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RESEARCH PAPERS

The potential of grafting with selected stone fruit varieties for management of almond witches' broom

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Summary. A lethal disease of stone fruit trees, characterized by proliferation of axillary shoots and witches' broom symptoms, has caused severe problems for more than 200,000 almond, peach and nectarine plants in Lebanon since the 1990s. The agent associated with almond witches' broom (AlmWB) was identified as '*Candidatus* Phytoplasma phoenicium', belonging to subgroup 16SrIX-B. Management of the disease has relied on uprooting of affected trees. Since no disease-resistant cultivars have been identified, grafting experiments in the field and in the greenhouse we performed to develop a new option for the integrated management of the disease. AlmWB-affected almond trees were grafted with apricot or plum scions, and their growth was symptomless for over 2 years in the field. Similarly, in greenhouse trials, grafting AlmWB-affected almond scions onto seedlings of plum and apricot resulted in growth of symptomless shoots. One year post-grafting in the greenhouse the phytoplasma was not detected by PCR in almond grafted on Angeleno and Red plum and Early Blush apricot used as rootstocks. The phytoplasma was, however, detected in almond scions grafted on Farclo apricot and Jawhara plum, although their growth was symptomless. Shoots developing from Farclo apricot grafted on AlmWB-affected trees in the field showed severe symptoms 2 months after grafting but recovered 3 months later, and remained symptomless for about 2.5 years. Similarly, in the greenhouse trial, the growth of phytoplasma-infected scions grafted on Early blush apricot developed symptoms 2 months after grafting, did not show symptoms 2 months later, and remained symptomless 1 year later. Quantitative PCR analysis of almond scions grafted on Early blush apricot seedlings confirmed reduction of phytoplasma titre from 44 GU/ng DNA to below detection level.

Key words: '*Candidatus* Phytoplasma phoenicium', qPCR, resistant cultivars, grafting.

Introduction

In the early 1990s, almond witches' broom (AlmWB) was discovered in three confined regions in Lebanon (mainly North and South Lebanon, and the Bekaa valley), where almond is a main cash crop (Abou-Jawdah *et al*., 2002). The disease spread and became epidemic affecting over 200,000 almond, nectarine and peach tress (Molino-Lova *et al*., 2011). It was also reported to cause severe almond losses in Iran (Verdin *et al*., 2003). Molecular identification and characterization established a strict association between the AlmWB disease and the presence of a phytoplasma belonging to the pigeon pea witches' broom group (16SrIX), subgroup 16SrIX-B (Abou-Jawdah *et al*., 2002). This AlmWB-associated phytoplasma was then classified as '*Candidatus* Phytoplasma phoenicium' (Verdin *et al*., 2003).

Phytoplasmas are obligate parasites characterized by the lack of cell walls, pleomorphic shape, and individual size ranging between 200 and 800 nm (Firrao *et al*., 2004). They inhabit plant phloem tissues, as well as haemolymph, gut lumen and saliva of insect vectors, which are mainly leafhoppers, planthoppers and psyllids (Weintraub and Beanland, 2006; Bertaccini and Duduk, 2010). Phytoplasmas are also

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known to be transmitted through vegetative propagation such as cuttings and grafting (Aldaghi *et al*., 2007), dodder (Přibylová and Špak, 2013) and possibly through seeds (Calari *et al*., 2011). Phytoplasmas are responsible for numerous diseases on crops worldwide, and disease symptoms vary depending on host species, crop variety, developmental stage, and environmental conditions (Dickinson *et al*., 2013; Bertaccini *et al*., 2014). In plants, phytoplasmas interfere with the balance of growth regulators, causing abnormalities in development and some metabolic processes leading to a group of characteristic symptoms such as witches' broom and virescence. Other common symptoms of phytoplasma infections are yellowing of the leaves, stunting of plants, phloem necrosis, sterility of flowers and abnormal internode elongation (Hogenhout *et al*., 2008; Bertaccini and Duduk, 2010; Salehi *et al*., 2016).

Management phytoplasma diseases is usually through insecticidof e applications to control insect vectors. However, pesticides are expensive, do not always decrease the infection rates, and may have negative impacts on the human health and the environment. Control methods such as planting resistant varieties or grafting resistant rootstocks are the best options. Breeding of a rootstock resistant to apple proliferation phytoplasma (AP), yet with favourable agronomic traits such as yield, size and quality of fruit, has been successfully accomplished through crossing *Malus seiboldii* with M9 rootstock (Jarausch *et al*., 2011).

Phytoplasmas are restricted to the phloem of host plants, and their titres are normally low in plant tissues, so sensitive detection methods such as PCR and quantitative PCR (qPCR) (Christensen *et al*., 2013) should be employed for their detection. '*Ca*. P. phoenicium' was detected in different tissues of almond and nectarine plants by a highly specific AlmWB phytoplasma qPCR assay (Jawhari *et al*., 2015), indicating that the phytoplasma titre is greatest in phloem and root tissue of almond, nectarine and peach.

To date, management of AlmWB in Lebanon depends mainly on eradication of infected trees in regions where the disease was introduced and where the infection rates are still low, while in regions where the disease is endemic, no control measures have been taken except trying to restrict its further spread.

