Phytopathologia Mediterranea
The international journal of the
Mediterranean Phytopathological Union

Firenze University Press
www.fupress.com/pm

Research Papers

Interactions between lime witches' broom phytoplasma and phytoplasma strains from 16SrI, 16SrII, and 16SrIX groups in periwinkle

Elham SALEHI[1], Keramatollah IZADPANAH[1,*], Seyed Mohsen TAGHAVI[1], Habibollah HAMZEHZARGHANI[1], Alireza AFSHARIFAR[1], Mohammad SALEHI[2]

[1] *Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz, Iran*
[2] *Plant Protection Research Department, Fars Agricultural and Natural Resources Research and Education Center, AREEO, Zarghan, Iran*
*Corresponding author: izadpana@shirazu.ac.ir

Summary. The interactions of lime witches' broom phytoplasma (LWBP, 16SrII-B) with alfalfa witches' broom phytoplasma (AWBP, 16SrII-C), tomato witches' broom phytoplasma (TWBP, 16SrII-D), sesame phyllody phytoplasma (SPhP, 16SrIX-C) and rapeseed phyllody phytoplasma (RPhP, 16SrI-B) were studied in periwinkle plants graft-inoculated either with each phytoplasma alone or with LWBP and a second phytoplasma. The latter was inoculated below or above the site of LWBP inoculation, and was applied either simultaneously or non-simultaneously. In all treatments and all replications, the plants doubly inoculated with LWBP + SPhP or LWBP + AWBP showed milder symptoms and lived longer than those singly inoculated with LWBP, SPhP, or AWBP. In plants with mixed infection by LWBP + RPhP or LWBP + TWBP, characteristic symptoms were present regardless of the grafting order or inoculation site. Analysis of quantitative PCR data showed that the mean concentrations of LWBP in all doubly inoculated plants were less than in plants inoculated with LWBP alone. In the SPhP inoculated plants, a substantial decrease in LWBP concentration was measured, followed in order of decreasing concentration in the AWBP, TWBP, and RPhP inoculated plants. In the mixed infections, greater reduction in LWBP concentration was found in non-simultaneous inoculations and when the second phytoplasma was grafted below the site of LWBP inoculation. Based on symptoms and quantitative PCR results, the interactions of LWBP with SPhP and AWBP resulted in greater cross-protection than interactions of LWBP with RPhP and TWBP.

Keywords. Mixed infections, symptomatology, quantitative PCR.

OPEN ACCESS

Citation: E. Salehi, K. Izadpanah, S.M. Taghavi, H. Hamzehzarghani, A. Afsharifar, M. Salehi (2020) Interactions between lime witches' broom phytoplasma and phytoplasma strains from 16SrI, 16SrII, and 16SrIX groups in periwinkle. *Phytopathologia Mediterranea* 59(2): 247-259. DOI: 10.14601/Phyto-11035

Accepted: May 11, 2020

Published: August 31, 2020

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Assunta Bertaccini, Alma Mater Studiorum, University of Bologna, Italy.

INTRODUCTION

Diseases caused by phytoplasmas are associated with significant yield losses in more than 1,000 plant species from different families, including many important field, vegetable and fruit crops, ornamental plants, timber and shade trees (McCoy *et al.,* 1989; Lee *et al.,* 2000; Bertaccini and Duduk,

2009). Characteristic symptoms include virescence, phyllody, abnormal proliferation of shoots and witches' broom, foliar yellowing, reddening and other discolorations, reduced leaf and fruit size, phloem necrosis, stunting and overall decline (Seemüller *et al.,* 1998; Bertaccini *et al.,* 2014). Phytoplasma diseases are difficult to control, mainly due to the specific plant-insect vector-pathogen relationship. Control recommendations for these diseases include phytosanitary and other preventive measures as well as control of the insect vectors, but none of these strategies have been shown to be satisfactory in field applications.

Cross-protection was reported as a strategy for control of fruit tree phytoplasma diseases when other approaches failed (Marcone *et al.,* 2010). This strategy has been regarded as induced resistance based on moderation of disease symptoms due to prior infection of host plants by closely related pathogen strains, usually with mild pathogenicity (Pennazio and conti, 2001). In plants mixed virus infections are common, and virus-virus interactions have been studied for many years and shown to be either antagonistic or synergistic (Syller, 2012).

Studies of interaction between mild and severe strains of phytoplasmas in insect vectors and plant hosts date back to the mid-20th century when these phytoplasmas were considered to be viruses (Kunkel, 1955; Freitag, 1964; Valenta, 1959). More recently, antagonistic effects of mild strains on severe strains have been reported for a number of phytoplasmas, including those associated with European stone fruit yellows (16SrX), ash yellows (16SrVI) and apple proliferation (16SrX) (Castelain *et al*., 2007; Kiss *et al*., 2013; Schneider *et al*., 2014; Sinclair and Griffiths, 2000). However, there is little information on interactions between different '*Candidatus* Phytoplasma' species or different 16S ribosomal groups.

Mexican lime [*Citrus aurantifolia* (Christsm.) Swingle)] is one of the most economically important horticultural crops in southern Iran, where it is cultivated in about 41,800 ha. with total annual production of about 400,000 tons (Anonymous, 2006). The lime witches' broom disease associated with the presence of '*Candidatus* Phytoplasma aurantifolia' (LWBP), a 16SrII-B strain (Zreik *et al.,* 1995), is the most devastating disease of Mexican lime and other citrus species in the southern Iran (Salehi *et al*., 2002), Oman and United Arab Emirates (Garnier *et al*., 1991). To date, no mild strains of this phytoplasma have been described, and its host range is expanded to other citrus varieties including grapefruit, sweet orange and mandarin (Mannan *et al.,* 2010). Traditional methods used to control these diseases are eradication, quarantine and use of chemicals (Salehi, 2016).

The aim of the present study was to investigate the interaction of lime witches' broom with four phytoplasma strains, including alfalfa witches' broom, tomato witches' broom, sesame phyllody and rapeseed phyllody, in periwinkle plants after graft inoculations.

MATERIALS AND METHODS

Experimental plants

A pink flower variety of periwinkle [*Catharanthus roseus* (L.) G. Don] was propagated from seed and grown in an insect-free greenhouse. Six-month-old plants were graft-inoculated with the phytoplasma strains described below, at greenhouse temperatures of approx. 30°C in the day time and 26°C at night, and with 15 h light / 9 h dark regime.

