

SHORT COMMUNICATION

**CANDIDATUS PHYTOPLASMA PYRI INFECTIONS IN PEAR ORCHARDS
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Campus Universitaire, Tunis El-Manar, 2092 Tunis, Tunisia***SUMMARY**

Samples from apparently healthy European pears (*Pyrus communis*) from Tunisian orchards and from trees showing symptoms recalling those of the “slow decline” form of pear decline, i.e. reduced growth, production of fewer and smaller leaves that turn reddish and drop early in autumn, were analyzed by polymerase chain reaction (PCR) using the universal phytoplasma primers P1/P7. A band with the expected size (1.8 kbp) was obtained from samples collected from symptomatic trees only. PCR products were used for restriction fragment length polymorphism (RFLP) analysis after digestion with the endonucleases *AluI*, *RsaI* and *SspI*. RFLP patterns obtained were consistent with those reported for *Candidatus* Phytoplasma pyri, thus providing evidence of the occurrence in Tunisia of this hitherto unrecorded pathogen.

Key words: Pear, pear decline, *Candidatus* Phytoplasma pyri, PCR, RFLP.

Pear (*Pyrus communis*) is widely grown in Tunisia, which ranks third among African countries, with an average annual fruit production (from 2001 through 2004) of approximately 60,000 metric tons, or 11% of the African production of European pears (Anonymous, 2004). Most of the crop is for local consumption, a small amount being exported, mainly to neighbouring countries. During the 1980s, new pear cultivars were introduced in Tunisia, mainly from Spain, where the phytoplasma-induced pear decline (PD), first identified in 1994 (Avinent *et al.*, 1997), occurs in all pear-growing areas of the country.

Recently, trees of the newly introduced varieties Williams and Alexander Lucas and of the local cultivars Miski-ahrech and Bouguedma growing in northern Tunisia showed symptoms similar to those of the “slow

decline” form of PD, i.e. reduced growth, production of few and small leaves that turn reddish and drop prematurely in autumn (Nemeth, 1986; Seemüller, 1992). These symptoms were more prominent in the introduced than in the local cultivars.

This prompted us to perform PCR assays using the universal phytoplasma primers P1 and P7 (Deng and Hiruki, 1991; Smart *et al.*, 1996) on DNA extracted from cortical scrapings (Ahrens and Seemüller, 1992), followed by RFLP analysis of the amplicons (Lorenz *et al.*, 1995; Kison and Seemüller, 2001).

Briefly, phloem tissues were ground twice in a mortar in the presence of ice-cold extraction buffer (10% sucrose, 1 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl, pH 7.2), the slurry was centrifuged in the cold (4°C) for 5 min at 3,000 rpm, and the supernatant centrifuged again for 25 min at 13,500 rpm. The pellet was resuspended in 1.5 ml of preheated (65°C) extraction buffer according to Doyle and Doyle (1990) (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). The preparation was incubated at 65°C for 30 min, and extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v:v). The supernatant was mixed with a two-third volume of precooled isopropanol (-20°C) and centrifuged at 13,500 rpm. The pellet was washed with 70% ethanol, dried at room temperature and dissolved in 100 µl of distilled water.

PCR was done in a 25 µl reaction volume containing 20 ng DNA, 0.4 mM of each primer, 0.25 mM of each dNTP, 1.5 mM MgCl₂ and 0.5 units of Taq DNA polymerase (Fermentas, Vilnius, Lithuania) in the buffer supplied by the manufacturer. Amplification was done in a Peltier thermocycler (Hybaid, Teddington, UK) for 40 cycles as follows: 45 sec denaturation at 94°C (3 min for the first cycle), 45 sec annealing at 55°C and 2 min of extension at 72°C. In the final cycle the extension step was extended to 10 min. PCR products were separated in 1% agarose gels containing 0.5 µg ml⁻¹ ethidium bromide and visualised with a UV transilluminator. The molecular weight of the PCR products was estimated by comparison with 1 kb DNA ladder (Invitrogen, Groningen, Netherlands).

For RFLP analysis, PCR products (10–12 µl) were digested with endonucleases *AluI*, *RsaI*, or *SspI* (Fermen-

Table 1. Orchards surveyed, number of trees sampled and symptoms shown.