'*Ca*. P. phoenicium' preliminary transmission tests were performed in Lebanon in mid-June 2001 by grafting AlmWB-affected almond and nectarine buds on almond, plum, cherry, apricot, peach and nectarine seedlings. After grafting onto almond, peach and nectarine, the scions and rootstocks both developed symptoms, including bush-like growth from the plant bases and weak light green leaves with short internodes. In contrast, AlmWB-affected buds grafted on seedlings of apricot and plum did not develop symptoms (Abou-Jawdah *et al*., 2003). In the present study, a short-term greenhouse trial and a long-term field trial were carried out to evaluate the effectiveness of grafting with resistant stone fruit varieties for management of AlmWB, with the aim of reintroducing almond production in AlmWB-affected areas, and to limit the negative economic consequences of the disease when it is accidentally introduced into new regions where eradication is difficult.

Materials and methods

Plant material and experimental layout

For the greenhouse trials, 38 2-year-old seedlings were obtained from a nursery located in the Bekaa region, in an area free from AlmWB. The plants consisted of eight almond seedlings, 18 plum seedlings (six Jawhara, six Angeleno and six Red plum), and 12 apricot seedlings (six Early Blush and six Farclo). The plums and apricots were grafted onto Myrobalan 29C. AlmWB-affected almond scions were collected from 5-year-old almond trees showing characteristic symptoms which also tested positive to '*Ca*. P. phoenicium' by PCR. Scions were wrapped in moist cloth, put in plastic bags and stored at 5°C until use. T-grafting on the above-mentioned stone fruit seedlings was carried out on 13 April, 2015 on minimum four seedlings per variety. A total of 25 seedlings were grafted with scions originating from '*Ca*. P. phoenicium'-infected trees and 13 seedlings served as negative controls (Table 1).

Individual seedlings were transplanted into 20 L capacity plastic pots which were placed inside a single span greenhouse (12 × 6 m) equipped with a double door and 50 mesh insect-proof nets. Pots were randomly distributed with approx. 1 m between pots to prevent contact between plants. Seedlings were monitored for symptom development every month; phytoplasma detection and quantification were performed by PCR and qPCR each month from July 2015 until September 2015, then in June 2016.

Table 1. Greenhouse grafting experiment: number of AlmWB phytoplasma-infected scions that successfully grew 2 months post grafting.

Treatments	Number of seedlings	Negative control	Grafted	Scion survival
Almond (Halawani)	8	3	5	4/5
Plum (Jawhara)	6	2	4	2/4
Plum (Angeleno)	6	2	4	3/4
Plum (Red)	6	2	4	2/4
Apricot (Early Blush)	6	2	4	2/4
Apricot (Farclo)	6	2	4	3/4

A long-term field experiment, commencing in April 2013, was carried out in Feghal, a region severely affected by AlmWB. Scions from different plum and apricot varieties were grafted on almond trees infected with AlmWB phytoplasma and exhibiting severe disease symptoms. Two infected almond trees were each grafted with one of the following varieties: plum Abou-Riha, plum Janarek, plum Fortune, plum Santarosa or apricot Farclo, resulting in five treatments with two replicates per treatment.

Total nucleic acid extraction

Total nucleic acids (TNAs) were extracted from 150 mg of leaf midribs from each test plant, using a CTAB-based protocol (Abou-Jawdah *et al*., 2002). The extracted TNAs were suspended in 50 µL of deionized water, analyzed in a 1% agarose gel electrophoresis and quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). The TNA extracts were stored at -20°C.

Phytoplasma detection by PCR

Total nucleic acids from plants in the greenhouse trials were extracted from leaf midribs of almond scion growths in July, August and September 2015, and June 2016. These were used as templates for PCR-AlmWB phytoplasma detection using specific primer pairs AW16sF / AW23sR that amplify a 492 bp region, as described by Jawhari *et al*. (2015). TNAs from plants not grafted were used as negative controls. TNAs from AlmWB-affected trees, growing in an infected orchard in Feghal and showing characteristic symptoms were used as positive controls (Quaglino *et al*., 2015).

Phytoplasma detection from samples grafted in the field was carried out using PCR on TNAs extracted at 2, 5 and 6 months after grafting, followed by yearly extractions until 2016. In these PCRs a '*Ca*. P. phoenicium' semi-specific primer pair, AlmF2 / AlmR2, amplifying a 390 bp region, was used (Abou-Jawdah *et al*., 2003). PCR products were separated in a 1% agarose gel by electrophoresis at 110 V for 30 min. and stained in ethidium bromide solution (0.5 g L^{-1}) for 30 min, followed by destaining in distilled water for 5–10 mins. Gel visualization was carried out in a Gel Doc XR+ system (Bio-Rad Laboratories).

