Identification, occurrence, incidence and transmission of phytoplasma associated with Petunia violacea witches' broom in Iran.

Occurrence, incidence and insect vector of '*Candidatus* Phytoplasma australasia' associated with *Petunia violacea* witches' broom in Iran

Chamran Hemmati[1,2*], Assunta Bertaccini[3], Mehrnoosh Nikooei[1]

[1]Department of Entomology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran

[2] Plant Protection Research Group, University of Hormozgan, Bandar Abbas, Iran

[3] Department of Agricultural Sciences, *Alma Mater Studiorum* - University of Bologna, Italy.

Corresponding author: Chamran Hemmati

Phone: +989177609307

Fax: +987633621491

Chamran.hemmati@hormozgan.ac.ir

ORCID: 0000-0001-9854-1873

Running title: Phytoplasma disease in *Petunia violacea*

Abstract

A petunia witches' broom (PvWB) disease, characterised by phyllody, virescence, witches' broom, little leaf and yellowing was observed in municipal lands and parks in Bandar Abbas, Hormozgan province, Iran with an average incidence of 20%. PCR and sequencing analysis showed that a phytoplasma was associated with the disease sharing 99% sequence identity with '*Candidatus* Phytoplasma australasia'-related strains. Furthermore this phytoplasma was transmitted to healthy petunia plants by the leafhopper *Orosius albicinctus* was then demonstrated to be a vector of this phytoplasma. This is the first report of the association between '*Ca*. P. australasia' and *Petunia violacea* witches' broom and of the insect vector of this disease.

Keywords: phytoplasma, purple petunia, 16SrII-D, *Orosius albicintus*, molecular identification

Introduction

Phytoplasmas which are obligate bacterial parasites of the plant are characterised by the lack of cell wall, pleomorphic or filamentous shape, with a diameter less than one micron (Hopkins 1977). Phytoplasmas are responsible for a significant economic loss in annual and perennial crops, bushes and fruit trees as well as ornamental species and natural landscapes around the globe. Phytoplasma-associated disease symptoms in several plant species are yellowing, stunting and phyllody as well as witches' broom (Bertaccini et al. 2014). To date, many phytoplasma strains have been identified and classified in ribosomal group and subgroups following examination of the restriction fragment length polymorphism (RFLP) of 16S rRNA gene (Lee et al. 1998). Phloem sap-sucking insect vectors belonging to Hemiptera order are responsible for the natural spread of phytoplasmas (Weintraub and Beanland 2006). Phytoplasma transmission could be achieved through grafting, seed and vegetative propagation through cutting, tubers, rhizomes or bulbs (Calari et al. 2011; Chung and Jeong 2014; Caglayan et al. 2019).

Petunia is an economically important ornamental species cultivated throughout the world. Most of the cultivated *Petunia* plants are produced through hybridisation between *Petunia violacea* with purple flowers and *Petunia axillaris* with white flowers. *P. violacea* is a perennial species in its natural environment but cultivated as annual plant under production systems. It is a low-spreading species very heat tolerant with purple flowers (Shams et al. 2012). Cultivated varieties have attractive and funnel-shaped flowers and are popular among gardeners. Also, seed-grown *Petunia violacea* are widely planted as an ornamental plants in urban areas and parks in Bandar Abbas, Hormozgan Province, Iran from November to March.

The presence of phytoplasmas in *Petunia violacea* was reported in India, however the phytoplasma identification was not achieved while a phytoplasma was experimentally transmitted from sesame

to *P. violacea* by *Orosius* spp. (Vasudeva and Sahambi 1955). An aster yellows (AY) phytoplasma was recently associated with *Petunia* flat stem disease in China and Korea (Chung and Huh, 2008). Faghihi et al. (2014) also reported the association of 16SrII-D phytoplasma with *P.* × *hybrida* in Iran.

In December 2017, typical symptoms of phytoplasma disease, including phyllody, virescence, witches' broom, little leaf and yellowing were observed in seed-grown *Petunia violacea* planted in municipal lands and parks of Bandar Abbas, Hormozgan province, Iran. This study was carried out to detect and identify the phytoplasma in *P. violacea* and its insect vector.

