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Detection of apple proliferation phytoplasma by ELISA and PCR in growing and dormant apple trees

Nachweis des Apfeltriebsucht-Phytoplasmas durch ELISA und PCR in wachsenden und ruhenden Apfelbäumen

J. Brzin[1] *, P. Ermacora[2], R. Osler[2], Nazia Loi[2], Maja Ravnikar[1], Nataša Petrovič[1, 3]

[1] Department of Plant Physiology and Biotechnology, National institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia.

[2] Dipartimento di Biologia applicata alla Difesa delle Piante, Università degli Studi di Udine, via delle Scienze 208, 33100 Udine, Italy.

[3] Current Address: Department of Plant Biology and Pathology, Rutgers, the State University of New Jersey, 59 Dudley Road, New Brunswick, NJ 08901-8520.

* Corresponding author: e-mail: jernej.brzin@nib.si

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Summary

Apple proliferation (AP), caused by a phytoplasma belonging to AP group, is an important vector-borne and graft-transmissible disease of apple trees in most European pome fruit-growing areas. Rapid and sensitive detection methods are required for its control. Recently developed monoclonal antibodies were evaluated for detecting AP phytoplasma by enzyme-linked immunosorbent assay (ELISA) during different seasons and in different tissues of apple tree, including dormant bud-wood and roots. ELISA was compared with polymerase chain reaction (PCR) on samples from 156 apple trees of 33 different cultivars. The sensitivity of ELISA was found to be high and AP phytoplasma could be reliably detected in samples of leaves, shoots and roots during the growing season and also in dormant bud-wood and roots. A simple and sensitive laboratory procedure for the detection of AP phytoplasma is proposed, based on ELISA, combined with simplified PCR analyses. The technique was successfully applied in a large-scale laboratory testing and should provide epidemiologists and state extension pathologists with a rapid and selective detection procedure during both growing and dormant seasons.

Key words: Apple proliferation phytoplasma; DAPI; DNA extraction; ELISA; immunofluorescence; PCR; quarantine

Zusammenfassung

Apfeltriebsucht (AP) wird durch ein zur AP-Gruppe gehörendes Phytoplasma verursacht und ist eine wichtige vektorbürtige und durch Pfropfung übertragbare Krankheit an Apfelblüten in den meisten europäischen Apfelanbaugebieten. Schnelle und empfindliche Nachweismethoden sind zur Bekämpfung unerlässlich. Der Nachweis von AP-Phytoplasmen mit Hilfe von kürzlich entwickelten monoklonalen Antikörpern durch ELISA während verschiedener Wachstumsphasen und in verschiedenen Geweben von Apfelbäumen wurde untersucht, einschließlich ruhender Knospen und Wurzeln. An Proben von 156 Apfelbäumen, die zu 33 Sorten gehörten, erfolgte ein Vergleich von ELISA mit PCR. Die Empfindlichkeit von ELISA erwies sich als hoch und AP-Phytoplasmen konnten zuverlässig in Proben von Blättern, Trieben und Wurzeln während der Wachstumsphasen und auch in ruhenden Knospen und Wurzeln nachgewiesen werden. Eine einfache und empfindliche Laboratoriumsmethode

auf der Basis von ELISA, kombiniert mit einer vereinfachten PCR-Analyse, wird vorgeschlagen. Diese Technik wurde erfolgreich in großem Maßstab im Laboratorium eingesetzt und somit steht Epidemiologen und Pflanzenschutzexperten eine schnelle und selektive Nachweismethode sowohl während der Vegetationsperiode als auch in Ruhestadien der Pflanzen zur Verfügung.

