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First report of *Candidatus* Phytoplasma prunorum infecting almonds in Tunisia

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Abstract *Candidatus* Phytoplasma prunorum was detected for the first time in almond (*Prunus dulcis* Mill.) cv. 'Abiod' in Tunisia. Infected trees showed emergence of new growth during dormancy and leafed out before flowers opened in addition to early defoliation in summer. Phytoplasma was detected by nested polymerase chain reaction (PCR) using universal phytoplasma primer pairs P1/P7 and F2n/R2. A band with expected size was observed in samples collected from five symptomatic, but not symptomless almond trees. PCR products (1.2 kbp) were used for restriction fragment length polymorphism (RFLP) analysis after digestion with endonucleases *Rsa*I and *Ssp*I. RFLP patterns obtained were similar to those reported previously for the European stone fruit yellows (ESFY, 16SrX-B). Identification has been further confirmed by PCR using ESFY specific primer pairs (ECA1/ECA2). This is the first report of *Ca.* Phytoplasma prunorum infecting almonds in Tunisia.

Keywords European stone fruit yellows (ESFY) · Polymerase chain reaction (PCR) · Restriction fragment length polymorphism (RFLP)

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Candidatus Phytoplasma prunorum (Seemüller and Schneider 2004), formerly European stone fruit yellows (ESFY), is the causal agent of many economically important decline diseases of stone fruit species in Europe (Jarausch *et al.* 1998). *Ca.* Phytoplasma prunorum belongs to subgroup B of the apple proliferation group (16SrX-B) (Lee *et al.* 1998). The most common hosts of ESFY phytoplasma include apricots (Morvan 1977), plum (Giunchedi *et al.* 1982), peach (Pollini *et al.* 2001) and almond (Lederer and Seemüller 1992; Lorenz *et al.* 1994). The characteristic disease symptoms of ESFY on almonds include early defoliation in summer and new sprouting in winter followed by dieback within a few years of the first symptoms (Lederer and Seemüller 1992; Lorenz *et al.* 1994).

Almond (*Prunus dulcis* Mill.) is widely grown in Tunisia, which ranks second among African countries and seventh in the world, with an annual fruit production of 60,000 tons, or 3% of the world almond production (Anon. 2011). Almonds represent 20% of Tunisian stone fruit production, most of which is for local consumption and only 4% of which is exported, mainly to Libya and European countries (Anon. 2011).

Recently, disease symptoms resembling those caused by ESFY phytoplasma were observed on almonds in the Ras Jebel area (northern Tunisia). This study aimed to confirm ESFY phytoplasma etiology of almond syndrome in Tunisia. To achieve this, PCR

analysis using universal and ESFY-specific primer pairs and restriction fragment length polymorphism (RFLP) analysis were applied.

Samples of leaves were collected during summer 2010 from almond trees of the local cultivar 'Abiod' growing in Ras Jebel area. Ten trees were sampled from the same almond orchard, five of which showed disease symptoms and the remaining five trees were symptomless. Phytoplasma reference strains were maintained on the experimental host *Catharanthus roseus* (periwinkle) and used as positive control in this study. These strains included apple proliferation (AP, 16SrX-A), European stone fruit yellows (ESFY, 16SrX-B) and pear decline (PD, 16SrX-C) phytoplasma (Lee *et al.* 1998).

Total DNA, used as template in PCR reactions, was extracted from approximately 1 g of freshly prepared midribs. DNA from phytoplasma strains used as controls was obtained from petioles and midribs of infected *C. roseus* plants. Nucleic acids were isolated according to the phytoplasma enrichment procedure described by Ahrens and Seemüller (1992).

Nested PCR amplification of phytoplasma 16S rDNA was carried out with universal primers P1/P7 (Deng and Hiruki 1991; Schneider *et al.* 1995) and F2n/R2 (Gundersen and Lee 1996). Non-ribosomal primers ECA1/ECA2 (Jarausch *et al.* 1998) were also used for ESFY-specific detection. The PCR reaction was performed in 25 µl reaction volumes containing 20 ng DNA, 0.4 mM of each primer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$ and 0.5 units of Taq DNA polymerase (MP Biomedicals, Illkirch, France) with the manufacturer's supplied buffer. Thermocycling was performed using a Peltier thermocycler (Hybaid, Teddington, UK) for 40 cycles with 1 min each for denaturation, annealing and extension. In the final cycle the extension step was extended to 10 min. PCR products were separated on 1% agarose gels containing 0.5 µg ml^{-1} ethidium bromide and visualized using a UV transilluminator. The molecular weight of the PCR products was determined by comparison with 1 Kb DNA ladder (Invitrogen, Toulouse, France).

For RFLP analysis, nested PCR products (10–12 µl) were digested with *Rsa*I and S*sp*I restriction enzymes (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Digested fragments were separated either on 5% polyacrylamide gel electrophoresis and stained on ethidium bromide or on 2% agarose gels containing 0.5 µg ml^{-1} ethidium bromide and visualized under the UV light. The molecular weight of the fragments was determined by comparison with 1 Kb DNA ladder (Invitrogen). RFLP patterns were compared with those obtained from the phytoplasma reference strains and the RFLP patterns previously published (Lee *et al.* 1998).

Almond trees of the local cultivar 'Abiod' showed disease symptoms recalling those caused by European stone fruit yellows (ESFY) phytoplasma. Infected trees exhibited emergence of new growth during dormancy and leafed out before flowers opened, in addition to early defoliation in summer.