Materials and Methods

Plant materials and disease incidence

Samples of symptomatic plants (10 samples) showing phyllody, virescence, witches' broom and little leaf and of asymptomatic *P. violacea* (5 samples) were collected from three boulevards in Bandar Abbas (Table 1). Simultaneously, insect samples were collected using a D-Vac aspirator to be checked for phytoplasma presence. Insects species, mainly leafhoppers, collected on the symptomatic plants were morphologically identified (Pakarpour Rayeni and Seraj 2016). In addition, insects were preserved in acetone until DNA extraction. Information about number of collected and infected insects is presented in Table 1. Disease incidence of planted *Petunia* was separately calculated in 3 boulevards of Bandar Abbas using the formula given below:

$$\%\ \text{disease (incidence)} = \frac{\text{number of infected plants}}{\text{population size}} \text{x } 100$$

A total of 3,000 plants were inspected based on symptoms including yellowing, witches' broom and phyllody for each boulevards (Table 1) and only considered infected if the plants showed all these symptoms. The disease incidence was about 20% at the time of the survey.

DNA extraction and amplification

Plant DNA was extracted from 0.5 g of the mid-vein of ten symptomatic plants and five symptomless plants by the cetyltrimethylammonium bromide (CTAB) extraction procedure described by Doyle and Doyle (1990). Total DNA was extracted from single leafhopper using an adjusted CTAB protocol (Reineke et al., 1998).

Phytoplasma presence was detected by PCR. The P1 and P7 primers (Deng and Hiruki 1991, Schneider et al. 1995) were used to prime phytoplasma-universal amplification of 16S rRNA gene sequences, followed in nested-PCR by the primer pair R16F2n/R16R2 (Gundersen and Lee 1996) amplifying on the 16S rRNA gene. PCR assays were performed in 50 µl reaction mixture containing one µM of each primer, 25 µl master mix (Ampliqon, cat. no. A190303, Denmark), 20 µl deionized distilled (dd) H_2O and 3 µl template DNA. Thirty five PCR cycles were conducted in a Peqlab thermocycler (Germany). The thermal conditions for P1/P7 were as reported (Hemmati et al., 2018). The obtained PCR products from the direct amplification were diluted with ddH_2O (1: 20) and then 1 µl of each dilution was used as template for nested PCR amplification carried out as described above except for the annealing temperature at 56ºC. The PCR products were separated on 1% agarose gel using a 3-kb plus molecular weight marker (DM23500, SMOBIO, Taiwan).

Afterwards, the nested-PCR products (three samples of infected plants, three samples of insects) were bidirectionally sequenced by Macrogen Sequencing Service (Republic of Korea) by using primer R16F2n/R16R2. Raw sequence chromatograms were assembled and then edited using DNASTAR version 5.1 to correct ambiguous bases or remove low quality stretches from termini of the sequences. Homologies to known sequences were detected using the BLASTN algorithms against the non-redundant GenBank database.

Transmission trial

For the leafhopper transmission assays, seeds of *Petunia violacea* plants were sown in pots and placed in insect-free greenhouse inside cages covered with plastic cylinder of 13 inch diameter tightened on the top with muslin cloth. Twenty seed-grown petunia plants were used for transmission trial. Before the start of the transmission trial, all plants were tested for the presence of phytoplasma by nested PCR using the aforementioned assay. In addition, plants were individually caged. The leafhoppers were collected from symptomatic plants at full flowering stage by using a D-Vac aspirator in early January 2017. A total of 65 adults of *Orosius albicinctus* were transferred to 15 cages with plants at four leaf stage. For each cage, 4-5 insect individuals were released. Five cages with healthy *P. violacea* plants where no leafhopper was released were used as control. The plants were maintained in the growth chamber (25 ± 1ºC, 16:8 L:D and 70 ± 5% RH) for monitoring the disease symptoms development and sampling for PCR assays up to 60-75 days post-inoculation.

Results

Naturally infected *Petunia* showed severe symptoms such as malformed leaves, yellowing and little leaf as well as witches' broom, virescence and phyllody. The target DNA of the expected size of approximately 1.25 kb was amplified using R16F2n/ R16R2 primer pair specific of phytoplasma 16S rRNA from all symptomatic plants. Likewise, the amplified products were obtained from positive control (‘*Candidatus* Phytoplasma phoenicium’) (Hemmati *et al*., 2018) samples, but not in assays of samples from both non-symptomatic plants and negative control (DNA template free). Sequence analysis of the 1,210 bp of ribosomal RNA gene revealed that the phytoplasma detected in *P. violacea* witches' broom disease (PvWB) (GenBank accession numbers MH450237-39) is related to '*Candidatus* Phytoplasma australasia' with highest identity 99.92% (1209 identical bases)

to the strain phs 2 from *Heliopsis helianthoides* from Iran (GenBank accession number MK255070) and strain CpSD1 from *Cicer aretinum* from India (GenBank accession number MK217101). The phylogenetic tree constructed using a neighbor-joining (NJ) phylogeny with MEGA 6 software (Tamura et al. 2013) allowed to cluster the detected sequences with phytoplasma strains related to '*Ca*. P. australasia' (White et al. 1998; Bertaccini and Lee 2018) enclosed in subgroup 16SrII-D (Fig. 2).

