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Asymmetrasca decedens (Cicadellidae, Typhlocybinae), a natural vector of '*Candidatus* Phytoplasma phoenicium'

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Abstract

'*Candidatus* Phytoplasma phoenicium' is associated with a lethal disease of almond, peach and nectarine named almond witches'-broom disease (AlmWB). The disease spread rapidly in Lebanon from coastal areas to elevations exceeding 1200 m, killing over 150,000 trees in a span of two decades. The mode of spread suggested the involvement of efficient vector(s) and *Asymmetrasca decedens* (Hemiptera, Cicadellidae) was suspected as it is the most abundant leafhopper species present in Lebanese stone fruit orchards. Living *A. decedens* specimens were collected from fields heavily infested by AlmWB and used in transmission trials on healthy peach almond hybrid GF-677 and peach GF-305 seedlings with an inoculation-access period of 30 days. PCR analysis supported by sequencing showed that *A. decedens* is a carrier of the phytoplasma, and that the phytoplasma was detected in insect salivary glands and in some inoculated GF-677 and GF-305 seedlings. One year post-inoculation, '*Ca.* P. phoenicium' was detected in newly emergent leaves of inoculated seedlings. However, the characteristic symptoms of witches'-broom were not observed. PCR amplified fragments from phytoplasma-positive seedlings and from *A. decedens* samples showed 99.9% nucleotide identity in their 16S RNA region and phylogenetic analysis using a neighbour jointing tree confirmed that the phytoplasmas detected in both insects and inoculated seedlings belonged to 16SrIX-B (D). The present manuscript is the first known report for a leafhopper vector of '*Ca.* P. phoenicium' and shows that the incubation period of the disease in plants may be longer than 1 year. The importance of phytosanitary control measures, the adoption of a national strategy and regional cooperation in order to contain the further spread of the disease are discussed.

Keywords

Asymmetrasca decedens; '*Ca*. Phytoplasma phoenicium'; leafhopper; phytoplasma transmission; phytoplasma vector; salivary glands.

Introduction

In the 1990s, a devastating disease on almond trees appeared in Lebanon, characterised by proliferation, small yellowish leaves, bushy growth, dieback and appearance of witches'-broom on the stems. Infected trees either did not produce any fruits, or produced a limited number of deformed fruits, resulting in practically 100% marketable yield loss. The disease was named almond witches'-broom (AlmWB), it spread rapidly and killed about 100,000 trees over a period of 10 years (Abou-Jawdah *et al.*, 2002). The disease was associated with '*Candidatus* Phytoplasma phoenicium' strains belonging to the pigeon pea witches'- broom (PPWB) group (16SrIX) (Abou-Jawdah *et al.*, 2002; Verdin *et al.*, 2003), subgroup 16SrIX-B (also called 16SrIX-D) and its genetic variants (subgroups 16SrIX-F and -G) (Molino Lova *et al.*, 2011). More recent surveys identified over 40,000 new almond, peach and nectarine trees infected with AlmWB (Molino Lova *et al.*, 2011). The disease epidemic spreads rapidly from coastal areas to high mountainous areas (>1200 m), encompassing several ecological niches. Furthermore, AlmWB was found to infect properly managed orchards, abandoned orchards and isolated wild trees. These observations suggested the presence of efficient aerial vectors.

Phytoplasmas are bacteria devoid of cell walls that are capable of growing in specific insect vectors as well as in the phloem tissue of host plants (Lee *et al.*, 2000). Phytoplasmas are mainly transmitted by phloem-feeding insects which belong to the families Cicadellidae, Cixiidae, Psyllidae, Cercopidae, Delphacidae, Derbidae, Meenoplidae and Flatidae in the order Hemiptera (Weintraub & Beanland, 2006). Of these families, only some species can act as vectors because of vector-pathogen-host specificity (Bosco *et al.*, 2009). The most common vectors of phytoplasmas appear to be leafhoppers (Cicadellidae), planthoppers (Cixiidae) and psyllids (Psyllidae) (Weintraub & Gross, 2013).