Phytoplasma strains

The phytoplasma strains used in this study were lime witches' broom phytoplasma (LWBP, 16SrII-B) from Nikshahr (Sistan-Baluchistan province) (Salehi *et al.,* 2002), alfalfa witches' broom phytoplasma (AWBP, 16SrII-C) from Abarkooh (Yazd province) (Esmailzadeh Hosseini *et al.,* 2015), sesame phyllody phytoplasma (SPhP, 16SrIX-C) from Fasa (Fars province) (Salehi *et al.,* 2017), tomato witches' broom phytoplasma (TWBP, 16SrII-D) from Borazjan (Bushehr province) (Salehi *et al*., 2014), and rapeseed phyllody phytoplasma (RPhP, 16SrI-B) from Zarghan (Fars province) (Salehi *et al.,* 2011). These phytoplasmas were transmitted from the original plant hosts to periwinkle via dodder (*Cuscuta campestris* Yank.), and were propagated and maintained in periwinkle by grafting for 2 years.

Graft inoculation

For graft inoculations of each phytoplasma strain, axillary shoots (each 3 cm long) containing two leaves from a symptomatic periwinkle plant were used as scions. These were side grafted on the main stems of healthy periwinkle plants grown from seed. Each graft site was 10 cm above the soil level. In mixed inoculations, the second scion was grafted at 6 cm below the first graft. Grafted areas were wrapped with parafilm, and the plants were covered with plastic bags for a week to maintain humidity. All grafted and non-graft-

ed experimental control plants were maintained in an insect-proof greenhouse.

Experimental treatments

A factorial experiment in a completely randomized design with three factors and four replicates, comprising a total of 84 plants (Table 2), was conducted to study interactions of LWBP with AWBP, TWBP, RPhP and SPhP. Factor A consisted of mixed infection of LWBP with either AWBP, TWBP, RPhP or SPhP; factor B was the grafting site of the second phytoplasma above or below the site of LWBP grafting on the stem; and factor C was the time of grafting of each phytoplasma in mixed infections, either simultaneous or non-simultaneous (2 weeks after the first grafting). Plants with single phytoplasma grafting, served as experimental controls for symptom expression. Comparisons were made by calculation of the area under phytoplasma concentration growth curves (AUCGC) at several times after grafting, using the following equation:

$$AUCGC = \sum_{i=1}^{n-1} \left[\frac{p_i + p_{i+1}}{2}\right][t_{i+1} - t_i]$$

where p_i and p_{i+1} are the phytoplasma concentrations obtained from grafted plants at consecutive times of t_i and t_{i+1}, and n is the number of times in which the concentration was estimated.

The simple and interactive effects of the factors on the total concentration of LWBP (AUCGC) was investigated using the procglm software, SAS 9.3 (Garret *et al.*, 2004). The mean of the treatments was compared using Duncan Multiple Range Test (DMRT) at the 5% significance level. At each inoculation time, four periwinkle plants were singly grafted with each phytoplasma as positive controls, and four healthy non-grafted periwinkle plants were included as negative controls.

Biological and molecular assessments

The evaluations of disease symptoms in the grafted periwinkle plants began 1 week after grafting, and was repeated every 2 weeks for 14 months. Nested PCR was used for detection, sequencing and identification of LWBP, AWBP, TWBP, RPhP and SPhP in plants, at 8 and 40 weeks after the grafting. Quantitative PCR (qPCR) assays were performed at 1 week after the grafting, and then repeated at 8-week intervals for 57 weeks.

Sampling, total nucleic acid extraction, PCR and sequence analysis

The sampling for phytoplasma detection and quantification started at day 7 after the grafting, and was repeated at 8-week intervals for 57 weeks. At each sampling time, 16 leaves from four shoots (four leaves from each shoot) grown above the grafting site, were collected from each grafted plant. The leaf midribs were separated, mixed and used for DNA extractions. Total nucleic acid (TNA) was extracted from 0.2 g of each sample using the small-scale method of Zhang *et al.* (1998), with the minor modifications proposed by Abou-Jawdah *et al.* (2002). Positive control samples consisted of nucleic acid from periwinkle plants singly grafted with each phytoplasma and negative control samples were nucleic acid from healthy periwinkle plants.

Phytoplasmas in grafted plants were detected by nested PCR using the universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995), followed by specific length primer pairs designed using online primer3 software (Untergasser *et al.*, 2012) (Table 1) in nested and qPCR assays. Each 50 µL PCR reaction mixture contained 100 ng of TNA from diseased or healthy plants, 0.4 µM of each primer, 0.2 mM of each dNTP and 1.25 U of Taq DNA polymerase (Cinagen, Iran) in l0× PCR buffer. The temperature profile for PCR consisted of a first denaturation step of 2 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min for annealing, and 2 min at 72°C. A final extension was carried out at 72°C for 3 min. The specificity of the primers was improved by changing the annealing temperatures as listed in Table 1. PCR products were electrophoresed through 1% agarose gel in 1× TBE buffer (67 mM Tris-HCl, 22 mM boric acid, 10 mM EDTA, pH 8.0), stained with ethidium bromide and visualized using a UV transilluminator (Sambrook *et al.*, 1989).

Nested PCR products of primer pairs LWB^F/LWB^R (146 bp), AWB^F/AWB^R (90 bp), TWB^F/TWB^R (96 bp), RPh^F/RPh^R (140 bp) and SPh^F/SPh^R (143 bp) were purified using GF-1 PCR Clean-Up Kit (Vivantis, Malaysia) according to the manufacturer's instructions, and were directly sequenced by Macrogen (South Korea) on both strands. The resulting consensus sequences were deposited in the GenBank database and used in BLAST search (http://blast.ncbi.nlm. nih.gov/Blast.cgi).

Quantitative polymerase chain reaction amplification

For qPCR, in addition to the specific length primer pairs for phytoplasmas, internal control primer pairs ($ef\text{-}1\alpha^F/ef\text{-}1\alpha^R$) (Table 1) were designed from the periwin-

Table 1. Primers used in nested PCR and qPCR assays.

Primer pairs	Sequence (5'→3')	Annealing temperature	Expected fragment size (bp)	References
P_1/P_7	AAGAGTTTGATCCTGGCTCAGG ATTCGTCCTTCATCGGCTCTT	55°C	1,800	Deng and Hiruki, 1991; Schneider *et al*., 1995
LWB^F/LWB^R	GTACACACCGCCCGTCAAAC GATCCATCCCCACCTTCCGG	63°C	146	Present study
AWB^F/AWB^R	GTTTGTACACACCGCCCGTC CCTTAGACAGCGCCCTCTCG	61°C	90	Present study
TWB^F/TWB^R	GCCGCGGTAAGACATAAGGG AGCGTTGCCATTACACCACTG	61°C	96	Present study
RPh^F/ RPh^R	GGAGGAGCTTGCGTCACATT ATTCCCTACTGCTGCCTCCC	61°C	140	Present study
SPh^F/ SPh^R	AGGAACACCAGAGGCGTAGG TCAGTACCGAGCCGAAACCC	63°C	143	Present study
ef-$1\alpha^F$/ ef-$1\alpha^R$	CTCTGCTTGCTTTCACCCTTGG GAGACCTCCTTCACAATTTCATC	55°C	115	Present study

