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Molecular identification of a phytoplasma associated with Russian olive witches' broom in Iran

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Abstract Russian olive trees (*Elaeagnus angustifolia*) showing witches' broom symptoms typical of phytoplasma infection were observed in the Urmia region of Iran. A phytoplasma named Russian olive witches' broom phytoplasma (ROWBp-U) was detected from all symptomatic samples by amplification of the 16S rRNA gene and 16S/23S rDNA spacer region using the polymerase chain reaction (PCR) which gave a product of expected length. DNA from symptomless plants used as a negative control yielded no product. The sequence of the 16S rRNA gene and 16S/23S rDNA spacer region of ROWBp-U showed 99% similarity with the homologous genes of members of the aster yellows group. We also detected a phytoplasma in neighboring alfalfa plants (AlWBp-U) showing severe witches' broom symptoms. An 1107 bp PCR product from the 16S rRNA gene showed 99% homology with the corresponding product in ROWBp-U, suggesting the presence of the same phytoplasma actively vectored in the area. Further observations showed that Russian olive trees with typical ROWB symptoms were present in an orchard near Tehran which is located over 530 km south-east of the original Urmia site. The corresponding sequence of this phytoplasma (ROWBp-T) showed 99% homology to that of the ROWBp-U. A sequence homology study based on the 16S rRNA gene and 16S/23S rDNA spacer region of ROWBp-U and other phytoplasmas showed that ROWBp-U is most closely related to the 16SrI group. To our knowledge, this is the first report of a phytoplasma infection in a member of the Elaeagnaceae.

Keywords *Candidatus* phytoplasma asteris · *Elaeagnus angustifolia* · Elaeagnaceae · PCR · Ribosomal RNA gene · Alfalfa

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Russian olive (*Elaeagnus angustifolia*, Elaeagnaceae) is a small tree with silvery twigs and foliage, and golden olive-like fruit, native to southeastern Europe and western Asia. In September 2003, we first observed witches' broom in self-sown Russian olive trees surrounding orchards at Vazirabad, in the Urmia (U) region. The symptoms included little leaves, short internodes, and shoot proliferation (Fig. 1a) followed by a decline suggesting a phytoplasma infection, which we named "Russian olive witches broom" (ROWBp-U). The disease is a threat to cultivation of Russian olives in the region, and affected trees may be a reservoir of infection for other crops. The objective of the present work was to determine if

ROWB is indeed associated with phytoplasma, and if so, of what kind, and whether local weed plants or crops also showing phytoplasma symptoms could be reservoirs of the same agent.

The incidence of the disease was sporadic. In 2006, Russian olive trees at our research station in Tehran (530 km south-east of Urmia) also showed ROWB symptoms, and samples were collected from these trees for phytoplasma tests, which proved positive (ROWBp-T). In addition, during a survey in 2005, three alfalfa (*Medicago sativa*) plants surrounding the infected Russian olive trees at Urmia were found to have witches' broom and little-leaf symptoms (Fig. 1b) and were collected for testing, again with positive results (AlWBp-U); samples were also collected from other weeds as well as from tomatoes showing big bud symptoms and growing nearby.

Total DNA was extracted from frozen phloem tissue of two Russian olive infected trees from each site following the cetyl trimethyl ammonium bromide (CTAB) procedure (Ahrens and Seemuller 1992). PCR amplification was performed using the universal phytoplasma primers P1/P7 (Schneider et al. 1995) followed by primers R16F2/R16R2 (Gundersen and Lee 1996) as nested primers after diluting the DNA from first step. Total DNA from Tomato big bud (TBB) phytoplasma was used as a positive control. DNA from healthy Russian olive plants or sterilized distilled water was used as negative controls in each PCR. The PCR products were analyzed by electrophoresis in agarose gels and visualized by UV following staining with ethidium bromide.

For nucleotide sequence analysis of the ROWBp-U isolate, the P1/P7 amplicon was used. This product was readily visible in agarose gels, while we had to perform a nested PCR to be able to visualize a product in agarose gels from ROWBp-T and AlWBp-U. DNA sequencing was performed in an Applied Biosystems model ABI 3100 sequencer using the Taq dideoxy terminator sequencing method (SeqLab, Germany). Individual sequence fragments were assembled using Seqman software (www.dnastar.com).