Stichwörter: Apfeltriebsucht; Phytoplasma; DAPI; DNA-Extraktion; ELISA; Immunofluoreszenz; PCR; Quarantäne

1 Introduction

Phytoplasmas, wall-less prokaryotes restricted to the phloem sieve tubes of the host, are associated with diseases of more than 1000 plant species (SEEMÜLLER et al. 2002). Apple proliferation (AP) phytoplasma causes one of the most important vector-borne and graft-transmissible diseases of apple tree in most pome fruit-growing areas of Europe. The characteristic symptoms of AP are witches' brooms, enlarged stipules, late growth in autumn and drastic reduction in fruit quality and yield. The main control measures for this quarantine disease (EPPO A2 list) are planting healthy material in new orchards and vector control (OSLER et al. 2001). For this reason, rapid and user-friendly methods are required to determine the health of commercial nursery stocks and plant propagation material and to speed-up field surveys.

The inability to culture phytoplasmas *in vitro,* their low concentration and uneven distribution in the host plant can make their detection difficult (KIRKPATRICK 1989). Traditionally, detection methods were based on direct examination of phloem tissues by either electron microscopy or fluorescent staining by 4',6'-diamidino-2-phenylindole hydrochloride (DAPI, SEEMÜLLER 1976). However, these methods are not specific and are not always sufficiently sensitive. Advances in DNA technology have helped in phytoplasma taxonomy (SEEMÜLLER et al. 2002) and provided more sensitive assays, but many problems still exist with the extraction of DNA from woody tissues, especially roots. Phytoplasmas can be enriched using extraction procedures for isolating cellular organelles, such as differential centrifugation (AHRENS and SEEMÜLLER 1992). While such methods are effective in decreasing the levels of compounds inhibitory to PCR, they are either time consuming or impractical when extracting from woody tissues, and are not suited for handling large numbers of samples.

For large scale routine testing, due to their simplicity and low cost, serological assays, particularly ELISA, are applicable. Until now, these assays were not applied to the best advantage for phytoplasma detection, mainly because of the difficulties encountered in obtaining good quality antisera. Recently, monoclonal antibodies to the AP phytoplasma were developed (LOI et al. 2002). The aim of this work was to develop a reliable, relatively simple and sensitive laboratory procedure for the detection of AP phytoplasma, based on ELISA, and combined with simplified PCR analysis, during both growing and dormant seasons. For this reason, ELISA was compared with PCR assay on samples of different tissues, including dormant bud-wood and roots, from more than 150 apple trees of different cultivars. In addition, the proposed detection procedure was applied to the large-scale testing of AP in 149 trees as part of the governmental survey in 2002. The suitability of the proposed laboratory procedure combining ELISA and PCR techniques for management of the disease will be discussed.

2 Material and methods

To evaluate the sensitivity and specificity of ELISA for AP detection in different tissues during the growth season, one symptomatic and one healthy apple tree of the highly susceptible cultivar 'Golden Delicious' were selected. Leaf, shoot and root samples were tested by ELISA, PCR, DAPI and immunofluorescence (IF) assays. For direct comparison of the sensitivity of ELISA and PCR assays, samples simulating low levels of AP infection were prepared: infected and healthy samples of *C. roseus* and apple tree leaves, shoots and roots were homogenized in ELISA extraction buffer (20 mmol/l Tris, 137 mmol/l NaCl, 2 % polyvinylpyrolidone-24, 0.05 % Tween 20, 2.68 mmol/l KCl: pH 7.4). Aliquots of infected tissue extract were serially diluted with extract of healthy tissue to obtain 1 : 100, 1 : 1000 and 1 : 10000 final dilutions of infected tissue.

To evaluate AP detection during dormant season, an apple tree of cv. 'Damasonski kosmač', showing typical AP symptoms on some shoots and none on the others, was chosen for sampling. Samples of roots and dormant bud-wood with and without symptoms of AP were taken monthly from November 2000 until April 2001. On average, three shoots or roots were collected and pooled to get one representative sample.

