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Institute of Plant Pathology, via F. Re 8, Bologna, Italy

Immunoenzymatic Detection of PCR Products for the Identification of Phytoplasmas in Plants

C. Poggi Pollini, L. Giunchedi and R. Bissani
Institute of Plant Pathology, via F. Re 8, I-40126, Bologna, Italy
With 1 figure
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Abstract

Polymerase chain reaction (PCR, with universal and specific primers designed on rRNA genes) provides a rapid, reliable method of diagnosing phytoplasmas (formerly mycoplasma-like organisms) in plants. However, to attain a better identification of these prokaryotes, it is often necessary to digest the PCR products with restriction endonucleases or to hybridize them with specific probes. The present study compared routine procedures for detecting PCR products against a new system, PCR-ELISA (Boehringer Mannheim), which enables immunoenzymatic detection of PCR products. The results show that this new system provides fast and highly sensitive detection of several phytoplasmas associated with certain trees and shrubs. Optimization of all parameters involved in the PCR-ELISA procedure and its advantages are reported and discussed.

Zusammenfassung

Immunenzymatischer Nachweis von PCR-Produkten zur Identifizierung von Phytoplasmen in Pflanzen

Die Polymerase-Kettenreaktion (PCR, durchgeführt mit universellen und spezifischen, an rRNA-Genen erzeugten Primern) ist ein schnelles, zuverlässiges Verfahren zur Diagnose von Phytoplasmen (frühere Bezeichnung: mykoplasmenähnliche Organismen) in Pflanzen. Um diese Prokaryonten besser identifizieren zu können, ist es jedoch oft notwendig, die PCR-Produkte mit Restriktionsendonucleasen zu spalten oder mit spezifischen Sonden zu hybridisieren. Die vorliegende Arbeit verglich Routineverfahren zum Nachweis von PCR-Produkten mit dem neuen System PCR-ELISA (Boehringer Mannheim), das eine immunenzymatische Detektion von PCR-Produkten ermöglicht. Die Ergebnisse zeigen, daß dieses neue System einen raschen und hochempfindlichen Nachweis verschiedener Phytoplasmen erlaubt, die mit bestimmten Bäumen und Sträuchern assoziiert sind. Die Optimierung aller Parameter der PCR-ELISA und die Vorteile des Verlahrens werden vorgestellt und diskutiert.

Introduction

Taxonomic insights into phytoplasmas (previously known as mycoplasma-like organisms) and diagnostic techniques for their detection have undergone considerable advances since the early 1990s. These developments are largely due to the application of new molecular biology techniques, especially polymerase chain reaction (PCR) and the cloning and sequencing of genomic parts.

While research in this field has concentrated on a small portion of the genome, i.e. the 16S ribosomal RNA gene and the 16S/23S DNA spacer region, the sequence homologies found between phytoplasmas from different plants have made it possible to divide them into various groups. The latter range from 6 to 10 depending on the classification criteria followed (Lee et al., 1993; Seemüller et al., 1994).

Although genomic probes are available today for diagnosis, there are most importantly 'group-specific' primers enabling characterization at a group level. At times, however, for a more accurate diagnosis, it is necessary to cut the amplified region with various restriction enzymes to analyse the resulting profiles (Lorenz et al., 1995).

Recently, certain diagnostic kits have come on the market (PCR-ELISA, Boehringer Mannheim, Germany; Gen-Eti-K-DNA Enzyme Immunoassay (DEIA); Sorin Biomedica, Italy; and Captagene GCN-4, Amrad, Australia) enabling immunoenzymatic determination of amplified products in the liquid phase, thereby simplifying the analysis of the results with an ELISA reader. These highly sensitive systems for the determination of amplified products, which obivate the need for electrophoresis and associated methods, have been used to excellent effect mainly in human medicine (Sakrauski et al., 1994; Polvsen and Jensen, 1996). In plant pathology they have been used to diagnose plum pox potyvirus (PPV) in plum trees and tobacco (Schonfelder et al., 1995) and the *Polymyxa betae* fungus in sugar-beet roots (Mutasa et al., 1996).

