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EPPO STANDARD - DIAGNOSTICS

PM 7/150 (1) '*Candidatus* Phytoplasma phoenicium'

Specific scope: This Standard describes a diagnostic protocol for '*Candidatus* Phytoplasma phoenicium'.

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.[1]

Specific approval and amendment: Approved in 2021–06. Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Phytoplasmas are phloem-restricted and can be transmitted by grafting and insect vectors. They can also spread to neighbouring hosts via natural root grafts. '*Candidatus* Phytoplasma phoenicium' ('*Ca*. P. phoenicium') strains are members of subgroup 16SrIX-B and its variants [(subgroups 16SrIX-D if per Wei et al., (2007), -F and -G if per Molino Lova et al., (2011)] (Quaglino et al., 2015). These strains are the etiological agents of a devastating disease of almond trees (*Prunus dulcis*). Peach (*P. persica*) and nectarine (*P. persica* var. *nucipersica*) may also be seriously affected by '*Ca*. P. phoenicium' (Jawhari et al., 2015). The common name of the disease is almond witches' broom. Although '*Ca*. P. phoenicium' infection occurs mainly in almond, peach and nectarine, it has occasionally been identified in *P. armeniaca* (apricot), *Prunus* × *amygdalo-persica* (main rootstock for almond and peach in Europe) and in wild plants such as *P. orientalis*, *P. scoparia*, *Anthemis* spp. and *Smilax aspera* (Abou-Jawdah et al., 2002; Salehi et al., 2015; Tedeschi et al., 2015; EPPO, 2017). *Anthemis* spp. and *Smilax aspera* are asymptomatic hosts. '*Ca*. P. phoenicium' is reported from Lebanon and Iran and is widespread where *Prunus* hosts are grown (EPPO, 2017). Recently, it has also been detected in almond plants in South-East Italy (Nigro et al., 2019).

All currently known or potential vectors of '*Ca*. P. phoenicium' are hemipteran leafhoppers (Cicadellidae) or planthoppers (Cixiidae) (EPPO, 2017). The leafhopper *Asymmetrasca decedens* (synonym *Empoasca decedens*) and the planthoppers *Tachycixius cypricus* and *T. viperinus* have already been confirmed as vectors (Abou-Jawdah et al., 2014; Tedeschi et al., 2015). *Asymmetrasca decedens* is highly polyphagous on a wide variety of plants and has a wide distribution including part of the EPPO regions (EPPO, 2017). *Tachycixius cypricus* and *T. viperinus* are not well studied.

A flow diagram describing the procedures for detection and identification is presented in Figure 1.

2 | IDENTITY

Name: '*Candidatus* Phytoplasma phoenicium'.
Notes on taxonomy: '*Ca*. P. phoenicium' strains are members of subgroup 16SrIX-B and its variants [(subgroups 16SrIX-D if per Wei et al., (2007), -F and –G if per Molino Lova et al., (2011)].
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*.
EPPO Code: PHYPPH.
Phytosanitary categorization: EPPO A1 List.

3 | DETECTION

3.1 | Disease symptoms

The most typical symptom on almond, peach and nectarine caused by '*Ca*. P. phoenicium' is shoot proliferation on the main trunk, roots or branches. Witches' broom symptoms may also appear (Figures 2, 3 and 4).

On almond, symptoms may include the development of many axillary buds on the branches, with small and yellowing leaves, and shoots becoming stunted with short internodes (rosetting). Early flowering and long peduncle of flowers or brownish-red leaves may be another indication. Almond trees decline rapidly and some die within 3–4 years following appearance of the first symptoms, while others may survive several years thereafter. The yield of infected trees is reduced. In the first year when symptom appear, fruits are few, small and dark, with shrivelled or sour almonds. Susceptibility among almond cultivars varies. '*Ca*. P. phoenicium' was also detected in asymptomatic almond trees.

