

Specific PCR and real-time PCR assays for detection and quantitation of '*Candidatus Phytoplasma phoenicium*'



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ABSTRACT

Almond witches' broom (AlmWB) is a fast-spreading lethal disease of almond, peach and nectarine associated with '*Candidatus Phytoplasma phoenicium*'. The development of PCR and quantitative real-time PCR (qPCR) assays for the sensitive and specific detection of the phytoplasma is of prime importance for early detection of '*Ca. P. phoenicium*' and for epidemiological studies. The developed qPCR assay herein uses a TaqMan[®] probe labeled with Black Hole Quencher Plus. The specificity of the PCR and that of the qPCR detection protocols were tested on 17 phytoplasma isolates belonging to 11 phytoplasma 16S rRNA groups, on samples of almond, peach, nectarine, native plants and insects infected or uninfected with the phytoplasma. The developed assays showed high specificity against '*Ca. P. phoenicium*' and no cross-reactivity against any other phytoplasma, plant or insect tested. The sensitivity of the developed PCR and qPCR assays was similar to the conventional nested PCR protocol using universal primers. The qPCR assay was further validated by quantitating AlmWB phytoplasma in different hosts, plant parts and potential insect vectors. The highest titers of '*Ca. P. phoenicium*' were detected in the phloem tissues of stems and roots of almond and nectarine trees, where they averaged from 10⁵ to 10⁶ genomic units per nanogram of host DNA (GU/ng of DNA). The newly developed PCR and qPCR protocols are reliable, specific and sensitive methods that are easily applicable to high-throughput diagnosis of AlmWB in plants and insects and can be used for surveys of potential vectors and alternative hosts.

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1. Introduction

A disease characterized by proliferation, small chlorotic leaves, witches' broom and followed by dieback and death of almond trees was first observed in the 1990s in North Lebanon [1]. This disease, commonly known as Almond witches' broom (AlmWB), was associated with a phytoplasma belonging to the 16SrIX group and designated '*Candidatus Phytoplasma phoenicium*' [2]. So far, AlmWB has been reported only in Lebanon and Iran [3].

A survey, conducted in 2009 and 2010, showed that the AlmWB disease had become widely spread in Lebanon and that epidemics had occurred not only on almond but also on peach and nectarine trees; at least 40,000 newly infected trees were observed [4]. Furthermore, genetic characterization based on 16SrRNA gene showed three typical subgroups using restriction fragment length polymorphism (RFLP) analysis, the predominant 16SrIX-D (B)

subgroup followed by subgroups-G, and -F [4]. Due to its rapid widespread and threat to the almond industry, the disease was considered of quarantine importance and the Ministry of Agriculture took unprecedented phytosanitary measures in an effort to mitigate its impact.

Sensitive and robust detection methods are required for phytoplasmas, which are phloem-limited and occur at very low titer levels inside the plant tissue [5]. While various techniques have been used for detection of phytoplasmas, nucleic acid-based assays, such as PCR, are most commonly used in surveys [6]. Several conventional or nested PCR protocols have been developed for universal or species-specific detection of phytoplasmas [7–10]. Over the last decade, quantitative real-time PCR (qPCR) methods for detection and quantitation of phytoplasma species were proven to be more specific, less time consuming and often equally or more sensitive than conventional nested PCR [11,12]. Several chemistries have been used for detection such as, SYBR Green [7], EvaGreen [10] and TaqMan[®] probes [13–15]. Real-time qPCR has been used extensively in quantitating phytoplasma in different plant parts and in vectors [16,17].

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In Lebanon, surveys for AlmWB were typically conducted by performing either direct or nested PCR. For the nested PCR assay, a universal primer pair (P1/P7) was used to amplify a 1800 bp product [18], followed by a second primer pair (R16F2n/R16R2), amplifying a 1250 bp fragment [19]. Alternatively, stone fruit samples could be tested by direct PCR using the primer pair ALW-F2/ALW-R2, which amplifies a DNA fragment of about 390 bp from 16SrIX phytoplasmas [20]. The latter primer pair also amplified other group IX phytoplasmas and was considered as a semi-specific primer for detection of AlmWB phytoplasma [20]. Another primer pair (AlmF1/AlmR1) designed by Verdin et al. [2] amplifies 1509 bp fragment of the rRNA sequence of 'Ca. P. phoenicium'. Although the direct and nested protocols described above effectively detect phytoplasmas, they are not able to confirm the presence of 'Ca. P. phoenicium', compelling researchers to undertake further analysis. RFLP patterns of the 1250 bp PCR products or DNA sequencing of the 390, 1509 or 1250 bp amplicons were required for confirmation of infections by 'Ca. P. phoenicium' [2,4,20]. Therefore, development of specific detection methods for 'Ca. P. phoenicium' will help in speeding surveys, especially when surveying for potential insect vectors or alternative host plants, reducing time and cost of detection.

A specific and rapid detection method for identification and quantitation of 'Ca. P. phoenicium' has not yet been reported. In this study, two PCR-based assays, conventional PCR and TaqMan[®]-based qPCR, were designed and validated. The assays were tested for the specific detection of 'Ca. P. phoenicium' in plants and an insect vector. Also, the population dynamics of 'Ca. P. phoenicium' were studied in different seasons and in samples from different plant parts.