Field surveys were conducted in AlmWB-infested almond orchards located in South and North Lebanon. *Asymmetrasca decedens* (Hemiptera, Cicadellidae, Typhlocybinae) was the most abundant hemipteran species representing over 82% of total leafhoppers caught in sticky yellow

traps and in malaise traps (Dakhil *et al.*, 2011). *Asymmetrasca decedens* is a polyphagous species which may feed on a wide variety of economic crops such as peach, almond, citrus, grapevine, beans, beet, cotton, lucerne and potatoes (Jacas *et al.*, 1997). PCR tests showed that *A. decedens* along with eight other leafhopper species carried 16SrIX phytoplasma and may represent potential vectors (Dakhil *et al.*, 2011). However, phytoplasmas may be acquired by insects but may not be transmitted during feeding (Marzachì *et al.*, 2004). Phytoplasmas are transmitted in a persistent propagative manner (Marzachì *et al.*, 2004). For an insect carrier to become a vector, an intimate association with the phytoplasma is required (Suzuki *et al.*, 2006). The phytoplasma must be able to multiply in the vector, circulate in the hemolymph, accumulate in the salivary glands and be secreted with the saliva upon feeding on plant phloem cells (Hogenhout *et al.*, 2008). Such a cycle may take several days to several months. For example, in the case of *Cacopsylla pruni*, the vector of '*Ca.* P. prunorum' (agent of European stone fruit yellows, ESFY), most transmissions occur only after an effective latency of 8 months (Th´ebaud *et al.*, 2009).

Only appropriate transmission tests can provide definitive evidence of the role of an insect as a vector, while the detection of a phytoplasma in an insect is just considered as a preliminary step. Moreover, controlled transmission tests are not always straightforward. Many vectors do not survive easily in captivity, and various life stages may vary in the efficiency of transmission. Symptom development on the inoculated plants and incubation period may also span from 1 week to more than 24 months (Hogenhout *et al.*, 2008). In the case of ESFY, it may take 4–5 months and some hosts may remain symptomless (Carraro *et al.*, 1998). Hence, molecular techniques may play an important role in phytoplasma detection in asymptomatic and susceptible hosts during the incubation or latent period (Mehle *et al.*, 2010). The major objective of this work was to investigate the capacity of *A. decedens* to transmit AlmWB phytoplasma.

Materials and methods

Plant material

Certified tissue culture seedlings of two stone fruit rootstocks were imported from Italy, peach almond hybrid 'GF-677' rootstock (Prunus persica×Prunus dulcis (Mill.) D.A. Webb.) and peach seedling 'GF-305'. The seedlings (30–35cm in length) were transplanted into 25 cm diameter pots containing a mixture of potting soil, sand and perlite (2:1:1) and maintained in insect-proof cages, within an insect-proof net house.

Leafhoppers collection and transmission trials

Insects were collected in two stone fruit orchards infested with AlmWB, an almond orchard in Feghal, North Lebanon, and a nectarine orchard in Kfarkela, South Lebanon. A hand-held mechanical aspirator (D-Vac Vacuum Insect Net-Model 122, Rincon-Vitova Insectaries, Ventura, CA, USA) was used to collect insects from AlmWB-infected trees. *Asymmetrasca decedens* leafhoppers were sorted out by mouth aspirator and transported to a cold room where they were counted and dispensed into falcon tubes. Transmission trials were initiated the day of insect collection. Collected insects were released either into 10 small insect-proof cages containing a single seedling each or into 4 large cages containing 6 seedlings per cage. Twenty-five leafhoppers were used for each seedling in individual cages and 150 leafhoppers were released into each of the bigger cages. The leafhoppers were allowed an inoculation access feeding on GF-677 and GF-305 seedlings for 30 days. Afterwards, the insects were sprayed with insecticides at 5-day intervals (spinosad and acetamiprid, in alternation). A total of 34 seedlings (15 GF-305 and 19 GF-677) were inoculated in these tests. Two types of controls were used, six healthy seedlings maintained in an insect-proof cage and six healthy seedlings maintained in another cage but subjected to feeding by a total of 150 leafhoppers collected from a nectarine orchard in Wata Al Jawz, an AlmWB-free region. Observations on symptom development were recorded at weekly intervals. Leaf samples were collected periodically (1, 2, 3 and 12 months post-inoculation [mpi]) from treatments and control seedlings and tested by polymerase chain reaction (PCR) for the presence of '*Ca*. P. phoenicium'. Five batches of samples each containing five leafhoppers were collected from each of the