Quantitative PCR protocol

Quantification of '*Ca.* P. phoenicium' was performed using qPCR for the shoots that developed from scions grafted on apricot cultivars Early blush and Farclo, and on plum cultivars Red, Angeleno and Jawhara, along with the positive control described above and negative controls from the seedlings that were not grafted. TNAs extracted from the above mentioned samples during July, August and September, 2015, and June 2016, were subjected to qPCR assays targeting '*Ca.* P. phoenicium' through primer pair AWsF / AWsR (spanning the intergenic spacer region and the 23SrRNA region of '*Ca*. P. phoenicium') amplifiying a 132 bp amplicon. The internal control gene was amplified by primer pairs Prun18S-F / Prun18S-R targeting the 18S rRNA gene of *Prunus dolcis* (Genbank accession number: DQ886376) (Pafundo *et al*., 2011). The qPCR assays were performed as described by Jawhari *et al*. (2015) in Hard-Shell® 96-Well PCR

plates (Bio-Rad Laboratories), in a CFX96 Touch thermal cycler (Bio-Rad Laboratories).

Generation of standard curves for qPCR

'*Ca.* P. phoenicium' and internal control standard curves were generated based on the protocol described by Jawhari *et al.* (2015). Plasmid copy number calculation was performed using the following formula: copy number = concentration of plasmid/ [(size of insert + size of vector) × 660)/ (Avogadro's number)], where size of insert = 579 bp, size of vector = 3015 bp and Avogadro's number = 6.022×10^{23} so, 127.8 ng μL^{-1} of pGEM-T easy vector (3,015 + 579) consists of 3.7×10^{10} plasmids.

For normalization of phytoplasma titre, a standard curve of plant internal control was generated from healthy almond phloem DNA., An ND-1000 spectrophotometer (NanoDrop Technologies) was used for DNA quantitation, and DNA was serially diluted from a starting quantity of 295 ng. The developed standard curve for '*Ca.* P. phoenicium' using the recombinant plasmid had an efficiency of 108.7%, and the curve obtained from healthy almond phloem DNA had an efficiency of 91.5% (Figure 1). Copy number calculation of phytoplasma was performed using the formula Nt=10^((Cq-b)/a), where Nt is the target copy number, Cq or Ct the quantitation cycle of each sample, and a and b are, respectively, the slope and the intercept of each standard curve (Jawhari *et al*., 2015). Efficiencies of the standard curves were calculated using the Bio-Rad CFX Manager software and the formula E= [10^(-1/slope) - 1] * 100 (Hren *et al*., 2007).

Statistical analyses

Statistical analyses were performed using SPSS, version 21. Almond scion recovery from phytoplasma infection was assessed using oneway ANOVA followed by Fischer's least significant test (P=0.05). For qPCR, each sample was tested in duplicate. Due to the small sample size, mean values were not used for statistical analyses.

Results

Short term greenhouse trials

In almond seedlings grafted with AlmWB phytoplasma-infected scions used as positive controls, survival of grafted scions was 80%. Jawhara and Red plum varieties had survivals of 50%, while Angeleno plum had 75% survival, Early blush apricot had 50% survival and Farclo apricot had 75% survival (Table 1). In some of these plants, symptom development started 2 months after grafting. In June 2015 one seedling from the positive control treatment showed small leaves. In July another seedling from the positive controls exhibited typical symptoms of AlmWB from the rootstock, but not from the new scion. Additionally, 2 months post-grafting, an almond scion grafted on an Early blush apricot seedling, and another grafted on Red plum developed bush-like growth, with stems having short internodes and small leaves. Both plants were PCR positive for '*Ca*. P. phoenicium'. However, the following month the same seedlings exhibited development of normal stems with normal leaves and internode distances, and gave negative PCR results for presence of the AlmWB phytoplasma (Table 2). In August and September 2015, about five months post-grafting, all shoots that developed from infected almond scions grafted on plum or apricot showed normal growth and were PCR negative, except for almond grafted on plum variety Jawhara and apricot variety Farclo that were PCR positive without showing symptoms. On the other hand, only one of the positive controls showed symptoms and was PCR positive for '*Ca*. P. phoenicium'.

In June 2016, one year and two months post-grafting, all the seedlings showed normal growth, whereas one of the positive controls showed characteristic disease symptoms and three of four positive controls were giving positive PCR results. However, two PCR positive samples were not showing disease symptoms (Table 2). Two samples of the almond growth grafted on apricot variety Farclo exhibited positive PCR results but did not develop symptoms. The new growing shoots from almond scions grafted on Angeleno, Jawhara or Red plums or Apricot Early Blush, were PCR negative and did not develop symptoms. Throughout the experiment, the plum Angeleno and the negative controls of each treatment showed no disease symptoms and tested negative for 'Ca. P. phoenicium' by PCR (Table 2). Furthermore, almond scions grafted on apricot Early blush and Red plum, which were PCR positive 2 months post-grafting, showed phytoplasma titres below the detection level, respectively, 4 and 14 months after grafting.

Table 2. Greenhouse experiment: symptom development and PCR detection of '*Ca*. P. phoenicium' in the almond scions grown from grafted seedlings.