2. Materials and methods

2.1. Plant material and phytoplasma sources

For experiments related to the specificity of the primers used in PCR and qPCR tests, a total of 97 leaf tissue samples of almond, peach and nectarine, either asymptomatic or showing characteristic symptoms of AlmWB disease were collected from North and South Lebanon.

For the quantitation of the phytoplasma, two commercial orchards with a history of AlmWB were selected for studying phytoplasma population dynamics. Three almond trees in Feghal area (North Lebanon) and three nectarine trees in Kfarkela (South Lebanon) showing typical AlmWB symptoms were selected to collect tissue samples. Different plant parts were sampled from

each tree. Samples from the stem phloem tissue, root phloem tissue and leaf midribs were collected during August. Samples were also collected from the three almond trees in December (winter), and again in late January, at flowering but before leaf emergence.

Samples from native, non-cultivated plant species, including annual and perennial plants (Table 3), as well as samples from the leafhopper *Assymetrasca decedens* (Hemiptera: Cicadellidae), a vector of AlmWB, were also collected and included in the detection tests.

Reference isolates of distinct phytoplasma groups were kindly supplied by Professors Assunta Bertaccini (UNIBO, Bologna, Italy) and Piero A. Bianco (UNIMI, Milano, Italy) as DNA extracts. The presence of phytoplasma in these samples was confirmed by nested PCR using universal primer pairs P1/P7 and R16F2n/R16R2 [18,19].

2.2. Total nucleic acid extraction

Total nucleic acids (TNAs) were extracted from 100 mg of leaf midribs, petals, stem and root phloem tissues using a CTAB-based protocol [1]. The TNAs extracted were re-suspended in 50 µl of deionized water, analyzed in a 1% agarose gel electrophoresis and quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The TNA extracts were stored at -20 °C. TNAs from insect samples were extracted as described previously [24].

2.3. Primers and probes design

Multiple sequence alignment using ClustalX [21] was performed on 56 rRNA (16S-ITS-23S) sequences obtained from GenBank, representing phytoplasmas belonging to eight different 16S rRNA groups (II; III; IV; V; VI; IX; X and XII). The aligned sequences were screened for hyper-variable nucleotides in the rRNA region among different groups to design primers for the specific detection of 'Ca. P. phoenicium' in PCR or qPCR. Primers (AW16sF/AW23sR) were designed for specific detection of 'Ca. P. phoenicium' in conventional PCR (Table 1). Multiple sequence alignment showed that the reverse primer was located in a hypervariable region (23S rRNA), whereas the forward primer was in a conserved region among different phytoplasma groups. The specificity of the reverse primer was confirmed by BLAST analyses (Fig. 1). Conventional PCR primers were designed using the Primer3 (<http://frodo.wi.mit.edu/>) tool program.

For the specific detection and quantitation of 'Ca. P. phoenicium', a qPCR protocol was developed using the primer pair AWsF/AWsR which amplifies a specific fragment of 132 bp spanning the hyper-

Table 1
List of primers and probes for detection and quantitation of 'Ca. P. phoenicium' designed in this study.

Target/name	Sequence (5' → 3')	Position	Amplicon (bp)
PCR			
'Ca. P. phoenicium' 16S-ITS-23S			492
AW16sF (forward)	ACAGTCTCAGTTCGGATT	1270–1287 ^a	
AW23sR (reverse)	CTTCCTTAATAAAGGTCCG	1742–1761 ^a	
Real-time PCR			
'Ca. P. phoenicium' ITS-23S			132
AWsF (forward)	AGGCCACCAAAACGCTTAA	1674–1693 ^a	
AWsR (reverse)	CCITCATCGGCTCTTAGTGC	1786–1805 ^a	
AW23plus (probe)	FAM -ACAAGAGAACAGCGACCTTTATTA- BHQplus	1731–1754 ^a	
<i>Prunus dulcis</i> 18S rRNA			
Prun18S-F (forward)	GGAGAGGGAGCCTGAGAAAC	284–303 ^b	109
Prun18S-R (reverse)	GAGCCCGTATTGTTATTTATTGTC	368–392 ^b	
Prun18S-Taq (probe)	FAM -CCACATCCAAGGAAGGCAGCAGCGG- BHQ1	309–333 ^b	

^a Based on accession no. AF390136.

^b Based on accession no. DQ886376.

AlmWB (IX-D (B))	5'-CTTCCTTTAATAAAGGTCGC-3'
PEY (IX-C)A..T..CTAT
RLL (IX-A)	A.....GTT.CA.AC.
NaxY (IX-C)	A.....GTTTCA.ACT
LactPh (IX-D)	A.....GTTTCA.TT
BBS3NJ (IX-E)	A.....GTTTCA.TT
ESFY (X)	A.....GTTTCA.ACT
AY (I)	A.....GTTTCA.ACT
WX (III)-.....A.AACT

Fig. 1. Multiple sequence alignment of the 16S rDNA regions showing the specificity of the AW23sR primer in relation to other phytoplasmas belonging to the 16SrIX group or occurring in stone fruit trees. The 16S rDNA groups are indicated in parentheses. The GenBank accession no. for Almond witches' broom (AlmWB), Picris echioides yellows (PEY), Rhynchosia little leaf (RLL), Periwinkle virescence (NaxY), Lactuca sativa phyllody (LactPh), Blueberry stunt (BBS3NJ), 'Ca. P. prunorum' (ESFY), 'Ca. P. asteris' (AY) and western X (WX) phytoplasmas are, AF515637, JQ868441, AF361019, HQ589191, DQ889748, AY265220, JF730310, KC000005 and AF533231, respectively.

