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RESEARCH PAPER

Identification of 16SrIX-C phytoplasmas in *Argyranthemum frutescens* in Italy

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Summary. Phytoplasmas are cell wall-less microorganisms associated with plant diseases worldwide. Many important food, vegetable and fruits crops as well as ornamental plants can be severely affected by these pathogens, with significant economic impacts. Phytoplasma diseases of ornamentals have been described worldwide in a wide range of plant genera, and 11 different 16Sr groups have been identified. In Italy, many ornamental plant species belonging to several botanical families have been found to be infected by phytoplasmas, classified into the ribosomal groups 16SrI, 16SrII, 16SrV and 16SrXII. During a survey carried out in commercial gardens in Rome, some marguerite daisy *(Argyranthemum frutescens)* plants showing symptoms of phytoplasma-like disease, were collected and submitted to molecular analyses. Cloning and sequencing of the portion of the 16S rRNA gene followed by BLAST analysis, real and virtual restriction fragment length polymorphism anlaysis with *Alu*I and *Rsa*I, allowed assignment of the detected phytoplasma to the 16SrIX-C group (*Picris echioides* yellows, PEY).

Key words: *Picris echioides* yellows, ornamentals, RFLP analysis, marguerite daisy, sequencing.

Introduction

Phytoplasmas are cell wall-less microorganisms belonging to the class *Mollicutes*, and are associated with plant diseases worldwide. Typically located in plant phloem tissues, they are transmitted by sap-sucking insect vectors. They induce symptoms that generally consist of phyllody, leaf yellowing, virescence, and appearance of bushy or witches' broom both due to the proliferation of shoots and the decreased length of the internodes (Bertaccini and Duduk, 2009). They cause general decline in host vigour which can often lead to plant death. On the basis of conserved 16S rRNA gene sequence similarity, the currently known phytoplasmas are classified into a number of different 16S ribosomal (16Sr) groups and subgroups (Duduk and Bertaccini, 2011; Dickinson *et al*., 2013).

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Many important food, vegetable and fruit crops, as well as ornamental plants, can be severely affected by these pathogens, with significant economic impacts (Bertaccini and Duduk, 2009), due to subsequent quantitative and qualitative crop losses. With particular regard to ornamental plants and commercial floricultural crops, numerous phytoplasma diseases have been described worldwide in a wide range of plant genera (Grimaldi and Grasso, 1988; Bellardi and Bertaccini, 2009; Chaturvedi *et al*., 2010; Montano *et al*., 2011; Bertaccini *et al*., 2013). Phytoplasmas belonging to 11 different 16Sr groups have been identified in ornamentals worldwide, and *'Candidatus* Phytoplasma asteris' (16SrI) has been reported as the major group associated with the described phytoplasma diseases (Chaturvedi *et al*., 2010).

In Italy, many ornamental plant species belonging to several botanical families have been found to be infected by phytoplasmas that were classified into ribosomal groups 16SrI, 16SrII, 16SrV and 16SrXII (Bertaccini, 1990; Bertaccini *et al*., 1990a, 1990b, 1996, 2013; Marzachì *et al*., 1999; Boarino *et al*., 2002; Davino

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et al., 2007; Bellardi and Bertaccini 2009). The majority of these plant species belong to the Asteraceae, which also includes the ornamental species *Argyranthemum frutescens* (L.) Webb et Berth, synonymous with and formerly known as *Chrysanthemum frutescens*, since it was originally included in the genus *Chrysanthemum*. This marguerite daisy, commonly cultivated for its white, pink or yellow flowers, has a prominent position in flower production of some Italian regions and, recently, new genotypes have been produced, thus enhancing its export niche (Beruto, 2013).

Phytoplasma infections in symptomatic *A. frutescens* plants have already been reported and associated with phytoplasmas belonging to the aster yellows (16SrI) and elm yellows (16SrV) ribosomal groups (Bertaccini *et al*., 1990a, 1992; Boarino *et al*., 2002).

During a survey carried out in the summer 2012 in a commercial garden centre in the Latium region (central Italy), which aimed to evaluate the occurrence of infection by systemic pathogens in ornamental plants that were for sale, marguerite daises (*A. frutescens*) were identified showing symptoms resembling those associated with phytoplasma diseases. In order to ascertain the possible presence of phytoplasmas in symptomatic plants, samples were collected and submitted to molecular analyses.