Treatments	14/07/2015		31/08/2015		21/09/2015		10/06/2016	
	PCR*	Symptom	PCR	Symptom	PCR	Symptom	PCR	Symptom
Almond	2/4	S/NS**	2/4	S/NS	3/4	S/NS/NS	3/4	S/NS/NS
Plum (Jawhara)	0/2	NS	1/2	NS	1/2	NS	0/2	NS
Plum (Angeleno)	0/3	NS	0/3	NS	0/3	NS	0/3	NS
Plum (Red)	1/2	NS	0/2	NS	0/2	NS	0/1	NS
Apricot (Early blush)	1/2	S	0/2	NS	0/2	NS	0/2	NS
Apricot (Farclo)	0/3	NS	0/3	NS	1/3	NS	2/3	NS

* PCR = Number of samples that tested positive for phytoplasma / total number of samples tested.
** S = symptom appearance on the PCR positive samples; NS = no symptom appearance.

In the qPCR tests, the Cq value of all the negative controls was greater than 30 cycles and was thus considered as not infected by '*Ca*. P. phoenicium' or below detection level (Table 3). Since the plum variety Angeleno remained qPCR negative during the 3 months tested, with Cq value greater than 30 cycles, data pertaining to the Angeleno qPCR results were not used in the statistical analysis and copy number calculation.

Data reported in Table 3 and Figure 1 were used to calculate the quantity of phytoplasma (genomic units (GU) ng^{-1} plant DNA). Results suggest that the detection limit of the qPCR technique used was about 0.5 GU ng^{-1} of plant DNA. In the positive control, the phytoplasma titre increased from 124 to 406 phytoplasma GUs ng^{-1} plant DNA from July to September 2015. In almond grafted on Early Blush apricot and on Red Plum, treatments that exhibited symptom disappearance, the qPCR data showed that the phytoplasma titre was, respectively, 44 and 3.1 phytoplasma GU ng^{-1} of plant DNA in July 2015 when the symptoms were present. These titres decreased to below detection level in August and September 2015, when external symptoms disappeared. The phytoplasma was not detected in almond grafted on Angeleno plum during the whole season. In almond scion growth on Farclo apricot, the phytoplasma titre was below detection limit in July 2015 and increased to 1.5 GU ng^{-1} plant DNA in September 2015. However, in Jawhara plum, the concentration varied between 11 and 42 GU ng^{-1} plant DNA, respectively, in July and September 2015. From August, all plum and apricot treatments did not exhibit symptom development.

During June 2016, the phytoplasma titre in the positive control was 3157 GU ng^{-1} plant DNA. In almond grafted on Early Blush apricot and Red Plum, the disappearance of symptoms was also confirmed by qPCR and the titre was below 0.5 GU ng^{-1} plant DNA, suggesting a reduction of phytoplasma titre to below detection levels or possibly absence of phytoplasma. Phytoplasma was also not detected in almond grafted on Angeleno plum also during this season. In scions grafted on Farclo apricot, the phytoplasma titres increased from 1.5 GU ng^{-1} plant DNA in September 2015 to 8.94 GU ng^{-1} in June 2016, but the plants exhibited no external symptoms. However, in Jawhara plum the concentration decreased from 42 GU ng^{-1} plant DNA in September 2015 to 6.25 GU ng^{-1} in June 2016. Pathogen titre values were significantly different ($F = 2.444$, $P=0.05$) among the treatments.

Results of long term field trials

When the severely AlmWB-affected almond trees were grafted with scions of various plum and apricot varieties, two results were observed. First, all ten scions from the five varieties used in the experiment developed very vigorous shoot growth in June and remained vigorous until September 2013. In one replicate, an almond tree with two main branches was cleft grafted with Janarek plum on both branches but only one scion survived. The growth of the surviv-

Table 3. Greenhouse experiment: detection and quantification of '*Ca.* P. phoenicium' in grafted and non-grafted (negative controls) stone fruit seedlings, during July, August and September 2015, and June 2016.

Treatment	Month	AlmWB DNA in plant DNA Cq (mean ± SD)	Quantity (GU)	Plant 18S rDNA Cq (mean ± SD)	Quantity (ng)	AlmWB DNA/ plant DNA (GU/ng)	Statistical/ analysis of mean AlmWB DNA/ plant DNA (mean GU/ng)
Negative controls							
Almond	July 2015	31.18 ± 1.22	1.42E+02	14.18 ± 0.43	1.23E+03	< 0.5	0.07 a
	August 2015						
	September 2015						
	June 2016	31.76 ± 0.98	9.4E + 01	14.18 ± 0.07	2.7E + 03		
Plum Jawhara	July	32.17 ± 2.59	6.93E+01	16.02 ± 0.06	8.09E+02	< 0.5	-
	August						
	September						
	June 2016	32.69 ± 0.79	4.7E+01	12.60 ± 0.07	7.5E+03		
Red Plum	July	30.93 ± 0.3	1.72E+02	13.72 ± 0.46	3.602E+03	< 0.5	-
	August						
	September						
	June 2016	30.81 ± 0.58	1.88E+02	11.78 ± 1.54	1.3E+04		
Early blush Apricot	July	30.90 ± 0.8	1.75E+02	13.23 ± 0.23	4.984E+03	< 0.5	-
	August						
	September						
	June 2016	31.28 ± 1.74	1.33E+02	13.19 ± 0.12	5.1E+03		
Farclo Apricot	July	32.8 ± 2.72	4.3E+01	14.75 ± 0.01	1.857E+03	< 0.5	-
	August						
	September						
	June 2016	33.12 ± 1.03	3.4E+01	14.62 ± 0.13	2E+03		
Grafted with AlmWB infected scions							
Almond	July	22.53 ± 0.88	8.3248E+04	16.32 ± 0.43	6.69E+02	124	112.47b
	August	23.42 ± 0.39	4.3254E+04	17.3± 0.11	3.54E+02	122.2	
	September	18.19 ± 0.16	2.023E+06	13.23 ± 0.06	4.98E+03	406	
	June 2016	16.7 ± 1.78	6E+06	14.74 ± 1.6	1.9E+03	3157	
Plum Jawhara	July	25.71 ± 0.21	7.8E+03	16.35 ± 0.99	6.6E+02	11.2	16.7a
	August	27.83 ± 0.47	1.7E+03	12.86 ± 0.47	6.34E+03	< 0.5	
	September	23.19 ± 0.48	5.1E+04	15.47 ± 0.42	1.2E+03	42.5	
	June 2016	24.82 ± 0.95	1.5E+04	14.33 ± 0.7	2.4E+03	6.25	