variable intergenic spacer region and the 23SrRNA region (Fig. 2). BLAST analyses and sequence alignments showed that the primers and probe can detect all 'Ca. P. phoenicium' isolates reported in GenBank (Fig. 2). At the 3' end, the TaqMan® probe was labeled with Black Hole Quencher plus (BHQplus™), which uses a duplex stabilizing technology permitting the synthesis of shorter oligonucleotides while maintaining optimal melting temperatures for PCR thermocycling and detection (Biosearch Technologies, Novato, USA). Samples infected with 'Ca. P. phoenicium' and other phytoplasmas belonging to different 16S rRNA groups and sub-groups were used for the specificity assays. For normalization of phytoplasma titer, a primer pair and probe was developed for the reference gene. The primers (Prun18S-F/Prun18S-R) and probe (Prun18S-TaqM) were designed based on the almond (*Prunus dulcis*) 18S rRNA gene (DQ886376). The TaqMan® probe was labeled with Black Hole Quencher-1 (BHQ-1) at the 3' end. The two probes were labeled with 6-carboxyfluorescein (FAM) at the 5' end.

All oligonucleotides were synthesized by Biosearch Technologies (Novato, CA). All qPCR primers and probes were assessed with Beacon Designer 7 (Premier Biosoft, Palo Alto, CA) or the web-tool RealTimeDesign software (www.biosearchtech.com).

2.4. Assay optimization

The conventional PCR protocol was optimized with primers designed for the specific detection of 'Ca. P. phoenicium'. Each amplification reaction was performed in 20 µl reaction mixture containing 2 µl of template DNA, 10 µl of REDTaq® ReadyMix™ PCR Reaction Mix (Sigma–Aldrich, MO, USA), 0.25 µM of each primer and 7 µl of molecular grade water. The cycling conditions were: 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, followed by a final extension for 7 min at 72 °C. Amplified

products were analyzed following electrophoresis in 1.5% agarose gel, stained in ethidium bromide (0.5 g/L) and visualized with a UV transilluminator.

The presence of phytoplasma DNA in all samples tested was confirmed using nested PCR with primers P1/P7 followed by R16F2n/R16R2. For comparison with other published protocols, primer pairs ALW-F2/ALW-R2 [20] and AlmF1/AlmR1 [2] were used as described previously.

Real-time qPCR was performed in 96-well hard plates using CFX96 Touch thermal cycler (Bio-Rad Laboratories, Hercules, USA). The reaction in 20 µl contained 2 µl of template DNA, 10 µl iQ Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.3 µM for each AWsF/AWsR primers and 0.2 µM of the AWs23plus probe, or 0.4 µM for each Prun18S-F/18S-R primer pair and 0.2 µM of the Prun18S-TaqM probe.

The optimized cycling conditions for the qPCR using primers, AWsF/AWsR, were: 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 63 °C for 15 s and 72 °C for 15 s. However, for the reference gene, primers Prun18S-F/R and Prun18S-TaqM probe, the optimized cycling conditions used a 2-step real-time protocol: 95 °C for 3 min, 40 cycles alternating between 95 °C for 10 s and 65 °C for 30s.

2.5. Standard curves

After partial amplification of 'Ca. P. phoenicium' rRNA with the primers AlwF2 and P7 [20,22] to get a 579 bp amplicon that contains the primer and probe sequences (AWsF/AWsR), the amplicons were purified with the Illustra™ GFX PCR DNA and Gel Band Purification kit (GE Healthcare, UK) and cloned using the pGEM-T Easy Vector System II (Promega, USA). The purified plasmid was quantified with the ND-1000 spectrophotometer (NanoDrop Technologies, USA). The number of plasmid copies was calculated based on molecular weight using the formula: number of copies = plasmid concentration/[(plasmid size + insert (bp) × 660)/(Avogadro's number)]. In this case, 80 ng of pGEM-T Easy (3015 + 579 bp) consists of approximately 2 × 10¹⁰ plasmids. For calculation of the number or genome units (GU), the copy number was divided by two, since it is expected to have about two rRNA operons in the genome of phytoplasmas [23].

Ten-fold serial dilutions (2.0 × 10⁸–200 GU/µl) of the purified plasmids were used to generate a standard curve for the absolute quantification of AlmWB rRNA targets in the leaf midribs, petals and the phloem tissues of stems and roots. The estimated number of phytoplasma was derived from the equation: N_T = 10^{Cq-a-b}, where N_T is the target copy number, Cq for any sample, a and b the slope and intercept of the regression line, respectively. The copy numbers obtained were normalized for input amount of DNA using the qPCR assay for plant 18S rRNA. For the plant DNA, a standard curve was constructed using total DNA from healthy almond plant. Total DNA