kle elongation factor 1-alpha gene, using online primer3 software (Untergasser *et al*., 2012). The qPCR was performed using a MyiQTM Single Color Real time-PCR Detection System (Bioneer, Exicycler™ 96) with qPCR GreenMaster with LowRox (Jena Bioscience) for quantification of the assayed phytoplasmas in periwinkle plants. The PCR reaction mixture contained the following components in a final volume of 20 µL: 10 µL qPCR Green Master with Low Rox, 0.6 µL of each 10 µM forward and reverse primers, 6.8 µL PCR-grade H_2O and 2.0 µL of DNA template. The parameters used for amplification were 2 min at 95°C followed by 40 cycles of a two-step protocol consisting of 15 s at 95°C and 1 min at 63°C, for the LWB^F/LWB^R and SPh^F/SPh^R primer pairs, or 61°C, for the AWB^F/AWB^R, TWB^F/TWB^R and RPh^F/RPh^R primer pairs. For each treatment, 8 qPCR assays were made at 8-week intervals, and changes in the concentrations of LWBP were recorded during the course of the study (57 weeks).

Statistical analyses

A complete randomized block design in the form of a factorial experiment with four replications was used to evaluate the presence of interactions of LWBP with the four other phytoplasmas, based on symptomatology and qPCR values. Repeated measures analysis of variance was used for detecting changes in the mean parameters at 1, 9, 17, 25, 33, 41, 49 and 57 weeks after the first grafting, using SAS 9.3 software for statistical analysis (Garret *et al.,* 2004). Areas under the concentration growth curves (AUCGC) were determined, and used in statistical analyses for the normalization of the concentration data.

RESULTS

Symptomatology in periwinkle

In all grafted plants, the scions remained alive and grew as expected. In periwinkle, LWBP induced more severe symptoms of witches' broom, little leaf, internode shortening and stunting than the AWBP, TWBP, RPhP or SPhP strains. Time to symptom appearance in periwinkle plants, separately grafted with each phytoplasma, was approx. 8 weeks (Table 2). Disease symptoms in periwinkle plants grafted with LWBP were yellowing, incomplete virescence and severe little leaf, shortened internodes, witches' broom and stunting. In plants singly grafted with AWBP, TWBP, RPhP or SPhP the main disease symptoms were flower virescence and phyllody, little leaf, shortened internodes, yellowing and stunting (Figures 1a-f, and Table 2). In periwinkle plants grafted with LWBP + AWBP and LWBP + SPhP, the symptoms were mild little leaf and yellowing regardless of the grafting time (simultaneous or non-simultaneous) or the relative position of scions (Figures 2a and b, and Table 2). At the end of the observations (57 weeks), periwinkle plants graft inoculated with phytoplasma mixtures produced normal flowers (Figure 2b, and Table 2).

All periwinkle plants grafted with LWBP + TWBP showed symptoms of yellowing, small leaves, shortened internodes, stem proliferation, virescence and phyllody after 20 weeks. At the end of the experiment (57 weeks) all LWBP + TWBP plants showed yellowing, shortened internodes, mild little leaf and rosetting without the virescence, phyllody or witches' broom symptoms which

Table 2. Summary of results obtained in the grafted periwinkle plants.

Treatment type	Grafting type	Symptomatology	Time to symptom appearance (weeks post inoculation)	Number of grafted plants
LWBP[a]	Alone	Y[d], IV, SLL, SSI, SWB, SST	8	4
AWBP	Alone	MY, LL, V, Ph, MST	8	4
TWBP	Alone	MY, LL, V, Ph, MST	8	4
SPhP	Alone	MY, LL, V, Ph, MST	8	4
RPhP	Alone	MY, LL, V, Ph, MST	8	4
LWBP+AWBP	S[b]&NS[c]	MLL, Y, NF	40	16
LWBP+SPhP	S & NS	MLL, Y, NF	40	16
LWBP+TWBP	S & NS	Y, SL, SI, SP, V, Ph, Y, MLL, Ro	20 57	16
		W, De	20	2
LWBP+RPhP	S & NS	MY, LL, SI	57	2
		SLL, SSI, SY, SLD	57	12

[a]AWBP, alfalfa witches' broom phytoplasma; LWBP, lime witches' broom phytoplasma; RPhP, rapeseed phyllody phytoplasma; SPhP, sesame phyllody phytoplasma; TWBP, tomato witches' broom phytoplasma.
[b] S, simultaneous.
[c] NS, non-simultaneous.
[d] Symptom abbreviations: De, death; IV, incomplete flower virescence; LL, little leaf; MLL, mild little leaf; MST, mild stunting; MY, mild yellowing; NF, normal flower; Ph, flower phyllody; Ro, rosetting; SI, shortened internodes; SL, small leaves; SLD, severe leaf drop; SLL, severe little leaf; SP, stem proliferation; SSI, severe shortened internodes; SST, severe stunting; SWB, severe witches' broom; SY, severe yellowing; V, flower virescence; W, wilting; Y, yellowing.

were present in the plants singly grafted with LWBP or TWBP (Figure 2c, and Table 2).

In the periwinkle plants infected with LWBP+RPhP, regardless of grafting time and site, the scions remained stunted. Two of the grafted plants wilted and died after 20 weeks (Figure 2d, and Table 2). At the end of observations (57 weeks), two plants showed mild yellowing, little leaf and very shortened internodes (Figure 2e, and Table 2) and the remaining 12 plants showed severe symptoms of little leaf, shortened internodes, yellowing and leaf drop (Figure 2f, and Table 2).

Detection of phytoplasma strains in graft inoculated periwinkle plants

Nested PCR gave specific amplification of expected 146 bp fragments from the plants inoculated with LWBP, 90 bp fragments from AWBP, 96 bp fragments from TWBP, 140 bp fragments from RPhP, or 143 bp fragments from SPhP (Table 1, Figures 3 and 4d). These fragments were sequenced and deposited in GenBank under accession numbers MH411203 (LWBP), MH647740 (AWBP), MH647741 (TWBP), MH647743 (RPhP) and MH647742 (SPhP), and all aligned with phytoplasma sequences. In all doubly grafted plants, fragments of expected lengths corresponding to those obtained from the inoculated phytoplasmas were amplified (Figure 4a-c), regardless of the grafting time and the relative position of the two grafting sites.