Universal nested-PCR assay yielded a characteristic band of approximately 1.2 kbp from all symptomatic almond samples tested and from samples infected by reference phytoplasma strains used as positive controls. No amplification was obtained from any of the symptomless trees (data not shown).

*Rsa*I and *Ssp*I restriction profiles obtained from diseased almond trees were indistinguishable from each other and similar to those of European stone fruit yellows (ESFY, *Candidatus* Phytoplasma prunorum) positive control (Fig. 1), but differed from the patterns given by the pear decline phytoplasma (PD, *Candidatus* Phytoplasma pyri) (Fig. 1a) and apple proliferation phytoplasma (AP, *Candidatus* Phytoplasma mali) (Fig. 1b).

PCR analysis using ESFY-specific primer pair ECA1/ECA2 revealed an amplification of 273 bp from all symptomatic almond samples tested. From ESFY phytoplasma used as a positive control, no amplification was observed with AP and PD phytoplasma positive controls.

These results were taken as evidence that all tested symptomatic almond trees of the local cultivar 'Abiod' were infected by *Ca*. Phytoplasma prunorum, a new record for Tunisia.

Candidatus Phytoplasma prunorum has been reported to infect several stone fruit species (Jarausch *et al.* 1998). Thus, knowledge on the transmission mode of the pathogen needs to be improved, in order to define efficient control strategies against ESFY infections in Tunisia. European stone fruit yellows phytoplasmas have been reported to be transmitted by *Cacopsylla pruni* (Carraro *et al.* 1998). Since *C. pruni* is not observed in Tunisia, other studies are needed to identify the *Ca*. Phytoplasma prunorum vector in Tunisia.

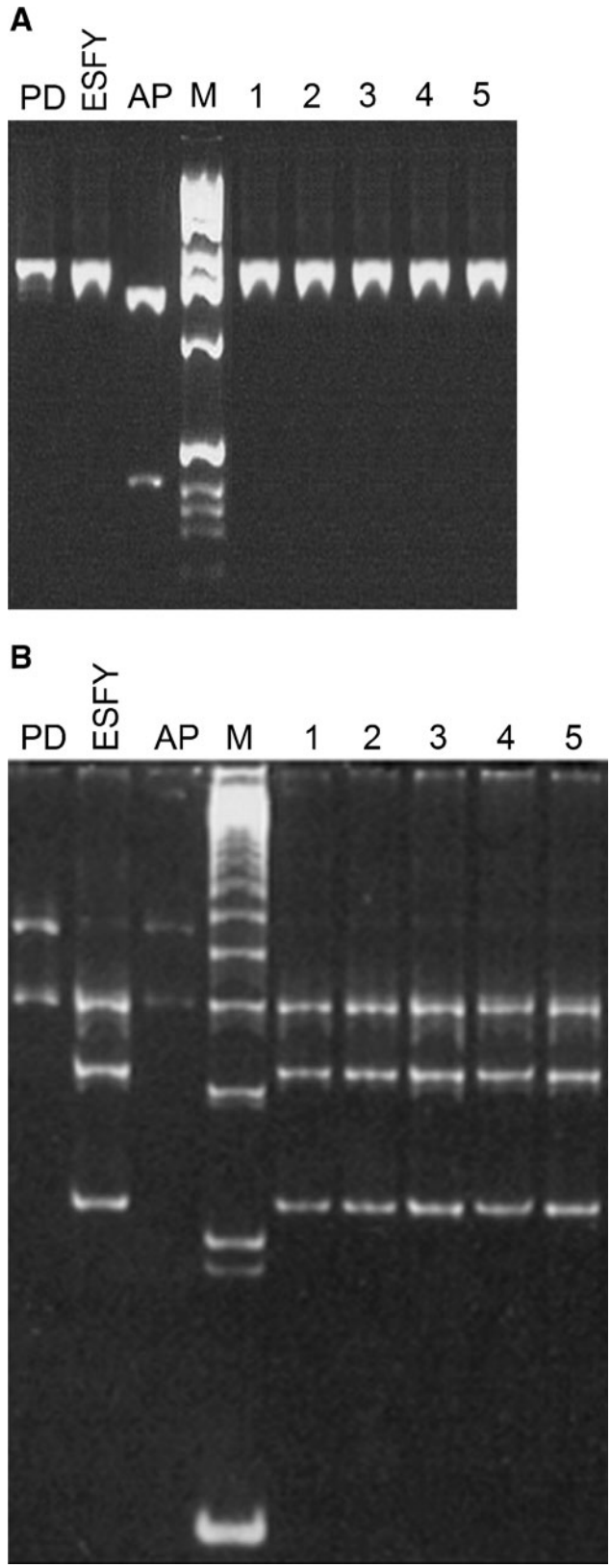

Fig. 1 *Rsa*I (**a**) and *Ssp*I (**b**) restriction profiles of phytoplasma ribosomal DNA amplified using the universal primer pairs P1/P7 and R16F2n/R2. Template DNA was from symptomatic almonds (*Prunus dulcis)* cv. 'Abiod' (1–5) or from periwinkle plants (*Catharanthus roseus)* infected with the following phytoplasma reference strains: AP, apple proliferation; ESFY, European stone fruit yellows; PD, pear decline. M, marker

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