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Molecular characterization, vector identification and partial host range determination of phytoplasmas associated with faba bean phyllody in Iran

Mohammad Salehi [a, *], Rasoul Rasoulpour [b], Keramatollah Izadpanah [b]

[a] Fars Agricultural and Natural Resources Research and Education Center, AREEO, Shiraz, Iran
[b] Plant Virology Research Center, Shiraz University, Shiraz, Iran

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ABSTRACT

During surveys of phytoplasma diseases, faba bean phyllody (FBP) was observed in several locations in Fars and Bushehr provinces (southern Iran). Samples of affected plants from Borazjan (Bushehr province) and Fasa (Fars province) were used to transmit the phyllody agent to faba bean, mung bean, pea, alfalfa and periwinkle by grafting, dodder and/or vector insect. *Orosius albicinctus* (Distant) was identified as the vector of Borazjan (BFBP) and Fasa (FFBP) faba bean phyllody. Naturally affected faba bean plants and all inoculated plants were positive for phytoplasma by direct PCR using the P1/P7 primer pair and nested PCR using P1/P7 and R16F2n/R16R2 primer pairs. Sequencing of PCR products identified the associated phytoplasmas as members of 16SrII phytoplasma group. Phylogenetic analysis using full length 16S rRNA gene sequences also confirmed similarity of the BFBP and FFBP phytoplasmas to the 16SrII group phytoplasmas. In this analysis the FFBP phytoplasma was grouped with '*Candidatus* Phytoplasma australasia', representative of 16SrII-D subgroup, while the BFBP phytoplasma formed a discrete group close to the 16SrII-C subgroup. Restriction fragment length polymorphism (RFLP) confirmed that BFBP and FFBP phytoplasmas belong to 16SrII group. Virtual RFLP confirmed that as members of peanut witches' broom (16SrII) phytoplasma group, BFBP and FFBP phytoplasmas belonged to 16SrII-C and16SrII-D subgroups, respectively. Phytoplasmas associated with BFBP and FFBP were shown to be serologically related to the Fars alfalfa witches'broom phytoplasma, a member of 16SrII-C subgroup. It seems that witches'broom affected alfalfa fields are natural reservoirs of the FBP phytoplasma in southern Iran.

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1. Introduction

Phytoplasmas are cell wall-less plant pathogenic prokaryotes of class Mollicutes (Razin, 1985; Weisburg et al., 1989). They are restricted to phloem tissues of infected plant hosts and are transmitted by leafhoppers, planthoppers and psyllids. Plants infected with phytoplasmas exhibit an array of symptoms that are suggestive of disturbances in the normal balance of plant hormones (Lee and Davis, 1992). These symptoms include virescence (development of green flowers), phyllody (development of floral parts into leaf-like structures), proliferation of axillary shoots resulting in witches' broom behaviour, sterility of flowers, yellowing and dieback of branches in woody plants. Phytoplasmas are associated with more than 1000 diseases in several hundred plant species (Seemüller et al., 1998; Lee et al., 2000; Bertaccini and Duduk, 2009).

The peanut witches' broom (16SrII) group consists of diverse phytoplasmas that are associated with numerous diseases in cultivated plants and weeds worldwide (Table 1).

Phytoplasma diseases of faba bean (*Vicia faba* L.) plant have been previously reported in Cuba (Arocha et al., 2007), Egypt (Hamed et al., 2014), India (Singh et al., 2013), Saudi Arabia (AL-Saleh and Amer, 2014), Spain (Castro and Romero, 2004) and Sudan (Alfaro-Fernández et al., 2012; Jones et al., 1984).

Phytoplasmas belonging to several 16S rRNA groups have been reported in association with faba bean phyllody. They include those of 16SrII group in Saudi Arabia (AL-Saleh and Amer, 2014) and Sudan (Alfaro-Fernández et al., 2012), 16SrIII group in Spain (Castro and Romero, 2004) and 16SrI in Cuba (Arocha et al., 2007).

* Corresponding author. Shiraz, Modarres Blvd, Mardani Street, Fars Agricultural and Natural Resources Research and Education Center, AREEO, Shiraz 0098-71555-617, Iran.
E-mail address: salehi_abarkoohi@yahoo.com (M. Salehi).

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Area under faba bean cultivation in Iran is about 100,000 ha with average dry seed yield of 2100 kg ha^{-1}. It is mostly cultivated in Golestan province in the north and Khuzistan, Bushehr and Fars provinces in the south of Iran. In a survey performed during 2001–2010, phyllody disease was observed in faba bean fields of Fasa and Kazeroun in Fars province and Borazjan in Bushehr province. The aim of this work was molecular and biological characterization including vector identification of phytoplasmas associated with faba bean phyllody in Borazjan and Fasa. A preliminary report on the presence of faba bean phyllody in Borazjan has been previously published (Salehi et al., 2005).

2. Methodology

2.1. Sources of the disease

Faba bean plants with typical symptoms of phyllody from Borazjan and Fasa were selected, transferred to a greenhouse sprayed with Metasystox at biweekly intervals and used as the sources of the faba bean phyllody (FBP) disease in molecular and biological studies. For the maintenance, the FBP agent was transmitted to Madagascar periwinkle (*Catharanthus roseus* L.) G. Don via dodder.

