SHORT COMMUNICATION

MOLECULAR CHARACTERIZATION OF PHYTOPLASMAS RELATED TO PEANUT WITCHES' BROOM AND STOLBUR GROUPS INFECTING PLUM IN IRAN

L. Zirak, M. Bahar and A. Ahoonmanesh

Division of Plant Pathology, Department of Plant Protection, College of Agriculture, Isfahan University of Technology, Isfahan, Iran

SUMMARY

Japanese plum trees showing symptoms of phytoplasma disease including little leaf, leaf rolling, rosetting, yellowing and shoot proliferation were observed in stone fruits growing areas in the central regions of Iran. DNA isolated from symptomatic and healthy plum trees was used to amplify 16S rDNA and 16S-23S rDNA intergenic spacer (IS) fragments by direct and nested PCR. Phytoplasmas were detected in symptomatic plum trees in the major stone fruit production areas in ChaharMahal-O-Bakhtiari, Isfahan and Tehran provinces in central Iran. Restriction fragment length polymorphism (RFLP) analyses of fragments amplified by nested PCR revealed that the plum phytoplama isolates were genetically different from each other. Sequence analyses of part of the 16S rRNA gene and IS region indicated that the phytoplasmas associated with plum diseases in these regions belong to peanut witches'-broom (16SrII) and stolbur (16SrXII) groups. This is the first report of the presence of phytoplasmas related to stolbur (*Candidatus* Phytoplasma solani) and peanut WB groups in Japanese plum trees in Iran.

Key words: '*Ca.* Phytoplasma solani', peanut WB group, plum, diagnosis, nested PCR

European stone fruit yellows (ESFY) phytoplasmas, members of Apple proliferation (16SrX) group, are important pathogens infecting Japanese plum (*Prunus salicina* Lindl.) and European plum (*P. domestica* L.) in Europe (Giunchedi *et al.*, 1978; Dosba *et al.*, 1991; Lorenz *et al.*, 1994). It has been reported that peach yellows (Jones *et al.*, 1974) and peach rosette (Kirkpatrick *et al.*, 1975) phytoplasmas which belong to elm yellows (16SrV) and X-disease (16SrIII) groups, respectively, cause diseases on plums in different areas of the world, and that in Italy Japanese plums are affected by a phytoplasma belonging to 16SrXII-A group (Paltrinieri *et al.*, 2004). Iran is one of the main origins of several stone fruit species. Plums (*P. salicina* and *P. domestica*) represent the most important fruits growing in Iran. Phytoplasmas are known to infect several trees, perennial and herbaceous plants in Iran (Salehi *et al.*, 2005). However, plum phytoplasma diseases in Iran have not been studied up to now. Recently, we observed Japanese plum trees showing symptoms thought to be related to infection by phytoplasmas in the central regions of Iran. The aim of this study was to detect and characterize phytoplasmas associated with plum diseases Our results show that Japanese plum trees in the central regions of Iran were affected with phytoplasmas belonging to peanut WB and stolbur groups.

During autumn (September to November) 2004 to 2005, leaf samples of 23 symptomatic and 3 symptomless Japanese plum trees showing little leaf, leaf rolling, rosetting, yellowing and shoot proliferation symptoms were collected from orchards of ChaharMahal-O-Bakhtiari (Saman), Isfahan (Daregan, Najafabad) and Tehran (Shareyar) provinces located in the centre of Iran.

DNA was extracted from leaf midribs of healthy and symptomatic trees as previously described by Murray and Thompson (1980). Leaf material was ground in liquid nitrogen and suspended in 1 ml pre-warmed (65°C) extraction buffer. The final DNA pellet was suspended in 50 µl sterile distilled water. Samples were stored at -20°C until used for PCR.

In primary PCR assays, the phytoplasma universal primers P1/P7 which amplify a 1784 bp DNA fragment (Schneider *et al.*, 1995) and PA2F/R which amplify a 1187 bp DNA fragment (Heinrich *et al.*, 2001) were used to initially detect phytoplasmas. The 20 µl reaction mixture contained 10-20 ng DNA template, 0.5 µM of each primer, 200 µM each of the four dNTPs, 2 mM $MgCl_2$, 1x polymerase buffer and 1 U of *Taq* DNA polymerase (Roche, Germany). Thermocycling parameters for primers P1/P7 and PA2F/R were identical to those previously described (Schneider *et al.*, 1995; Heinrich *et al.*, 2001). The primary PCR products were then diluted 5 to 50 times and subjected to nested PCR amplification. In nested PCR the primers P1/P7 were used fol-