Orosius albicinctus (Distant, 1908) was the only infected leafhopper collected from the three boulevards in Bandar Abbas (Table 1). The three sequences obtained from insects were aligned (Clustal Omega) were deposited in GenBank under the accession numbers MH463460-62 and showed 100% identity with those obtained from the symptomatic plants. In the transmission trials, after 8-10 weeks from inoculation, thirteen out of fifteen *P. violaceae* plants fed on by the *O. albicinctus* showed symptoms such as yellowing, little leaf, stunting, phyllody and witches' broom (Fig 3). Phytoplasma presence in the symptomatic insect-inoculated plant was verified by nested-PCR and sequencing as described above. All plants showing symptoms were positive in PCR assay. The sequences obtained from experimentally infected plants (GenBank Accession numbers MH068682-4) were aligned with those obtained from symptomatic plants and insects and showed 100% identity. The plants that were not exposed to *O. albicinctus* were growing normally.

Discussion

In this study the association between symptomatic *P. violacea*, a '*Ca.* P. australasia' –related strain and the insect vector of the disease was confirmed by PCR assays and transmission trials respectively. In Iran, group 16SrII-D phytoplasmas have been identified in association with many diseased vegetable crops such alfalfa witches' broom, tomato witches' broom, cucumber and squash phyllody, parsley witches' broom, pomegranate little leaf (Salehi et al. 2014, 2015, 2016a,

2016b; Esmailzadeh-Hosseini et al. 2016). Since the symptoms affect the architecture and aesthetics of private and public gardens and parks, phytoplasma infected ornamentals are of an economic importance. In addition, phytoplasma-infected *P. violacea* plants may pose an epidemiological threat to the other ornamentals or nearby plant species.

Based on the results *O. albicinctus* is a vector of this disease in *P. violaceae* in Bandar Abbas. As reported by other researchers (Esmailzadeh-Hosseini et al. 2007; Ikten et al. 2014, Omidi et al. 2010; Salehi et al. 2015; Yadav et al. 2015), *O. albicinctus* can vector diverse phytoplasmas to different plant species; the insect is widely distributed in Iran, Australia, Fiji, Indonesia, New Britain and Giava, Israel, Egypt, India (Pakarpour Rayeni and Seraj 2016).

Although the association between phytoplasmas and *P. violacea* has not been reported yet, the association between *Petunia* × *hybrida* and phytoplasmas has been previously verified by Chung et al. (2013) and Faghihi et al. (2014) who indicated that the species was infected by "stolbur" phytoplasmas and '*Ca.* P. australasia'-related strains in Korea and Iran, respectively. Chung and Jeong (2014) showed that the phytoplasma associated with *P.*× *hybrida* in Korea had seed transmission. The high prevalence of this disease in Bandar Abbas is in agreement the seed transmission possibility since about 20% of the plants resulted phytoplasma-infected, even though the role of insect vector should not be ignored in the case reported here. In addition, it should be taken into account that in the current research, only plants with severe and visible symptoms were considered as infected, therefore the real disease incidence could be underestimated, and the disease incidence may be higher if tested by PCR. The possible presence of seed transmission may explain the incidence observed in the boulevards and municipal parks that were surveyed. Moreover usually the petunia plants are cultivated in nurseries first and then transplanted in the parks where the active insect vector, *O. albicinctus*, could infect many plants and increase the

incidence of the disease. This is the first report of a '*Ca.* P. australasia'-related strain (16SrII-D subgroup) associated with *P. violacea* witches broom' disease and of its insect vector in Iran and in the world.

Compliance with Ethical Standards:

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Conflict of interests: all authors declare that they have no conflict of interest.

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Table 1. Location, coordinates, insects and disease incidence of *Petunia violacea* witches' broom in Bandar Abbas, Iran.

Location	Coordinates	Collected insects[a]	No. of infected *O. albicinctus* / no. of collected *O. albicinctus*	Disease incidence %	No. of symptomatic plants /No. of plants
BLVD1	N27º18'44" E56º30'84"	1, 2, 3	21/25	17.2	215/1,250
BLVD2	N27º17'88" E56º28'59"	2, 3, 4, 5	15/19	23.2	198/850
BLVD3	N27º18'89" E56º28'82"	1, 2, 5, 6	18/26	20.8	188/900

a: 1: Coccinellidae; 2: *Orosius albicinctus* (Cicadellidae); 3: Lygaeidae; 4: Miridae; 5: Aphididae; 6: *Asymmetrasca decedens* (Cicadellidae)

Figure 1. Symptoms of witches' broom, little leaf (A), phyllody and virescence (B) in diseased plants in comparison with healthy *Petunia violacea* (C).