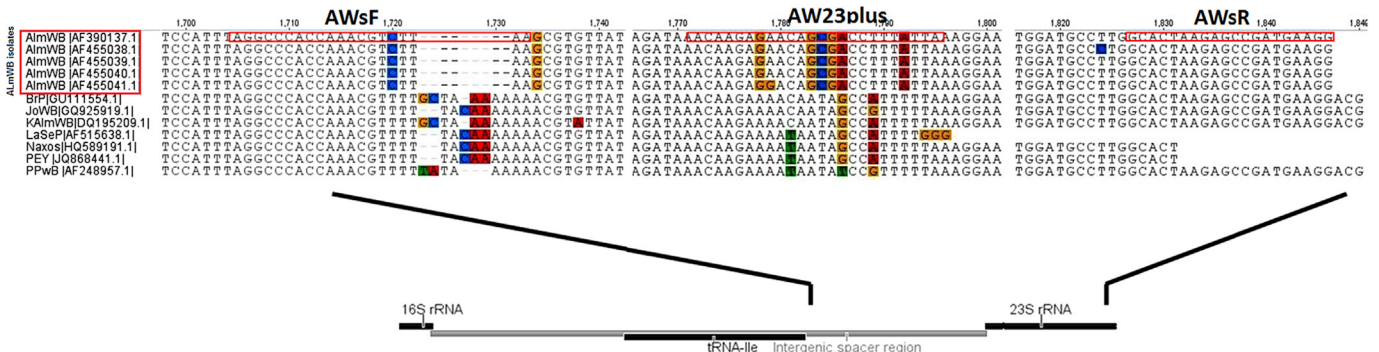


Fig. 2. Primer and probe position on the aligned partial sequences of the 16S-ITS-23S rRNA of phytoplasmas from the pigeon-pea group (16SrIX).

of the sample was quantified by Nanodrop and was serially diluted 10-fold over a six point range (640 ng–6.4 pg). The standard curves were constructed by plotting the \log_{10} of the target copy number against their respective Cq. 'Ca. P. phoenicium' titer in plant organs was expressed as genome units (GU) of phytoplasma per nanogram (ng) of plant DNA. Efficiency of each standard curve was calculated by the Bio-Rad CFX Manager software using the following equation:

$$E = \left[10^{\left(\frac{-1}{\text{slope}}\right)} - 1 \right] * [100].$$

2.6. Specificity and sensitivity of the developed PCR and qPCR assays

For the specificity assay, DNA extracts from 17 phytoplasma strains belonging to 11 different 16Sr RNA groups were used as DNA template in PCR assays. DNA of field collected samples from infected and healthy stone fruits, weeds, and *A. decedens*, an insect vector of AlmWB [24], were also tested with the specific and the semi-specific primer pairs described earlier. Each PCR assay was run according to the optimized protocol for each set of primer pairs. All tests included a positive control, a negative control and a no template control. Nested PCR tests using the universal primer pair

P1/P7 followed by primers R16F2n/R16R2 were conducted as a benchmark to compare the specificity and sensitivity of the developed diagnostic tests.

The sensitivity of the different detection methods was based on 10-fold serial dilutions of TNAs extracted from the stem phloem tissue of an AlmWB-infected almond tree. Subsequently, different primer pairs were used in PCR and qPCR to test and compare the sensitivity of each assay. Conventional PCRs were performed with three different primer pairs; two previously reported primer pairs, ALW-F2/ALW-R2 [20] and AlmF1/AlmR1 [2], and the primer pair (AW16sF/23sR) designed in this study for the specific detection of AlmWB phytoplasma. The qPCRs were performed using the primer pairs and probes designed in this study.

3. Results

3.1. Evaluation and comparison of the specificity of PCR and qPCR assays

BLAST analyses in the NCBI GenBank database against all the available sequences of 'Ca. P. phoenicium' showed that the designed primer pairs AW16sF/AW23sR and AWsF/AWsR matched the 'Ca. P.

Table 2
Comparison of the specificity of previously reported primers and new primers designed in this study.

