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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 10^5 to 10^6 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.

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)	CTTCCTTTAATAAAGGTCGC	1742–1761[a]	
Real-time PCR			
'*Ca*. P. phoenicium' ITS-23S			132
AWsF (forward)	AGGCCCACCAAACGTCTTAA	1674–1693[a]	
AWsR (reverse)	CCTTCATCGGCTCTTAGTGC	1786–1805[a]	
AW23plus (probe)	**FAM**-ACAAGAGAACAGCGACCTTTATTA-**BHQplus**	1731–1754[a]	
Prunus dulcis 18S rRNA			109
Prun18S-F (forward)	GGAGAGGGAGCCTGAGAAAC	284–303[b]	
Prun18S-R (reverse)	GAGCCCGGTATTGTTATTTATTGTC	368–392[b]	
Prun18S-Taq (probe)	**FAM**-CCACATCCAAGGAAGGCAGCAGGCG-**BHQ1**	309–333[b]	

[a] Based on accession no. AF390136.
[b] Based on accession no. DQ886376.