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Research Paper

## Identification of phytoplasma strains associated with witches' broom and yellowing in *Ziziphus jujube* nurseries in Iran

GHOBAD BABAIEI<sup>1,\*</sup>, SEYYED ALIREZA ESMAEILZADEH-HOSSEINI<sup>2</sup>, MAHBOBEH ZANDIAN<sup>1</sup>, VAHID NIKBAKHT<sup>3</sup>

<sup>1</sup> Plant Protection Research Department, Chaharmahal and Bakhtiari Agricultural and Natural Resources Research and Education Center, AREEO, Shahrekord, Iran

<sup>2</sup> Plant Protection Research Departments, Yazd Agricultural and Natural Resources Research and Education Center, AREEO, Yazd, Iran

<sup>3</sup> Chaharmahal and Bakhtiari Agricultural Organization, Shahrekord, Iran

\*Corresponding author: [ghobad.babae@gmail.com](mailto:ghobad.babae@gmail.com)

**Summary.** Phytoplasma symptoms, including proliferation, witches' broom, leaf rolling and yellowing, were observed in jujube (*Ziziphus jujube*) nurseries in the East of Iran. Total nucleic acid was extracted from symptomatic and symptomless plants, and was tested for phytoplasma presence using nested PCR. Amplicons of about 1.8 kb (primer pair P1/P7) and 1.25 kb (R16F2n/R16R2) were obtained from all symptomatic plants but not from symptomless plants. Restriction fragment length polymorphism (RFLP) analysis of R16F2n/R2 amplicons using *Kpn*I, *Hae*III, *Rsa*I, *Alu*I, *Hpa*II, *Hha*I, *Taq*I, *Mse*I, *Bfa*I and *Tha*I restriction enzymes showed two RFLP patterns referable to 16SrI and 16SrVI phytoplasma groups. The consensus sequences of *Z. jujube* yellowing and witches' broom of six samples correspond to 'Candidatus Phytoplasma asteris' and 'Candidatus Phytoplasma trifolii'-related strains. Two R16F2n/R16R2 16S rDNA sequences representative of each RFLP profile, one each from witches' broom (accession number MK379605) and yellowing (MK379604) host symptoms, were submitted to the GenBank. Phylogenetic analysis confirmed that the phytoplasma strains associated with jujube yellowing clustered within the 16SrI phytoplasma clade, and those associated with witches' broom clustered within the 16SrVI clade. Restriction analysis confirmed that virtual RFLP patterns of the jujube yellowing and witches' broom phytoplasma strains were identical to the reference pattern of 16SrI-B and 16SrVI-A. This is the first report of these phytoplasma strains associations with witches' broom and yellowing in jujube plants.

**Keywords.** Aster yellows phytoplasma, clover proliferation phytoplasma, jujube.

### INTRODUCTION

Jujube (*Ziziphus jujube* Mill) is a small shrub or tree with small fruits about in the size of date, that is native to southern and central Asia and is grown in the northeast and central parts of Iran. Jujube has different cultivars, the fruit is used as food, and the plants can be used as a vegetable and for medicinal purposes.

Jujube witches' broom (JWB) was first reported as a graft transmissible disease (Kim, 1965), as phyllody, lack of fruit production and dieback in China (La and Woo, 1980). The disease symptoms included little leaf, phyllody and leaf yellowing, and the disease is a severe production-limiting factor, distributed in all Asian countries including China, Korea and Japan. Yield losses up to of 80% have been reported, and rapid tree death can result (Tsai *et al.*, 1988; Lee, 1988; Ohashi *et al.*, 1996).

Phytoplasma agents associated with JWB belong to subgroup 16SrV-B, and showed the same 16S ribosomal sequence in China and Korean strains (Zhu *et al.*, 1998; Han and Cha, 2002). This phytoplasma had been described as '*Candidatus* Phytoplasma ziziphi' (Jung *et al.*, 2003), and it is transmitted by the leafhoppers *Hishimonoides chinensis* and *H. sellatus* (La and Woo, 1980). Jujube trees with symptoms of proliferation, yellowing and witches' broom were reported from *Z. jujube* and *Z. nummularia* in India, and were associated with '*Ca. P. ziziphi*'-related strains (Khan *et al.*, 2008). Mixed infections of jujube by two phytoplasmas of groups of 16SrI and 16SrV-B were reported in Korea (Lee *et al.*, 2009).

