OCCURRENCE OF APPLE PROLIFERATION AND PEAR DECLINE PHYTOPLASMAS IN DISEASED PEAR TREES IN HUNGARY

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SUMMARY

Pear trees with proliferation and decline symptoms were sampled in orchards in Hungary.We describe the results obtained with different approaches in detecting phytoplasmas associated with the symptoms.Analyses with primers designed on ribosomal and non-ribosomal DNA sequences and the restriction profiles of the amplified products revealed the presence of phytoplasmas belonging to the apple proliferation group. This appears to be the first report on the presence of mixed infections of apple proliferation and pear decline phytoplasmas in diseased pear trees in Hungary.

RIASSUNTO

Rinvenimento di peri infetti da fitoplasmi in Ungheria. Campioni di pero con sintomi di deperimento sono stati collezionati in Ungheria. Nel presente lavoro vengono riportati i risultati ottenuti impiegando differenti tecniche molecolari per rilevare e caratterizzare i fitoplasmi associati alla sintomatologia. L'impiego della PCR e dell'analisi dei profili di restrizione enzimatica hanno permesso di assegnare al gruppo dendrologico «Apple Proliferation» i fitoplasmi presenti nei tessuti di pero saggiati. È questa la prima segnalazione di infezione mista di fitoplasmi della proliferazione del melo e della moria del pero in peri infetti in Ungheria.

Key words: pear, phytoplasmas, apple proliferation cluster, detection.

INTRODUCTION

Phytoplasmas cause severe damage and heavy economic loss in many european perennial fruit crops (Ahrens *et al.*, 1993; Lee *et al.*, 1995).This state of affairs is mainly due to use of infected planting material that constitutes the primary source for natural field spread by vectors.

Pear Decline (PD) is one of the most important diseases caused by phytoplasmas in pear, mainly due to its efficient and active spread by psyllid *Cacopsilla* spp. Symptoms differ according to the rootstock and the variety. The most susceptible cultivars reported in literature are 'Comice', 'Williams', 'Abate Fetel' and 'Kaiser' (Davies *et al.*, 1994; Giunchedi *et al.*, 1994). Infected trees bear few, small leaves that may become reddish in autumn. They roll along the longitudinal axis and drop earlier than normal. Infected tree may live for many years or die in a short time.

In Hungary typical pear decline symptoms were observed on 'Williams' indicators in a graft transmission experiment made with budwood from different pear cultivars in 1976 (Nemeth, 1979), but at that time there was no adequate laboratory method for identification of the pathogen. Since then infection of pear cultivars by PD has not been detected (Nemeth, unpublished).

Diseased pears show disorders, more conspicuous in autumn, such as off-season flowering and fruit production, and sometimes proliferation caused by premature development of axillary buds (Osler and Loi, 1986; Osler *et al.*, 1996).

Apple proliferation disease (AP) is widespread in apple orchards in Hungary (Nemeth, 1979), but it has not been found in pears so far (Lorenz *et al.*, 1995). AP and PD have been shown to be caused by two distinct microorganisms: pear decline and apple proliferation phytoplasmas, both belonging to the apple proliferation cluster (Lorenz *et al.*, 1995).

The present study describes the results obtained with different approaches in detecting phytoplasmas associated with diseased pear trees sampled in Hungarian orchards. This is, to our knowledge, the first report on the occurrance of an infection of phytoplasmas belonging to the Apple Proliferation group in pear orchards in Hungary.

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MATERIALS AND METHODS

Source of plant material. The samples, consisting of leaves and shoots from seven symptomatic pear plants of cvs Williams and Esperen's Bergamotte, were collected in a nursery at Alsòtekeres, Hungary.

Healthy and PD-infected pear (ISPAVE.1) as well as healthy periwinkles and those experimentally infected with european aster yellows (EAY, isolate kindly provided by Dr. G. Boccardo, IFA-CNR, Turin, Italy) phytoplasmas were used as controls. The apple proliferation strain, maintained in periwinkle, was also used (AP, isolate kindly, provided by Prof. R. Osler, Istituto di Patologia Vegetale, University of Udine, Italy) .

DNA Extraction. Total nucleic acids were extracted from symptomatic and symptomless leaves and green tissue scraped from phloematic tissue under the bark of shoots. About 2 g of tissue, pulverized with liquid nitrogen, was homogenized in 14 ml of tissue suspension buffer (TSB= 0.1 M phosphate buffer pH 7.8 containing 10% sucrose, 2% polyvinylpyrrolidone MW 10,000, 50 mM ascorbic acid), stirred for 30 s and filtered through miracloth. After centrifugation (15 min at 10,000 *g*), the pellet was resuspended in extraction buffer containing 2% CTAB (hexadecyltrimethyl ammonium bromide) and processed according to Doyle and Doyle (1990).