To evaluate ELISA for the large-scale laboratory detection of AP, ELISA was compared to PCR assay on samples from 156 apple trees of 33 cultivars from commercial nurseries and orchards in five locations in Slovenia and one in Italy (Table 1). Leaves and sometimes also shoots and roots were sampled from July until November in 2000 and 2001 from trees with or without symptoms of AP.

In order to use a simple common initial step for both ELISA and PCR extract preparation, two extraction methods were compared: a phytoplasma enrichment (PE) extraction procedure (Ahrens and Seemüller 1992), and a simplified, one-step and less costly standard extraction for ELISA sample preparation. The midribs of leaves and phloem tissue from 3 to 10 mm thick shoots and roots were prepared as described by Ahrens and Seemüller (1992). In the PE extraction, half of the enriched pellet prepared from 1.5 g of fresh tissue was used to extract DNA and the other half was resuspended in 1 ml of ELISA extraction buffer to obtain sample for ELISA. Alternatively, using standard sample extraction for ELISA, 0.5 g of fresh tissue was cut into small pieces and homogenized in 5 ml of ice cold ELISA extraction buffer (see above) in homogenization bags (U-form, Bioreba) using a Homex homogenizer (Bioreba) to obtain ELISA extract. To obtain sample for PCR, 1 ml of the extract was centrifuged at 10000 × *g* for 10 min at 4 °C and DNA was extracted from the resulting pellet (Ahrens and Seemüller 1992).

ELISA protocol was applied as recommended by the manufacturer (Bioreba, Switzerland). The absorbance at 405 nm (A_{405}) after 20, 60 and 120 min was measured using a Dynatech MR5000 plate reader. All samples were tested twice. A sample was considered to have a positive reaction in the test if its mean A_{405} value exceeded twice the mean of the healthy control. Samples with A_{405} values between 1.5–2 times the mean of the healthy control were considered borderline positive.

PCR of 30 cycles was performed in 40 µl reaction volume using the AP group-specific pair of primers fO1/rO1 (Lorenz et al. 1995) and 2 µl of DNA as template. Negative samples were further tested in the nested PCR assays, where amplification products obtained after 35 cycles in 40 µl reaction volume with the universal primer pair P1/P7 (Seemüller et al. 1996) were diluted 1 : 100 in water and re-amplified with AP group-specific primers fO1/rO1 as described above. All sets of reactions included DNA samples from healthy plants and controls lacking template DNA. Samples (15 µl) of PCR product were analyzed by 1 % agarose gel electrophoresis and visualized by staining with ethidium bromide (2×10^{-4} mg ml^{-1}) and UV illumination.

Shoots and roots of a healthy and AP infected 'Golden Delicious' tree (Table 2) were analyzed simultaneously by IF assay and DAPI staining. For each sample, two 1-cm pieces of tissue were fixed overnight at 4 °C in 4 % paraformaldehyde in PBS. Twenty µm tick longitudinal sections were cut by a cryomicrotome (Leitz Jung 1500) and incubated 1 h at 37 °C with anti-AP phytoplasma monoclonal antibody diluted 1 : 1000 in PBS. After washing with PBS, sections were incubated 30 min at 37 °C with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma). Sections were incubated in dark for 10 min with DAPI (1×10^{-3} mg ml^{-1} PBS, Sigma), washed and observed under a Nikon Diaphot TMD fluorescence microscope. Sets of 450–490 and 330–380 nm excitation filters with 520 and 420 nm barrage filters were used to visualize FITC and DAPI, respectively.

3 Results

3.1 Comparison of ELISA with PCR analysis and the symptoms of apple proliferation

Results of AP detection in 156 apple trees by ELISA and PCR, compared with the symptoms of AP, are presented in Table 1. All samples taken from 26 symptomatic trees were positive by ELISA and PCR. Among 130 samples from trees without symptoms of the disease, three were positive by ELISA and PCR and three only by PCR. Two trees without symptoms were found positive only in roots (Table 1).