The aim of the present study was to assess the presence of phytoplasmas in diseased jujube, and to identify the phytoplasma strains associated with proliferation, witches' broom and yellowing in jujube nurseries in the Eastern part of Iran. This information was required to plan appropriate disease management strategies.

## MATERIALS AND METHODS

### Plant sampling

During surveys in 2017 in the Razavi Khorasan Province in eastern Iran, witches' broom, proliferation, leaf rolling and yellowing symptoms (Figure 1) were observed in jujube nursery plants, and 22 symptomatic and four asymptomatic jujube plants were sampled and subjected to molecular analyses for phytoplasma detection and identification.

### DNA extraction and nested PCR amplification

Total DNA was extracted from 0.2 g of midrib tissue of fresh leaves from symptomatic and symptomless *Z. jujube* nursery plants, following the procedure described by Li *et al.* (2005). Midrib tissue samples were ground in liquid nitrogen and homogenized in 3M CTAB buffer. After chlorophorm/isoamyl alcohol (24: 1) treatment, the aqueous phase was mixed with 5M NaCl and maintained for 1 h at -20°C. After precipita-

tion by centrifugation, the resulting pellet was washed with 70% ethanol, and the final DNA pellet was dissolved in 100 µL of sterilized water. The total DNA extracted from *Prunus persica* yellowing and decline in Iran ('*Ca. P. omanense*'-related strain) (Esmailzadeh Hosseini *et al.*, 2017b) was used as positive control. The DNA quality and concentration were estimated by spectrophotometer and agarose gel electrophoresis, and 100 ng of nucleic acids was used for each PCR reaction with universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) to amplify parts of the rRNA operon. This included the 16S rRNA gene, 16S-23S rRNA spacer region (SR) and the 5' end of the 23S rRNA gene. The amplified products (1 µL) were diluted in 30 µL with sterile deionized water, and 1 µL of the resulting solution was used as template in nested PCR with the primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). The PCR reactions were performed in 50 µL mixtures containing 0.4 µM of each primer, with EmeraldAmp PCR master mix (Takara). The reaction cycled 30 times in a programmable thermocycler (QuantaBiotech), with the following parameters: denaturation for 30 sec at 94°C (2 min in the first round), annealing for 30 sec at 55°C and primer extension for 1 min at 72°C (10 min in the final cycle). The PCR conditions for the nested PCR reaction were the same except that the annealing temperature was 58°C. Five µL of each reaction mixture were analyzed by electrophoresis in a 1% (w/v) agarose gel in TBE 1X buffer and visualized with a UV imaging system (Isogen Life Science) after ethidium bromide staining. The sizes of the PCR products were estimated by comparison with a 100 bp DNA ladder (Biobasic).

### Restriction fragment length polymorphism analyses

Restriction fragment length polymorphism (RFLP) analyses of R16F2n/R16R2 amplicons, using *KpnI*, *HaeI*, *RsaI*, *AluI*, *HpaII*, *HhaI*, *TaqI*, *MseI*, *BfaI* and *ThaI* restriction enzymes, were performed according to the instructions of the manufacturer (Thermo). The restriction products were then separated by electrophoresis through an 8% polyacrylamide gel, stained with ethidium bromide and visualized with a UV imaging system (Isogen Life Science). The resulting RFLP patterns were compared with those published for the same 16S rDNA amplicons of other phytoplasmas (Lee *et al.*, 1998).

### Sequencing and phylogenetic analysis

R16F2n/R16R2 primed PCR products (1.2 kb) from six samples of symptomatic *Z. jujube* plants from nurs-



**Figure 1.** Phytoplasma disease symptoms on jujube plants: (A) and (B) proliferation and witches' broom, (B) and (C), (D) yellowing, leaf rolling and stunting of an infected plant.

eries in Razavi Khorasan province in eastern Iran were sequenced from both directions. Consensus assembled sequences were compared with deposited sequences in the GeneBank database using BlastN (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and aligned with Bio edit tools. The 1,250 bp of 16S rDNA sequences of the two strains associated with witches' broom and yellowing of *Z. jujube* were aligned using MEGA7 software (Kumar *et al.*, 2016), and a phylogenetic tree was constructed using the neighbor-joining method. *Acholeplasma laidlawii* was used as an out group to root the tree. Bootstrapping was performed 1,000 times to estimate branch stability.