The potential epidemic threat posed by phytoplasmas

justifies the increasing efforts to develop diagnostic methods combining high sensitivity and reliability with application in mass tests. The present study investigated the use of the PCR-ELISA kit for the diagnosis of phytoplasmas in various plant species and assessed its potential advantages in comparison to other techniques.

Materials and Methods

Phytoplasma sources

Field samples from 10 *Prunus armeniaca* apricots affected by apricot chlorotic leaf roll (ACLR), 10 Japanese *Prunus salicina* plums affected by plum leptonecrosis (PLN) and 10 *Pyrus communis* pears affected by pear decline (PD) were collected in various orchards near the northern Italian city of Bologna. The diseases of the *Prunus* spp. in Europe are induced by a relatively uniform organism for which the term European stone fruit yellows (ESFY) has been proposed (Lorenz et al., 1994). The other field samples were five *Rubus fruticosus* blackberry plants affected by rubus stunt (RS), which were collected at various sites in north-eastern Italy, and five *Olea europea* olives affected by yellowing (hereinafter referred to as OY), which were collected in the western part of the Trentino Region.

The ESFY and the PD phytoplasmas belong to the apple proliferation phytoplasma group and the others to the elm yellows (EY) group (Seemüller et al., 1994; Poggi Pollini et al., 1996). DNA was extracted using the phytoplasma-enrichment procedure after Ahrens and Seemüller (1992) from *c.* 1.0 g fresh tissue of petiolesand midribs or with a knife from the phloem of stem parts after scraping off the bark. Five healthy plants of each species were employed as negative controls.

DNA amplification and incorporation of digoxigenin in amplified products

Immunoenzyme determination of amplified products with the PCR-ELISA kit is a procedure involving three key stages: (1) incorporation of digoxigenin as DIG-dUTP during amplification; (2) liquid-phase hybridization of the 'labelled' amplified product with a specific biotinylated probe at the 5′ end inside the amplified product and binding of the complex to ELISA plates coated with streptavidine; and (3) immunoenzymatic reaction between the bound hybrid and the specific antibody and the colorimetric variation read on the ELISA plate at 405 nm following enzyme–substrate interaction.

Amplification for each sample was performed using 5 μl of extracted DNA (150 ng), 250 nM of the primers fP1 and zP7, universal for phytoplasmas (Marcone et al., 1996), 250 μM of PCR-DIG labelling mix (Boehringer) containing the four nucleotides, one of which bound to digoxigenin, 1 unit of the Taq enzyme polymerase (Boehringer) and 100 μl of mineral oil. The amplification conditions were 60 s at 94°C, 75 s at 55°C and 90 s at 72°C for 35 cycles (Marcone et al., 1996).

Hybridization of amplified product and specific probe

All the reagents in the last two stages, except for the specific probes, are supplied with the PCR-ELISA DIG detection kit (Boehringer). Ten μl of amplified product, or of appropriate dilutions in sterile water, were added to the 20-μl denaturation solution and incubated for 10 min at room temperature. The hybridization solution (220 μl) containing the various dilutions of the specific probe was added to this solution. Two hundred μl of this mixture was then placed in a well on an ELISA plate coated with streptavidine (kit-supplied) and incubated over varying times.

Oligonucleotide probes of 20 or 21 bases were used for specific diagnosis. These corresponded to segments of the spacer region, i.e. rULWS, a group-specific primer used for the diagnosis of phytoplasmas related to elm yellows (EY) (Smart et al., 1996), rPRUS for the specific diagnosis of PLN and ACLR (Ahrens et al., 1994) and rPDS for the specific diagnosis of PD (Lorenz et al., 1995). Primer synthesis and their biotinylation at the 5′ end was performed by Life Technologies (UKi).