Host plant	Phytoplasma/bacterial strain	16Sr group	DNA source	No. of samples	18S P1P7 (Lee et al., 1998)	AlwF2/R2 (Abou-Jawdeh et al. 2003)	AlmF1/R1 (Verdin et al., 2003)	AW16sF/23sR this study	Avg Cq value	
									Prun18S-F/R this study	AWsF/R this study
<i>Catharanthus roseus</i>	Severe strain of Western Aster Yellow	I-B	DNA, A. Bianco	1	+	–	–	–	15.3 ± 0.1	–
	Clover phyllody	I-C	DNA, A. Bianco	1	+	–	+	–	14.4 ± 0.2	–
	Phytoplasma									
	Crotalaria juncea	II-A	DNA, A. Bianco	1	+	–	+	–	13.2 ± 0.1	–
	Witches' broom									
	Plum leptonecrosis	III	DNA, Bertaccini	1	+	–	–	–	13.6 ± 0.1	–
	Brinjal little leaf	IV-D	DNA, A. Bianco	1	+	–	+	–	14.2 ± 0.2	–
	phytoplasma									
	Elm yellows phytoplasma	V-A	DNA, A. Bianco	1	+	–	+	–	12.9 ± 0.1	–
	Elm witches broom	V-A	DNA, A. Bianco	1	+	–	+	–	13.5 ± 0.1	–
	Beet leafhopper-transmitted	VI-A	DNA, A. Bianco	1	+	–	+	–	12.2 ± 0.2	–
	Virescence									
	Ash yellows	VII-A	DNA, Bertaccini	1	+	–	–	–	13.9 ± 0.2	–
	Picris echinoides yellow	IX-C	DNA, Bertaccini	1	+	+	–	–	18.2 ± 0.1	–
	Naxos periwinkle	IX-C	DNA, Bertaccini	1	+	+	–	–	14.7 ± 0.1	–
	virescence									
	Peach yellow leaf roll	X	DNA, Bertaccini	1	+	+	–	–	13.5 ± 0.1	–
European Stone	X-B	DNA, Bertaccini	1	+	–	–	–	12.5 ± 0.1	–	
Fruit Yellows										
Pear decline	X-C	DNA, Bertaccini	1	+	–	–	–	14.6 ± 0.1	–	
Stolbur Disease of Pepper	XII-A	DNA, A. Bianco	1	+	–	–	–	15.5 ± 0.1	–	
Stolbur phytoplasma	XII-A	DNA, A. Bianco	1	+	+	–	–	12.7 ± 0.1	–	
strain C										
Bermuda grass white leaf	XIV	DNA, Bertaccini	1	+	–	–	–	15.1 ± 0.1	–	
<i>Prunus dulcis</i>	Almond Witches' broom	IX-D	plant, field sampling	29	+	+	+	12.1 ± 2.3	22.1 ± 3.2	
<i>Prunus persica</i>	Almond Witches' broom	IX-B (D)	plant, field sampling	21	+	+	NT ^a	10.8 ± 3.5	24.8 ± 3.5	
<i>Prunus persica</i>	Almond Witches' broom	IX-B (D)	plant, field sampling	9	+	+	NT	11.8 ± 2.5	23.3 ± 1.2	
<i>Prunus persica</i>	var. <i>nucipersica</i>									
<i>Prunus dulcis</i>	Asymptomatic samples		plant, field sampling	20	–	–	NT	9.6 ± 2.1	–	
<i>Prunus persica</i>	Asymptomatic samples		plant, field sampling	11	–	–	NT	11.4 ± 2.5	–	
<i>Prunus persica</i>	var. <i>nucipersica</i>									
<i>Prunus persica</i>	Asymptomatic samples		plant, field sampling	7	–	–	NT	11.1 ± 1.9	–	
<i>Prunus persica</i>	var. <i>nucipersica</i>									
<i>Quercus</i> sp.		IX-C	plant, field sampling	2	+	+	NT	–	NT	
<i>Pistacia palaestina</i>		IX-C	plant, field sampling	1	+	+	NT	–	NT	
<i>Rhamnus punctata</i>		IX-C	plant, field sampling	1	+	+	NT	–	NT	
<i>Bryonia multiflora</i>		IX-C	plant, field sampling	1	+	+	NT	–	NT	
<i>Assymetrasca decedens</i>		IX-B (D)	Insect, field sampling	80	+	+	NT	+	22.9 ± 4.1	

^a NT: Not tested.

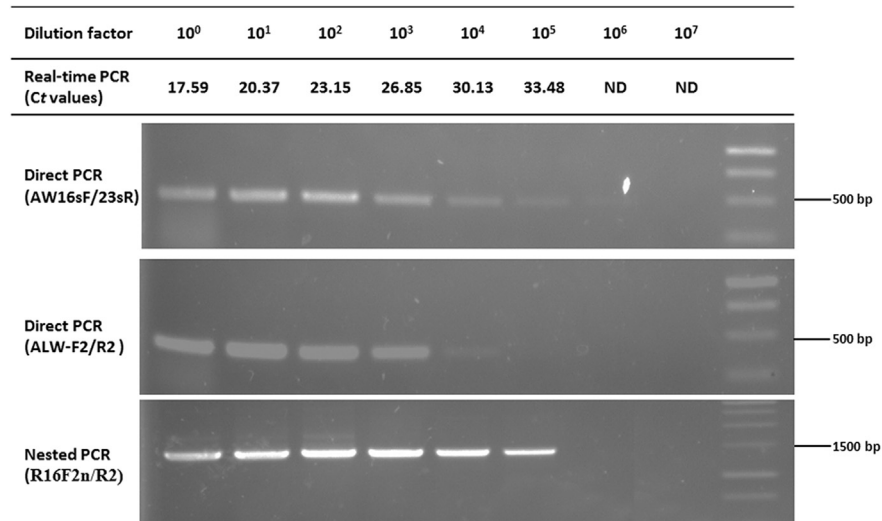


Fig. 3. Comparison of the sensitivity of the real-time PCR protocol (using AWsF/AWsR + AW23plus) with the sensitivities of two conventional PCRs (using the primers AW16sF/AW23sR or ALW-F2/ALW-R2) and with nested PCR using universal primers R16F2n/R16R2). Tenfold serial dilution ($1-10^7$ folds) of DNA samples extracted from AlmWB infected almond samples.

'phoenicum' sequences reported from the three *Prunus* species: almond, peach and nectarine. The specificity resides in the primer AW23sR which differed from the '*Picris echioides* yellows' (PEY) sequences by 6 point mutations, knowing that PEY shares 99% nucleotide identity with AlmWB (Fig. 1). While for the qPCR assay, the specificity resides in both the forward (AWsF) primer and the probe (Fig. 2). It is worth mentioning that in the BLAST analysis of the designed primers, no other organism than '*Ca. P. phoenicum*' was detected.