Phytoplasma concentration

In all treatments, the graft inoculated phytoplasmas were present in the plants although at different concentrations. The mean of eight determinations showed that in single phytoplasma graft inoculations, the LWBP concentration was greater than that of the other phytoplasma strains (Figure 5). There were also clear reductions of LWBP concentration in doubly grafted plants compared with singly grafted plants. These differences were consistent in all samplings during the course of the study. The analysis of variance (ANOVA) of the AUCGC for LWBP in mixed and singly grafted periwinkle plants is shown in Table 3. Experimental factors such as treatment, grafting time and grafting site, as well as the interactions of the treatment × grafting site and the grafting site × inoculation time were statistically significant, at least at 5%, on the reduction of the AUCGC for LWBP (Table 3). Among the four phytoplasma strain combinations, LWBP + SPhP showed the greatest reduction of mean concentration of LWBP (Figure 6). The ranking order (from greatest to least) of the examined phytoplasmas for reducing the concentration of LWBP was SPhP, AWBP, TWBP and RPhP (Figure 6).

Figure 1. Disease symptoms in periwinkle plants following graft-inoculation with lime witches' broom phytoplasma (LWBP), alfalfa witches' broom phytoplasma (AWBP), tomato witches' broom phytoplasma (TWBP), sesame phyllody phytoplasma (SPhP) and rapeseed phyllody phytoplasma (RPhP). (A) plants grafted with LWBP showing yellowing and severe symptoms of little leaf, shortened internodes, witches' broom and stunting. (B-D) plants with yellowing, shortened internodes, virescence and phyllody due to AWBP (B), TWBP (C) and SPhP (D). (E) yellowing, shortened internodes, little leaf and stem proliferation due to RPhP. (F) healthy periwinkle plant. Arrows in B and D indicate flower phyllody and virescence.

Figure 2. Disease symptoms in periwinkle plants doubly grafted with lime witches' broom phytoplasma (LWBP) and another phytoplasma strain. (A) mild yellowing in a periwinkle plant grafted with LWBP and alfalfa witches' broom phytoplasma (AWBP). (B) mild yellowing and shortened internodes with normal flowers in a periwinkle plant grafted with LWBP and sesame phyllody phytoplasma (SPhP). (C) mild yellowing, internode shortening, little leaf, and rosetting, in a periwinkle plant grafted with LWBP and tomato witches' broom phytoplasma (TWBP). (D) wilting, rosetting. (E) mild yellowing, shortened internodes, little leaf, crown proliferation. (F) severe symptoms of little leaf, yellowing and leaf drop in periwinkle plants grafted with LWBP and rapeseed phyllody phytoplasma (RPhP). In (A), right and left arrows indicate, respectively, scions from LWBP and AWBP.

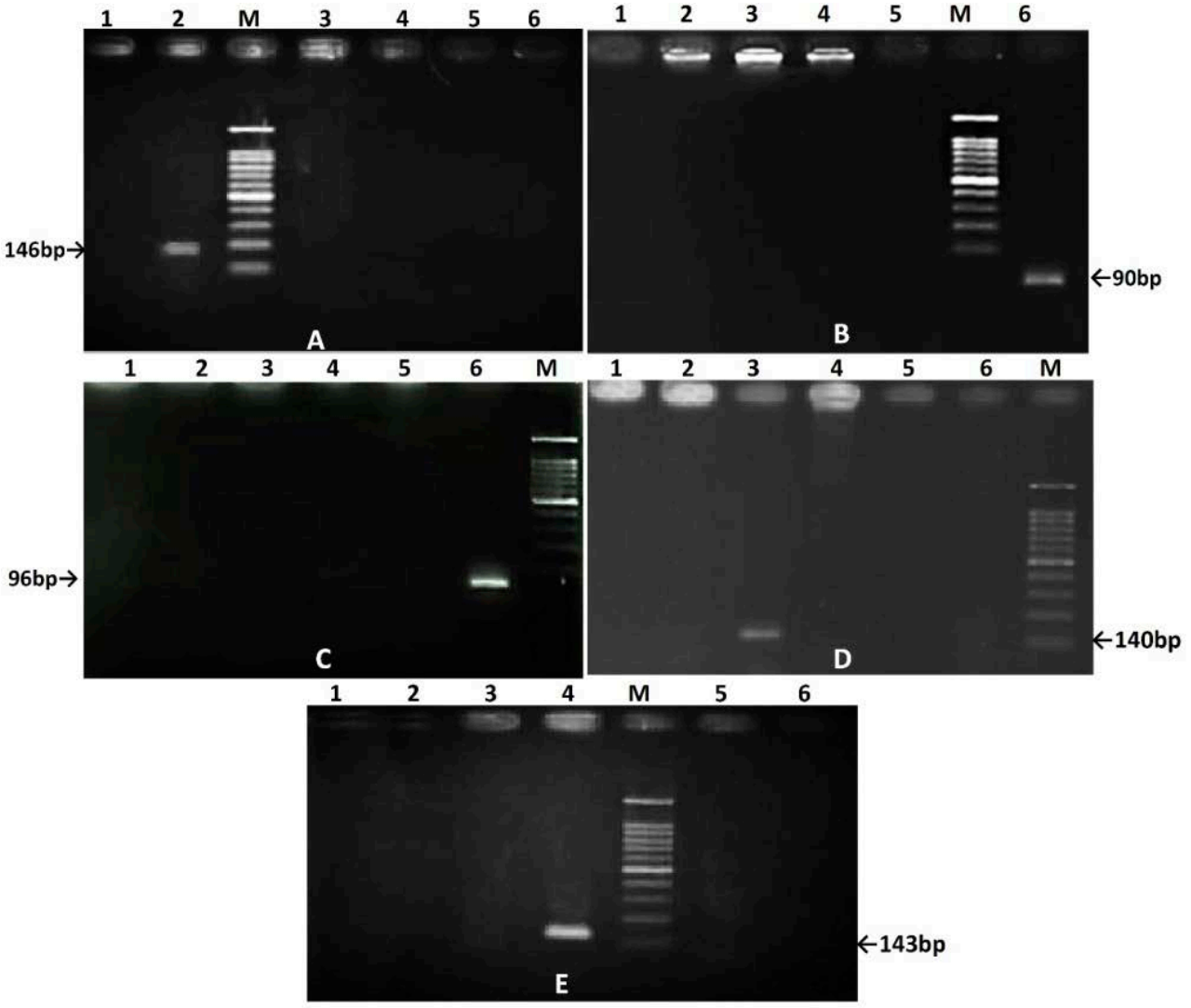

Figure 3. Agarose gel electrophoresis pattern of nested PCR products using P1/P7 primer pair followed by primer pairs LWB^F/LWB^R (146 bp) for detection, in singly graft-inoculated periwinkle plants, of lime witches' broom phytoplasma (LWBP) (A); AWB^F/AWB^R (90 bp), for alfalfa witches' broom phytoplasma (AWBP) (B); TWB^F/TWB^R (96 bp), for tomato witches' broom phytoplasma (TWBP) (C); RPh^F/RPh^R (140 bp), for rapeseed phyllody phytoplasma (RSPh) (D); and SPh^F/SPh^R (143 bp), for sesame phyllody phytoplasma (SPhP) (E). In A, B, D, and E, lanes 1, 2, 3, 4, 5, 6 are from, respectively, healthy periwinkle, LWBP, RPhP, SPhP, TWBP or AWBP graft-inoculated periwinkle plants. In C, lanes 1, 2, 3, 4, 5, 6, are from, respectively, healthy periwinkle, LWBP, RPhP, SPhP, AWBP or TWBP graft-inoculated periwinkle plants. M, 100 bp DNA marker fragments (Fermentas, Vilnius, Lithuania).