On peach and nectarine early flowering (Figure 5) and development of buds, abnormal flowers (phyllody), smaller light green leave, and early senescence may also be

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

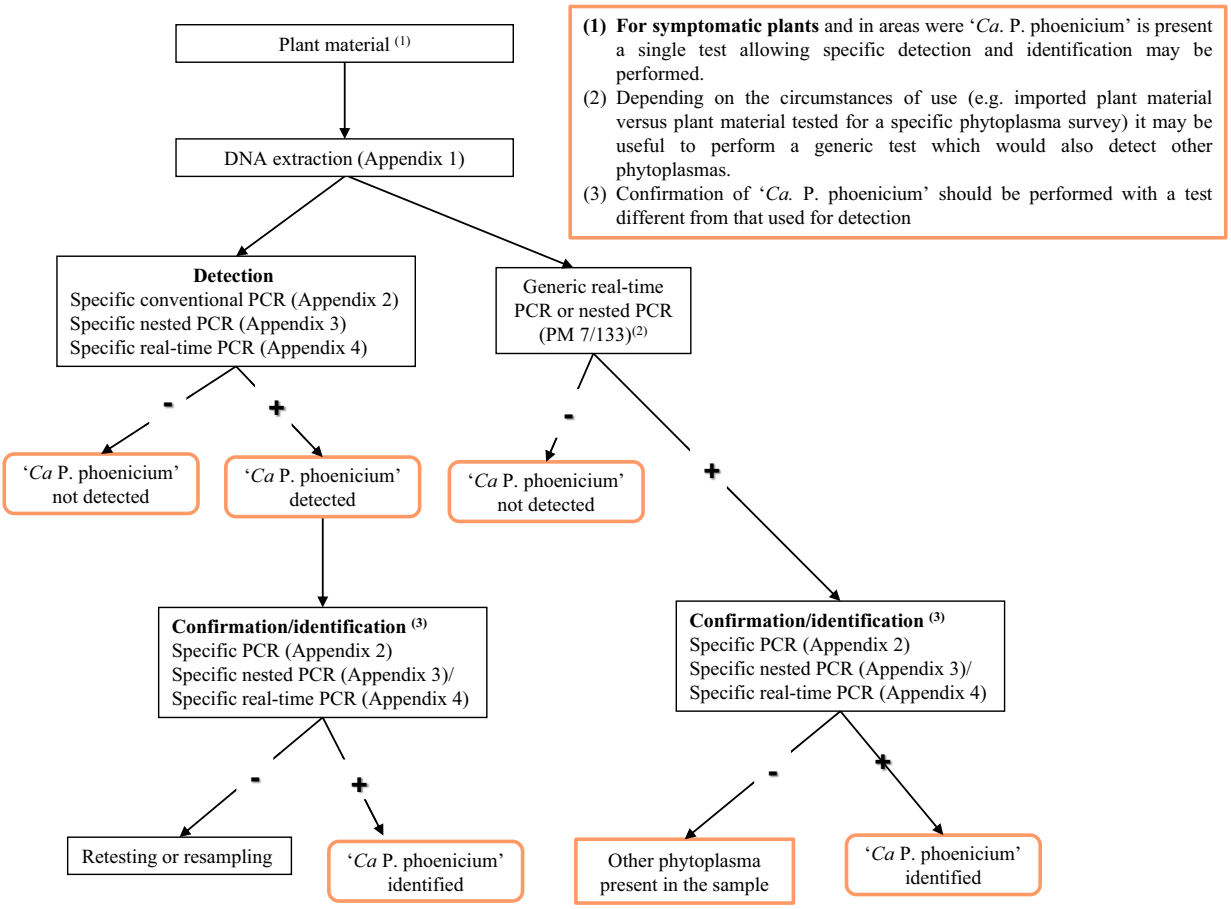

FIGURE 1 Flow diagram for the detection and identification of '*Ca.* P. phoenicium'

FIGURE 2 Almond witches' broom symptoms in August (left). Courtesy: Piero A. Bianco (Univ. of Milan) and Marina Molino Lova (AVSI-Lebanon) and in December (right) Courtesy: Piero A. Bianco (Univ. of Milan)

observed. Symptoms initially affect only some branches and in subsequent years they affect all branches. In the year when symptoms appear, most infected trees do not set any fruits, but some trees bear a limited number of deformed fruits (Figure 6), which are usually elongated and curved. The year following the first appearance of symptoms, fruit production stops.