AlmWBinfested almond and nectarine orchards or from AlmWBfree regions to be tested by PCR, as well as one batch that was taken from each cage during the transmission studies. Moreover, the salivary glands of nine selected *A. decedens* specimens were dissected out of the insects and analysed by PCR in order to assess presence of '*Ca*. P. phoenicium' and to get further confirmation of the insect vectorship capability. Briefly, leafhopper heads were removed from the rest of the insect body, and the salivary glands from subsets of three insects were dissected and pooled into a microfuge tube (1.5 mL) containing 25 µL Sodium Chloride-Tris-EDTA (STE) Buffer. DNA in the samples was extracted and used for phytoplasma detection by PCR.

Molecular diagnosis

Total nucleic acid extraction

For plant samples, the total nucleic acids (TNA) were extracted from 100mg of leaf midribs following the CTAB protocol as described previously (Abou-Jawdah *et al.*, 2002). Samples from leafhoppers collected from AlmWB-infested orchards or from AlmWB-free orchards were also tested by PCR. Groups of five A. decedens insects were put in a 1.5mL Eppendorf tube and the TNAs were extracted according to the procedure described by Marzachì *et al.* (1998). The final TNA precipitate was suspended in 50 µL of sterile water. TNA extracts were analysed in a 1% agarose gel electrophoresis to determine their quality. Total DNA were quantified using a NanoDrop 2000c (NanoDrop Technologies, Wilmington, DE, USA) and stored at –20°C.

Phytoplasma detection by polymerase chain reaction

The semi-specific primer pair, ALW-F2/ALW-R2, which amplifies a DNA fragment of 390 bp from 16SrIX phytoplasmas, was used in PCR assays as described previously (Abou-Jawdah *et al.*, 2003). Each amplification reaction was performed in 20 µL reaction mixture containing 2 µL of template DNA (20–50 ng), 10 µL of REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, St Louis, MO, USA), 0.25 µM of each primer and 7 µL of sterile water. Amplifications were done

147 with a Bio-Rad Thermal Cycler 1000 (Bio-Rad Laboratories, Hercules, CA, USA). Positive
148 leafhoppers and inoculated plant samples detected by previous direct PCR, were retested by nested
149 PCR using forward primer P1 (Deng & Hiruki, 1991) and reverse primer P7 (Smart *et al.*, 1996)
150 followed by R16F2n/R16R2 (F2n/R2) to confirm phytoplasma attribution to '*Ca*. P. phoenicium'
151 following nucleotide sequence analyses (Lee *et al.*, 1998; Abou-Jawdah *et al.*, 2003). The F2n/R2
152 amplicons were purified with the Illustra™ GFX PCR DNA and Gel Band Purification kit (GE
153 Healthcare, Waukesha, UK) and cloned using the pGEM-T Easy Vector System II (Promega,
154 Madison, WI, USA). Sequencing of the cloned PCR products was performed at Macrogen Inc.
155 (Seoul, South Korea) in both forward and reverse directions. The nucleotide sequence data were
156 assembled by employing the Contig Assembling programme of the sequence analysis software
157 BIOEDIT, version 7.0.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html).
158
159 *16S rRNA gene analysis*
160 F2n/R2 fragments amplified from insect bodies, salivary glands and inoculated seedlings were
161 sequenced and a representative sequence from each host was submitted to GenBank. Sequences
162 were compared with the Gen- Bank database using the algorithm BLASTN (http://www.
163 ncbi.nim.nih.gov/BLAST/) in order to determine the best sequence identity hit and to establish the
164 species affiliation of phytoplasmas detected in *A. decedens* specimens and in plants used in
165 transmission trials. Multiple alignment using Geneious R6 (v6.0.5, Biomatters, Auckland, New
166 Zealand) was performed for sequences obtained from the insect vectors and the inoculated seedlings
167 to ascertain that the same phytoplasma species occur in both hosts.
168 The alignments were exported to the MEGA 6 software (Tamura *et al.*, 2013) for distance and
169 phylogenetic analyses. Neighbour-Joining (NJ) (Saitou & Nei, 1987) tree was constructed using 500
170 replicates for bootstrap analysis (Felsenstein, 1985) and *Acholeplasma laidlawii* was used as an out
171 group.
172