Relative grafting site also affected LWBP concentration. All mixed graftings below the position of LWBP infected scions were more effective in decreasing the LWBP concentration than those above the site of LWBP inoculation (Figure 7). A decrease in concentration of LWBP in mixed graft-inoculations was also affected by grafting times: non-simultaneous grafting in mixed infections was more effective in reducing the mean total concentration of LWBP than simultaneous grafting (Figure 8).

DISCUSSION

Previous studies on the interactions between phytoplasmas have been limited to a number of closely related strains (Castelain *et al.,* 2007; Kiss *et al.,* 2013; Schneider *et al.,* 2014; Sinclair and Griffiths, 2000). In the present research, the interaction of LWBP with four phytoplasma strains classified in different 16S ribosomal groups or subgroups (AWBP, SPhP, TWBP and RPhP) was studied.

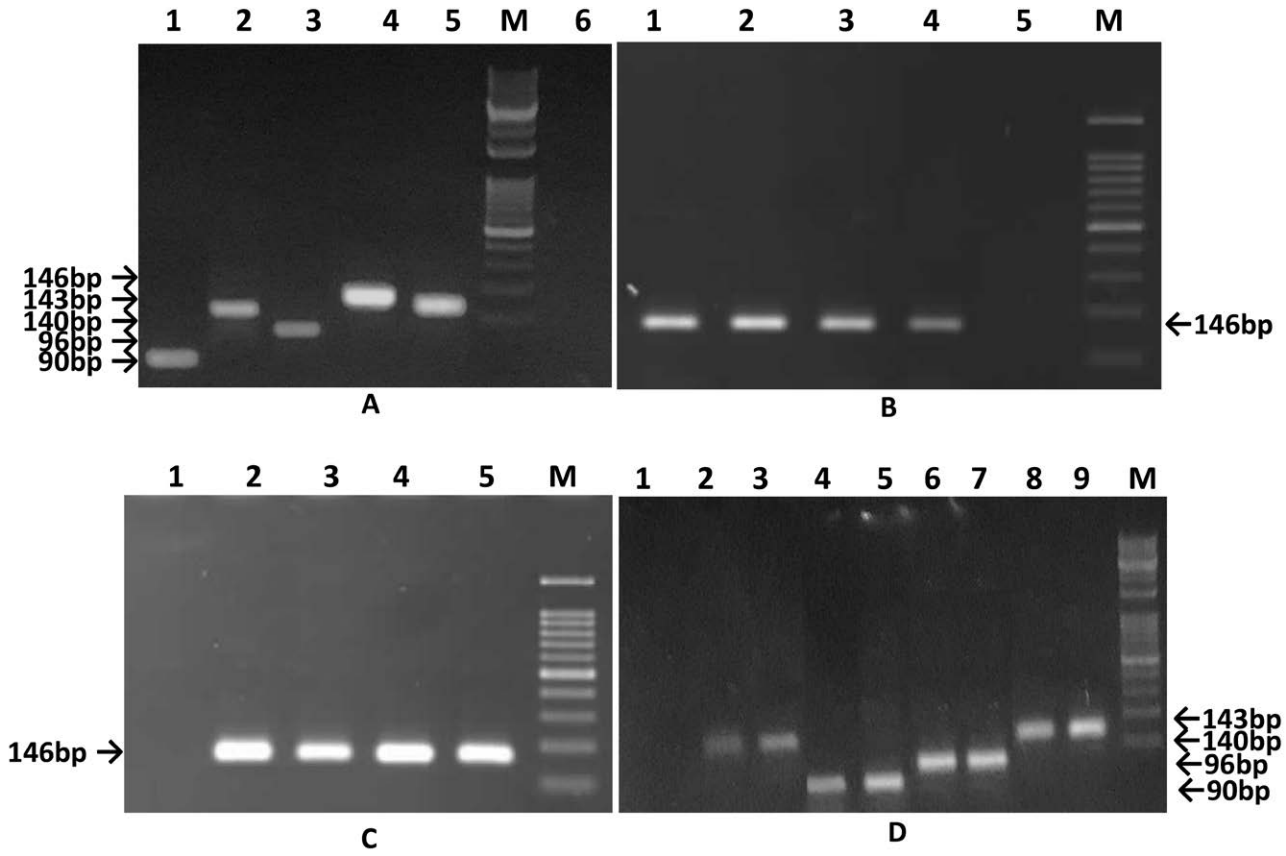

Figure 4. Agarose gel electrophoresis pattern of nested PCR products using P1/P7 primer pair followed by lime witches' broom phytoplasma (LWBP), alfalfa witches' broom phytoplasma (AWBP), tomato witches' broom phytoplasma (TWBP), rapeseed phyllody phytoplasma (RPhP), or sesame phyllody phytoplasma (SPhP) primer pairs amplifying, respectively, 146 bp, 90 bp, 96 bp, 140 bp and 143 bp fragments. (A) lanes 1-5 electrophoresis pattern of nested PCR products from plants singly grafted with, respectively, AWBP, RPhP, TWBP, LWBP or SPhP. (B) lanes 1-4 in simultaneous grafting, and (C) lanes 2-5 in non-simultaneous grafting, 146 bp band of LWBP amplified from periwinkle plants having mixed infection with, respectively, AWBP, TWBP, RPhP or SPhP. (D) pairs of bands from simultaneous (left bands) and non-simultaneous (right bands) grafting of RPhP (lanes 2 and 3), AWBP (lanes 4 and 5), TWBP (lanes 6 and 7) and SPhP (lanes 8 and 9) in mixed infection with LWBP. Lanes 6 in (A), 5 in (B) and 1 in (C) and (D) are from healthy periwinkle plants. M, 100bp DNA marker fragments (Fermentas, Vilnius, Lithuania).