The DNA extracts obtained from plants infected by 17 phyto-plasmas belonging to 11 different groups were tested by nested PCR using the primer pair (P1/P7) followed by (R16F2n/R2). All the samples gave positive results with the expected amplicon size of about 1250 bp and thus were all included in the specificity tests (Table 2).

In conventional PCR, primer pair AW16sF/AW23sR consistently detected only '*Ca. P. phoenicum*', while the other phyto-plasmas were not detected. In addition, this primer pair was tested on 59 infected samples from three *Prunus* species (almond, peach and nectarine) which were collected from various regions. Non-specific amplification was not observed neither in the healthy plants nor in

the 11 groups of phyto-plasmas. The semi-specific primer pair ALW-F2/ALW-R2 amplified three phyto-plasmas belonging to 16S rRNA group IX. Faint bands were also observed with a phyto-plasma from group X and another from group XII-A. On the other hand, the primer pair AlmF1/AlmR1 [2], reported to be specific for '*Ca. P. phoenicum*', detected only '*Ca. P. phoenicum*' within group IX, but gave amplification products from phyto-plasmas belonging to groups I, II, V, VI and XII (Table 2).

The specificity of the developed qPCR protocol was also confirmed by several tests as shown in Table 2. '*Ca. P. phoenicum*' was detected in almond, peach, nectarine and the insect vector *A. decedens*. Samples from native non-cultivated plants (*Quercus* sp., *Pistacia palaestina*, *Rhamnus punctate* and *Bryonia multiflora*) collected from AlmWB infested orchards that tested positive with both the universal and the semi-specific primer pairs, but tested negative by the newly designed specific primers were further analyzed. Sequencing of the resulting amplicons showed that these plant samples were infected with PEY phyto-plasma which belong to group IX-C. No amplification signals were detected in the DNA extracts of 11 different phyto-plasma groups or within the 16Sr-IX

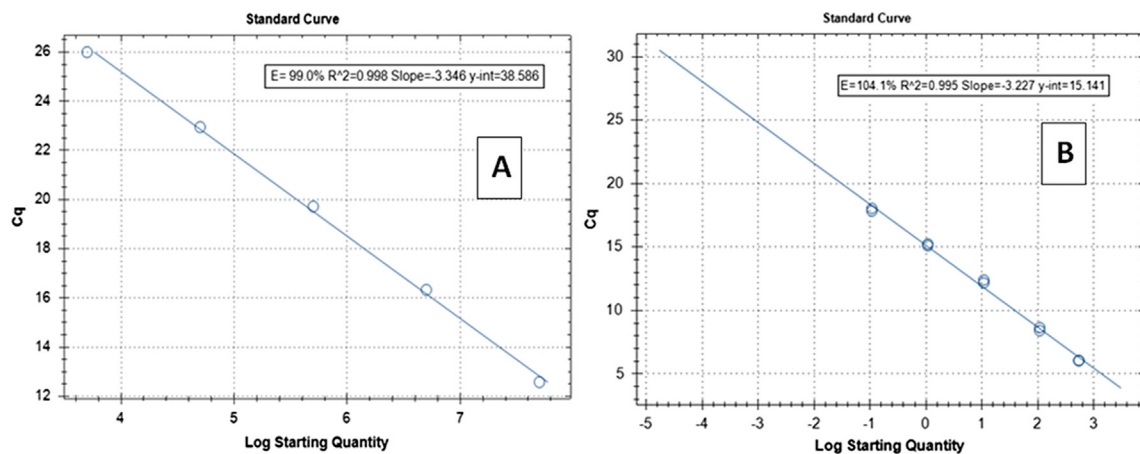


Fig. 4. Standard curves of two real-time PCR assays showing the linear regression line of the Cq on the log dilution of plasmid containing an insert from 16S rDNA from '*Ca. P. phoenicum*' (A) and from 18S rDNA of total nucleic acids extract from a healthy almond plant (B).

Table 3
Quantitation of 'Ca. P. phoenicium' titer during August using qPCR primers and probe (AWsF/R) in almond (A, B and C) and nectarine (D, E and F) plant tissues, using 3 replicates per sample with duplicate testing of each.