(Continued)

Table 3. (Continued).

Treatment	Month	AlmWB DNA in plant DNA Cq (mean ± SD)	Quantity (GU)	Plant 18S rDNA Cq (mean ± SD)	Quantity (ng)	AlmWB DNA/ plant DNA (GU/ng)	Statistical/ analysis of mean AlmWB DNA/ plant DNA (mean GU/ng)
Red Plum	July	28.47 ± 0.75	1.05E+03	17.35 ± 0.24	3.38E+03	3.1	1.1a
	August	29.76 ± 0.06	4.1E+02	14.89 ± 0.60	1.7E+03	< 0.5	
	September	31.51 ± 2.15	1.12E+02	16.14 ± 0.52	7.53E+02	< 0.5	
	June 2016	30.51 ± 0.74	2.3E+02	14.46 ± 0.51	2.2E+03	< 0.5	
Early blush Apricot	July	21.84 ± 0.23	1.4E+05	13.92 ± 0.68	3.2E+03	44	11.2a
	August	31.50 ± 1.19	3.2E+03	14.14 ± 0.34	2.76E+03	1.15	
	September	31.07 ± 1.05	8.6E+01	15.87 ± 0.50	8.97E+02	< 0.5	
	June 2016	30.99 ± 0.68	1.65E+02	15.61 ± 0.21	1.01E+03	< 0.5	
Farclo Apricot	July	30.74 ± 0.78	2.4E+02	14.80 ± 0.42	1.2E+04	< 0.5	2.7a
	August	30.33 ± 0.18	1.75E+02	11.79 ± 0.59	2.5E+03	< 0.5	
	September	30.97 ± 0.81	6.4E+02	13.57 ± 0.64	4.2E+02	1.5	
	June 2016	25.64 ± 1.44	8.4E+03	15.76 ± 0.18	9.4E+02	8.94	

[a] A Cq over 30, is considered qPCR negative, i.e. phytoplasma below detection limit or not present.
Angeleno plum was qPCR negative during the period of the experiment and was not used in the statistical analysis.

ing Janarek scion 2 months post-grafting (June 2013) was very vigorous, while the almond growth on the second branch was weak and developed characteristic disease symptoms. Five months post-grafting (September 2013) the Janarek growth was still very vigorous while the almond growth had died (Figure 2). In June and September 2013, PCR results showed that all almond trees used as rootstocks were infected with for '*Ca*. P. phoenicium', but growth from the plum scions were not (Table 4).

In June, growth that developed from Farclo apricot scions was highly symptomatic, with proliferation, stunted growth and leaf curl and was for positive for 'Ca. P. phoenicium' by PCR. However, in September 2013, symptoms had disappeared, and the main branches grew vigorously with only a few small leaves remaining on the bases of the shoots (Figure 3). When tested by PCR, the vigorous shoots gave negative results while the small leaves tested positive for phytoplasma. All almond trees grafted with plums died after a very dry summer. Only the trees grafted with Farclo apricot variety survived because they were indirectly irrigated. Three years post-grafting the growth from the two trees, F2P2 and F2P4 grafted with Farclo apricot, did not show disease symptoms. PCR analysis using AlmF2/R2 primers was positive for Farclo F2P4 but negative for Farclo F2P2 (Table 4).

Discussion

AlmWB is a devastating stone fruit disease, often resulting in 100% yield losses in almond, peach and nectarine trees within a year of early symptom appearance. Consequently, the disease could be economically very significant for Mediterranean farmers who depend on stone fruit crops for their livelihoods. In Lebanon, almond production has been greatly reduced since introduction of the AlmWB phytoplasma in the early 1990s. Field and greenhouse experiments were performed to develop a potential management option for farmers in endemic areas of the disease or their vicinities.