Corresponding author: L. Zirak
Fax: +98.411.5282962
E-mail: lzirak@alumni.iut.ac.ir

lowed by universal primers R16F2/R2 which amplify a 1239 bp DNA fragment (Lee *et al*., 1993) and phytoplasma specific primers P1/WXint which amplify an approximately 1600 bp DNA fragment and P1/PYLRint which amplify an approximately 1550 bp DNA fragment (Smart *et al*., 1996). Also for the second round, the primers PA2F/R followed by universal primers NPA2F/R which amplify a 485 bp DNA fragment (Heinrich *et al*., 2001), were employed. Nested PCRs were performed as previously described (Lee *et al*., 1993; Smart *et al*., 1996; Heinrich *et al*., 2001). The reaction mixture containing DNA template of healthy plum trees was used as negative control. PCR products were electrophoresed in 1.2% agarose gel (using TBE buffer) and stained with ethidium bromide (5 μg/ml). DNA bands were visualized with a UV transilluminator.

In RFLP analyses, 5 μl of DNA fragments (150-200 ng) amplified by primers NPA2F/R were digested individually with *Alu*I, *Rsa*I and *Tru*9I restriction enzymes (Fermentas, Lithuania) at 37°C overnight. Restricted fragments were analyzed in a vertical 8% polyacryamide gel and stained with silver nitrate.

The DNA fragments amplified by primers NPA2F/R from plum phytoplasma isolates PJ19 and PJ15I and a fragment amplified by primer pair R16F2/R2 from isolate PJ15I were purified using GENECLEAN®III kit (Qbiogene, UK) and cloned into pGEM®–T Easy vector (Promega, USA) according to the manufacturer′s instructions. The ligation mixtures were used to transform competent *Escherichia coli* MC1061. Then partial 16S rDNA and IS region were sequenced at SEQLAB laboratory (Göttingen, Germany). The nucleotide sequence of a 1481 bp fragment, region between primers R16F2 and NPA2R, from isolate PJ15I and a 409 bp fragment amplified using primers NPA2F/R from isolate PJ19 were submitted to GenBank under accession numbers FJ409624 (isolate PJ15I) and FJ204400 (isolate PJ19). Cladistic analyses were performed with DNAMAN version 4.02 software (BBA, Germany) and a phylogenetic tree was constructed by parsimony analyses of nearly similar length (about 410 bp) of phytoplasma 16S rRNA gene and IS region sequences. *Acholeplasma laidlawii* was selected as the out-group to root the tree and the stability of relationships was assessed by bootstraping in 1000 replications.

In primary PCR assays using primers P1/P7 or PA2F/R from 23 symptomatic plum samples, no PCR product was obtained. This could be related to the low concentration of pathogen or presence of high levels of putative PCR inhibitors in the new leaves of the infected trees (Lepka *et al*., 1999; Heinrich *et al*. 2001). Therefore, primary amplification products were used for nested PCR In nested PCR assays using phytoplasma specific primers P1/WXInt (designed for Western X-disease phytoplasmas) and P1/PYLRInt (designed for apple proliferation group phytoplasmas) no product was amplified from any of the symptomatic and healthy plum DNA samples. Therefore, any phytoplasmas associated with diseased plum trees in the central Iran were not belonging to Western X-disease and apple proliferation group phytoplasmas. Nested PCR using primers P1/P7 followed by primers R16F2/R2 amplified an expected product from one plum DNA template (isolate PJ15I). Our results showed that the use of primers PA2F/R in primary PCR followed by primers NPA2F/R in nested PCR provided a sensitive method to detect plum phytoplasmas in central Iran. Primers PA2F/R followed by primers NPA2F/R amplified products from 13 symptomatic plum DNA samples (Fig. 1). However, blast searches of NPA2F/R-amplified sequences indicated that some phytoplasma isolates produced PCR products of different sizes from the expected 485 bp fragment described by Heinrich *et al.* (2001). No band appeared when DNA from healthy plum trees was exam-