Materials and methods

Plant material, DNA extraction and phytoplasma reference controls

Eight symptomatic plants showing general yellowing and stunting, little-leaf and/or abnormal proliferation of axillary shoots resulting in the appearance of witches' broom and reduced flower size (Figure 1) were sampled in a commercial garden centre in Rome, and molecularly analysed to verify the presence of phytoplasmas. For each plant, total DNA (TDNA) was extracted from 0.5 g of leaf tissue, following the procedure described in Doyle and Doyle (1990), with slight modifications. The final pellet was resuspended in 100 µl of sterile distilled water and submitted to molecular analysis.

The following phytoplasmas were used as references in molecular tests: severe aster yellows (SAY,

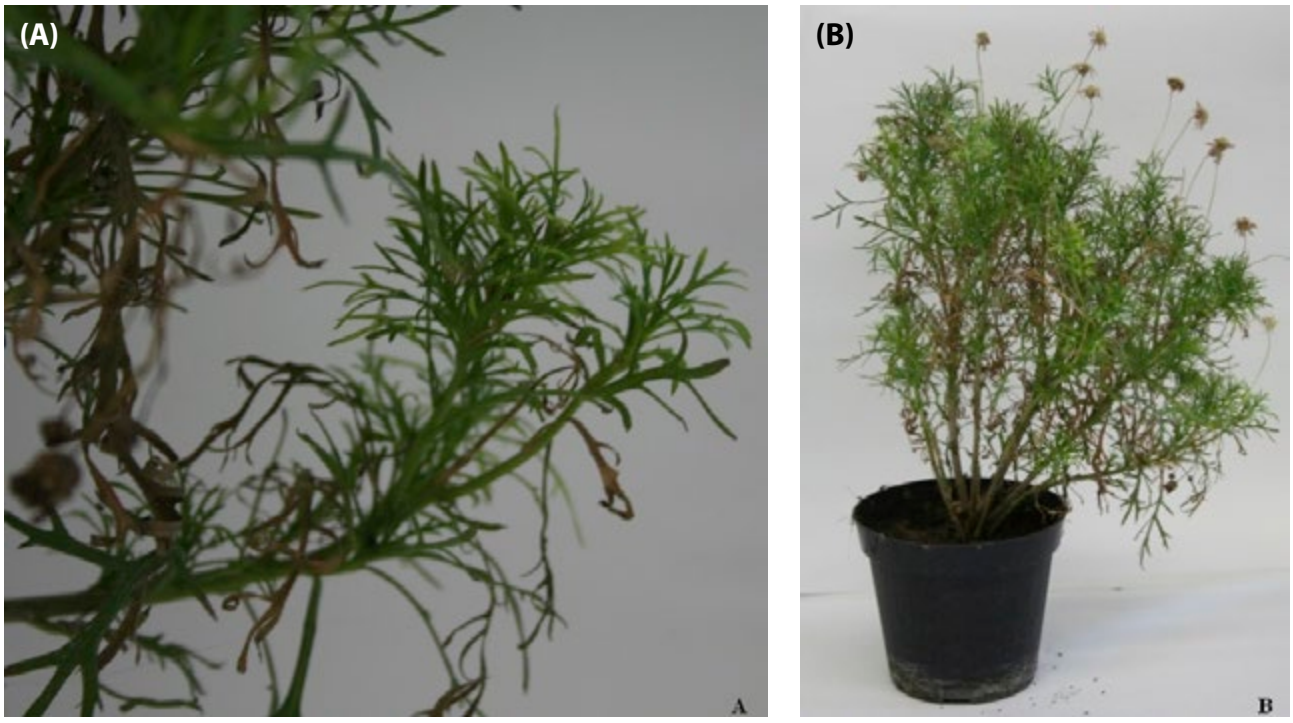

Figure 1. Proliferation of axillary shoots (A) resulting in the appearance of witches' broom (B) in *Argyranthemum frutescens* plants infected by *Picris echioides* yellows phytoplasmas (16SrIX-C).

16SrI-B), elm yellows (EY, 16SrV-A), "flavescence dorée" (FD, 16SrV-C) and "stolbur" (STOL, 16SrXII-A), from the CRA-PAV collection and *Picris echioides* yellows (PEY, 16SrIX-C) (Bertaccini, 2010). DNA from an asymptomatic marguerite daisy was used as a negative control.