Polymerase Chain Reaction. Different primers, designed on ribosomal and non-ribosomal DNA sequences, were used for PCR experiments: fU5/rU3 as universal primers, f01/r01 and fPD/r01, specific for the AP cluster, and fPD/rPDS specific for PD. Amplification conditions were those reported by Lorenz *et al.* (1995).

Restriction Fragment Length Polymorphism. Amplified products (10µl), obtained with universal (fU5/rU3) and cluster specific (f01/r01) primers, were digested with *Rsa* I at 37°C for 2 h in 20 µl of final volume.

Amplified products, obtained with fPDr01 primers, were digested with S*sp* I, S*fc* I and *Bsa* AI (Bio Labs) as above. All digested products were run in agarose and polyacrylamide gel (5%) electrophoresis, stained with ethidium bromide or silver nitrate and visualized on a UV transilluminator.

PCR-ELISA. Products obtained with the fP1/rP7 universal primers (Kirkpatrick, 1994) (amplifying both 16S rRNA gene and the space region between 16S rRNA and 23S rRNA), labelled with digoxigenin during the amplification process, were hybridized, after denaturation, with the rPDS oligonucleotide. This specific capture probe complementary to the inner part of the amplification product was biotilinated in the 5' position.

The hybridization product (20 pmoles of capture probe + 20 µl of amplified product made up to 220 µl with hybridization solution) was incubated in an ELISA plate previously coated with streptavidin. The test was performed according to manufacture's instructions (Boehringer Mannheim) following the protocol of Poggi-Pollini *et al.* (1996).

Amplified products, from PD-infected Italian pear, the AP strain and healthy pear and apple were compared as controls.

RESULTS

DNA Extraction. The extraction method yielded sufficient DNA, both from leaves or phloem tissue under the bark, to be used successfully.

Polymerase Chain Reaction. Amplified products of 880 bp from DNA, of six symptomatic pear samples and AP and EAY periwinkle experimentally infected, were obtained using universal primers fU5/rU3 (Fig. 1a). Other amplified fragments of about 1000 bp and 900 bp were obtained, from the same samples, using f01/r01 and fPD/r01 primers, respectively. No amplification was observed either from healthy pear and periwinkle or EAY-infected periwinkle.(Fig.1b and 1c).

Only two Hungarian samples, among the six processed with primers specific for PD (fPD/rPDS) and the italian isolate of PD gave band of the expected size (1400 bp) (Fig.1d). Those of the two Hungarian isolates were very weak (Fig.1d)

Restriction Fragment Length Polymorphism. In all samples, except one that appeared to be healthy and was not considered further, restriction profiles, obtained with *Rsa* I after amplification with primers fU3/U5 and f01/r01, showed bands specific for phytoplasmas found, until now, only in pome fruit trees (Fig.2a and 2b).

Furthermore, a band of 717 and of 792 bp were observed when amplified products obtained with fPD/r01 were digested with *Ssp* I and *Sfc* I, respectively, indicating the presence of AP phytoplasmas in all samples (Fig. 2c).

PCR-ELISA. Among the amplified products obtained with universal primers fP1/rP7 (Fig.1e), four Hungarian samples reacted with the biotilinated rPDS probe indicating the presence of PD phytoplasmas (Table 1).