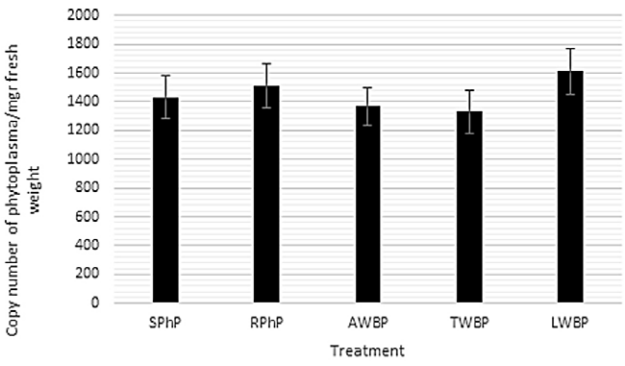

Figure 5. Mean concentrations of lime witches' broom phytoplasma (LWBP), tomato witches' broom phytoplasma (TWBP), alfalfa witches' broom phytoplasma (AWBP), rapeseed phyllody phytoplasma (RPhP) and sesame phyllody phytoplasma (SPhP) in singly grafted periwinkle plants. Bars represent standard deviations.

In all doubly grafted plants, the concentration of LWBP was less than that in the singly grafted plants. A strong antagonistic interaction was observed in mixed infections of LWBP with AWBP or SPhP. The antagonistic relationship was mutual, as the symptoms of both phytoplasmas were greatly reduced. Mixed infections of LWBP + TWBP or LWBP + RPhP resulted in suppression of LWBP concentration, but the symptoms were not suppressed. It is possible that the extent of reduction in concentration of LWBP was not sufficient to affect symptom expression. Another possibility is that there were synergistic effects of these combinations in the host plants for symptom expression, despite the lowered concentrations of the phytoplasmas. Alternatively, other factors besides phytoplasma concentration, such as genes involved

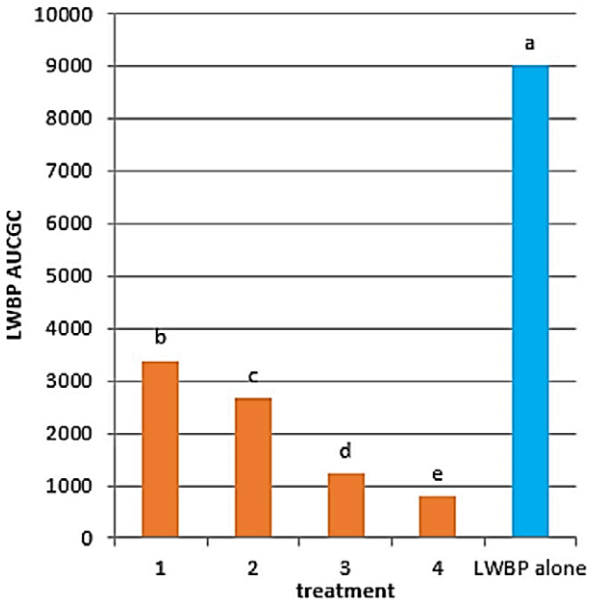

Figure 6. Mean concentrations of lime witches' broom phytoplasma (LWBP) in four mixed infections and a single infection in periwinkle plants, based on areas under concentration growth curves (AUCGC). 1, LWBP (16SrII-B) + rapeseed phyllody phytoplasma (RPhP) (16SrI-B); 2, LWBP + tomato witches' broom phytoplasma (TWBP) (16SrII-D); 3, LWBP + alfalfa witches' broom phytoplasma (AWBP) (16SrII-C); and 4, LWBP + sesame phyllody phytoplasma (SPhP) (16SrIX-C). Means were compared using Duncan Multiple Range Test. Means accompanied by different letters are significantly different ($P \leq 0.05$).

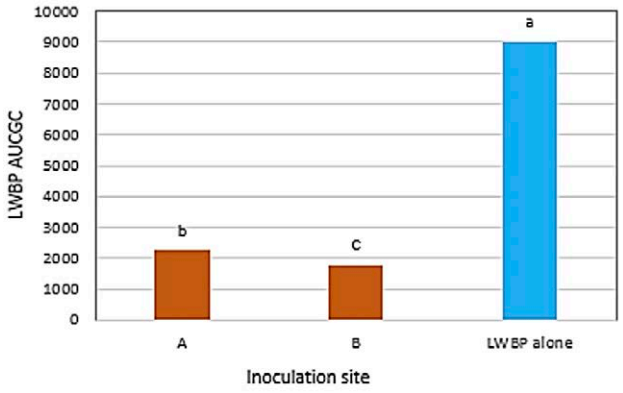

Figure 7. Effect of grafting site on concentration of lime witches' broom phytoplasma (LWBP) (16SrII-B) in mixed grafting and alone: LWBP AUCGC, area under concentration growth curve of LWBP; A, inoculation of the alfalfa witches' broom phytoplasma (AWBP) (16SrII-C), rapeseed phyllody phytoplasma (RPhP) (16SrI-B), sesame phyllody phytoplasma (SPhP) (16SrIX-C) and tomato witches' broom phytoplasmas phytoplasma (TWBP) (16SrII-D) phytoplasmas above the site of the LWBP grafting and B, inoculation of the AWBP, RPhP, SPhP and TWBP below the site of the LWBP grafting. A and B are means of the four mixed inoculations. Means with different letters are significantly different ($P \leq 0.05$).

in pathogenicity and/or virulence, may have affected symptom expression.

The results of these experiments show that effects of possible interactions varies with different phytoplasmas. Previous studies have shown that different strains of the same '*Candidatus* Phytoplasma' species may differ in their effectiveness for conferring cross protection (Schneider *et al.*, 2014; Kiss e*t al.*, 2013). Kiss *et al.*, (2013) found a cross effect between a mild strain of apple proliferation (AP, 16SrX-A) and a related phytoplasma strain, Germany stone fruit yellows phytoplasma (16SrX-B), but not between AP and aster yellows (16SrI-B) phytoplasmas. A mutual antagonistic relationship between LWBP and lettuce phyllody phytoplasma (16SrIX-D, DQ889749) in periwinkle plants inoculated via dodder has been reported previously (Salehi *et al.*, 2018).

Cross protection with mild strains has been used to control severe strains of viruses (Pennazio and Conti, 2001). The use of this strategy may be justified for controlling severe phytoplasma epidemics if no alternative disease management strategies are available.

Table 3. Analysis of variance (using SAS 9.3 software) of areas under concentration growth curves of LWBP in mixed and singly grafted periwinkle plants.

Source	DF	Type III SS	Mean Square	F value	Pr>F
Treatment	3	71047487.66	23682495.89	105.63	<.0001**
Grafting site	1	3375487.56	3375487.56	15.06	0.0003**
Grafting time	1	5871171.30	5871171.30	26.19	<.0001**
Treatment × grafting site	3	2271979.20	757326.40	3.38	0.0257*
Treatment × grafting time	3	921103.94	307034.65	1.37	0.2634 ns
Grafting site × grafting time	1	2959690.14	2959690.14	13.20	0.0007**
Treatment × grafting site × grafting time	3	1412122.09	470707.36	2.10	0.1126 ns

ns, non-significant; *, $P \leq 0.05$; **, $P \leq 0.01$.