PCR amplifications

The presence of phytoplasma was ascertained by amplification of the 16S rRNA gene using the universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and R16F2n/R16R2 (Lee *et al*., 1993; Gundersen and Lee, 1996), in direct and nested PCR assays respectively, and the universal primers fU5/rU3 (Lorenz *et al*., 1995) in direct PCR.

Direct PCR amplifications using P1/P7 primer pair were performed by adding 1 μL of template DNA (20 μg μL^{-1}) to a reaction mixture containing 1× Master mix buffer (Promega), 0.4 μM of each primer and DNase free water to a final volume of 25 μl. The amplification reaction was conducted for 35 cycles in a thermocycler (PTC-2000 DNA engine) under the following conditions: denaturation at 94°C for 1 min (2 min first cycle), annealing at 55°C for 1 min, and extension at 72°C for 2 min (10 min last cycle). PCR products (2 μL) of the first amplification diluted 1:30 were used as a template in the nested-PCR assay carried out with the R16F2n/R16R2 primer pair under the conditions detailed in Gundersen and Lee (1996).

Direct PCR using fU5/rU3 primers was performed in a final volume of 25 μl in a reaction mixture containing: 1 μL of template DNA (20 μg μL^{-1}), 1× *Taq* DNA buffer (Promega), 200 μM dNTP mixture, 0.4 μM each primer, 1.5 U *Taq* DNA polymerase (Promega), and DNase free water. Amplification was carried out as detailed in Lorenz *et al*., (1995).

Cloning and sequencing

Amplification products obtained from R16F2n/R16R2 nested PCR assays were purified using an Amicon® Ultra 100K device (Millipore Corporation) and cloned into a pGEM –T easy vector system (Promega) according to the manufacturers' instructions. For each analysed sample, DNA fragments from five recombinant clones were sequenced on both strands using the T7 and SP6 RNA polymerase promoters, after plasmid extraction by Quantum Prep® Plasmid Miniprep Kit (Bio-Rad).

For each cloned fragment, nucleotide sequences from both strands were aligned using ClustalW2 (Larkin *et al*., 2007) and assembled. The obtained sequences were analysed by multiple alignments with ClustalW2, and compared with sequences retrieved from GenBank using BLAST software (Altschul *et al*., 1997) on the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/).

Restriction Fragment Length Polymorphism (RFLP) analysis

Amplified fragments from R16F2n/R16R2 nested PCR assays were submitted to RFLP analysis after enzymatic digestion. Ten microliters of PCR products were separately digested with the restriction endonucleases *Taq*I, *Rsa*I and *Mse*I (New England BioLabs® Inc.). Digestion reactions were performed overnight according to the manufacturer's instructions. Digested products were loaded on vertical 5% polyacrylamide gel in TBE 1× buffer (45 mM Tris-borate, 1 mM EDTA, pH 8). Restriction profiles were visualized under UV light after staining with ethidium bromide.

A virtual RFLP analysis was also performed on the nucleotide sequences obtained from R16F2n/R16R2 cloned fragments using *i*PhyClassifier software (Zhao *et al*., 2009), after simulated digestion with *Alu*I and *Rsa*I. Nucleotide sequences of representative strains of the new delineated 16SrIX-F (HQ407532) and -G (HQ407514) subgroups (Molino Lova *et al*., 2011) not included in the *i*PhyClassifier database were retrieved from GenBank and exported to *i*PhyClassifier program.

Results

PCR amplifications and sequence analysis

Amplicons of the expected size were obtained from three (GN4, GN5, GN6) out of eight tested samples, both in R16F2n/R16R2 nested PCR assays and in fU5/rU3 direct PCR assays. No bands were obtained from the remaining samples or from the asymptomatic and water controls.