2.2. Dodder and graft transmission

Dodder (*Cuscuta campestris* Yank.) seeds collected on a healthy seed-grown tomato plant in insect-free greenhouse were germinated in a sterile plastic Petri dish on a moist filter paper at room temperature. In order to establish healthy dodder source, newly-emerged seedlings were transferred to healthy seed grown sugar beet plants. After four weeks, detached dodder stems from sugar beet plants were placed on naturally phyllody-affected faba bean plants for establishment and subsequent connection to five healthy periwinkle seedlings. Three weeks later, periwinkle plants were freed of dodder and maintained in the greenhouse for observation of possible symptoms and PCR assays.

For propagation and maintenance of the FBP agent, scions (tiny shoots with 2–3 leaves) were prepared from symptomatic dodder-inoculated periwinkle plants and side-grafted on seed-grown periwinkle plants. Graft inoculation was also used to transmit FBP agent from a diseased to healthy faba bean plant. The graft-inoculated plants were maintained in the greenhouse during the studies.

2.3. Insect vector identification

Leafhopper samples were collected in faba bean fields by a sweeping net and sorted by their gross morphology. The insects (3 per sample) were tested for phytoplasma by nested PCR using primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) followed by R16F2n/R2 (Gundersen and Lee, 1996). Leafhoppers of PCR-positive species were tested for their ability of transmitting faba bean phyllody, immediately after collection in phyllody affected fields or after three weeks of acquisition access feeding on diseased faba bean plants under greenhouse condition. In the first transmission trial, PCR positive leafhoppers were collected on Borazjan diseased faba bean fields and directly caged on healthy faba bean plants (10 insects per plant). In the second experiment, colonies of PCR positive species were developed by transferring fertilized females from healthy colonies to phyllody-affected faba bean plants for subsequent development. Young adults of inoculative colonies of each leafhopper species were caged on faba bean, pea (*Pisum sativum* L.), alfalfa (*Medicago sativa* L. cv. Yazdi) and mung bean (*Vignaradiata* (L.) R. Wilczek) (20 leafhoppers per 5 plants of each species in a pot) for 14 days of inoculation access time. The plants were then sprayed with insecticide and kept in the greenhouse for PCR assays and development of the disease symptoms. Controls were set up with leafhoppers from non-inoculative colonies caged on healthy, seed-grown faba bean, pea, alfalfa and mung bean plants.

2.4. Serological relationship between faba bean phyllody phytoplasmas and Fars alfalfa witches' broom phytoplasma

Possible serological relationships between Borazjan and Fasa FBP (BFBP and FFBP, respectively) phytoplasmas and the phytoplasma agent of alfalfa witches' broom of Fars province (FAWB), were investigated using a polyclonal antiserum prepared against FAWB phytoplasma (Salehi et al., 2011b) by dot immunobinding assay (DIBA) according to the method of Hibi and Saito (1985).

2.5. Total DNA extraction, PCR amplification and RFLP analysis

Total DNA was extracted from 0.25 g midrib tissue of 10 naturally phyllody affected faba bean plants from Borazjan and Fasa (five plant per area) and symptomatic experimentally inoculated plants using Zhang et al. (1998) procedure and from insect samples (3 insects per batch) following the protocol of Doyle and Doyle (1990). Total DNAs extracted from symptomless alfalfa, faba bean, mung bean, pea and periwinkle plants and insects from non-inoculative colonies were used as negative controls and DNA from a symptomatic periwinkle plant infected with Fars alfalfa witches' broom phytoplasma (Salehi et al., 1995) was used as a positive control. Direct PCR using P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) primer pair and nested PCR using P1/P7 and R16F2n/R16R2 (Gundersen and Lee, 1996) primer pairs were performed as previously described (Salehi et al., 2011a).

2.6. Identification of phytoplasmas associated with Borazjan and Fasa faba bean phyllody diseases

RFLP analysis was employed for the preliminary identification of phytoplasmas in diseased plants (Lee et al., 1998). Each nested PCR product (8 μl, almost 200 ng DNA) was digested separately with the restriction enzymes *Alu*I, *Hae*III, *Hin*fI, *Hpa*II, *Mse*I, *Rsa*I, and *Taq*I according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). The digested products were then separated by electrophoresis through a 2% agarose gel and stained in ethidium bromide solution. DNA bands were visualized with a UV transilluminator. The resulting digestion patterns were compared with those reported by Lee et al. (1998).

Virtual RFLP analysis using *i*PhyClassifier (Zhao et al., 2009) was used to determine subgroup affiliation of FBP phytoplasmas. RFLP profile of 1.25 kb fragment (R16F2n/R16R2 amplicon of 16S rRNA gene) of BFBP and FFBP phytoplasmas were compared to those of 16SrII described subgroups. Each aligned DNA fragment was digested *in silico* with 17 distinct restriction enzymes: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I (*Sau*3AI), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I (Lee et al., 1998).

2.7. DNA sequencing and phylogenetic analysis

The amplified PCR products using primer pairs P1/P7 and R16F2n/R16R2 from naturally diseased faba bean plants and vector insect were ligated into pTZ57R/T vector and cloned in *Escherichia coli* DH5α using InsT/Aclone PCR Cloning Kit (Fermentas, Vilnius, Lithuania) according to manufacturer's instructions. Plasmid DNA from three recombinant colonies was purified using a High Pure Isolation kit (Roche, Germany) according to the manufacturer's instructions. Purified plasmids harbouring DNA inserts were

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