Table 1. AP detection in 156 apple trees from commercial orchards in various locations in Slovenia and Italy in 2000 and 2001. Leaves and sometimes also shoots and roots were sampled from July until November from trees with or without symptoms of apple proliferation. Results of ELISA are compared with the symptoms of apple proliferation and PCR analysis

Cultivar	Location	Number of trees			
		Tested	With symptoms	Positive in ELISA	Positive in PCR[1)]
'Bohnapfel'	Celje	4	0	0	0
'Braeburn'	Maribor, Pohorski Dvor	3	0	0	0
'Damasonski kosmaè'	Ljubljana	2	1	1	1
'Elstar'	Celje, Pohorski Dvor	22	4	4	5
'Florina'	Udine (Italy)	5	3	3	3
'Gala'	Maribor, Pohorski Dvor	6	0	0	0
'Gloster'	Maribor, Pohorski Dvor	4	1	1	2
'Golden Delicious'	Maribor, Pohorski Dvor	26	9	10	11
'Golden Winter'	Celje, Ljubljana	5	0	0	0
'Idared'	Pohorski Dvor	18	0	0	0
'Jonagold'	Celje, Pohorski Dvor	12	2	2	2
'Lord Lambourne'	Celje, Mozirje	3	0	0	0
'Rootstock M11'	Maribor	2	0	0	0
'Rootstock M9'	Celje, Maribor	13	0	1[2)]	1[2)]
'Majda'	Celje, Mozirje	3	2	2	2
'Melrose'	Pohorski Dvor	3	1	1	1
'Mutsu'	Pohorski Dvor, Mozirje	2	1	1	1
'Peinischer Krummstiel'	Celje	2	0	0	0
'Prima'	Udine (Italy)	1	1	1	1
'Red Belle de Boskoop'	Celje, Mozirje	4	1	1	1
'Reinette du Canada'	Celje	3	0	0	0
'Steirischer Maschanzler'	Celje	3	0	0	0
'White transparent'	Ljubljana, Celje	2	0	1[2)]	1[2)]
Other[3)]	Celje, Mozirje	10	0	0	0
Total		156	26	29	32

1) PCR performed with AP group-specific primers fO1/rO1. Negative samples were additionally tested with nested PCR: amplification products obtained in PCR with the universal primer pair P1/P7 were re-amplified with primers fO1/rO1.
2) Positive in roots and negative in leaves and shoots.
3) 'Alkmene', 'Cox's Orange', 'Estivar Delbar', 'James Grieve', 'Jonadel', 'Jonathan', 'Kronprinz Rudolf', 'M106', 'Vesna' and 'Yellow Bellefleur'; one tree of each cultivar was tested.

3.2 Evaluation of ELISA in different tissues

As shown in Table 2, it was possible to detect the presence of AP phytoplasma in leaves, shoots and roots of a symptomatic 'Golden Delicious' apple tree with ELISA, PCR, IF and DAPI techniques. The degree of infection as evaluated by IF and DAPI assays was correlated to A_{405} values in ELISA. Typically, the highest A_{405} values were obtained with the extracts of shoots, then of leaves and finally of roots. Dilutions of the tissue extracts in the extraction buffer gave proportionally lower A_{405} values (data not shown), indicating no inhibition of antibody binding.

The sensitivity of ELISA was compared to PCR in the assays where AP-infected tissue extracts were diluted with healthy tissue extracts (Table 3). AP phytoplasma could be detected by ELISA in 1 : 100 final dilutions of infected tissues except in the roots where it could be detected in 1 : 10 dilution. Dilutions 10 times higher than the detection limit gave borderline positive results. PCR analysis could detect AP phytoplasma even in 1 : 10000 final dilutions of infected tissues.