Cloned sequences of the R16F2n/R16R2 fragment were obtained from all positive samples. For each sample, sequences from five recombinant clones were analysed by multiple alignment, which revealed 100% nucleotide identity in all cases. When

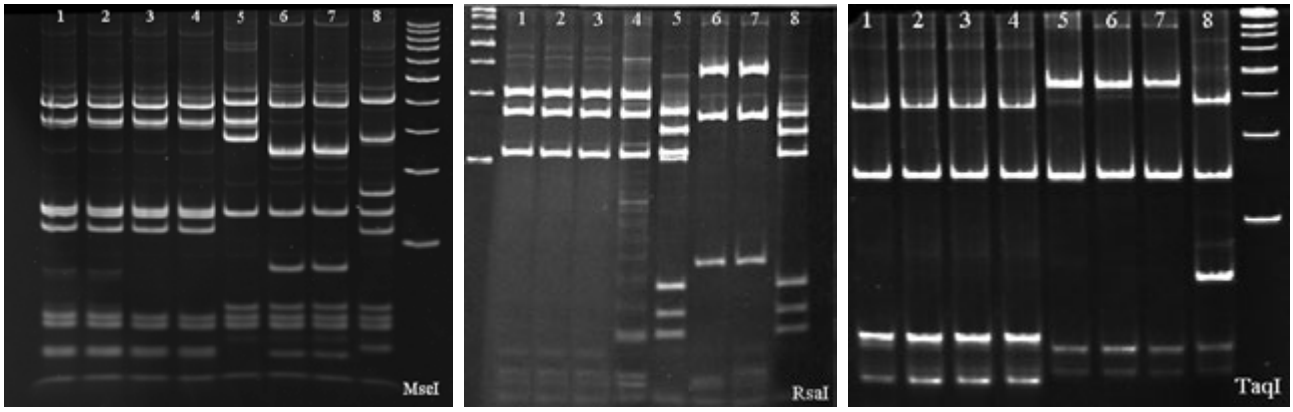

Figure 2. Polyacrylamide gel (5%) showing the RFLP profiles obtained from R16F2n/R16R2 - nested PCR amplicons after digestion with *Rsa*I, *Taq*I, and *Mse*I restriction enzymes. Lanes 1-3: symptomatic *Argyranthemum frutescens* samples GN4, GN5, GN6; lane 4: *Picris echioides* yellows (PEY, 16SrIX-C); lane 5: severe aster yellows (SAY, 16SrI-B); lane 6: elm yellows (EY, 16SrV-A); lane 7: "flavescence dorée" (FD, 16SrV-C); lane 8: "stolbur" (STOL, 16SrXII-A). DNA marker: GeneRuler™ 100 bp DNA Ladder (Fermentas).