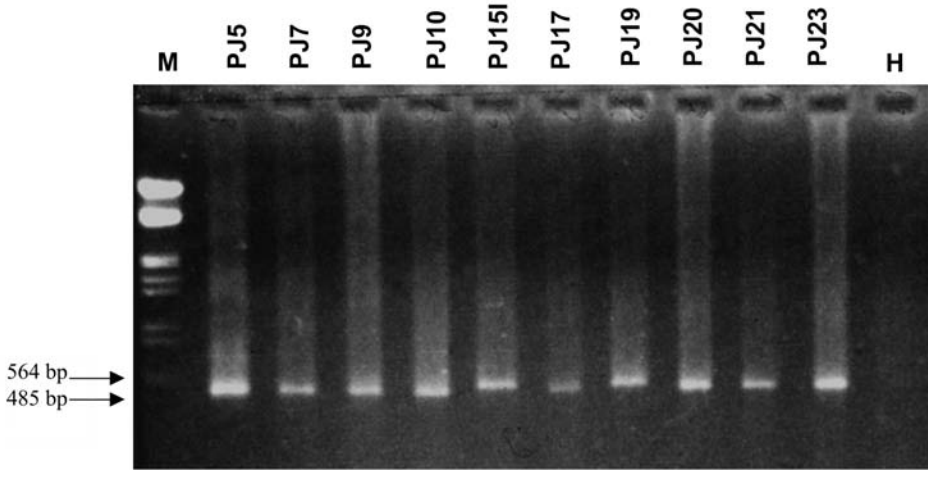

Fig. 1. Amplification using the primer pair PA2F/R followed by primer pair NPA2F/R in nested PCR from infected Japanese plum trees DNA samples. Control H is PCR product of healthy plum and lane M is Marker III.

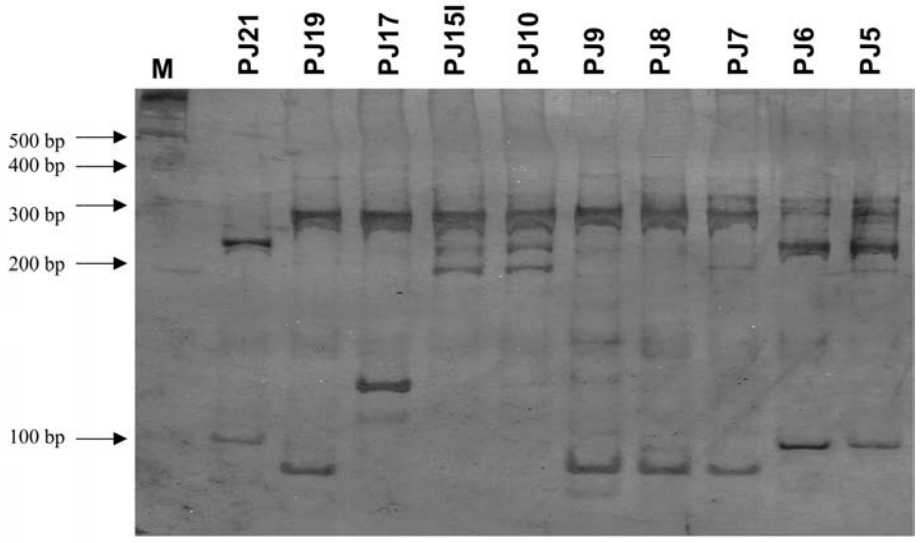

Fig. 2. RFLP analyses of fragments amplified by nested PCR with primer pair NPA2F/R from Japanese plum phytoplasmas using *Tru*9I restriction enzyme. The primary PCR was performed with PA2F/R primer pair. Restricted fragments separated by electrophoresis through 8% polyacrylamide gel and stained in silver nitrate. Lane M is 100 bp DNA ladder.

