitivity 10–100 times over one-round PCR. Many authors have reservations to the use of nested PCR as a diagnostic tool because of the greater chance of a false positive being generated due to contamination. In this case, to offset a 2.5-fold difference in the concentration of recovered DNA, it may be enough to raise the number of PCR cycles to a maximum of 37–40. In conclusion, a 2.5-fold increase of sensitivity may not be worth the use of a labour-intense DNA extraction method when the diagnostic protocol includes a PCR or a nested PCR assay.

The competitive PCR used in this work as a tool to quantify phytoplasma DNA obtained by different methods of extraction can also be used to examine the phytoplasma-host interaction, for example by evaluating the relationship between the phytoplasma titer in infected plant tissue and pathogenicity. It can also make possible to run several controls at different times of the year in order to find a quantitative response to the seasonal variations of these phloem pathogens.

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