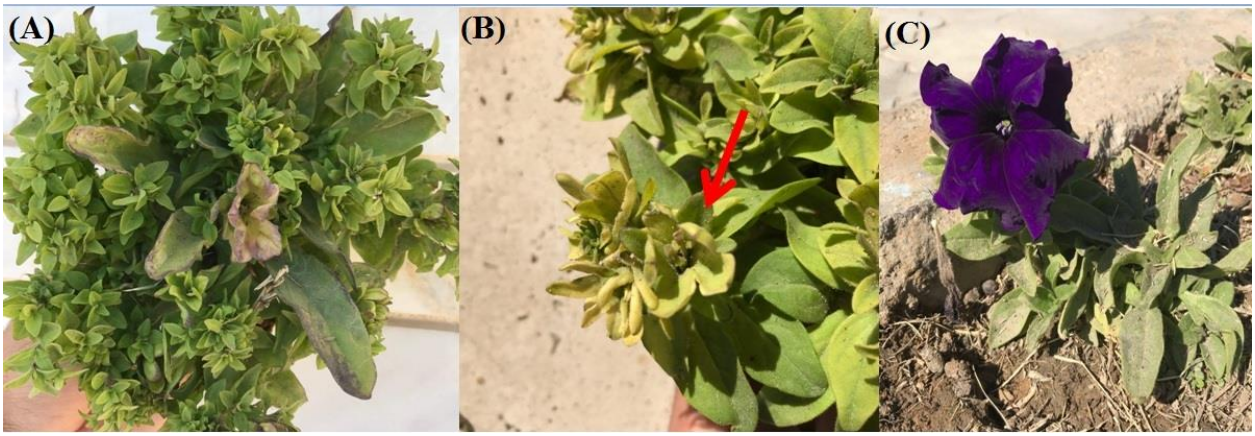

Figure 2. Phylogenetic tree of partial 16S rDNA gene sequences from *Petunia violacea* witches' broom phytoplasma strains (marked in bold) and selected phytoplasma sequences from GenBank. GenBank accession numbers are shown in brackets, and 16S ribosomal groups are annotated to the right. *Acholeplasma laidlawii* was used as outgroup to root the tree. The analysis involved 19 nucleotides sequences. The bar indicates the number of nucleotides substitution per site. Bootstrap values are shown at nodes with greater than 50% support.