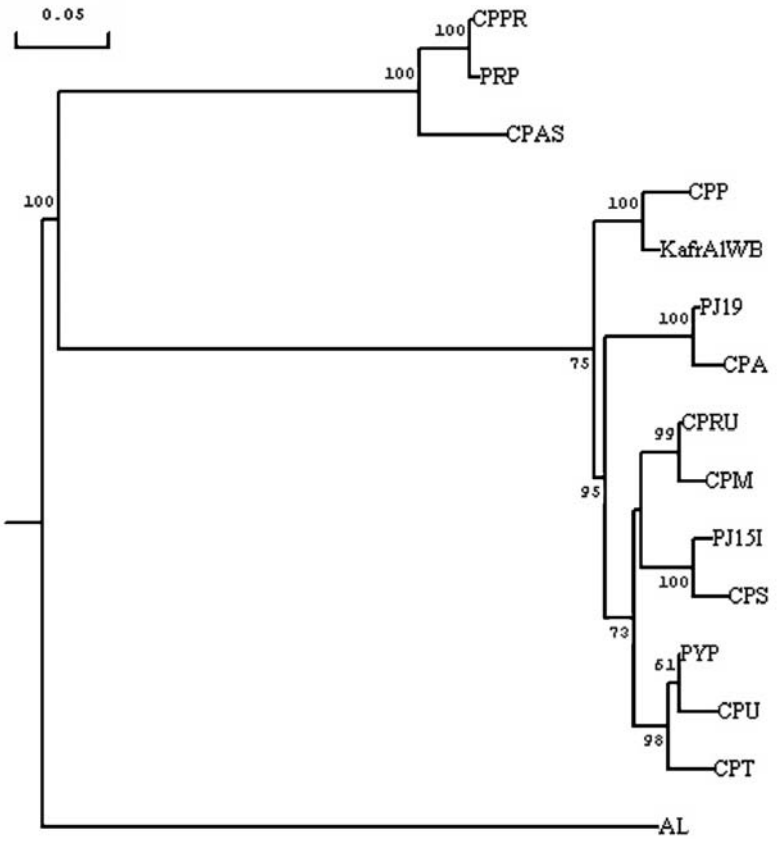

Fig. 3. Phylogenetic tree constructed by parsimony analyses of nearly similar length of partial 16S rRNA gene and IS region sequences (about 410 bp) from two plum phytoplasma isolates, 12 representative phytoplasmas from groups infecting several stone fruits and *Acheleplasma laidlawii* as the out-group. Cladistic analyses were performed with DNAMAN version 4.02 software. Phytoplasmas abbreviations and sequences accension number: CPPR: '*Candidatus* Phytoplasma pruni' (EU753605); PRP: Peach rosette phytoplasma (AF236121); CPAS: '*Ca.* Phytoplasma asteris' (peach red leaf phytoplasma) (AY635144); CPP: '*Ca.* Phytoplasma phoenicium' (AF515637); KhafrAWB: Khafr (Iran) almond witches'-broom phytoplasma (DQ195209); PJ19: Plum phytoplasma isolate (FJ204400); CPA: '*Ca.* Phytoplasma aurantifolia' (EU024405); CPRU: '*Ca.* Phytoplasma prunorum' (European stone fruit yellows phytoplasma) (AJ542544); CPM: '*Ca.* Phytoplasma mali' (EF392654); PJ15I: Plum phytoplasma isolate (FJ409624); CPS: '*Ca.* Phytoplasma solani' (EU814645); PYP: Peach yellows phytoplasma (AY197694); CPU: '*Ca.* Phytoplasma ulmi' (EU184021); CPT: '*Ca.* Phytoplasma trifolii' (AB279597); AL: *Acholeplasma laidlawii* (AY740435).

ined in both of the primary PCR and nested PCR amplifications.

In the RFLP analyses *Alu*I, *Rsa*I and *Tru*9I restriction enzymes were used to digest fragments amplified using primer pair NPA2F/R from 10 plum phytoplasma isolates. Polymorphism could be observed when restriction enzyme *Tru*9I was used. The result of RFLP analyses indicated that different phytoplasmas were associated with diseased plum trees in central Iran (Fig. 2). Therefore, two phytoplasma isolates that were associated with different symptoms in plum trees and showed distinct RFLP patterns were selected for the sequence analyses.

Sequence analyses of the partial 16S rRNA gene and IS region indicated that plum phytoplasma isolate PJ15I from Saman, which caused little leaf and yellowing in infected tree, was closely related to *Ca.* Phytoplasma solani (accession No. EU814645) with 99% identity. However, isolate PJ19 from Najafabad which caused little leaf, leaf rolling and shoot proliferation was related to Cuban tobacco phytoplasma (accession No. EU328255), a member of the peanut WB group, with 99% identity. The phylogenetic tree constructed by parsimony analyses of partial 16S rRNA gene and IS region sequences from two plum phytoplasma isolates PJ15I and PJ19, 12 representative phytoplasmas and *A. laidlawii* showed that the isolate PJ15I and *Ca.* Phytoplasma solani clustered together, whereas isolate PJ19 clustered with *Ca.* Phytoplasma aurantifolia (Fig. 3).

It has previously been reported that phytoplasmas related to peanut WB (such as '*Ca.* Phytoplasma aurantifolia') and stolbur (such as '*Ca.* Phytoplasma solani') groups have wide host ranges in fruit trees, perennial and herbaceous plants in central Iran (Salehi *et al.*, 2005), whereas they have not been reported to be associated with plum diseases in Iran. Also, the phytoplasmas associated with Japanese plum diseases in Iran are different from phytoplasmas which have previously been reported to infect plums elsewhere (Jones *et al.,* 1974; Kirkpatrick *et al.,* 1975; Giunchedi *et al.*, 1978; Dosba *et al.*, 1991; Lorenz *et al.,* 1994; Paltrinieri *et al.*, 2004). This is the first report of presence of phytoplasmas related to stolbur (*Ca.* Phytoplasma solani) and peanut WB groups in Japanese plum trees in Iran. The role of insect vectors in the outbreak of these diseases is still unknown.

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