Results

Symptom development

The transmission trials using leafhoppers were initiated on May 2012, and symptoms were monitored at weekly intervals. Symptoms started to develop on 16 inoculated seedlings within 25 days post-inoculation (dpi). By 30 dpi, 4 out of 15 GF-305 seedlings and 12 out of 19 GF-677 seedlings had developed symptoms. The observed symptoms were not typical of AlmWB phytoplasma; they consisted mainly of downward leaf curling or rolling and proliferation of new growth at the leaf axils. The curled leaves were smaller than normal leaves but were not chlorotic; moreover, many growing tips were burned. Similar symptoms were observed on some of the control plants which were inoculated with leafhoppers originating from an area free of AlmWB phytoplasma.

In August, 3 mpi, a new flush of growth appeared which looked normal. During winter, the leaves dropped and in early March the new growth had vigorous growth that was similar to that emerging from healthy controls.

Molecular diagnosis

At 1 and 2 mpi, PCR tests showed that out of the 34 inoculated seedlings, 16 gave positive results using the AlmWB semi-specific primer pair, ALW-F2/R2 (Table 1). When the new summer flush appeared in August (about 3 mpi), the new growth looked normal. Young leaf samples were collected and the PCR results showed that only three samples of the GF-305 seedlings were positive out of the four that were positive at 2 mpi, while with the GF-677 seedlings, only 8 seedlings tested positive out of the 12 seedlings that were previously positive. Both populations of A. decedens collected from almond or nectarine orchards were able to transmit the phytoplasma (Table 2). The direct PCR results were confirmed by nested PCR, sequencing and BLAST analyses. During winter, all the leaves dropped. In the following spring season, new growth emerged which appeared normal. PCR tests were repeated and all the seedlings, whose summer flush tested positive, were also

positive with the new spring growth (Tables 1 and 2, Fig. 1). Therefore out of a total of 34 inoculated seedlings only 11 seedlings got infected as revealed by PCR tests about 1 year post-inoculation; however, none developed AlmWB-associated symptoms.

Leafhoppers collected from Wata Al Jawz, an AlmWBfree area, gave negative PCR results using the semispecific primer pair, ALW-F2/R2. The 14 composite samples of A. decedens leafhoppers used in the inoculation tests gave positive results (Fig. 2). When the salivary glands of three representative *A. decedens* samples, each composed of pooling salivary glands of three insects collected from the AlmWB-infested orchard also tested positive for Alm WB (Fig. 2).

Sequences of the F2n/R2 amplified products from four samples, one sample each from the insect body (GenBank accession number: KF359551), the salivary glands (KF488577), the inoculated GF-677 seedlings (KF500029) and from GF-305 (KF500030) were deposited in GenBank. BLAST analysis showed 99.9% identity with '*Ca*. P. phoenicium'. Results obtained by the NJ tree showed that phytoplasmas present in the insects and in the inoculated seedlings were all similar and were members of the species '*Ca*. P. phoenicium', subgroup 16SrIX-B (D) (Fig. 3).

Discussion

'*Candidatus* Phytoplasma phoenicium' is associated with a devastating and lethal disease of almond, peach and nectarine that has so far only been reported in Lebanon and Iran (Abou-Jawdah *et al.*, 2002; Verdin *et al.*, 2003). '*Candidatus* Phytoplasma phoenicium' has all the characteristics of a severe quarantine pathogen. It is associated with a lethal disease of three major stone fruit crops; cannot be controlled by classical control measures, has the potential to occupy different ecological niches, and its unaided transmission across natural barriers seems limited because it has been reported in only two countries. The rapid spread of AlmWB in Lebanon suggests the presence of one or more efficient vectors. Previous surveys carried out in Lebanese orchards showed that several leafhopper species are carriers and may represent potential vectors of '*Ca*. P. phoenicium'

(Dakhil *et al.*, 2011). In this study, transmission trialswere performed with *A. decedens*, the most dominant leafhopper detected in stone fruit orchards, to investigate its vectoring activity.