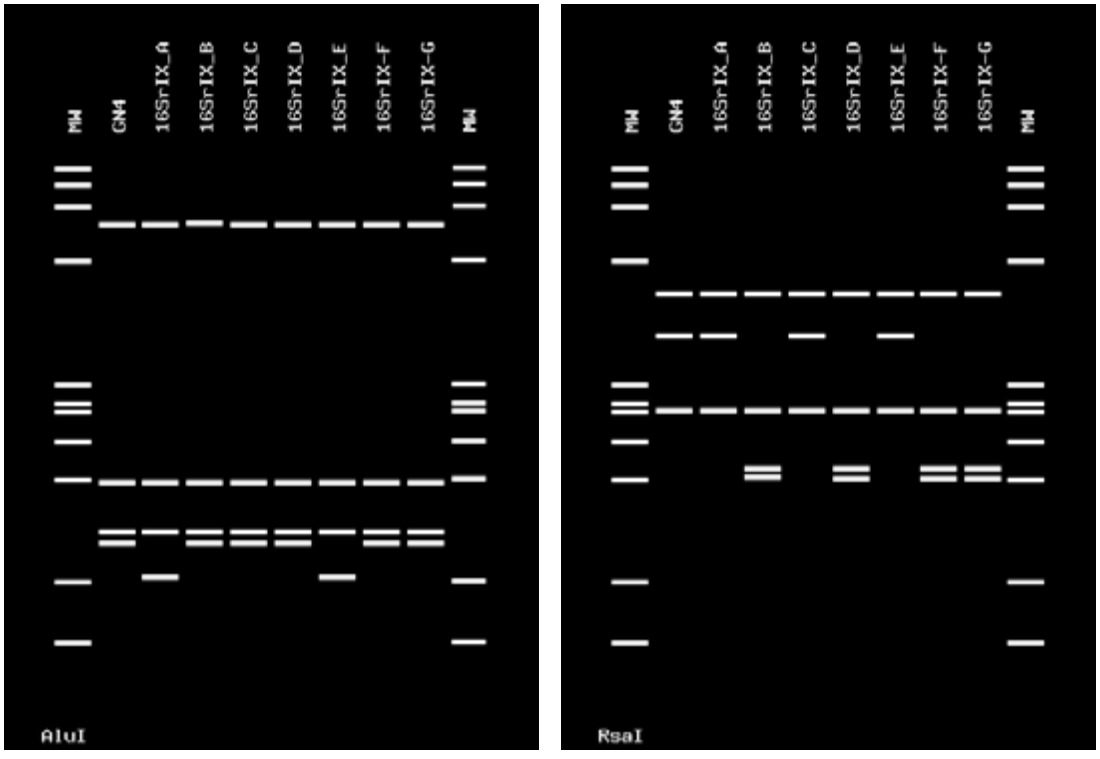

Figure 3. Virtual RFLP profiles obtained after simulated digestion with *Alu*I (left) and *Rsa*I (right) restriction enzymes of R16F2n/R16R2 nucleotide fragments from GN4 sample and from representative strains of 16SrIX subgroups.

sequences from different samples were compared, 100% nucleotide identity was found.

When compared with the NCBI database sequences, all positive samples exhibited 99% nucleotide identity with the sequence Y16389.1 obtained from *Picris echioides* yellows phytoplasma identified

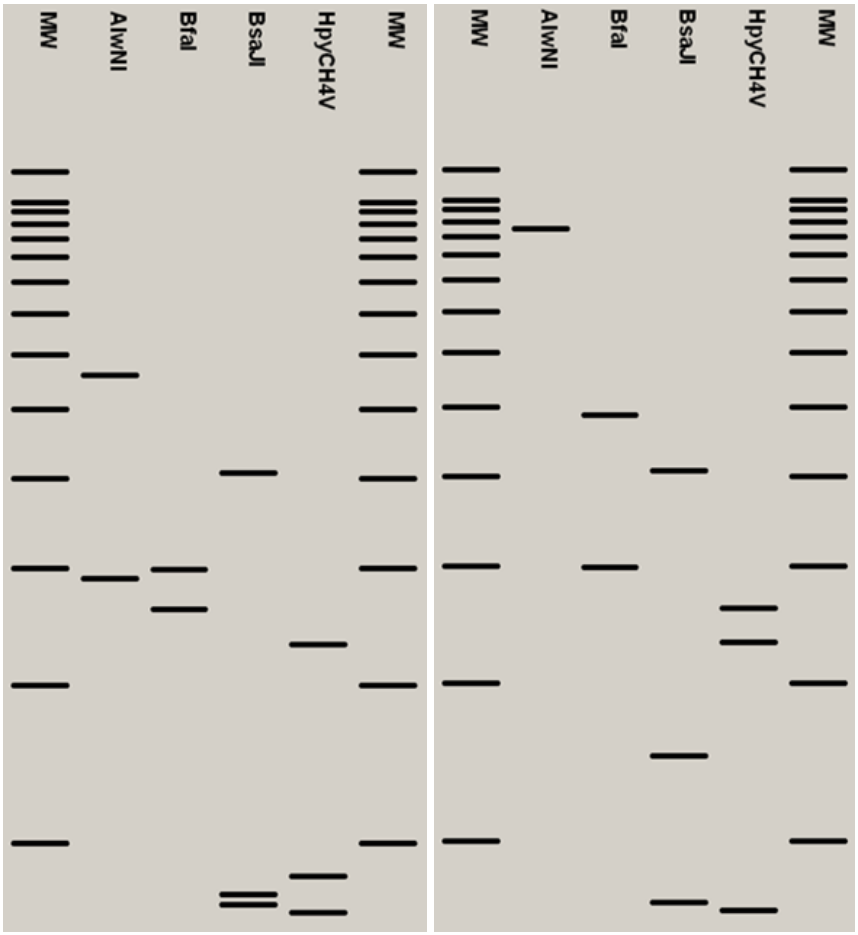

Figure 4. Virtual RFLP profiles generated by pDRAW32 software after digestion with *Alw*NI, *Bfa*I, *Bsa*JI, and *Hpy*CH4V restriction enzymes of 16S rDNA sequence KJ825886 from phytoplasma strain GN4 (left) and Y16389.1 (right). Line MW: Invitrogen 100 bp DNA Ladder, fragments size (bp) from top to bottom: 2072, 1500, 1400, 1300, 1200, 1100, 1000, 900, 800, 700, 600, 500, 400, 300.

in *Picris echioides* L. (Asteraceae) and belonging to 16SrIX-C group (Lee *et al*., 2012). Compared with this, all sequences obtained from *A. frutescens* phytoplasmas exhibited four SNPs at positions, 275, 284, 485 and 961 always consisting in a transition T → C. The nucleotide sequence of GN4 as representative phytoplasma was deposited in GenBank with the accession number KJ825886.