Symptoms	Cultivars	Orchards surveyed (No.)	Age of orchard (years)	Trees sampled (No.)
Reduced canopy, leaf reddening and early drop in autumn	Williams	5	8	20
	Alexander Lucas	2	5	16
Reduced leafing	Bouguedma	1	14	7
	Miski-ahrech	1	18	9
No apparent symptoms	Williams, Alexander Lucas, Bouguedma, Miski-ahrech	9	8-18	18

tas, Vilnius, Lithuania) according to the manufacturer's instructions. Digested fragments were separated on 1.5% agarose gels, visualized, and their molecular weight estimated as described above. RFLP patterns were compared with those previously published, obtained from phytoplasma reference strains (Seemüller and Schneider, 2004).

Nine pear orchards in the Ras-Jebel area (northern Tunisia) were surveyed in the autumn of 2003 and 2004. Shoots were collected from symptomatic and symptomless pear trees of the four foreign and local varieties, all grafted on quince (*Cydonia oblonga*) rootstock, as detailed in Table 1. During the two years of observations, the number of symptomatic trees of cvs Williams and Alexander Lucas increased, unlike those of the two local cultivars (Bouguedma and Miski-ahrech), which seemed to be more tolerant to the disease (Table 2).

Table 2. Incidence of symptomatic pear trees in the two years of survey.

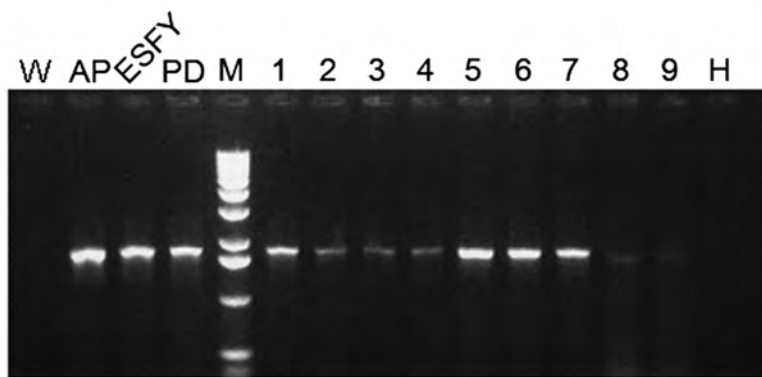
Cultivar	Trees surveyed	Symptomatic trees (%)	
		2003	2004
Williams	120	15	20
Alexander Lucas	40	3	6
Bouguedma	60	2	1
Miski-ahrech	30	1	0.5

Whereas PCR assays yielded a characteristic band of approximately 1.8 kb from all symptomatic pear samples tested and from samples infected by known phytoplasmas (apple proliferation, European stone fruit yellows and pear decline) used as positive controls, no amplification was obtained from any of the symptomless trees (Fig. 1).

As shown in Fig. 2, all RFLP patterns obtained from pear trees of local or newly introduced cultivars were indistinguishable. All profiles belonged to the apple proliferation group (Fig. 2a). In particular, the restriction profile was identical to that of the PD phytoplasma (*Candidatus phytoplasma pyri*), but differed from the patterns given by the European stone fruit yellows phytoplasma (ESFY, *Candidatus phytoplasma prunorum*) (Fig. 2b) and apple proliferation phytoplasma (AP, *Candidatus phytoplasma mali*) (Fig. 2c).

These results were taken as evidence that all symptomatic pear trees tested, regardless of the variety, were infected by *Candidatus phytoplasma pyri*, a new record for Tunisia.

In the course of the survey, an increase in the number of symptomatic trees was observed in the orchards of cvs Williams and Alexander Lucas, suggesting natural spread of PD. Pear decline is known to be transmitted by two *Cacopsylla* species of which *C. pyricola* is the vector in North America (Jensen *et al.*, 1964; Hibino *et al.*, 1971) and England (Davies *et al.*, 1992), whereas *C. pyri*

**Fig. 1.** PCR detection of phytoplasmas using the universal primers P1/P7. Templates consisted of DNA extracted from symptomless pears (H), different pear cultivars from Northern Tunisia (1-9) or from periwinkle plants infected with the following phytoplasma reference strains: apple proliferation (AP), European stone fruit yellow (ESFY) and pear decline (PD). W, water control; M, marker.