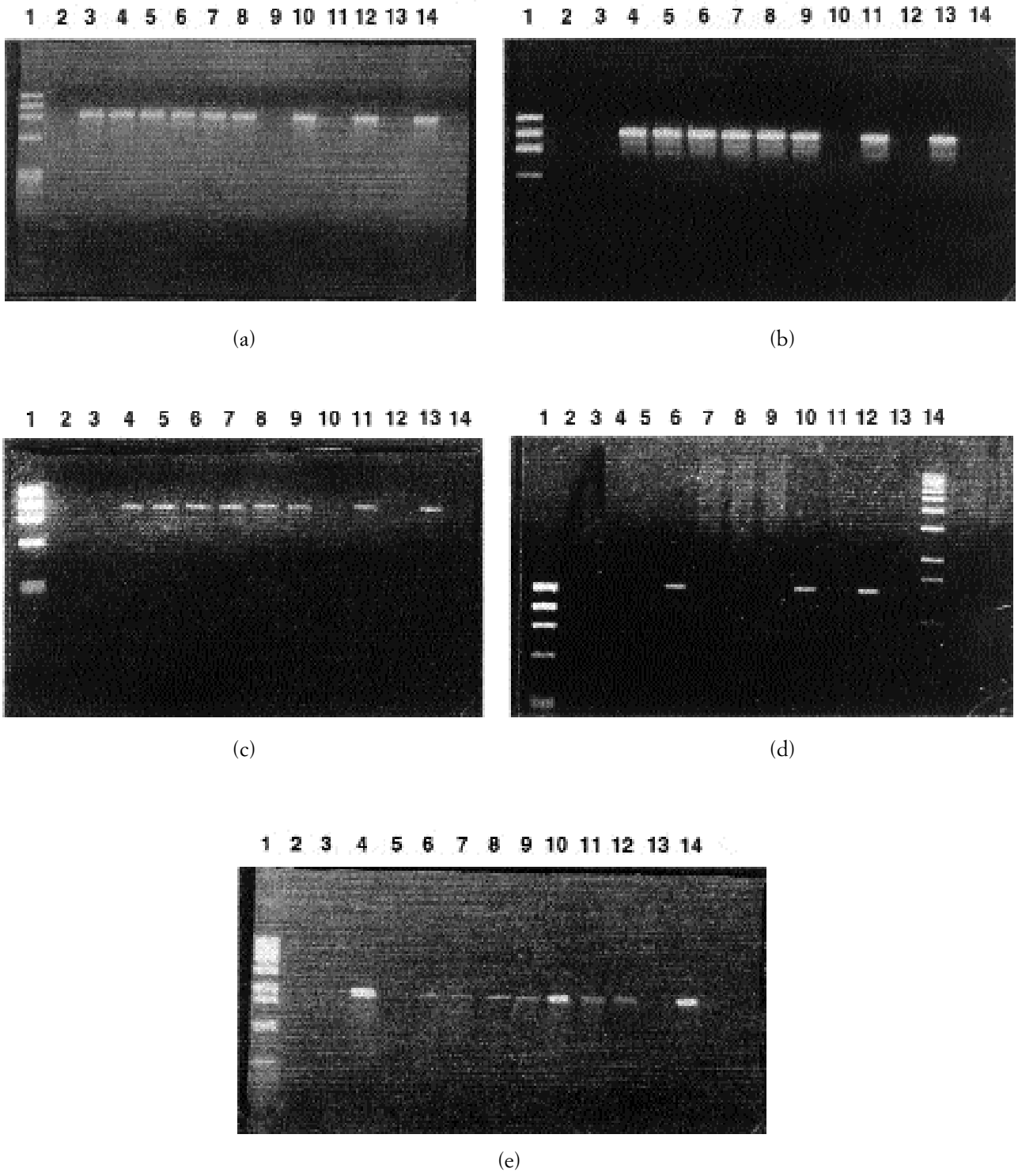

Fig.1. Agarose (1%) gel electrophoresis of amplified products with the primers: **(a)** fU5/rU3; lanes 1 = marker (1F174, Gibco BRL), 3-9 = Hungarian samples, 10 = Italian PD infected pear, 11 = healthy pear, 12 = AP strain infected periwinkle, 13 = healthy periwinkle, 14 = EAY infected periwinkle. **(b)** f01/r01 and **(c)** fPD/rO1; lanes 3-9 = Hungarian samples, 10 = healthy pear, 11 = Italian PD infected pear, 12 = healthy periwinkle, 13 = AP strain infected periwinkle. **(d)** fPD/rPDS; lanes 1 = marker (1F174, Gibco BRL), 2-9 = Hungarian samples, 10 = Italian PD infected pear, 11 = healthy pear, 12 = AP strain infected periwinkle, 14 = marker (1kb ladder, Gibco BRL). **(e)** fP1/rP7; lanes 1 = marker (1kb ladder, Gibco BRL), 3 = healthy pear, 4 = Italian PD infected pear, 5-12 = Hungarian samples, 13 = healthy periwinkle, 14 = AP strain infected periwinkle.