Table 2. Detection of apple proliferation phytoplasma in different tissues of apple trees by ELISA, PCR, IF and DAPI techniques. Samples were collected from a symptomatic and a healthy 'Golden Delicious' apple tree in October 2000. The representative results of the repeated experiments are shown

Tissue analyzed		ELISA[1]	IF[2]	DAPI[2]	PCR[3]	Nested PCR[4]
Symptomatic apple tree	Leaves	+ (0.487)	NT[5]	NT	+	NT
	Shoot	+ (1.089)	+++	++	+	NT
	Root	+ (0.380)	+	+	+	NT
Healthy apple tree	Leaves	– (0.012)	NT	NT	–	–
	Shoot	– (0.049)	–	–	–	–
	Root	– (0.034)	–	–	–	–

1) A_{405} after 60 min; values are means from two wells.
2) +++ = strong, ++ = moderate, + = weak and – = no infection as evaluated by IF and DAPI assays.
3) PCR performed with AP group-specific primers fO1/rO1.
4) Amplification products obtained in PCR with the universal primer pair P1/P7 were re-amplified with AP group-specific primers fO1/rO1.
5) Not tested.

Table 3. Comparison of the sensitivity of ELISA and PCR in different tissues of a 'Golden Delicious' apple tree and the test plant *Catharanthus roseus* L. The extracts of the infected tissues were prepared by ELISA extraction (ratio of tissue in extraction buffer 1 : 10) and diluted with the extracts of the healthy tissue to obtain lower levels of infection (final dilutions of infected tissue 1 : 100, 1 : 1000 and 1 : 10000). The representative results of the repeated experiments are shown.

Tissue analyzed	Assay	Final dilutions of the infected tissue				
		1 : 10	1 : 100	1 : 1000	1 : 10000	Healthy
Apple tree leaves	ELISA	+[1]	+	+/–	–	–
	PCR[2]	+	+	+	–	–
Apple tree shoot	ELISA	+	+	+/–	–	–
	PCR	+	+	+	+	–
Apple tree root	ELISA	+	+/–	–	–	–
	PCR	+	+	+	+	–
C. roseus leaves	ELISA	+	+	+/–	–	–
	PCR	+	+	+	+	–

1) + = positive, – = negative, +/– = borderline positive in ELISA (A_{405} 1.5–2 times the mean of the healthy control).
2) PCR performed with AP group-specific primers fO1/rO1 on DNA preparations from ELISA extracts.

3.3 Evaluation of the extraction methods

The efficiency of phytoplasma DNA extraction by simpler ELISA extraction procedure was similar to the PE procedure as determined by PCR using serially diluted DNA templates. The same titer of DNA templates prepared by both extraction procedures could be detected although PCR using PE extracts yielded slightly more amplification products at higher dilutions as viewed under UV illumination on ethidium bromide-stained gel (Fig. 1). As presented in Figure 1, occasionally inhibition of PCR occurred in DNA extracts from roots prepared by both extraction procedures. The inhibition could be overcome by diluting DNA extracts in water. ELISA A_{405} values of positive samples were the same or even lower with the PE extracts than with ELISA extracts (data not shown). ELISA and PCR analysis of extracts after storage at –20 °C for several months revealed high stability of the phytoplasma antigen and DNA in the frozen extracts (data not shown).