#### Virtual RFLP

Virtual RFLP analysis using *iPhyClassifier* (Zhao *et al.*, 2009) was used to confirm subgroup affiliation

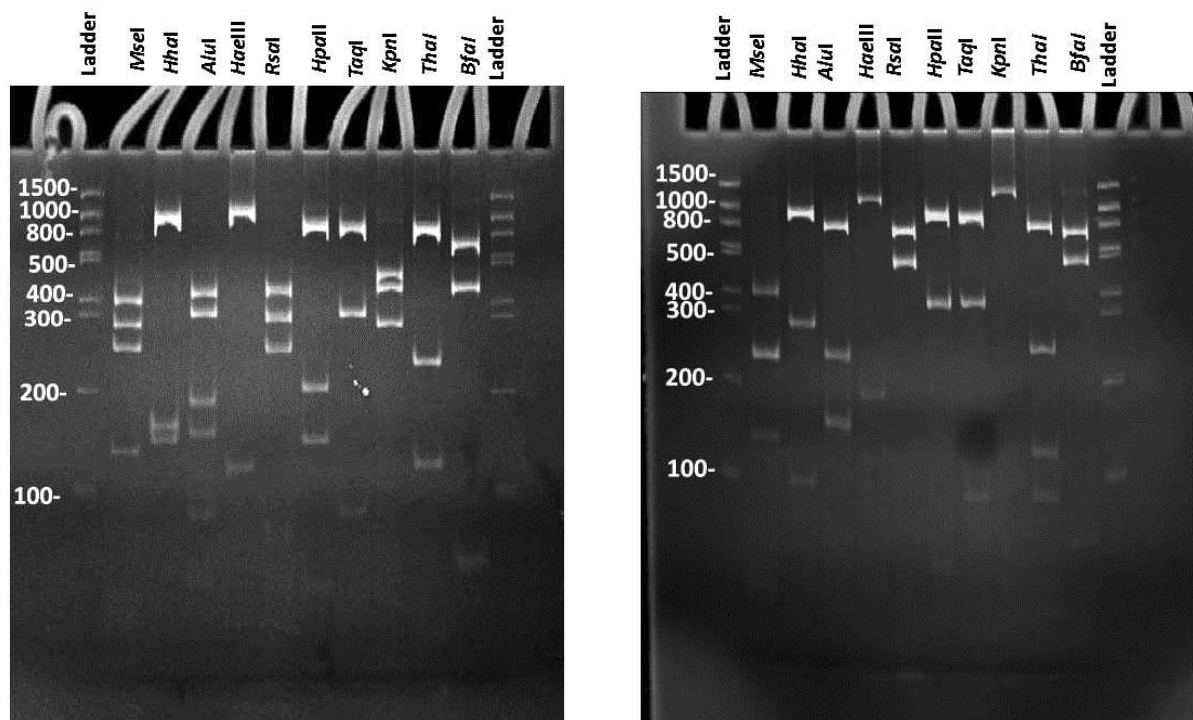
of phytoplasmas detected in *Z. jujube* plants displaying witches' broom and yellowing. The DNA fragment was digested *in silico* with 17 distinct restriction enzymes: *AluI*, *BamHI*, *BfaI*, *BstUI* (*ThaI*), *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *MboI* (*Sau3AI*), *MseI*, *RsaI*, *SspI* and *TaqI*.