The initial symptoms, observed 1 mpi, were not attributed to phytoplasma infection. They were most likely correlated with leafhopper feeding, because leafhoppers feed mainly on leaves and cause a symptom known as the 'hopperburn' (Backus *et al.*, 2005). In eastern Spain, a high infestation of *A. decedens* in almond orchards induced stunted shoots with small curled leaves that were only observed on young flush. The damage was mainly destructive to nursery seedlings, young nonbearing trees and over-grafted plants (Jacas *et al.*, 1997).

Transmission trials performed in this study showed that 11 out of 34 inoculated stone fruit seedlings got infected with '*Ca*. P. phoenicium', as evidenced by PCR detection in emergent tissue 3 and 12 mpi. PCR data were confirmed by BLASTN and iPhyClassifier analyses of nucleotide sequences, highlighting that the same phytoplasma '*Ca*. P. phoenicium', subgroup 16SrIX-B (D) was detected in the leafhoppers used in transmission trials and in the inoculated seedlings. The detection of '*Ca*. P. phoenicium' in leafhoppers and in inoculated certified seedlings provides strong evidence for the role of *A. decedens* as a vector of '*Ca*. P. phoenicium'. Interestingly, both populations of *A. decedens* collected from almond or nectarine orchards located in two different regions were able to transmit the phytoplasma. A possible explanation for getting negative PCR results, 3 and 12 months after inoculation, from five seedlings which showed positive results, 1 and 2 mpi, is that the vectors successfully inoculated the leaf tissues but the phytoplasma failed to induce systemic infection and thus was not detected in the new growth that emerged later on.

Two important features resulting from transmission experiments should be discussed. (1) The number of insects used in transmission trials and (2) The long incubation period. First, the high number of insects used per seedling led to transient phytotoxicity symptoms. In future tests, to reduce phytotoxicity symptoms, a lower number of leafhoppers should be used per plant and one seedling per cage may be preferable. However, it is worth mentioning that *A. decedens* was the most abundant species in a surveyed almond orchard and 544, 2760 and 3901 insects were collected

on six yellow sticky traps during the months of March, April and May 2002, respectively (Dakhil *et al.*, 2011). These results were confirmed in a recent survey with a slight difference in timing, whereby 3 800, 11 700 and 7 200, were trapped in May, June and July 2012 (H. Abdul-Nour, personal communication). This experiment was conducted in insect-proof cages under greenhouse conditions, and a large number of leafhoppers died within 2 weeks of transfer to the insect-proof cages, suggesting that the survival potential of *A. decedens* under the experimental conditions was limited. Moreover, in an effort to study the transmission characteristics, mainly the latency period in A. decedens, several attempts failed to rear this leafhopper in insect-proof cages.

Even though several leafhopper species belonging to the Cicadellidae family and sub-families were reported to transmit phytoplasmas, only one report mentions *A. decedens* as a potential phytoplasma vector based on transmission trials (Pastore *et al.*, 2004). Moreover, most leafhoppers in the subfamily Typhlocybinae are reported to be mesophyll feeders (Nault & Rodriguez, 1985). This characteristic reduces their potential to act as phytoplasma vectors. However, *A. decedens* and its close relative, *Empoasca decipiens*, the two predominant species in stone fruit orchards in Lebanon, were found to be carriers of AlmWB phytoplasma (Abou-Jawdah *et al.*, 2011; Dakhil *et al.*, 2011). In Italy, these two species were also found to be positive for ESFY in PCR assays. Preliminary trials showed that *Empoasca decedens* (a synonym to *A. decedens*) may transmit ESFY from *Prunus armeniaca* L. to *P. armeniaca* (Pastore *et al.*, 2004), however, more recent trials failed to confirm it (Pastore *et al.*, 2001). In Cuba, 67 *Empoasca* spp. samples were examined by PCR and 63 were found carrying '*Ca*. P. aurantifolia' (Arocha *et al.*, 2006).