RFLP analysis

Restriction profiles always corresponding to the profile exhibited by the 16SrIX-C (PEY) reference phytoplasma were obtained from all positive samples when R16F2n/R16R2 amplified fragments was digested by the endonuclease *Taq*I, *Rsa*I and *Mse*I (Figure 2).

Virtual RFLP analysis performed with *i*PhyClassifier on R16F2n/R16R2 cloned sequences with *Alu*I and *Rsa*I restriction sites confirmed the presence of a 16SrIX-C profile in all positive samples, ruling out the presence of phytoplasmas belonging to the 16SrIX subgroups -A, -B, -D, -E, -F and -G (Figure 3). Virtual RFLP analyses using the software pDRAW32 (www.acaclone.com) on the cloned fragments employing a panel of 110 different endonucleases allowed selection of four restriction enzymes generating distinguishing RFLP profiles (Figure 4). These

were *Bsa*JI (nt. 275), *Hpy*CH4V (nt. 284), *Alw*NI (nt. 485) and *Bfa*I (nt. 961).

Discussion

Molecular analyses performed on *A. frutescens* plants showing typical phytoplasma symptoms highlighted the presence of *Picris echioides* yellows phytoplasma (16SrIX-C) in three out of eight tested samples, all collected from the same plot. The negative results obtained from five symptomatic plants may be due to the severe alteration of plant tissues exhibited by these plants which probably negatively affected the efficiency of DNA extraction and/or PCR amplifications.

Phytoplasmas of the 16SrIX-C group have already been reported for the family Asteraceae associated with the species *Echinacea purpurea* (L.) Moench, which is widely cultivated as a medicinal plant (Bertaccini *et al*., 2009). High sequence homology (99%) was observed with 16SrIX-C phytoplasmas from the wild species *Picris echioides* L. Nevertheless SNP was found and specific enzymes to be used as markers associated with a possible host specificity were identified for the phytoplasmas infecting *A. frutescens*.

The identification of infected plants in a commercial garden centre which is devoted only to the marketing of plants and where plants are maintained only for short periods, makes it more probable that infection originated at the nursery (flower company and/or growers). As *A. frutescens* is commonly propagated by cuttings, the use of originally infected propagation material could be the basis of these infections. However, in some major flower production areas in Italy, such as Liguria (north Italy), significant amounts of *A. frutescens* plants produced annually are cultivated under open air conditions (Beruto, 2013), so that natural infection by possible insect vectors in the field may also occur.

Many phytoplasmas affecting ornamentals are spread by insects and can potentially infect a wide range of plants. Thus, phytoplasmas in ornamentals are of particular importance, not only for direct economic impacts due to yield and/or quality losses, but also for the roles that these plants could play as alternative natural hosts for the spread of these pathogens to other economically important plant species. The risk of the possible involvement of ornamentals as alternative natural hosts for systemic pathogens is substantially greater when plants are produced under open air conditions, as is the case of *A. frutescens*. This indicates the need to investigate insect populations associated with these crops to verify the presence of potential insect vectors.

Epidemics of chicory phyllody have recently been reported in north-east Italy, associated with 16SrIX-C phytoplasmas (Martini *et al*., 2012). Phytoplasma strains identified in almond trees in Iran showing witches' broom symptoms shared a similarity coefficient >99% with the representative strain of the 16SrIX-C (*Picris echioides* yellows phytoplasma, PEY) subgroup (Molino Lova *et al*., 2011). This highlights the possible ability of this phytoplasma to also infect *Prunus* species.

The increased globalization of trade occurring over the last decade has greatly facilitated the movement of pathogens that threaten ornamental plants. Vegetative propagation used with many ornamental species has also greatly increased the risk of rapid spread of these pathogens. Cataloguing of unreported phytoplasma diseases in ornamentals and the identification of the phytoplasmas and their potential vectors is crucial in order to prevent economic losses and the possible spread of these pathogens to other economically important crops.

Acknowledgements

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