On apricot trees in Iran, '*Ca.* P. phoenicium' causes leaf roll and proliferation. On rootstock *Prunus* × *amygdalo-persica* internode shortening,

FIGURE 3 Almond witches' broom symptoms in August. Courtesy: Piero A. Bianco (Univ. of Milan)

FIGURE 4 witches' broom symptoms on peach Courtesy: Piero A. Bianco (Univ. of Milan)

chlorosis, reduced size of leaves, proliferation, witches' broom, stunting and dieback were observed. On *P. scoparia*, '*Ca*. P. phoenicium' caused yellowing, witches' broom on different parts of the tree, decline, dieback and death (Abou-Jawdah et al., 2002, 2010; Molino Lova et al., 2011; Salehi et al., 2011, 2015; EPPO, 2017; Nigro et al., 2019).

FIGURE 5 Early flowering in nectarine (tree in the middle already at the end of flowering while other trees are still flowering). Courtesy: Piero A. Bianco (Univ. of Milan) and Marina Molino Lova (AVSI-Lebanon).

FIGURE 6 Fruit deformations on nectarine (on the left). Courtesy: Piero A. Bianco (Univ. of Milan) and Marina Molino Lova (AVSI-Lebanon).

3.2 | Test sample requirements and sample preparation

'*Ca*. P. phoenicium' can be found in flower petals, leaf petioles or midribs, but the highest concentration is in the phloem tissue of stems and roots (Jawhari et al., 2015). In Lebanon, '*Ca*. P. phoenicium' concentration remains high in the phloem tissue of stems and roots during all seasons except autumn (Jawhari et al., 2015). It is not known if this would be the same under different environmental conditions. Note that phytoplasmas may be unevenly distributed throughout the tree, requiring several different parts of the tree to be sampled.

3.2.1 | Sampling of asymptomatic plants

Although it has been possible to detect '*Ca*. P. phoenicium' in some asymptomatic almond trees, there is limited experience with testing asymptomatic plants. As for other phytoplasmas, it is recommended to sample stems or roots from at least three different parts of the tree. Sampled parts should each be at least 10 cm long. The sample may consist of a mixture of stems (with or without leaves) and roots.

3.2.2 | Sampling of symptomatic plants

Samples of roots and/or stems should be collected from trees showing symptoms (see Section 3.1). It is recommended to sample stems (with or without leaves) or roots from at least three different parts of the tree. Samples should not be collected from parts with necrotic areas. Sampled parts should each be at least 10 cm long. The sample may consist of a mixture of stems and roots.

3.2.3 | Sample preparation

Approximately 1–1.5 g of leaf petioles, leaf mid-vein tissue and/or vascular tissue (phloem) from stems or roots should be taken. To obtain phloem tissue from stems, the surface bark is removed and the vascular tissue collected using a scalpel. Roots should be washed thoroughly to eliminate the soil. Tiny roots can be used as the whole. In case of thicker roots, the upper surface should be removed and vascular tissue collected. There is no experience with pooling of samples from different plants.

Material for testing should be used fresh or stored at −20°C (or lower depending on the storage time, e.g. −80°C for more than 2 years).

3.2.4 | Vectors

Although some performance characteristics are available in Jawhari et al., (2015) for a conventional and a real-time PCR test, there is insufficient experience with insect testing to include it in this version of the protocol.

3.3 | Screening tests

3.3.1 | Molecular methods

Different molecular tests for phytoplasma detection are available. The tests recommended in this diagnostic protocol are listed below. They have been evaluated during a test performance study (TPS) organized in the framework of the Euphresco project DIPCAPP (2017-F-234) and were shown to be suitable for either detection or identification (see Section 4.1.1). DNA extraction from plant material is described in Appendix 1.

- Specific conventional PCR test targeting the 16S-ITS-23S rDNA gene (Jawhari et al., 2015), described in Appendix 2.
- Specific nested PCR test targeting the *inmp* gene (Quaglino et al., 2015), described in Appendix 3.
- Specific real-time PCR test targeting the ITS-23S rDNA gene (Jawhari et al., 2015), described in Appendix 4.