Second, phytoplasma symptoms can start to appear on plants as soon as 7 days after the insect has introduced the phytoplasma, but this is not always the case because the symptoms may also take 6 to over 24 months to develop depending on both the phytoplasma and the plant host species (Hogenhout *et al.*, 2008). Even in grafting experiments, symptoms may take a long time to appear, for example, it took around 18 months for the ESFY symptoms to appear on patch grafted 3-yearold plum and peach seedlings (Pastore *et al.*, 2001). Flavescence dor´ee of grapevine is symptomless in

some cultivars, and it also has a long (up to three years) latent period before symptoms can be seen (Belli *et al.*, 2010). These data may be explained by the fact that phytoplasmas live inside plants as symbiont but they can become pathogens in later stages when suitable conditions occur such as special weather conditions or changes in the production practices (Mehle *et al.*, 2010). The long incubation period poses a problem in early visual disease detection, and may have played a role in the spread of the AlmWB disease to distantly isolated regions in Lebanon, through the production of AlmWB-infected asymptomatic seedlings. This observation necessitates stricter phytosanitary control measures on stone fruit nurseries andmother stock plants. For this reason, specific AlmWB detection methods based on PCR and qPCR are being developed to survey accurately the plant materials within the stone fruit nurseries.

The rapid spread of the disease over distantly located regions, and the detection of AlmWB phytoplasma in eight other leafhopper species (Dakhil *et al.*, 2011) may indirectly represent a hypothesis that other potential vectors for AlmWB phytoplasma may be present. Effectively, for many phytoplasma diseases more than one vector was reported. For example, '*Ca*. Phytoplasma solani' (16SrXII-A), agent of the bois noir (BN) disease of grapevine (Quaglino *et al.*, 2013), is transmitted by Cixiidae; *Hyalesthes obsoletus* is the major reported vector, but recently *Reptalus panzeri* was reported also as a natural vector of the disease, and several other vectors are suspected (Cvrkovi´c *et al.*, 2013). The other potential vectors of AlmWB phytoplasma may not be common pests of stone fruits, but may infest stone fruits only during part of their life cycles or occasionally when their natural hosts become limited. For example, even though the vector of 'bois noir' (BN) *Hyalesthes obsoletus* cannot live on grapevines, it feeds on different crops and has been proved to accidentally transmit the phytoplasma from weeds to grapevine (Maixner, 1994; Weintraub *et al.*, 2009). Therefore, the preferred host(s) for some suspected phytoplasma vectors may be weeds or other plants (Maniyar *et al.*, 2013). In view of the concurrent results that Cixiidae may play a role in '*Ca*. P. phoenicium' transmission (R. Tedeschi, personal communication) from 'wild' or alternative

hosts to stone fruits, it seems that *A. decedens* plays a major role in spreading the disease within or to nearby stone fruit orchards.

In conclusion, the detection of '*Ca*. P. phoenicium' in the salivary glands of *A. decedens* along with the transmission trial results confirm that this leafhopper is a vector of '*Ca*. P. phoenicium', the suspected causal agent of AlmWB. This constitutes the first report of *A. decedens* as a vector of AlmWB disease and another experimental proof that it may act as a phytoplasma vector in stone fruits (Pastore *et al.*, 2004). Further research is needed on the modality of transmission (efficiency of different life stages, latency period), and the possibility of the occurrence of other potential vectors. Therefore, further studies must be conducted on the epidemiology of the disease including its alternative hosts and their relative importance in disease spread. In addition to vector control, screening for resistant germplasms may also represent a possible option to perform, although all the almond varieties present in Lebanon are susceptible. In view of the importance and severity of AlmWB disease, regional and international cooperation should be established in order to develop an integrated pest management approach to contain the disease, prevent its further spread and to reduce its negative impact on the stone fruit industry.