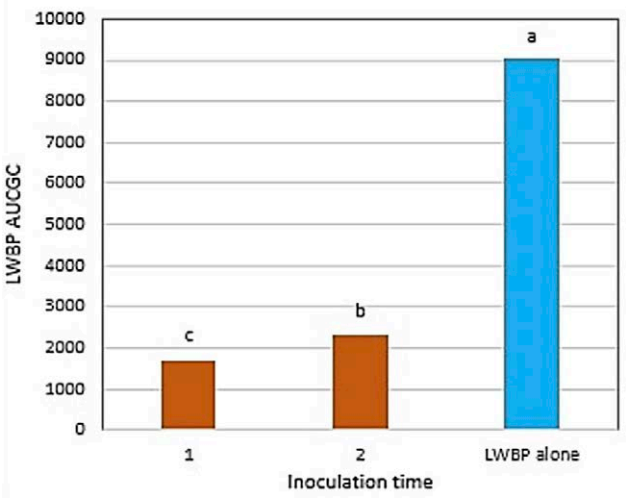

Figure 8. Effect of grafting time (simultaneous and non-simultaneous) on concentration of lime witches' broom phytoplasma (LWBP) in mixed infection with alfalfa witches' broom, rapeseed phyllody, sesame phyllody and tomato witches' broom phytoplasmas as compared with those grafted with LWBP alone: LWBP AUCGC, area under concentration growth curve of LWBP. 1, non-simultaneous and 2, simultaneous grafting. 1 and 2 are means of the four mixed inoculations. Means accompanied by different letters are significantly different ($P \leq 0.05$)

Phytoplasma strains from different 16Sr groups have been reported in citrus plants showing huanglongbing symptoms in *'Candidatus* Liberibacter asiaticus'-free samples or in mixed infection with this pathogen. These phytoplasma strains include 16SrI (Chen *et al.*,2009; Arratia-Castro *et al.*, 2014), 16SrII (Alhudaib *et al.,* 2009; Lou*et al.*, 2013; Saberi *et al.,* 2017), 16SrIII (Wulff *et al.*, 2018), 16SrVI (Das *et al.,* 2016), 16SrIX (Teixeira *et al.,* 2008; Abbasi *et al.*, 2019) and 16SrX-IV (Ghosh *et al.*, 2019). In a survey in some Caribbean countries, phytoplasma strains of 16Sr groups -I, , -III, -IV, -VI, -VII, -XI, and -XII were reported in citrus trees showing "huanglongbing" symptoms, either alone or, more commonly, in mixed infections with '*Ca.* L. asiaticus' (Bertaccini *et al.*, 2019).

The present study has shown that the interactions of LWBP with SPhP or AWBP were more effective in reducing disease symptoms and pathogen titre than those of LWBP with RPhP or TWBP

ACKNOWLEDGEMENTS

This study was part of the first author's PhD research, supported by the Shiraz University Graduate School. The authors declare that they have no conflicts of interest.

LITERATURE CITED

Abbasi A., Hasanzadeh N., Sobh H., Ghayeb Zamharir M., Tohidfar M., 2019. Identification of a group 16SrIX '*Candidatus* Phytoplasma phoenicium' phytoplasma associated with sweet orange exhibiting decline symptoms in Iran. *Australasian Plant Disease Notes* 14: 11.

Abou-Jawdah Y., Karakashina A., Sobh H., Martini M., Lee I-M., 2002. An epidemic of almond witches' broom in Lebanon: classification and phylogenetic relationships of the associated phytoplasma. *Plant Disease* 86: 477–484.

Alhudaib K., Arocha Y., Wilson M., Jones P., 2009. Molecular identification, potential vectors and alternative hosts of the phytoplasma associated with a lime decline disease in Saudi Arabia. *Crop Protection* 28: 13–18.

Anonymous, 2006. Agricultural Statistics of the Year 2006. The Org. of Statistics and information Pub., Tehran, Iran.

Arratia-Castro A. A., Santos-Cervantes M. E., Fernández Herrera E., Chávez-Medina J. A., Flores-Zamora G. L.,…Leyva-López N. E., 2014. Occurrence of '*Candidatus* Phytoplasma asteris' in citrus showing huanglongbing symptoms in Mexico. *Crop Protection* 62:144–151.

Bertaccini A., Duduk B., 2009. Phytoplasma and phytoplasma diseases: a review of recent research. *Phytopathologia Mediterranea* 48: 355–378.

Bertaccini A., Duduk B., Paltrinieri S., Contaldo N., 2014. Phytoplasmas and phytoplasma diseases: a severe threat to agriculture. *American Journal of Plant Sciences* 5:1763–1788.

Bertaccini A., Satta E., Luis-Pantog M., Paredes-Tomás C., Yuneau Y., Myrie W. 2019. '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter' species detection in citrus. *Phytopathogenic Mollicutes* 9 (1): 186-188.

Castelain C., Jullian J.P., Lemaire J.M., Morvan G., 2007. Lutte biologique contre l'ECA: la prémunition. *Arboriculture* 611: 33–38.

Chen J. Pu. X., Deng X., Liu S., Li H., Civerolo E., 2009. A phytoplasma related to '*Candidatus* phytoplasma asteris' detected in citrus showing "huanglongbing" (yellow shoot disease) symptoms in Guangdong, P.R. China. *Phytopathology* 99: 236–242.

Das A. K., Nerkar S., Thakre N., Kumar A., 2016. First report of '*Candidatus* Phytoplasma trifolii' (16SrVI-group) in Nagpur mandarin (*Citrus reticulata*) showing "huanglongbing" symptoms in central India. *New Disease Reports* 34: 15.

Deng S., Hiruki C., 1991. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. *Journal of Microbiological Methods* 14: 53–61.

Esmailzadeh-Hosseini S.A., Khodakaramian G., Salehi M., Fani S.R., Bolok Yazdi H.R., Bertaccini A., 2015. Status of alfalfa witches' broom phytoplasma disease in Iran. *Phytopathogenic Mollicutes* 5: S65–S66.

Freitag J.H., 1964. Interaction and mutual suppression among three strains of aster yellows virus. *Virology* 24: 401–413.

Garnier M., Zreik L., Bové J-M., 1991. Witches' broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. *Plant Disease* 75: 546–555.

Garrett K.A., Madden L.V., Hughes G., Pfender W.F., 2004. New applications of statistical tools in plant pathology. *Phytopathology* 94: 999–1003.