Phytoplasmas may occasionally be identified in plants other than their typical host. Therefore, depending on the circumstances of use (e.g. imported plant material versus plant material tested for a specific survey), it may be useful to perform a generic test (PM 7/133 *Generic detection of phytoplasmas*). Generic tests for phytoplasma detection are described in PM 7/133, however, only the real-time PCR test developed by Christensen et al., (2004) was evaluated in the framework of the Euphresco project DIPCAPP (2017-F-234). This test is suitable for detection of '*Ca*. P. phoenicium and is described in Appendix 3 of PM 7/133 (EPPO, 2018). *In silico* analysis was performed for the real-time PCR test of Hodgetts et al. (2009), described in PM 7/133 *Generic detection of phytoplasmas* (EPPO, 2018) based on sequences available in NCBI (National Institute of Biology, November 2018). This *in silico* analysis indicates that the test of Hodgetts et al. (2009) is likely to perform similarly to the test of Christensen et al., (2004) (Mehle, pers. comm., 2020).

4 | IDENTIFICATION

Confirmation of '*Ca*. P. phoenicium' should be performed with a test different from the test used for detection. In areas where '*Ca*. P. phoenicium' is present, a single test allowing specific detection and identification may be sufficient for symptomatic plants.

4.1 | Molecular methods

4.1.1 | Molecular tests recommended

Molecular tests recommended for the identification of species are:

- A specific conventional PCR test targeting the 16S-ITS-23S rDNA region (Jawhari et al., 2015), described in Appendix 2.
- A specific nested PCR test targeting the *inmp* gene (Quaglino et al., 2015), described in Appendix 3.
- A specific real-time PCR test targeting the ITS-23S rDNA region (Jawhari et al., 2015), described in Appendix 4.

These tests have been selected on the basis of the results of the TPS organized in the framework of the Euphresco project DIPCAPP (2017-F-234).

4.1.2 | Tests considered but not recommended

Molino Lova et al. (2011) have developed a nested PCR test targeting the 16S rRNA gene using generic phytoplasma primers (P1/P7 in direct PCR, F1/R0 in nested PCR), followed by *TaqI*-RFLP analysis of patterns to identify '*Ca*. P. phoenicium'. Based on personal communication from Dr R.E. Davis (USDA-ARS, Beltsville, MD) it is recommended to modify the sequence of the F1 primer (5'-AAGACGAGGATAACAGTTGG-3′), originally published in Lee et al. (1995), to (5'-AGGAC GAGGATAACAGTTGG-3′). The modified test of Molino Lova et al. (2011) has been evaluated in the TPS organized in the framework of the Euphresco project DIPCAPP (2017-F-234). It gave lower relative accuracy than the above-listed specific tests. Taking into account this result and the fact that nested PCR and RFLP are labour intensive, this test is not recommended in this Standard.

In the framework of the Euphresco project DIPCAPP (2017-F-234), DNA barcoding of phytoplasmas using P1-ATT (M13-tagged) and P625r (M13-tagged) [PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2020)] was evaluated. Based on *in silico* analysis using the available sequences in NCBI and EPPO-Q-bank (August 2019), the test was not considered suitable for identification of '*Ca*. P. phoenicium' (i.e. it was not possible to distinguish '*Ca*. P. phoenicium' from other phytoplasmas in subgroup 16SrIX-C). In addition to the analytical specificity problem observed, the relative accuracy was the lowest among all evaluated tests. Furthermore, during preliminary studies, primers targeting *Tuf* genes described in PM 7/129 (EPPO, 2021) were tested on two different positive samples and no amplification was obtained (Loiseau & Mehle, pers. comm.). Therefore, the latter primers were not included in the TPS and are not recommended for detection or identification of '*Ca*. P. phoenicium'.

5 | REFERENCE MATERIAL

'*Ca*. P. phoenicium' isolates are available from:

Prof Dr Yusuf Abou-Jawdah, Faculty of Agricultural and Food Sciences, American University of Beirut, Beirut, Lebanon, abujawyf@aub.edu.lb.

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data is also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on these organisms can be obtained from:

Prof Dr Yusuf Abou-Jawdah, Faculty of Agricultural and Food Sciences, American University of Beirut, Beirut, Lebanon, abujawyf@aub.edu.lb.

Dr Siampour Ashkavandi Majid, Department of Plant Protection, College of Agriculture, Shahrekord University, Shahrekord, Iran, siampour@agr.sku.ac.ir.