Acknowledgements

The AUB team is greatly thankful to his Excellency the Minister of Agriculture for his personal follow up on this important problem and to the Ministry Officers, Mrs. Lama Haidar andMrs. Rola Al-Achi for their devotion and cooperation. Our thanks are also extended to the AVSI officers, Mrs. Chantal Mahfoud and Mrs. Nadine Hanna, who greatly facilitated the progress of these activities and to Dr. Imad Saoud for his critical review of the manuscript. This research was partially funded by a grant from the Italian Cooperation and the Association of Volunteers in International Service (AVSI) as well as by the National Program for the Improvement of Olive Oil's Quality and Actions against the Diffusion of Stone Fruit Phytoplasma 'The Project' (Project No. AID 9627) implemented by the Lebanese Ministry of Agriculture.

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Table 1 PCR detection of '*Ca*. P. phoenicium' with ALW-F2/ALW-R2 primers in stone fruit seedlings 1, 2, 3 and 12 mpi using A. decedens as a vector

Variety	Seedling code	1 mpi	2 mpi	3 mpi[a]	12 mpi[a]
GF-305	AF3	+	+	+	+
	AF7	+	+	+	+
	AK4	+	+	+	+
	AK5	+	+	–	–
	AK10, 11, 12, 13, 14, 15	–	–	–	–
	AF8, 9, 10, 11, 12	–	–	–	–
GF-677	AF1	+	+	–	–
	AF2	+	+	+	+
	AF4	+	+	–	–
	AF5	+	+	+	+
	AF6	+	+	–	–
	AK1	+	+	+	+
	AK2	+	+	+	+
	AK3	+	+	–	–
	AK6	+	+	+	+
	AK7	+	+	+	+
	AK8	+	+	+	+
	AK9	+	+	+	+
	AK16, 17, 18	–	–	–	–
	AF13, 14, 15, 16	–	–	–	–
Total		16/34	16/34	11/34	11/34

[a]Results for 3 and 12 mpi are for leaf samples collected from new growths that were not subjected to direct leafhopper feeding.

Table 2 Transmission of '*Ca*. P. phoenicium' by *A. decedens* to seedlings of two stone fruit rootstocks (GF-305 and GF-677)

	No. of PCR positive/total tested	
	GF-305	GF-677
Single seedling/cage	0/1[a]	1/1[a]
	1/1[b]	1/1[b]
	0/1[a]	0/1[a]
		1/1[a]
		1/1[a]
		1/1[b]
		0/1[b]
Multiple seedlings/cage	2/6[a]	2/6[a]
	0/6[b]	1/6[b]
Total	3/15	8/19

The leafhoppers were collected from AlmWB-infested almond or nectarine orchards and the inoculations were conducted using either single or multiple seedling(s) per cage.

[a]*Asymmetrasca decedens* collected from nectarine orchard, Kfarkela region, South Lebanon.

[b]*Asymmetrasca decedens* collected from almond orchard, Feghal region, North Lebanon.