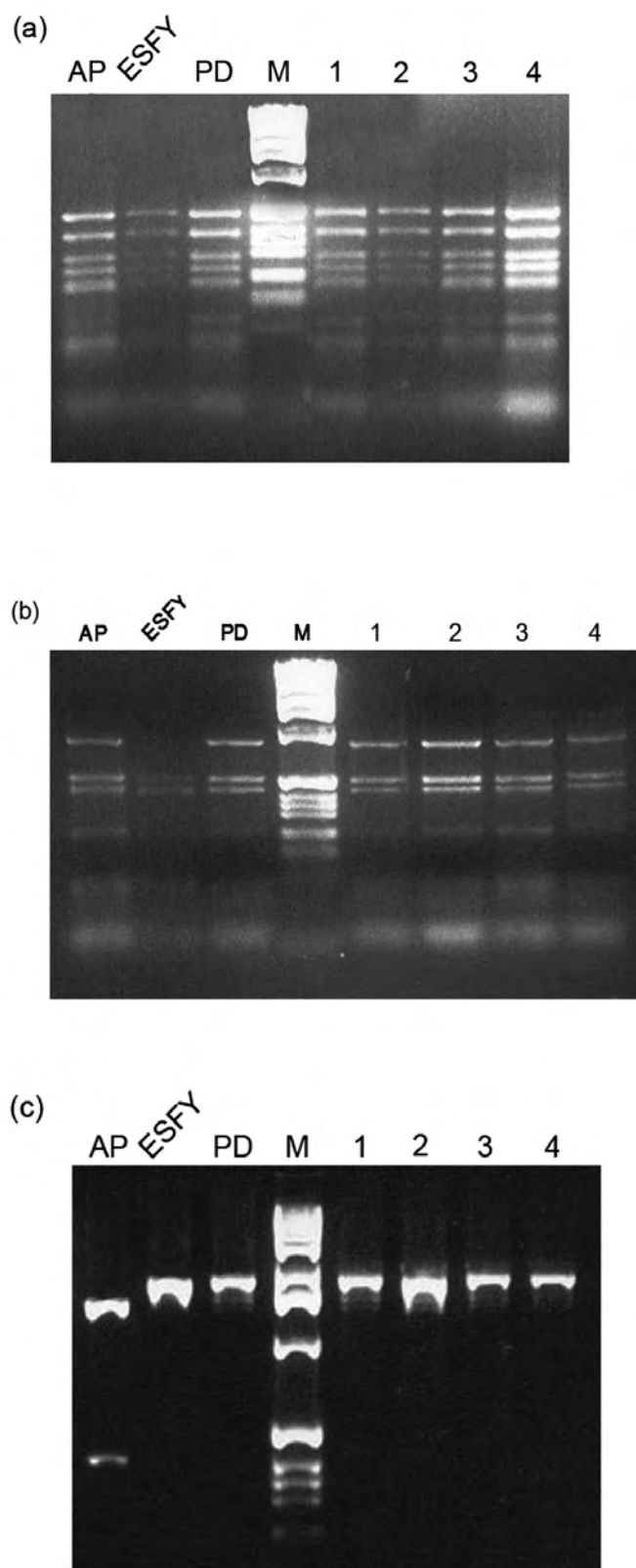


Fig. 2. *AluI* (a), *RasI* (b) and *SspI* (c) restriction profiles of phytoplasma ribosomal DNA amplified using the universal primers P1/P7. Template DNA was from different pear cultivars from Northern Tunisia (1-4) or from periwinkle plants infected with the following phytoplasma reference strains: AP, apple proliferation; ESFY, European stone fruit yellows and PD, Pear decline. M, marker.

transmits the disease in Italy (Giunchedi *et al.*, 1994) and Spain (Avinent *et al.*, 1997). *C. pyri* is the most common psyllid in Tunisian pear orchards (Boulahia Kheder and Jerraya, 2001), so it may well be the PD vector in this country, although transmission trials remain to be done.

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