Further information on recommended tests can be obtained from:

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M. Loiseau, ANSES-LSV Plant Health Laboratory, Angers, France, marianne.loiseau@anses.fr.

N. Mehle, National Institute of Biology, Ljubljana, Slovenia, natasa.mehle@nib.si.

9 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

If you have any feedback concerning this Diagnostic Standard, or any of the tests included, or if you can provide additional validation data for tests included in this Standard that you wish to share, please contact diagnostics@eppo.int.

10 | STANDARD REVISION

An annual review process is in place to identify the need for revision of Diagnostic Standards. Standards identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

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This protocol was prepared by an Expert Working Group composed of N Mehle (lead author), National Institute of Biology, Ljubljana (SI), M Loiseau, ANSES-LSV

Plant Health Laboratory, Angers (FR), and PA Bianco, Dipartimento di Scienze agrarie e ambientali – Produzione, Territorio, Agroenergia, Università degli Studi (IT). It was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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APPENDIX 1 – DNA EXTRACTION FROM PLANT MATERIAL

Extraction procedures are presented below. Other extraction procedures may be used but should be validated in combination with the PCR test to be used.

CTAB procedure (modified from Doyle and Doyle, 1990)

Several different CTAB-based protocols may be used.

The CTAB-based protocol described by Abou-Jawdah et al. (2002) has been shown to be appropriate in combination with the PCR and real-time PCR tests described in Appendices 2–4. This protocol is an optimization of a method described by Doyle and Doyle (1990) for extraction of DNA from woody plants and is presented below as described in PM 7/133 (1) *Generic detection of phytoplasmas* (EPPO, 2018).

Nucleic acids can be extracted from fresh or frozen (−20 or −80°C) tissues as described in Section 3.2.

Grind 1 g of tissue in 10 mL of 3% CTAB buffer [3% cetyl-trimethyl-ammonium bromide (CTAB) in 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 1.4 M NaCl] at room temperature. Transfer 1 mL of the suspension to an Eppendorf tube and add 2 µL of 2-mercaptoethanol (for a final concentration of 0.2%). Vortex briefly and incubate for 20 min at 65°C. Then add an equal volume of chloroform:isoamyl alcohol (24:1). Vortex and centrifuge at 10000 *g* for 10 min. The nucleic acids are in the aqueous phase (top layer). Transfer the aqueous phase carefully to a new tube and precipitate the nucleic acids with an equal volume of cold isopropanol. Shake by inversion and centrifuge at 10 000 *g* for 15 min to recover the precipitate. Wash the pellet with 70% ethanol, air dry and dissolve in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8) or nuclease-free water.

DNA should preferably be stored at approximately −20°C.

Alternative method

In addition to CTAB another procedure for DNA extraction applicable to a large number of samples is described in PM7/133 (1) *Generic detection of phytoplasmas* (EPPO, 2018). This procedure combines a simple and quick homogenization step of crude extracts with DNA extraction based on the binding of DNA to magnetic beads. This extraction procedure has been validated in combination with the phytoplasma universal real-time PCR test Christensen et al. (2004) described in PM 7/133, and with real-time PCR tests for the detection of other fruit tree phytoplasmas [PM 7/62 (EPPO, 2017)]. It has also been used with other molecular tests (e.g. nested PCR) and performed well, but validation data has not yet been published (Mehle, pers. comm., 2020).

One gram of leaf mid-vein tissue or vascular tissue (phloem) from bark or roots is homogenized in 2 mL of extraction buffer (264 mM Tris, 236 mM Tris-HCl, 137 mM NaCl, 2% PVP K-25, 2 mM PEG 6000, 0.05% Tween 20, pH 8.2) or lysis buffer (from a QuickPick™ SML Plant DNA kit, Bio-Nobile) using a tissue homogenizer (e.g. FastPrep®-24 with TN 12 × 15-TeenPrep™ Adapter, MP Biochemicals). Alternative grinding procedures include liquid nitrogen using a mortar and pestle or homogenization in extraction bags using a Homex 6 homogenizer (BIOREBA).

Total DNA can be reliably extracted using a QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (e.g. KingFisher® mL, Thermo Scientific) (Mehle et al., 2013).