## RESULTS

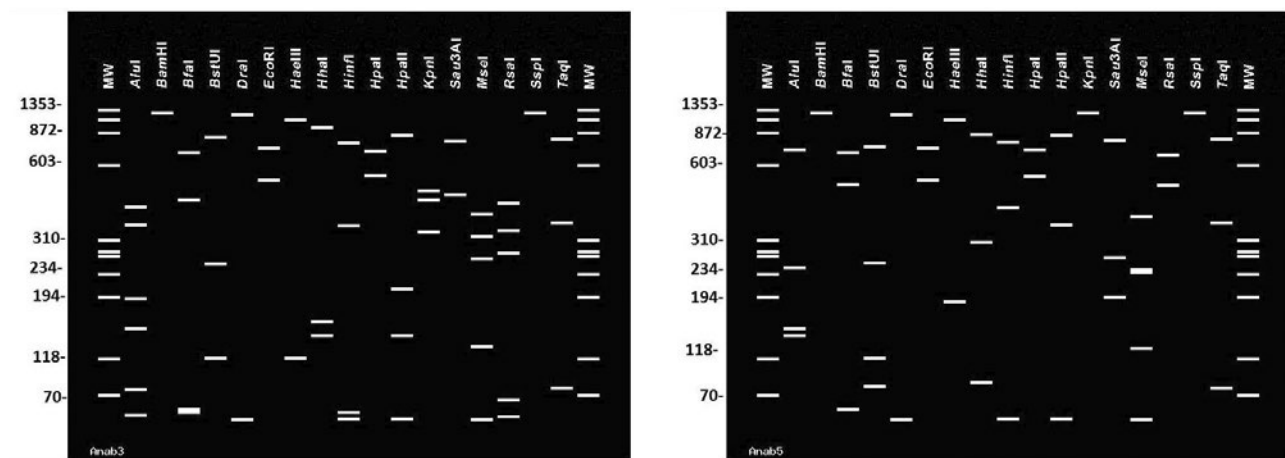
### *Symptoms and disease incidence*

Diseased *Z. jujube* plants with witches' broom, little leaf, yellowing or leaf roll symptoms in Razavi Khorasan province (Figure 1) were present in 6% of the plants in the jujube nurseries, and 4% of the plants with yellowing and thickening of leaves symptoms.





**Figure 2.** Polyacrylamide gel showing RFLP profiles of 16S rDNA amplified by nested PCR using P1/P7 followed by R16F2n/R16R2 primer pairs from (left) yellowing and (right) witches' broom symptomatic jujube plants. PCR products were digested by the enzymes indicated at the top of the figures; Ladder, 100 bp DNA ladder (Biobasic, Canada).

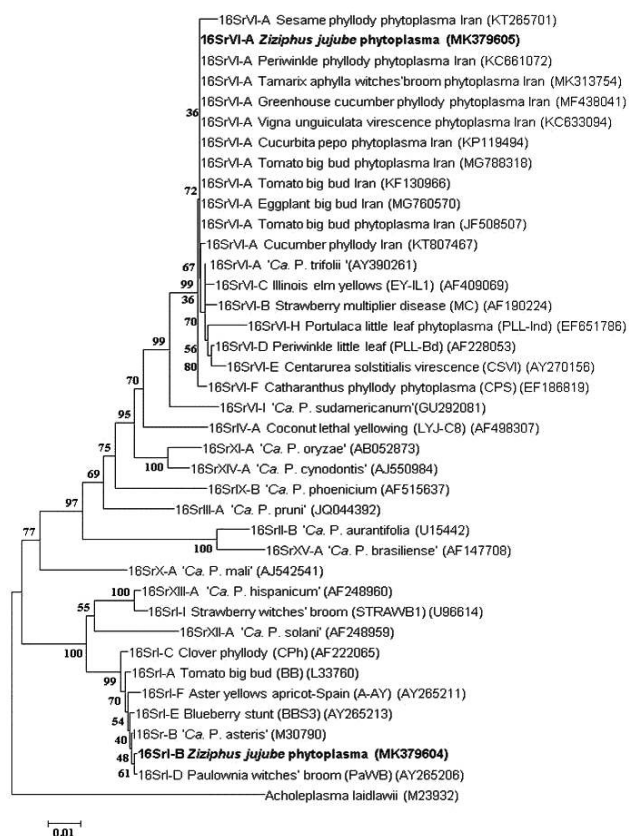


**Figure 3.** Virtual RFLP generated with program *iPhyClassifier* from *in silico* digestion of the R16F2n/R16R2 fragments of the (left) jujube yellowing (GenBank accession number MK379604) and (right) jujube witches' broom phytoplasma (GenBank accession number MK379605).

#### PCR-RFLP analyses

P1/P7 faint PCR amplicons of about 1.8 kb and R16F2n/R16R2 nested-PCR amplicons of 1.25 kb, were obtained from all symptomatic *Z. jujube* plants, but not from the symptomless plants. RFLP analysis of

R16F2n/R16R2 amplicons using *KpnI*, *HaeIII*, *RsaI*, *AluI*, *HpaII*, *HhaI*, *TaqI*, *MseI*, *BfaI* and *ThaI* restriction enzymes showed two RFLP patterns, one identical to those of the 16SrI-B phytoplasma subgroup and the other identical to the 16SrVI-A subgroup (Lee *et al.*, 1998) (Figure 2).