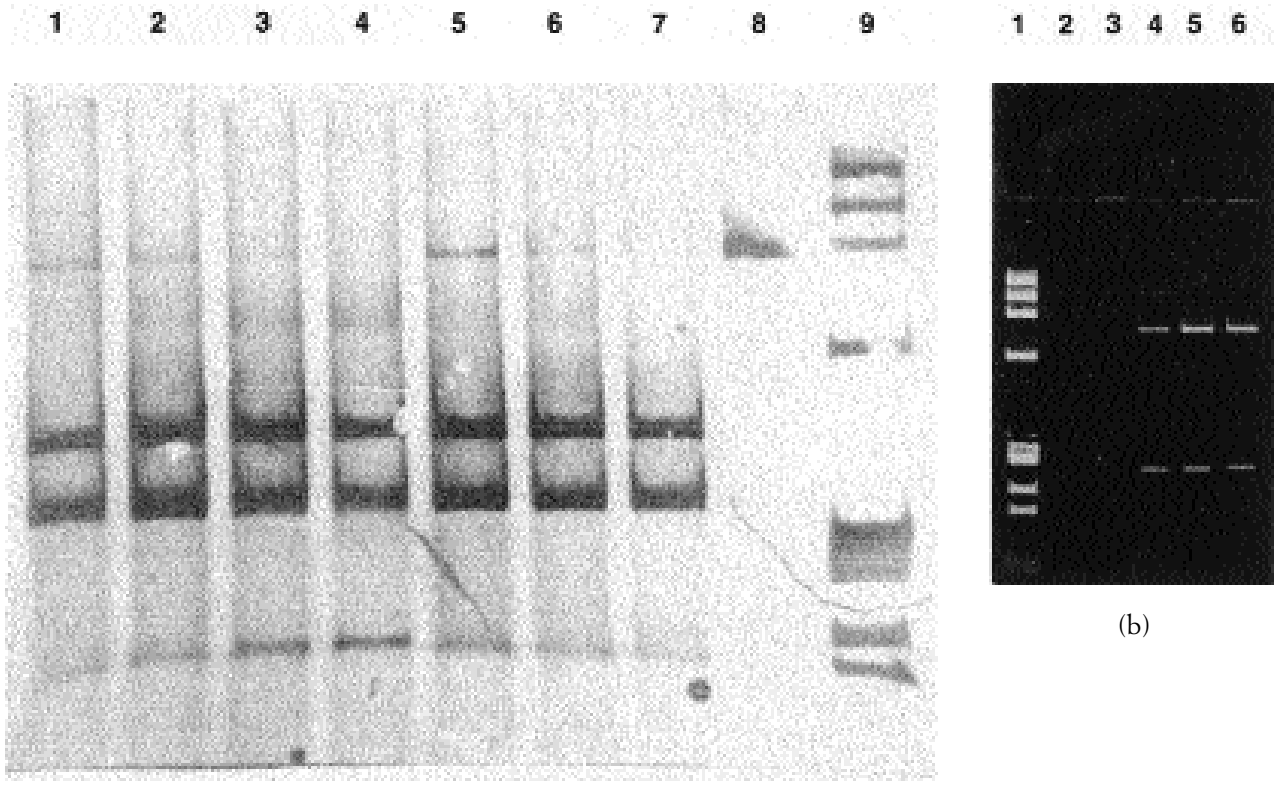

Fig.2. Profiles of fU5/U3 **(a)** and fO1/rO1 **(b)** amplified products restricted with *Rsa* I. **(a)** 5% polyacrylamide gel electrophoresis stained with silver; lanes 1 = Italian PD-infected pear, 2-7 = Hungarian samples, 8 = not digested, 9 = marker (1F174, Gibco BRL), **(b)** 1% agarose gel electrophoresis stained with ethidium bromide; lanes 1 = marker (1F174, Gibco BRL),2 = healthy pear, 3 = healthy periwinkle, 4 = Hungarian sample, 5 = Italian PD-infected pear, 6 = AP strain-infected periwinkle.