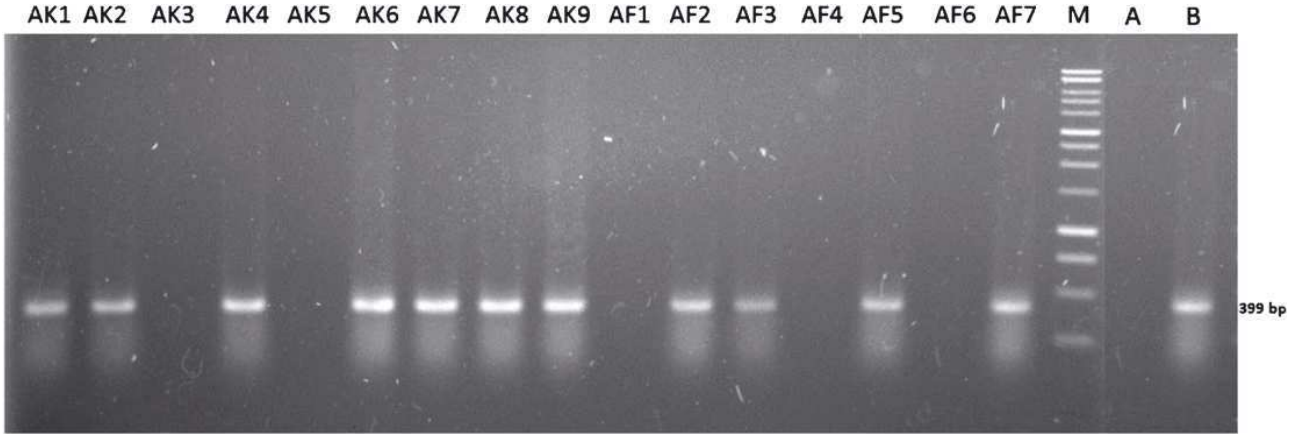

Figure 1 Agarose gel electrophoresis of PCR products using the semi-specific primer pair ALWF2/ALWR2 amplifying an amplicon of about 390 bp of the 16S-ITS23S region. DNA samples were extracted at 12 mpi, from 16 seedlings inoculated with *A. decedens* carrying '*Ca*. P. phoenicium. A: Healthy seedling, B: '*Ca*. P. phoenicium' positive control, M: 1 Kbp ladder.

Figure 2 Agarose gel electrophoresis of PCR products using the semispecific primer pairALWF2/ALWR2 amplifying an amplicon of about 390 bp of the 16S-ITS23S region. DNA samples were extracted from A: body and B: salivary glands of *A. decedens* collected from AlmWB-infested orchard, C: *A. decedens* collected from healthy orchard, D: healthy control, E: '*Ca*. P. phoenicium' positive control, M: 1 Kbp ladder.

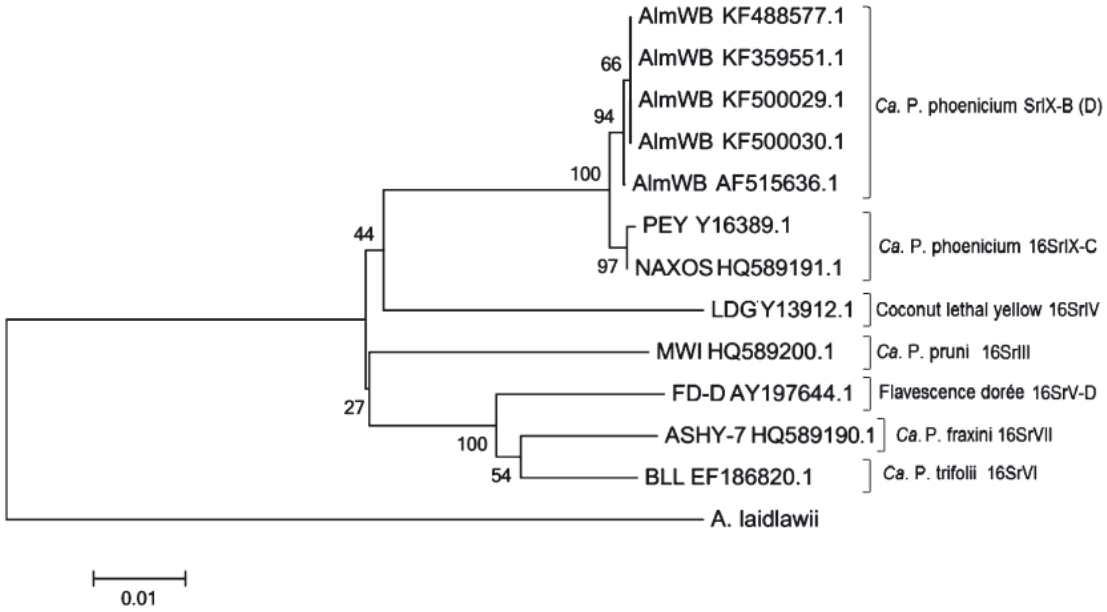

Figure 3 Neighbour-joining tree of R16F2n/R16R2 amplified fragment of the 16S rRNA gene. Numbers at the nodes indicate bootstrap values; bars, substitutions per nucleotide position; 16S rRNA GenBank sequence accession number is indicated following the strain acronym; 16S rRNA group and subgroup are indicated following the phytoplasma strain; *A. laidlawii* (NR074448.1) was used as an outgroup. AlmWB sequences are from insect body (KF359551) and the salivary glands (KF488577) of *A. decedens*, from the inoculated seedlings of GF-677 (KF500029) and GF-305 (KF500030). The AlmWB reference strain (AF515636) was also used as well as seven more reference phytoplasmas closely related to AlmWB.