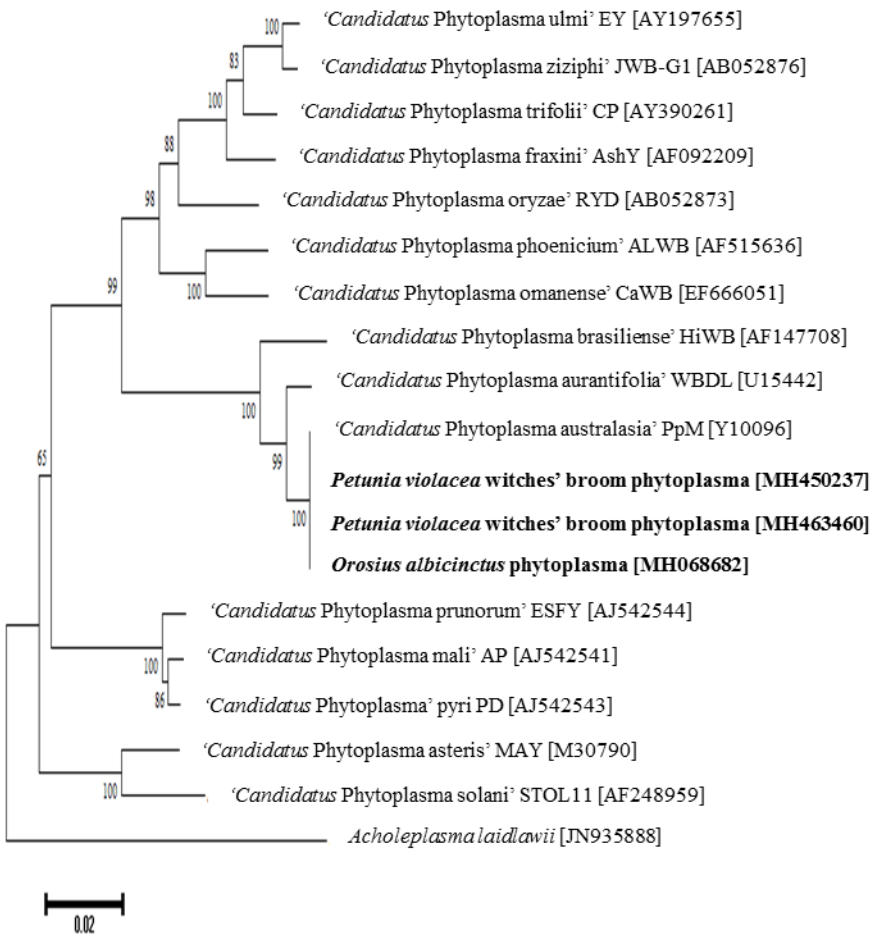

Figure 3. Symptoms of *Petunia* witches' broom in experimentally inoculated plants. (A) Little leaf and severe witches' broom; (B) yellowing, phyllody and virescence; (C) the vector *Orosius albicinctus;* and (D) healthy plants.

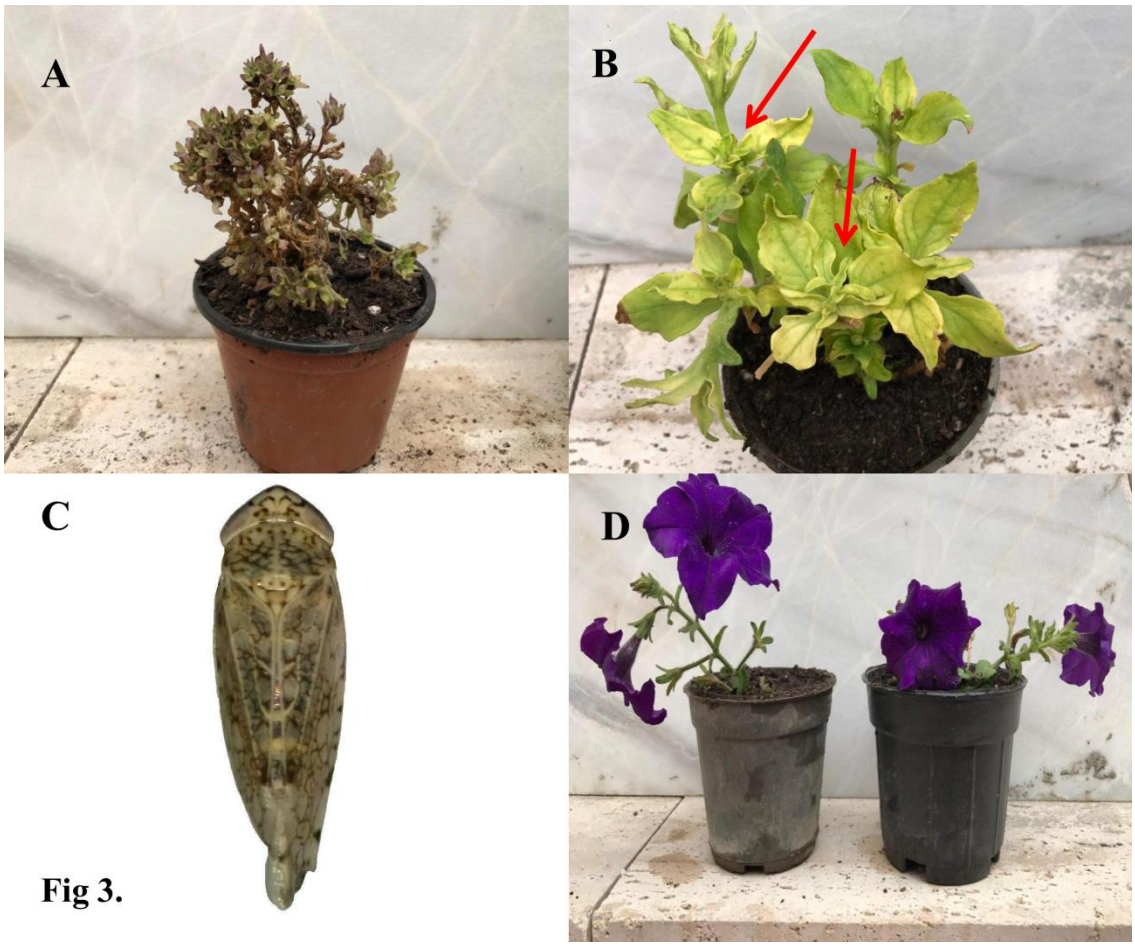

Fig 3.