The 1.8 kb sequence of the 16S rRNA gene and 16S/23S rDNA spacer region from ROWBp-U has been deposited in GenBank under accession number EU886968, while 1.25 kb sequence (partial sequences) of the 16S rRNA gene of ROWBp-T (1,102 nucleotides) and AlWBp-U (1,107 nucleotides) were deposited under FJ788515 and FJ788514, respectively. The 16S rRNA gene sequence of ROWBp-U, ROWBp-T, and AlWB-U were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), after having refined them to the same length, with those of other phytoplasma strains available in Genbank. 16S rRNA sequence of ROWBp-U, ROWBp-T and AlWBp-U showed 99% identity among each other, and with Black-eyed Susan phyllody (DQ855286), a phytoplasma reported from Iran, and classified as a 16SrI-A group member. ROWBp-U showed the highest sequence identity (99%) with Epilobium phyllody (AY101386), Dog fennel yellows (DQ381534), and periwinkle virescence phytoplasmas (DQ381535).

Samples from the following weed species growing around the infected trees in Urmia tested negative for phytoplasmas: *Cynodon dactylon, Malva sylvestris, Polygonium aviculare, Sorghum halepense* and *Tragopogon pretense.* Tomato samples showing big-bud symptoms and growing in the same area carried a phytoplasma belonging the clover proliferation group (16SrVI-A) (unpublished). We did not detect any other phytoplasmas in our samples.

In Iran, 16SrI phytoplasmas (aster yellows group) have been detected in association with little leaf of periwinkle, phyllody of oilseed rape (Salehi et al. 2005), yellows of spinach, virescence of canola

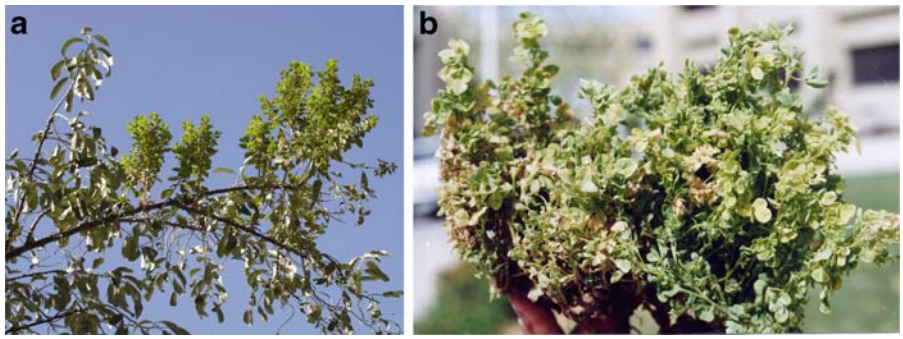

Fig. 1 Russian olive trees (**a**) and alfalfa (**b**) showing witches-broom symptoms of small leaves and shoot proliferation

(Asghari Tazehkand et al. 2006), as well as in ornamental and weed plants (Babaie et al. 2007), and in cherries (Zirak et al. 2006). A 16SrI-A subgroup phytoplasma has been detected in black-eyed susan phyllody (Babaie et al. 2007).

We conclude that phytoplasmas of the Aster yellows (16srI) group are involved in the Russian olive witches' broom disease we detected in Urmia and Tehran provinces. Our limited analysis suggests that ROWBp-U and ROWBp-T are indistinguishable from a phytoplasma in Alfalfa with witches' broom symptoms. This confirms that 16SrI phytoplasmas are widespread in Iran, and are hosted by several plant species (Babaie et al. 2007), and the possibility that alfalfa may present a reservoir of the phytoplasma cannot be ruled out. Since 2003, the occurrence of ROWBp has gradually increased in the Urmia region and currently 4% of trees are infected. No weed reservoir of the pathogen was identified in our surveys. ROWB symptoms were also observed in the trees grown in the Eastern Azarbaijan (Zamharir and Mohammadipour 2007) and Arak.

All these results together show that the disease is a threat for the cultivation of Russian olives. Although a number of specific phytoplasma vectors have been detected in Iran (Siampour et al. 2004), nothing is known about the presumed leafhopper that transmits phytoplasmas to Russian olive and, probably, alfalfa.

A preliminary report of our work has appeared (Rashidi et al. 2006).

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