In addition to the healthy samples, the controls employed consisted of amplified products diluted in the hybridization buffer without the specific probe. The controlled experimental conditions were 1 h and 3 h incubation with probe at 40, 45, 50 and 54 °C (± 1 °C) and probe concentration 10, 30, 50 pmoles/ml incubation buffer; either no shaking or shaking at moderate speed was also applied.

Immunoenzymatic reaction and spectrophotometry determination

The wells were washed after incubation in washing solution (3 washings of 3 min each). The digoxigenin specific antiserum was then added, bound to radish peroxidases (anti DIG-POD) and diluted 1/100 in the conjugated buffer. The incubation time was 1 h or 4 h at 37 °C with moderate shaking. The wells were again washed as above and 200 ml ABTS substrate were added to each well; the reading was recorded after a variable time by a standard ELISA reader at 405 nm.

The serial dilutions 1/10, 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640, derived from some amplified products, were used to check PCR-ELISA sensitivity with respect to agar-gel electrophoresis. In addition, some samples were amplified with the primer pairs fBI/rULWS (50°C annealing temperature) for all members of the EY group (Smart et al., 1996), fAT/rPRUS (55° annealing temperature) for ACLR and PLN (Ahrens et al., 1994) and fPD/rPDS (55° annealing temperature) for PD (Lorenz et al., 1995). Electrophoresis was performed with undiluted and diluted amplified products as above.

Results

PCR-ELISA reliability

PCR-ELISA exhibited findings that are in agreement with those for electrophoresis (Table 1). This method proved to be highly reliable, the infected samples always showing very high absorption, visible even to the naked eye (Fig. 1). Moreover, the rPDS probe for PD hybridized only to PCR products from pear samples with PD symptoms and not to those from symptomatic plants of the other species tested. The PRUS probe for ACLR and PLN hybridized only to amplicons from symptomatic

Table 1
Results of PCR-ELISA

Phytoplasma source	Absorption at 405 nm (O.D.)[a,b] with oligonucleotides rULWS	rPDS	rPRUS
Olive (OY)	1.52	0.15	0.15
Rubus (RS)	1.36	0.12	0.13
Pear (PD)	0.16	1.71	0.17
Japanese plum (PLN)	0.12	0.15	1.17
Apricot (ACLR)	0.15	0.17	1.20

[a]Each value is the mean of at least 5 samples; [b]the samples of healthy controls and those used without probes never exceeded a value of 0.15 O.D.

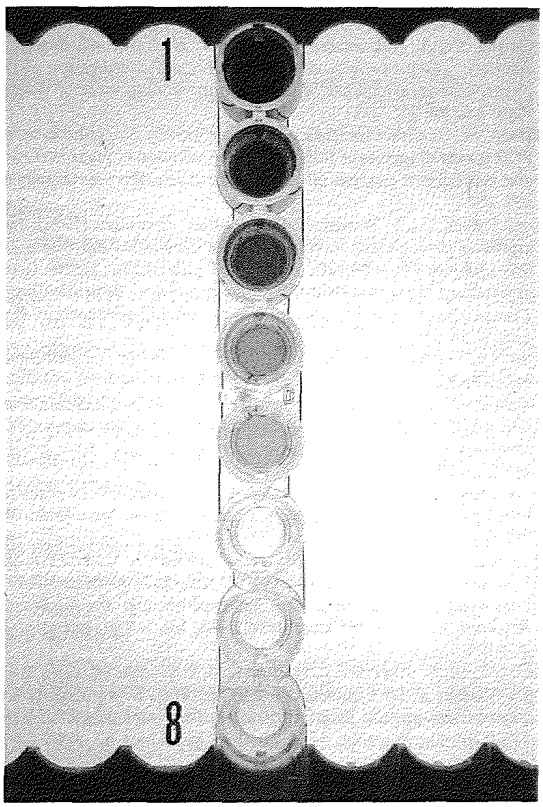

Fig. 1 Colorimetric detection of PCR products hybridized with biotin-rPDS probe: 1–5, serial dilution (1/10, 1/20, 1/40, 1/80, 1/160) of samples (source: pear tree with PD); 6: undiluted sample (source: Japanese plum with PLN); 7: undiluted sample (source: healthy pear tree); 8: undiluted sample (absence of probe during hybridization) (source: as in 1–5)