The field experiment showed that scions from three plum varieties and an apricot variety grafted onto almond trees that were severely infected by AlmWB phytoplasma developed vigorously and did not show any symptoms for a year or more. Many

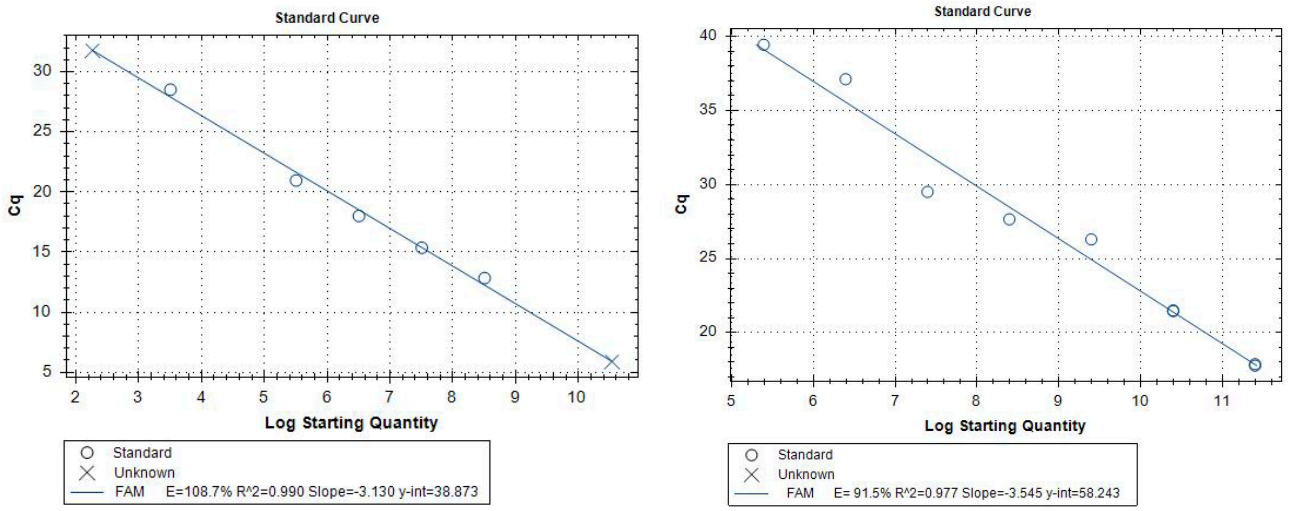

Figure 1. Standard curves for AlmWB phytoplasma plasmid (A); and for total nucleic acids (TNAs) extracted from healthy almond (B).