Total DNA extract is eluted in 200 µL of elution buffer (QuickPick™ SML Plant DNA kit +KingFisher). For leaf mid-vein tissue and bark/root phloem tissue 10-fold diluted DNA is suitable for testing.

DNA should preferably be stored at approximately −20°C.

APPENDIX 2 – '*CA*. P. PHOENICIUM' SPECIFIC CONVENTIONAL PCR TARGETING THE 16S-ITS-23S RDNA GENE (JAWHARI ET AL., 2015)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. The conventional PCR test is performed for the detection and identification of '*Ca*. P. phoenicium'.

1.2. The test is based on primers published by Jawhari et al. (2015).

1.3. The forward primer is located in a 16S rRNA region, whereas the reverse primer is in a 23S rRNA region (positions based on Genbank accession no. AF390136: 1270-1287 for the forward primer and 1742-1761 for the reverse primer).

1.4. Oligonucleotides.

	Primer	Sequence	Amplicon size
Forward primer	AW16sF	5′-ACAGTCTCAG TTCGGATT-3′	492 bp
Reverse primer	AW23sR	5′-CTTCCTTTAAT AAAGGTCGC-3′	

1.5. The test has been successfully performed using the GoTaq G2 DNA Polymerase (Promega), MyFitTM Mix (Bioline), ScreenMix-HS (Evrogen) or REDTaq ReadyMixTM PCR (Sigma-Aldrich). Note: the procedure described below is when GoTaq G2 DNA Polymerase (Promega) is used. If other enzymes are used the master mix and/or PCR conditions might need to be adapted.

2. METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. DNA extraction methods that are described in Appendix 1 may be used.

2.2. Conventional PCR.

2.2.1. Master mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	16.125	NA
Green GoTaq Reaction Buffer (Promega)	5×	5.0	1×
dNTPs (Promega)	10 mM	0.5	200 µM
AW16sF	10 µM	0.625	0.25 µM
AW23sR	10 µM	0.625	0.25 µM
GoTaq G2 DNA polymerase (Promega)	5 U μL^{-1}	0.125	0.625 U
Subtotal		23.0	
DNA extract		2.0	
Total		25.0	

NA, Not applicable.

2.2.2. PCR conditions: initial denaturation step at 94°C for 3 min; 35 cycles consisting of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C; final extension at 72°C for 7 min.

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product[2]). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive control PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example the universal eukaryotic 28S rRNA gene primers of Werren et al. (1995).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract (the same matrix spiked with nucleic acid from the target organism).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC (and if relevant IC): a band of 492 bp is visualized.

When these conditions are met

- A test will be considered positive if a band of 492 bp is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. PERFORMANCE CHARACTERISTICS AVAILABLE

4.A. Validation data available from Jawhari et al. (2015)

4.A.1. Analytical sensitivity data

Aliquots of a 10-fold serial dilution from DNA extracts of a severely infected almond tree were tested. The test allowed detection of '*Ca*. P. phoenicium' in DNA extracts in up to a dilution of 10^{-5}.

4.A.2. Analytical specificity data

In the BLAST analysis of the designed primer pair, no other organism than '*Ca*. P. phoenicium' was detected. The specificity resides in the primer AW23sR which differed from the sequence of Picris echioides yellows phytoplasma (PEY; phytoplasma belongs to subgroup 16SrIX-C) by six-point mutations, knowing that PEY shares 99% nucleotide identity with '*Ca*. P. phoenicium'. BLAST analyses and sequence alignments showed that the primer pair can detect all '*Ca*.

[2]Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks and whole genome amplicons).

P. phoenicium' isolates reported in GenBank at the time when the test was designed. These reported sequences were from the three *Prunus* species: almond, peach and nectarine.

Inclusivity: 100%

Ca. P. Phoenicium was detected in 59 infected samples of *Prunus dulcis*, *P. persica* var. *persica* and *P. persica* var. *nucipersica* collected in the field.

The tested samples were collected from various regions in North and South Lebanon.