Ghosh D.K., Motghare M., Kokane A., Kokane S., Warghane A., Ladaniya M. S., 2019. First report of a '*Candidatus* Phytoplasma cynodontis'-related strain (group 16SrXIV) associated with Huanglongbing disease on *Citrus grandis*. *Australasian Plant Disease Notes* 14: 9.

Kiss E., Seemuller E., Sule S., 2013. Cross-protection test with *'Candidatus* Phytoplasma mali' 1/93 strain against closely and distantly related phytoplasma strains. *Acta Phytopathologica et Entomologica Hungarica* 48: 207–218.

Kunkel L.O., 1955. Cross protection between strains of aster yellows. *Advances in Virus Research* 3: 251-273.

Lee I-M., Davis R. E., Gundersen-Rindal D. E., 2000. Phytoplasma: Phytopathogenic mollicutes. *Annual Review of Microbiology* 54: 221–255.

Lou B., Bai X., Bai Y., Deng C., Roy Chowdhury M., Song Y., 2013. Detection and molecular characterization of a 16SrII-A* phytoplasma in grapefruit (*Citrus paradise*) with huanglongbing like symptoms in China. *Journal of Phytopathology* 162: 387–395.

Mannan S. H., Ibrahimi M., Chohan S. H. N., Holford P., 2010. Detection of phytoplasma in citrus orchard of Pakistan. *Journal of Phytology* 2: 49-54.

Marcone C., Jarausch B., Jarausch W., 2010. '*Candidatus* Phytoplasma prunorum', the causal agent of European stone fruit yellows: an overview. *Journal of Plant Pathology* 92: 19–34.

McCoy R. E., Caudwell A., Chang C. J., Chen T. A., Chiykowski L. N., Seemüller E., 1989. Plant disease associated with mycoplasma- like organisms. In: *The Mycoplasmas, Vol. V, Spiroplasmas, Acholeplasmas and Mycoplasmas of Plants and Insects* (R.F. Whitcomb, J.G. Tully, ed.), Academic Press, New York, NY, USA, 545–640.

Pennazio R. P., Conti M., 2001. A history of plant virology. Cross protection and application. *Microbiologica* 24: 99–114.

Saberi E., Alavi A. M., Safaie N., Moslemkhany C., Azadvar M., 2017. Bacterial pathogens associated with citrus huanglongbing-like symptoms in southern Iran. *Journal of Crop Protection* 6: 99–113.

Salehi Abarghouei E., Izadpanah K., Taghavi S. M., Hamzezarghani H., Afsharifar A., 2018. Interaction of witches' broom disease of lime phytoplasma and lettuce phyllody phytoplasma in mixed infection of periwinkle plants. *Iranian Journal of Plant Pathology* 54: 131–146.

Salehi E., Salehi M., Taghavi S. M., Izadpanah K., 2014. A 16SrII-D phytoplasma strain associated with tomato witches' broom in Bushehr province. *Journal of Crop Protection* 3: 377–388.

Salehi M., 2016. Control strategies of witches' broom disease of lime in Iran. In: *Proceedings of 22nd Iranian Plant Protection Congress, College of Agriculture and Natural Resources*, *2016,* University of Tehran, Karaj, Iran, 364.

Salehi M., Izadpanah K., Taghizadeh M., 2002. Witches' broom disease of lime in Iran: new distribution areas, experimental herbaceous hosts and transmission trails. In: *Proceedings 15th Conference of International Organization of Citrus Virologists* (IOCV), Riverside, CA, USA, 293–296.

Salehi M., Izadpanah K., Siampour M., 2011. Occurrence, molecular characterization and vector transmission of a phytoplasma associated with rapeseed phyllody in Iran. *Journal of Plant Pathology* 159: 100–105.

Salehi M., Esmailzadeh-Hosseini S. A., Salehi E., Bertaccini A., 2017. Genetic diversity and vector transmission of phytoplasmas associated with sesame phyllody in Iran. *Folia Microbiologica* 62: 99–109.

Sambrook J., Fritsch E. F., Maniatis T., 1989. *Molecular Cloning: A Laboratory Manual. II ed.* Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, NY, USA.

Sanches M. M., Wulff N. A., Ferreira E. A., Santos J. F., Angarten M. B. O., …Martins O. A., 2016. Survey for phytoplasmas and '*Candidatus* Liberibacter' sp. from HLB-like symptomatic citrus plants in Brazil. *Citrus Research and Technology* 37: 88–93.

Schneider B., Seemüller E., Smart C. D., Kirkpatrick B. C., 1995. Phylogenetic classification of plant pathogenic mycoplasma like organisms or phytoplasmas. In: *Molecular and Diagnostic Procedures in Mycoplasmology* (S. Razin, J. G. Tully, ed.) Academic Press, San Diego, CA, USA, 369–380.

Schneider B., Sule S., Jelkmann W., Seemüller E., 2014. Suppression of aggressive strains of '*Candidatus* Phytoplasma mali' by mild strains in *Catharanthus roseus* and *Nicotiana occidentalis* and identification

of similar action in apple trees. *Phytopathology* 104: 453–461.

Seemüller E., Marcone C., Lauer U., Ragozzino A., Göschl M., 1998. Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology* 80: 3–26.

Sinclair W. A., Griffiths H. M., 2000. Variation in aggressiveness of ash yellows phytoplasmas. *Plant Disease* 84: 282–288.

Syller J., 2012. Facilitative and antagonistic interactions between plant viruses in mixed infections. *Molecular Plant Pathology* 13: 204–216.

Teixeira D. C., Wulff N. A., Martins E. C., Kitajima E. W., Bassanezi R., Bové J-M., 2008. A phytoplasma closely related to the pigeon pea witches' broom phytoplasma (16SrIX) is associated with citrus huanglongbing symptoms in the state of Sao Paulo, Brazil. *Phytopathology* 98: 977–984.

Untergasser A., Cutcutache I. K., Koressaar T., Ye. J., Faircloth B. C., Rozen S. G., 2012. Primer3-new capabilities and interfaces. *Nucleic Acids Research* 40: 115.

Valenta V., 1959. Interference studies with yellows-type plant viruses. II. Cross protection tests with European and American viruses. *Acta Virologica* 3: 145–152.

Wulff N. A., Fassini C. G., Marques V. V., Martins E. C., Coletti D. A. B., Bové J-M., 2018. Molecular characterization and detection of 16SrIII group phytoplasma associated with Huanglongbing symptoms. *Phytopathology* 109: 366-374.

Zhang Y., Uyemoto J. K., Kirkpatrick B. C., 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. *Journal of Virological Methods* 71: 45–50.

Zreik L., Carle P., Bové J-M., Garnier M., 1995. Characterization of the mycoplasma like organism associated with witches' broom disease of lime and proposition of a candidate taxon for the organism, *'Candidatus* Phytoplasma aurantifolia'. *International Journal of Systematic Bacteriology* 45: 449–453.