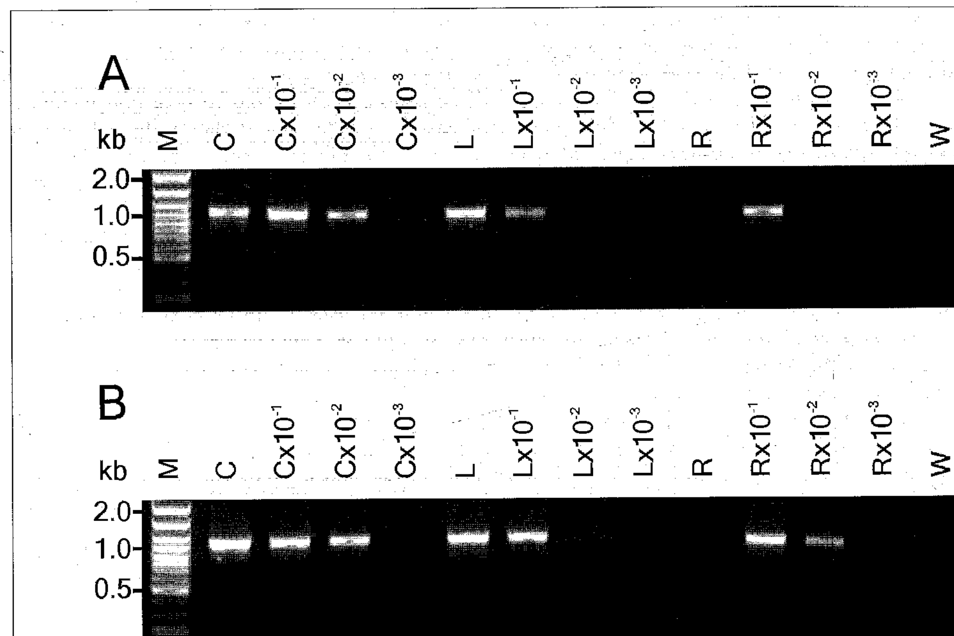

Fig. 1. Gel electrophoresis of PCR amplification products obtained with AP group specific primers fO1/rO1 from diluted (10^{-1}, 10^{-2} and 10^{-3}) and undiluted DNA extracts from *Catharanthus roseus* leaves (C), apple tree leaves (L) and apple tree roots (R). DNA was isolated from ELISA extracts (A) and by phytoplasma enrichment procedure using differential centrifugation (B). Samples in (A) and (B) were run on the same gel in two rows; M, molecular size marker (100 bp DNA Ladder Plus, MBI Fermentas, Lithuania); W, water control.

3.4 AP phytoplasma detection during the dormant season

In ELISA, all samples of symptomatic dormant bud-wood taken monthly from November until April were positive (A_{405} after 60 min: 0.234–0.386). Samples without any visible symptoms were negative (A_{405} after 60 min: 0.032–0.058) except one sample collected in April (A_{405} after 60 min: 0.402). Most of the root samples were positive (A_{405} after 60 min: 0.234–0.240) on all the sampling dates. However, depending on the site of sampling some root samples were negative (A_{405} after 60 min: 0.008–0.054). PCR gave identical results as ELISA in all the samples.

4 Discussion

The specificity of recently developed monoclonal antibody (Loi et al. 2002) to AP phytoplasma was demonstrated as AP phytoplasma could be reliably detected by ELISA in leaves, shoots and roots of infected apple trees. Healthy samples of all the tested tissues gave low background and there were no false positives, which was confirmed by direct and nested PCR, IF and DAPI techniques. The experiments in which infected tissue extracts were serially diluted with healthy tissue extracts demonstrate high sensitivity of ELISA as positive results were obtained in 1 : 100 and borderline positive in 1 : 1000 final dilutions of infected tissues (Table 3). As expected, the sensitivity of PCR was shown to be higher than that of ELISA.

In a large scale laboratory testing, where 156 apple trees of 33 cultivars were analyzed by ELISA and PCR, were both techniques sufficiently sensitive to detect AP phytoplasma in samples from symptomatic trees. AP phytoplasma was detected even in some samples from trees without any visible symptoms (Table 1). The detection of such latent infections is important because they are a potential