stone fruit trees and the rULWS probe for the detection of all EY-related phytoplasmas only to PCR products from symptomatic olive and blackberry plants (Table 1). The absorption values obtained with the control samples from healthy plants and with the amplified products used without probes were always less than 0.20 P.D.

The values reported in Table 1 refer to optimum and identical method conditions for the three probes, i.e. 3 h incubation with probe, moderate shaking at 40°C, probe concentration of 30 pmoles/ml incubation buffer, 1 h incubation with antiserum and moderate shaking.

Incubation temperature was a particularly critical factor because over 45°C the final absorption value markedly dropped (from 1.70–0.70 with rPDS, from 1.35–0.51 with rULWS); the decrease of probe concentration (to 10 pmoles/ml incubation buffer) and the absence of shaking had but little effect on the final outcome. The increase in probe concentration from 30 to 50 pmoles/ml incubation buffer and of incubation time (up to 4 h) with the antiserum, did not significantly improve absorption values.

PCR-ELISA sensitivity

Comparing the dilution limits of this method to those for electrophoresis after amplification with universal primers fP1/rP7, or with specific primers, PCR-ELISA exhibited a greater sensitivity (Table 2). Some blackberry samples with rubus stunt symptoms were not amplified with the fBI/rULWS pair, while the fAT/rPRUS pair in some cases amplified samples containing PD. These cross-reactions were never found with the PCR-ELISA.

Discussion

The basic criteria for assessing a new diagnostic test, as was the aim of the present study, are reliability, specificity, sensitivity, ease of use and applicability for mass tests, cost, hazards for the operator and for the environment because of the substances used (Powell, 1987). The results indicate that the PCR-ELISA kit is particularly reliable and specific since the amplified products from infected plants were hybridized from group- or pathogen-specific probes and the assessment of the results revealed no problems. The specificity of the technique was also highlighted by the absence of cross-reactions that can occur with PCR, as in the case of PD phytoplasma amplified by the fAT/rPRUS pair (Smart et al., 1996).

PCR-ELISA's detection of PCR products was 5–15 times more sensitive than that by electrophoresis. This enhanced sensitivity is comparable to the results reported in the medical and plant pathology with other immunoenzymatic kits (Sakrauski et al., 1994; Schonfelder et al., 1995).

The PCR-ELISA kit is simple to use, and the determination of amplified products can be adapted to the size and the simple instruments required by ELISA. The great ease with which the results can be read (little background, values expressed numerically) makes the method particularly suited to large-sample screening. The manipulation of the kit is certainly simpler than electrophoretic procedures, and the substances used are less toxic for the operator and the environment, especially if further product amplification is necessary (nested-PCR). At present, the kit's major drawback is certainly the cost, altough it is easy to envisage a reduction linked to its excellent performance and its use on an ever greater scale in human medicine (Schonfelder et al., 1995).