Plant code ^a	Plant organ	AlmWB DNA in plant DNA Cq (mean ± SD)	Quantity (GU; mean ± SD)	Plant 18S rDNA fragment Cq (mean ± SD)	Quantity (ng; mean ± SD)	AlmWB DNA/Plant DNA (GU/ng)
A	Leaf midribs	34.18 ± 0.16	1.04E+01 (±1.11E+00)	16.33 ± 0.09	4.28E-01 (±2.59E-02)	2.43E+01
	Root phloem	26.72 ± 0.26	1.78E+03 (±3.18E+02)	19.11 ± 0.15	5.93E-02 (±6.27E-03)	3.00E+04
	Stem phloem	29.64 ± 0.16	2.37E+02 (±2.53E+01)	22.66 ± 0.15	4.71E-03 (±4.98E-04)	5.50E+04
B	Leaf midribs	25.31 ± 0.12	4.67E+03 (±3.86E+02)	6.16 ± 0.08	6.09E+02 (±3.38E+01)	6.75E+00
	Root phloem	27.12 ± 0.12	1.35E+03 (±1.11E+02)	24.79 ± 0.13	1.03E-03 (±9.30E-05)	1.31E+06
	Stem phloem	15.88 ± 0.19	3.08E+06 (±4.04E+05)	8.88 ± 0.11	8.73E+01 (±7.04E+00)	1.31E+06
C	Leaf midribs	31.34 ± 0.15	7.35E+01 (±7.50E+00)	13.84 ± 0.09	2.53E+00 (±1.53E-01)	2.91E+01
	Root phloem	29.2 ± 0.15	3.08E+02 (±3.15E+01)	26.57 ± 0.12	2.89E-04 (±2.47E-05)	2.57E+06
	Stem phloem	20.07 ± 0.12	1.72E+05 (±1.42E+04)	16.86 ± 0.10	2.94E-01 (±2.07E-02)	5.85E+05
D	Leaf midribs	30.23 ± 0.18	1.59E+02 (±1.92E+01)	12.96 ± 0.10	4.75E+00 (±3.35E-01)	3.34E+01
	Root phloem	24.23 ± 0.13	9.80E+03 (±9.05E+02)	19.08 ± 0.16	6.05E-02 (±4.57E-03)	1.62E+05
	Stem phloem	15.31 ± 0.13	4.53E+06 (±3.97E+05)	13.18 ± 0.09	4.07E+00 (±2.67E-01)	1.12E+06
E	Leaf midribs	31.13 ± 0.11	8.50E+01 (±6.20E+00)	13.63 ± 0.12	2.96E+00 (±2.53E-01)	2.874E+01
	Root phloem	25.76 ± 0.80	3.43E+03 (±3.33E+02)	20.43 ± 0.02	2.30E-02 (±3.49E-04)	1.49E+05
	Stem phloem	16.26 ± 0.06	2.36E+06 (±9.15E+04)	13.20 ± 0.05	4.01E+00 (±1.42E-01)	5.85E+05
F	Leaf midribs	33.20 ± 0.07	2.04E+01 (±9.90E-01)	16.69 ± 0.11	3.32E-01 (±2.67E-02)	6.15E+01
	Root phloem	25.16 ± 0.08	5.15E+03 (±2.77E+02)	18.19 ± 0.10	1.14E-01 (±8.02E-03)	4.52E+04
	Stem phloem	28.24 ± 0.12	6.20E+02 (±5.15E+01)	24.05 ± 0.06	1.74E-03 (±7.91E-05)	3.57E+05

^a A, B and C are almond samples; D, E and F are nectarine samples.

group, except for 'Ca. P. phoenicium'. No false positive signals were observed from over 38 negative samples tested.

3.2. Comparison of the sensitivity between different assays

Aliquots of a 10-fold serial dilution from DNA extracts of a severely infected almond tree were tested by PCR and qPCR using different primer pairs reported in the literature or in-house designed primers according to the protocols described previously.

The different protocols and primer pairs designed in this study were compared against each other and against a previously reported primer pair and the universal nested primer pairs (Fig. 3). The specific PCR and qPCR assays showed a similar detection limit to the nested PCR, which was one order of magnitude higher than that of the semi-specific PCR primers (ALW-F2/ALW-R2) (Fig. 3). The developed protocols allow detection of the AlmWB phytoplasma in DNA extracts in up to a five-fold dilution.

3.3. Quantitation of 'Ca. P. phoenicium' in plant organs

The efficiency of the qPCR assays presented by the standard curve using a plasmid including an insert of 'Ca. P. phoenicium' DNA was 99% (Fig. 4A) and the efficiency for the plant 18S rRNA was 104.1% (Fig. 4B). The qPCR assays were used to determine the distribution of phytoplasma within six infected stone fruit trees. In both almond and nectarine, the highest titer during all seasons tested was observed in both root and stem phloem tissues and ranged from 3×10^4 to 10^6 GU/ng. Phytoplasma concentration in stem and root phloem tissues was about three orders of magnitude higher than in those of the leaf midribs which, among the tissues sampled, contained the lowest concentration, ranging from 7 to 33 GU/ng (Table 3). The phytoplasma was also detected in flower petals at levels that ranged from 72 to 130 GU/ng (Data not shown). This assay was also used to determine the titer change of AlmWB phytoplasma in plant organs during three periods: summer (August), winter (December, during dormancy) and late January at almond flowering time before leaf emergence. The 'Ca. P. phoenicium' phytoplasma concentration remained high in the phloem tissues of stems and roots during the three seasons with minor variation. During the summer it ranged from 10^4 to 10^6 GU/ng and 10^4 to 10^5 during December and January, respectively (Data not shown).

4. Discussion

Almond witches' broom disease has been causing significant losses to almond, peach and nectarine production in Lebanon and Iran. In Lebanon, the disease seems to be spreading at an unprecedented rate [4]. Insect vectors are suspected to be largely responsible for this spread. Recently, the spread to distant geographic regions through asymptomatic seedlings infected by AlmWB phytoplasma has also been suspected. Early detection of new introductions accompanied by appropriate measures for local eradication or containment of the disease is of prime importance. While several methods have been developed for detection of phytoplasma, the most commonly used methods in surveys are PCR-based methods. Phytoplasmas are phloem limited, thus their concentration is relatively low and most often a nested PCR protocol is used for their detection. The nested PCR method using universal primer pairs allows phytoplasma detection but not identification. The amplicons resulting from nested PCR may be either sequenced or analyzed with restriction digestion by 17 enzymes for identification of the phytoplasma 16S rRNA group [19]. Over thirty 16S rRNA groups were described recently based on actual or *in silico* RFLP analysis of the PCR amplicons [25–27]. While these techniques are highly valuable for the initial detection and characterization of novel phytoplasmas, the development of specific detection tests may considerably reduce the cost and time in surveys for a specific phytoplasma.