**Figure 4.** Phylogenetic tree constructed by the Neighbor-Joining method of 16S rRNA gene sequences from 31 phytoplasmas and *Acholeplasma laidlawii*, as the outgroup. The JWB phytoplasmas are indicated in bold font. Numbers at the nodes are bootstrap values based on 1,000 repetitions.

#### Analysis of nucleotide sequences

The 1.2 kb DNA fragments amplified from six samples of *Z. jujube* and two from yellowing symptoms) had sequence identity with '*Ca. P. trifolii*' related strains (four samples from witches' broom symptoms) and '*Ca. P. asteris*'-related strains (two samples from yellowing symptoms). One R16F2n/R16R2 16S rDNA sequence of each symptom was submitted to GenBank with accession numbers MK379605 and MK379604.

#### Phylogenetic analysis and virtual RFLP

The restriction analysis indicated that the detected phytoplasma strains were classified in the 16SrI-B and 16SrVI-A subgroups (Figure 3). Similarity coefficient and phylogenetic analyses confirmed that the phytoplasma strain associated with the yellowing symptoms was related to '*Ca. P. asteris*' with 99.7% similarity to the refer-

ence strain (GenBank accession number: M30790), while the phytoplasma strain associated with witches' broom symptoms was related to the '*Ca. P. trifolii*' group with 99.8% similarity with the reference strain (GenBank accession number AY390261) (Figure 4).

## DISCUSSION

Direct and nested PCR assays using phytoplasma universal primers confirmed the presence of phytoplasmas in jujube plants with witches' broom and yellowing symptoms. RFLP analyses showed that phytoplasma strains associated with JWB belong to the clover proliferation ribosomal group (16SrVI-A), while jujube yellowing phytoplasmas belong to the aster yellows ribosomal group (16SrI-B). However the phytoplasma subgroup 16SrV-B ('*Ca. P. ziziphi*') is the agent associated with JWB disease in different jujube varieties in China, Korea, Japan and India (Ohashi *et al.*, 1996; Zhu *et al.*, 1998; Fan *et al.*, 2008; Khan *et al.*, 2008). Phytoplasmas in the 16SrI group have only been reported in jujube trees in Korea in mixed infections with phytoplasmas of group 16SrV (Lee *et al.*, 2009). Therefore, the present study is the first demonstration of the presence of these two phytoplasmas in jujube nurseries.

Phytoplasma groups 16SrI and 16SrVI have broad host plant ranges (Lee *et al.*, 2004; Hiruki *et al.*, 2004), and have also been reported from numerous and different plant hosts in Iran (Babaie *et al.*, 2007; Salehi *et al.*, 2016; 2018; Asghari Tazehkand *et al.*, 2010; Rashidi *et al.*, 2010; Esmailzadeh Hosseini *et al.*, 2015, 2016, 2017a; Fattahi *et al.*, 2016). This indicates that various reservoir host plants besides jujube nurseries are likely to be present.

Planning for phytoplasma disease management requires the identification of phytoplasma subgroups, to better identify the plant reservoirs and the pathways through insect vectors for entry into jujube nurseries. The phytoplasmas in 16SrI-B and 16SrVI-A in Iran are mostly transmitted by leafhopper species including *Circulifer haematoceps*, *Macrosteles* sp. and *Neoaliturus haematoceps* (Salehi *et al.*, 2011; 2016). These insects are present in Iran and probably feed on other plant hosts and transmit phytoplasmas from reservoir hosts to jujube plants in nurseries. Selecting distance of nurseries from infected reservoir plants and protecting nursery plants from insect vectors is likely to assist disease management, together with the use of barrier plants or insect proof nets. Jujube cultivars resistant to phytoplasmas have been reported (Liu *et al.*, 2006), and further research with Iranian cultivars may identify resistant varieties that can help in the disease management. How-

ever, a certification and sanitation programme for producing healthy plant material is needed in jujube nurseries to prevent transmission of phytoplasma infections to new jujube orchards and to more geographical areas.

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