It is worth noting that our laboratory set-up could enable the determination of mixed infections caused by different phytoplasmas in the same plant by using the same PCR product (derived from the fP1/rP7 universal primer pair) and merely varying the specific probe in the

Table 2
Sensitivity of PCR-ELISA method compared with gel electrophoresis

Disease examined	PCR-ELISA[a]	Dilution limits with: electrophoresis (amplif. with P1/P7)	Electrophoresis (amplif. with specific primers)
OY	1/160	1/10	1/20
RS	1/160	1/10	1/20
PD	1/160	1/20	1/20
PLN	1/80	1/10	1/20
ACLR	1/80	1/10	1/20

[a]Absorption values of at least 3 times higher than the healthy control absorption value were considered as positive.

hybridization phase. While such mixed infections went undetected in our experiments, identification of doubly infected plants via nested-PCR assays, in which the fP1/rP7-elicited amplicons were re-amplified with different group- or pathogen-specific primer pairs, has been reported (Marcone et al., 1996). Further investigations are needed to determine the full potential of PCR-ELISA in detecting phytoplasma-mixed infections, although the nested-PCR techniques involve a notable increase in cost and complexity along with a higher risk of carry-over contamination (Sakrauski et al., 1994).

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Literature

Ahrens, U. E. Seemüller (1992): Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S RNA gene. Phytopathology **82,** 828–832.

Ahrens, U., K.H. Lorenz, H. Kison, R. Berges, B. Schneider, E. Seemüller (1994): Universal, cluster-specific, and pathogen-specific PCR amplification of 16S DNA for detection and identification of mycoplasmalike organisms. IOM Lett. **3,** 250.

Lee, I. M., R. Hammond, R. Davis, D. Gundersen (1993): Universal amplification and analysis of pathogen 16S DNA for classification and identification of mycoplasmalike organisms. Phytopathology **83,** 834–842.

Lorenz, K. H., C. Dosba, C. Poggi Pollini, G. Llacer, E. Seemüller (1994): Phytoplasma diseases of *Prunus* species in Europe are caused by genetically similar organisms. J. Plant Dis. Prot. **101,** 567–575.

Lorenz, K. H., B. Schneider, U. Ahrens, E. Seemüller (1995): Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomial and non-ribosomial DNA. Phytopathology **85,** 771–776.

Marcone, C., A. Ragozzino, B. Schneider, U. Lauer, C. Smart, E. Seemüller (1996): Genetic characterisation and classification of two phytoplasmas associated with spartium witches'-broom disease. Plant Dis. **80,** 365–371.

Mutasa, E., D. Chwarszczynska, M. Asher (1996): Single-tube, nested PCR for the diagnosis of *Polymyxa betae* infection in sugar beet roots and colorimetric analysis of amplified products. Phytopathology **86,** 493–497.

Poggi Pollini, C., R. Bissani, L. Giuncedi, E. Vindimian (1996): First report of phytoplasma infection in olive trees (*Olea Europea* L.). J. Phytopathol. **144,** 109–111.

Polvsen, K., J. Jensen (1996): Detection and biovar determination of *Ureaplasma* (*U.*) *urealyticum* by PCR and liquid hybridisation. IOM Lett. **4,** 94–95.

Powell, C. (1987): Detection of three plant viruses by dot-immunobinding assay. Phytopathology **77,** 306–309.

Sakrauski, A., B. Weber, H. Kessler, K. Pierer, H. Doerr (1994): Comparison of two hybridisation assays for the rapid detection of PCR amplified HSV genome sequences from cerebrospinal fluid. J. Virol. Methods **50,** 175–184.

Schonfelder, M., G. Adams, E. Maiss (1995): Detection of plum pox potyvirus (PPV) by DNA enzyme immunoassay. Acta Hort. **386,** 391–395.

Seemüller, E., B. Schneider, R. Maurer, U. Ahrens, X. Daire, H. Kison, H.H. Lorenz, G. Firrao, L. Avinent, B. Sears, E. Stackebrandt (1994): Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomial DNA. Int. J. Syst. Bacteriol. **44,** 440–446.

Smart, C. D., B. Schneider, C.L. Blomquist, L. Guerra, N.A. Harrison, U. Ahrens, K.-H. Lorenz, E. Seemüller, B.C. Kirkpatrick (1996): Phytoplasma-specific PCR primers based on sequences of the 16S-23S RNA spacer region. Appl. Environ. Microbiol. **62,** 2988–2993.