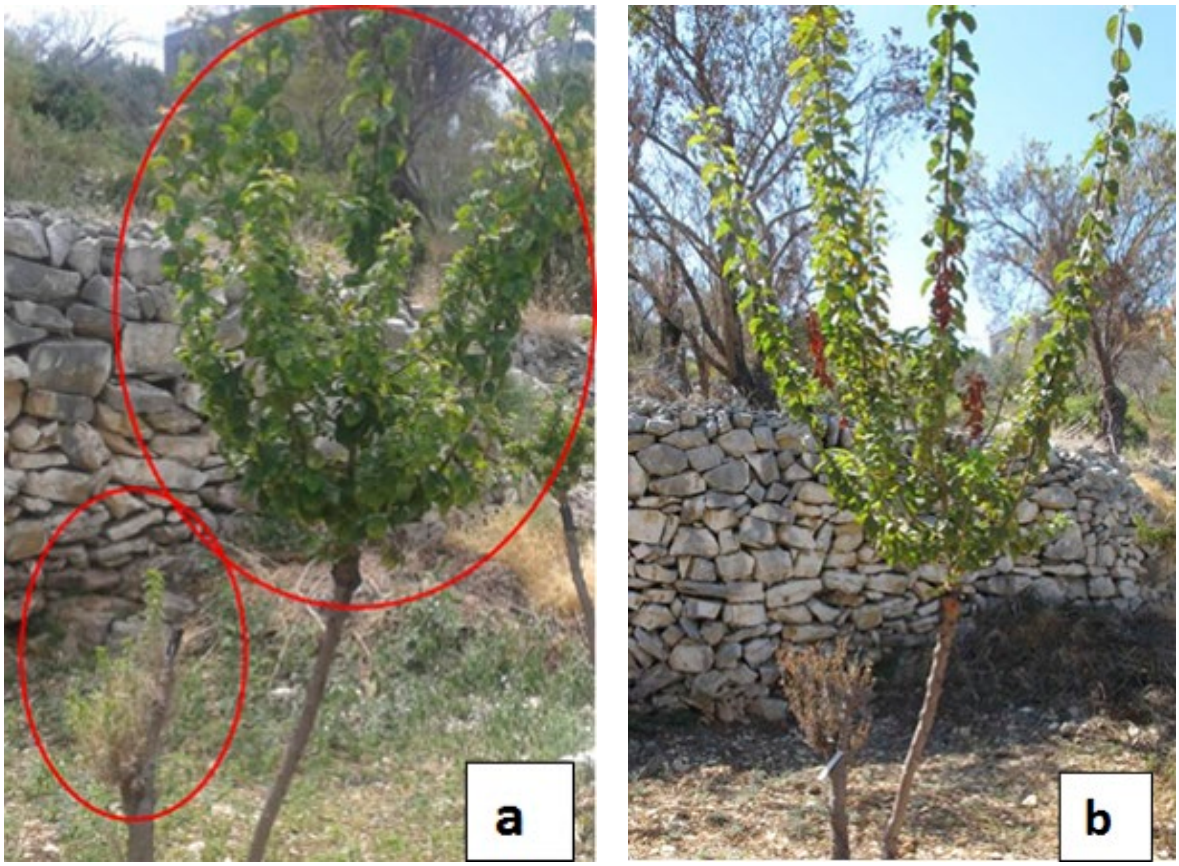

Figure 2. Two branches of the same tree, one showing almond growth (bottom left) and one showing Janarek plum growth (top right); photos taken in June (a) and in September (b), 2013.

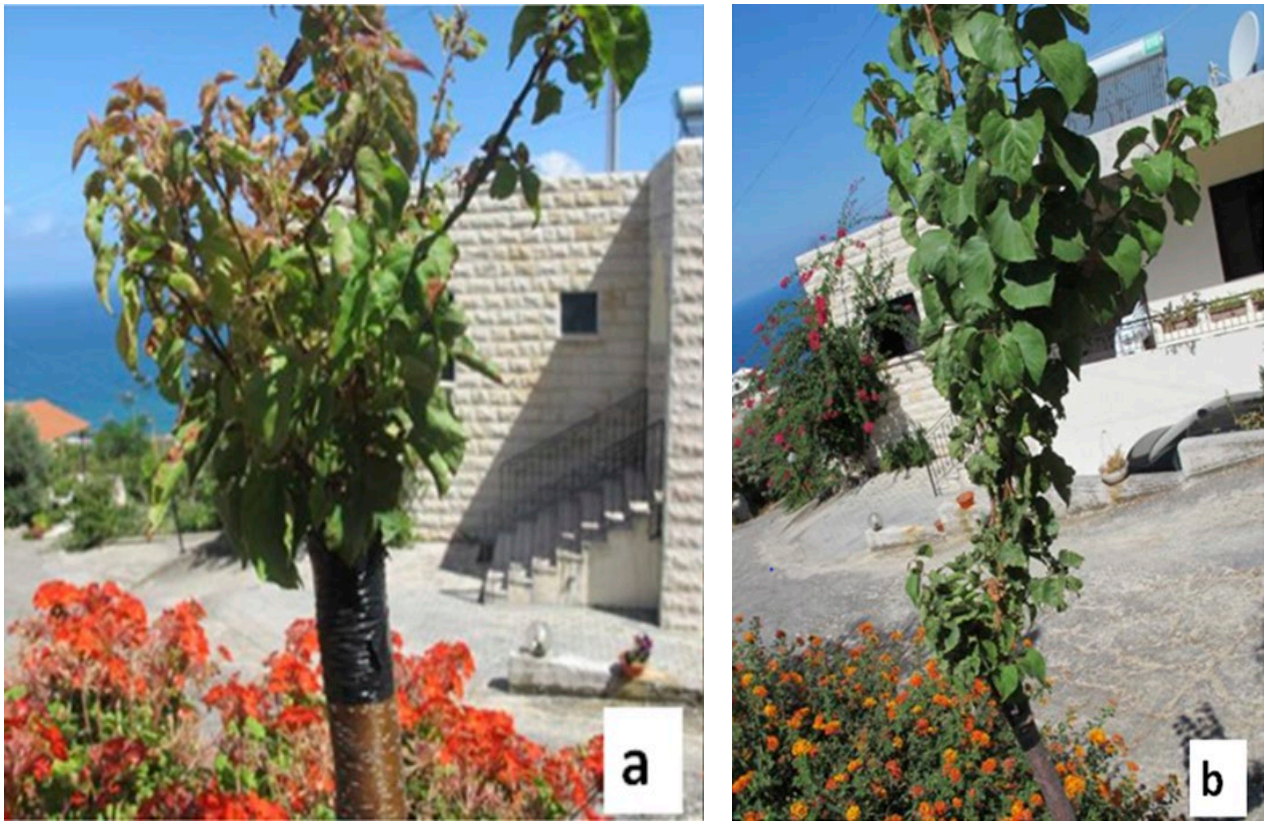

Figure 3. 'Ca. P. phoenicium'-infected almond rootstocks in the field, grafted with Apricot Farclo scions. In June 2013, the growth from scions showed clear symptoms of proliferation (a). The same tree in September, when the remission from symptoms was observed (b).

farmers removed infected almond trees and a year later replanted new almond seedlings. However, the phytoplasma symptoms appeared 5–6 years after replanting the almond seedlings. Therefore, grafting of the newly infected almonds with plum or apricot may reduce the economic losses to farmers.