In Lebanon, in spite of the limitations involved, the semi-specific primer pair, which detects mainly group IX phytoplasma, was used in direct PCR for country-wide surveys of 'Ca. P. phoenicium' agent of the AlmWB phytoplasma, since no other phytoplasma in group IX was reported to infect stone fruits. However, when the surveys were extended to include alternative host plants or potential insect vectors which may play a major role in the disease epidemiology, the importance of development of specific detection methods became evident. Multiple alignment of 56 sequences of 16S rRNA originating from eight phytoplasma groups allowed the identification of hypervariable regions that may be used for designing primers suitable for the specific detection of 'Ca. P. phoenicium' in PCR or qPCR assays. The same region was used to design primers for specific detection of phytoplasmas from the apple proliferation group [9] and was recommended for designing species specific PCR

assays. The designed primer pairs AW16sF/23sR and AWsF/AWsR were validated by BLAST analysis against all prokaryotic genes available in the GenBank, where only '*Ca. P. phoenicium*' could be detected. The Blast analysis results were validated in laboratory tests whereby 97 stone fruit samples (59 infected and 38 healthy) were tested using the locally designed primer pairs. The results were similar to those obtained using the universal nested PCR primers, all positive samples were positive and all negative samples were negative.

The specificity of detection was also tested by performing PCR on 17 phytoplasmas belonging to 11 16S rRNA groups. This primer pair detected only '*Ca. P. phoenicium*', and was more specific than the previously reported primers [2,20]. Furthermore, it showed a similar level of sensitivity to nested PCR.

Furthermore, in surveys for alternative weed hosts for '*Ca. P. phoenicium*', four weed species infected by PEY phytoplasma gave negative results using these primers, while positive results have been obtained by the semi-specific primer pair and was further confirmed by sequencing.

Several qPCR protocols for the specific detection or for quantitation of phytoplasmas were reported previously [7–10,28]. In this study, after preliminary design of three different qPCR assays (data not shown), the best specificity, sensitivity and efficiency were obtained with a qPCR assay based on TaqMan[®] probe labeled with a BHQplus[™] at the 3' end. The latter assay detected '*Ca. P. phoenicium*' only. It was also as sensitive as the nested PCR protocol allowing detection of the phytoplasma after a five-fold dilution of TNAs. The development of qPCR allowed the absolute quantitation of AlmWB phytoplasma in plants, a factor that may be of great assistance in studying some aspects of disease epidemiology.

The phytoplasma titer was highest in the phloem tissue of the stem and roots. However, the titer in leaf midribs was significantly lower, by about three orders of magnitude. The phytoplasma titer averaged from 10 to 10² GU/ng in leaf midribs and petals, and from 10⁵ to 10⁶ GU/ng in stem and root phloem tissues of almond and nectarine. These results are close to those reported for European stone fruit yellows (ESFY) phytoplasma in apricots which can host about 2 × 10⁴ cells of ESFY phytoplasmas per ng of plant DNA [29]. Seasonal variations in the concentration and distribution of phytoplasmas were reported previously. For example, apple proliferation (AP) and pear decline (PD) phytoplasmas disappear from the aboveground tree parts during winter and survive in the roots until the following spring when they recolonize the trunk and the branches [30–32]. However, in the case of '*Ca. P. phoenicium*', the situation is quite different; the phytoplasma concentration remains high in the winter season in the phloem tissues of both the root and stem. These results are in line with those reported for the ESFY phytoplasma which can persist in the stem of *Prunus* taxa in the dormant (winter) season [31,33]. This is of prime importance for quarantine detection, since stone fruit seedlings are shipped during the winter season. The detection of phytoplasma in flower petals allows detection of phytoplasma very early in the season, allowing taking eradication actions before considerable transmission activity of insect vectors takes place.

In conclusion sensitive, specific and reliable detection methods are needed for early detection of '*Ca. P. phoenicium*' in nurseries and orchards, since eradication decisions will be based on the results of the used technique [34]. Furthermore, phytoplasma epidemics results from efficient interaction between the cultivated host plants, the insect vectors and the alternative hosts under a conducive environment. The development of specific PCR assays will allow faster identification of potential alternative hosts and vectors, while quantitative assays offer an advantage for better understanding some aspects of the disease epidemiology and offer a good tool for identification of potential insect vectors. In studies related to

variation in vector competency of *Euscelidius variegatus*, only about 700 *Chrysanthemum* yellows phytoplasma (CYP) cells per ng of insect DNA were detected in the head of non-vector leafhoppers, versus 4000 to 8000 cells in the head of vectors [35]. Therefore, higher concentrations of phytoplasma in the salivary glands may indicate a potential role in transmission. This is the first report that describes a PCR and a qPCR method for the specific detection and for quantitation of '*Ca. P. phoenicium*' in plants and insects that will help in surveys and in studying the disease epidemiology.

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