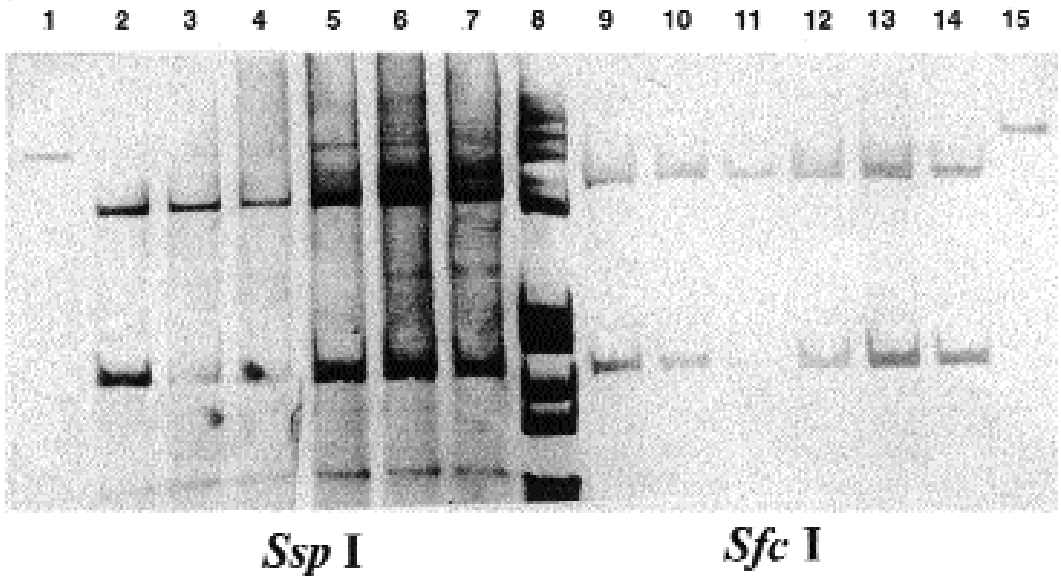

Fig. 3. Polyacrylamide (5%) gel electrophoresis of profiles of fPD/r01 amplified products restricted with *Ssp* I and *Sfc* I stained with silver nitrate; lanes 1 = not digested, 2 = AP strain-infected periwinkle, 3-7 = Hungarian samples, 8 = marker (1F174, Gibco BRL), 9-13 = Hungarian samples, 14 = AP strain-infected periwinkle, 15 = not digested.

Table 1. Absorbance values at A_{405nm} of fP1/rP7 amplified products tested by PCR-ELISA

Samples	A_{405nm}	Presence of PD
1	0.260	–
2	0.870	+
3	0.600	+
4	0.300	+
5	0.180	–
6	1.017	+
Italian isolate	0.600	+
Healthy pear	0.150	–
AP strain	0.300	+
Healthy apple	0.150	–
Buffer	0.135	
Kit check	> 2	

DISCUSSION

This is the first time that phytoplasmas have been detected by molecular methods in pear trees in Hungary. In fact, the PCR amplification with universal primers clearly indicated the presence of phytoplasma DNA.

PCR with universal primers (fU5/rU3) followed by RFLP analysis indicated that the phytoplasmas were in the apple proliferation cluster. Primers specific for this group gave amplified products whose restriction profile proved that these phytoplasmas belong to the pome fruit subgroup. In addition RFLP analysis, performed with amplified products obtained with fPD/r01 (subgroup-specific primers), confirmed the presence of AP, but not of PD phytoplasmas. On the other hand PCR, using specific primers, and PCR-ELISA detected PD phytoplasmas although in different extent.

The latter technique showed higher sensitivity, in terms both of signal intensity and number of samples found infected, so that four plants appeared to carry mixed infection of both phytoplasmas, although in PCR-ELISA the AP strain gave only a weak signal.

Mixed infections of phytoplasmas belonging to the same or different clusters not surprising since it has already been reported by several authors (Bianco *et al.*, 1993; Lee *et al.*, 1995; Lorenz *et al.*, 1995). From hybridization with the rPDS probe and from amplification with primers fPD/rPDS, both supposed to be PD-specific, there is evidence that at least some of the trees were also infected with the PD phytoplasma. It is possible that the PD phytoplasma was not detected by the RFLP analysis shown in Fig.3 because its concentration was too low.In all other gels the sizes of the fragments derived from AP and PD would not be different.Thus, it is clear that the trees were doubly infected or that the probes and primers supposed to be PD-specific cross-hybridized with AP DNA.

Furthemore pathogen-specific non-ribosomal primers for AP and PD phytoplasmas fail to detect all strains of both pathogens (Lorenz *et al.*, 1995). These facts emphasize the problem of how to distinguish these two microorganisms and whether they can be considered distinct, in order to detect specifically the pathogens associated with these two dangerous diseases.

AKNOWLEDGEMENTS

The authors are grateful to Prof. A. Quacquarelli (Istituto Sperimentale per la Patologia Vegetale, Rome, Italy) for his suggestions, Dr. E. Seemueller (Biologische Bundesanstalt Institut fur Pflanzenschutz, Obstau, Germany) for critically reading the manuscript and Prof. R. Osler (Istituto di Patologia Vegetale, University of Udine, Italy) and Dr. G.Boccardo (IFA-CNR, Turin, Italy) for kindly providing the AP and EAY strains in periwinkle.

This work was supported by the Italian Ministry of Agriculture (MiPA), Project «Biotechnology» n°457.

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Received 10 September 1997
Accepted 2 March 1997