Furthermore, results of the greenhouse experiment, which mimicked early infections by AlmWB phytoplasma, were promising. Over one year following grafting of AlmWB-infected scions on apricot and plum cultivars, growth from the almond scions was vigorous and did not develop disease symptoms. The AlmWB phytoplasma was not detected in the almond growths grafted on any the three plum varieties, Angeleno, Jawhara or Red Plum, while it was detected in three out of the four positive controls grafted on almond. The growth of infected almond scions grafted on the two apricot varieties was also symptomless, but the phytoplasma was detected in two out of threes scions grafted on Farclo. Grafting almond on Angeleno plum is likely to be most promising in conferring resistance to AlmWB phytoplasma, since the phytoplasma was not detected by PCR at any growth stage in these treatments. Therefore, in AlmWB endemic areas we recommend planting resistant plum or apricot varieties then grafting almond onto these, or purchase of triple grafted seedlings (for example: Myrobalan/ Angeleno/almond) as a method to control AlmWB and re-establish almond orchards. During more than 15 years of field observations, all the almond trees that developed specific AlmWB symptoms remained symptomatic or died. The present study is the first to report symptom remission in AlmWB-affected almond trees, a condition that has been induced by grafting on apricot or plum. Similar results were observed in field trials in which Farclo apricot scions were grafted on two severely affected almond trees. The initial apri-

Table 4. Field experiment: symptom development on scions and PCR results using 'Ca. P. phoenicium' primers (AlmF2/R2), 2 and 5 months post grafting and 1 and 2 years post grafting.

Tree Code	Scion variety	June 2013			September 2013		2014, 2015 and 2016
		PCR scions	PCR Rootstock	Symptoms	PCR scions	Symptoms	PCR scions
F1P2	Plum Abou Riha	-	+	Mild[a]	-	Very mild	Dead
F1P3	Plum Abou Riha	-	+	Mild	-	Scion dead	Dead
F1P4	Janarek plum	-	+	Mild	-	No	Dead
F1P5	Janarek plum	-	+	Mild	-	No	Dead
F3P2	Fortune plum	-	+	Mild	-	Mild	Dead
F3P1	Fortune plum	-	+	Mild	-	Mild	Dead
F1P1	Plum Santarosa	-	+	Mild	-	No	Dead
F3P4	Plum Santarosa	-	+	Mild	-	No	Dead
F2P4 (A)[c]	Apricot Farclo	-	+	Witches' broom	- (NS[b])	Combination: no symptoms and symptoms	+ (NS)
F2P4 (B)[c]	Apricot Farclo	+	+	Witches' broom	+ (S[b])		
F2P2 (A)[d]	Apricot Farclo	-	+	Witches' broom	- (NS)	Combination: no symptoms and symptoms	- (NS)
F2P2 (B)[d]	Apricot Farclo	+	+	Witches' broom	+ (S)		

[a] Mild symptoms: Mild nutrient deficiency like symptoms.
[b] NS: No symptom appearance; S: symptom appearance.
[c] F2P4 A and B: same tree some branches asymptomatic (A) and others symptomatic (B). Data based on June 2013.
[d] F2P2 A and B: same tree some branches asymptomatic (A) and others symptomatic (B). Data based on June 2013.

cot growth showed severe AlmWB symptoms but the new growth had most of the shoots free from the symptoms within 2–3 months and remained so for 3 years. '*Ca*. P. phoenicium' was detected by PCR in December 2014 and in June 2016 in the Farclo scions in one tree, but not in the other tree. Disappearance of symptoms from previously symptomatic trees has also been observed in cases of infection with the phytoplasma diseases apple proliferation, European stone fruit yellows, pear decline and grapevine yellows. This change could have been either temporary or permanent, and the causes of recovery are yet to be fully elucidated (Carraro *et al*., 2004).

Results from the present study also suggest that '*Ca*. P. phoenicium' titre and symptom appearance may not be directly related. Based on symptom monitoring and calculation of phytoplasma GU ng^{-1} of plant DNA, we postulate that symptom appearance is not only related to titre of phytoplasma in plant tissue, since this may also vary with the host crop and/or cultivar scion/rootstock combinations, and with environmental factors. For example in Early Blush, a phytoplasma titre of 44 GU ng^{-1} DNA was associated with symptoms, while in the Jawhara plum variety, this titre did not lead to appearance of symptoms.

AlmWB phytoplasma in asymptomatic almond tissue was detected by PCR in the present study. This corroborates with previous reports by Abou Jawdah *et al*., (2014), where '*Ca*. P. phoenicium' was detected in young almond seedlings using molecular tech-

niques but disease symptoms were not expressed in the seedlings. This indicates that long quarantine periods are required, or PCR testing of almond seedlings should be carried out, before seedlings are introduced into a region.

In conclusion, preliminary field and greenhouse results described here show potential for grafting to contribute to AlmWB disease management, and this approach may give hope to farmers who plan to replant almond in infected regions. However, disease resistance alone is not sufficient to ensure success. If grafting on various stone fruit rootstocks is to be used as a disease management tool, then horticultural characteristics, including yield potential, resistance to drought, tolerance to calcareous soils and adaptation to climatic and edaphic conditions are important factors to be considered. Therefore, long-term field trials are planned in Feghal region, a severely AlmWB-affected region in North Lebanon, where healthy almond scions from the variety Halwani will be grafted on plum or apricot rootstocks. These experiments could give hope to Lebanese farmers for planting almond seedlings into infected and endangered nearby regions.

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