source for the spread of the disease in the propagation material. On the other hand, the detection can be difficult even in symptomatic shoots, since the phytoplasma populations progressively decline during the winter until the vegetation restarts (SEEMÜLLER 1988). LOI et al. (2002) could not detect phytoplasma in shoots collected in April using a combination of DAPI and IF detection methods. However, in our analysis of samples during dormant season it was possible to detect phytoplasmas in symptomatic shoots including in April. This may be due to the higher sensitivity of the ELISA and PCR detection methods or simply to the milder winter temperatures, which allowed the phytoplasmas to survive in the canopy. The detection of AP in the sample without any visible symptoms of witches' brooms in April may indicate that at that time the reinvasion of the phytoplasmas from the roots into the canopy had already started. The phytoplasmas are present in the roots of infected apple trees all the year round (LOI et al. 2002) and the ability to detect AP phytoplasma in the roots was demonstrated both by ELISA and PCR. The importance of AP detection in the roots is further demonstrated by the presence of phytoplasmas in the roots of rootstock M9 that did not show any symptoms of the disease (Table 1). To obtain representative samples however, several major roots should be sampled because of the irregular distribution of phytoplasmas in the roots (JARAUSCH et al. 1996).

Based on the results of this and previous studies (LOI et al. 2002; SEEMÜLLER 1988), we recommend AP phytoplasma detection in the leaf veins from June until leaf fall since this material can be obtained from various locations in the canopy and is easily homogenized. Using phloem preparations of the shoots, the detection can be reliably carried out during this period and also during the dormant season. At the restart of the growth season, in April and May, root samples should be tested to gain reliable results.

In order to simplify the phytoplasma detection in a large-scale laboratory testing, the efficiency of the laborious and expensive phytoplasma enrichment step was evaluated both in DNA and ELISA extraction procedures. PCR, with the use of specific AP group primers, confirmed that DNA preparations from simple ELISA extracts of leaf, shoot and root tissues of apple trees are good templates for amplification of phytoplasma sequences (Table 3). In agreement with the report by PALMANO (2001), the phytoplasma enrichment step did not significantly improve the efficiency of phytoplasma DNA extraction nor did it prevent the inhibition of PCR in root samples (Fig. 1). Likewise in ELISA, the use of the enriched extracts did not result in higher A_{405} values. These results suggest that a simple, one-step standard ELISA extraction procedure could serve as a common initial step in sample preparation for both ELISA and DNA extraction for PCR analysis. Furthermore, as the phytoplasma antigen and DNA were shown to be stable in the frozen ELISA extracts, the long-term storage of samples until a sufficient number is reached for batch processing is possible. Therefore, a simple assay for AP phytoplasma detection in different tissues of apple tree is proposed; ELISA extracts are prepared and analyzed by ELISA, fresh or after storage in the freezer. After obtaining the results of large-scale ELISA analysis, DNA is isolated from frozen extracts of borderline positive samples (at or slightly below the threshold) and optionally from negative samples (depending on the required sensitivity of the test) and analyzed by PCR. By such a combination of the two methods, a relatively simple and very sensitive assay for the detection of AP phytoplasma is provided. The combination of the methods may be especially valuable in diagnosis of roots where inhibitors can compromise PCR analysis.

The developed procedure, using ELISA as a first step analysis, followed by a simplified PCR as a confirmation analysis was applied to the large-scale testing of 149 trees in 2002, as part of the governmental program. All 48 symptomatic apple trees were positive by ELISA. Among 101 trees without typical symptoms of AP, six were positive, two borderline positive and 93 negative by ELISA. PCR confirmed AP in both borderline positive samples and nested PCR in only two of 81 samples negative by ELISA. This testing further confirmed our hypothesis that ELISA could be used as very reliable first test for the large-scale testing, and PCR as an additional confirmation method in cases of ELISA doubtful results.

Our experiments have shown that, with ELISA, reliable AP phytoplasma detection is possible, not only from the leaf veins but also from the shoots and the roots. This is especially important if dormant bud-wood is to be tested and for the detection of latent infections in roots. ELISA, combined with simplified PCR analysis, enables sensitive detection of AP phytoplasma during both active growth and dormant seasons. The assay was successfully applied in a large-scale laboratory testing and should provide a valuable tool for the certification programs, epidemiological studies and phytoplasma eradication programs.

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