Exclusivity: 100%

Evaluated with

- DNA extracted from *Catharanthus roseus* plants infected with phytoplasma belonging to groups/subgroups: 16SrI-B, 16SrI-C, 16SrII-A, 16SrIII, 16SrIV-D, 16SrV-A, 16SrVI-A, 16SrVII-A, 16SrIX-C, 16SrX, 16SrX-B, 16SrX-C, 16SrXII-A and 16SrXIV.
- DNA extracted from five field-collected samples of *Quercus* sp., *Pistacia palaestina*, *Rhamnus punctata* and *Bryonia multiflora*, infected with 16SrIX-C.

4.A.3. Selectivity

100% evaluated with 38 DNA extracts from field-collected asymptomatic (healthy) plants of *Prunus dulcis*, *P. persica* var. *persica* and *P. persica* var. *nucipersica*.

4.A.4. Repeatability and reproducibility

Not determined.

4.A.5. Other information

Using this test '*Ca*. P. phoenicium' was detected in 80 samples of *Asymmetrasca decedens* (an insect vector of '*Ca*. P. phoenicium') collected in the field.

4.B. Validation data available from the test performance study in 2019 (Euphresco: DIPCAPP; 2017-F-234)

The six participating laboratories analysed a total of 12 blind DNA samples (six target, six non-target). The samples consisted of total DNA extracted from one healthy peach and one healthy almond tree, from three samples infected by phytoplasmas of other groups ('*Ca*. P. mali' infected apple, '*Ca*. P. prunorum' infected peach and '*Ca*. P. solani' infected grapevine), from one sample of periwinkle infected with phytoplasma of subgroup 16SrIX-C, and from three '*Ca*. P. phoenicium' infected peaches and from three '*Ca*. P. phoenicium' infected almond trees. The estimated concentration of '*Ca*. P. phoenicium' in these six DNA samples was medium.

4.B.1. Diagnostic sensitivity data

100%

4.B.2. Diagnostic specificity data

100%

4.B.3. Data on repeatability

Not available.

4.B.4. Data on reproducibility

Reproducibility 100%

Calculated as concordance.

APPENDIX 3 – 'CA. *P. PHOENICIUM*' SPECIFIC NESTED PCR TARGETING THE INMP GENE (QUAGLINO ET AL., 2015)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. The nested PCR protocol is performed for the detection and identification of '*Ca*. P. phoenicium'.

1.2. Two sets of primers are used: inmpF1/inmpR1 primers for first PCR and inmpF2/inmpR2 for second PCR (nested PCR) (Quaglino et al., 2015).

1.3. The inmpF1/inmpR1 and inmpF2/inmpR2 primers amplify the integral membrane protein coding region (*inmp* gene).

1.4. Oligonucleotides.

	Primer	Sequence	Amplicon size
Forward primer	inmpF1	5′-AGTAATTAATTTTCAATA TTGGACTG-3′	668 bp
Reverse primer	inmpR1	5′-TCACATCATCCTCATTCA TTTTTGAAGC-3′	
Forward primer	inmpF2	5′-AGAAATCTTATCAGTGG TATCAGTC-3′	413 bp
Reverse primer	inmpR2	5′-TCTTTATCTATTGTTTTA TATGCCAC-3′	

1.5. The test has been successfully performed using GoTaq G2 DNA Polymerase (Promega), ScreenMix-HS (Evrogen) and Platinum™ *Taq* DNA Polymerase (Thermo Fisher Scientific). Note: The procedure described below is for GoTaq G2 DNA Polymerase (Promega). If other enzymes are used the master mix and/or PCR conditions might need to be adapted.

2. METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. DNA extraction methods that are described in Appendix 1 may be used.

2.2. Conventional PCR, followed by nested PCR.

2.2.1. Master mix for PCR.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	15.375	NA
Green GoTaq Reaction Buffer (Promega)	5×	5.0	1×
dNTPs (Promega)	10 mM	0.5	200 µM
inmpF1	10 µM	1.0	0.4 µM

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
inmpR1	10 µM	1.0	0.4 µM
GoTaq G2 DNA polymerase (Promega)	5 U/µL^{-1}	0.125	0.625 U
Subtotal		23.0	
DNA extract		2.0	
Total		25.0	

NA, Not applicable.

2.2.2. PCR conditions: initial denaturation step at 94°C for 5 min; 35 cycles consisting of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C; final extension at 72°C for 10 min.

2.2.3. Master mix for nested PCR.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	15.375	NA
Green GoTaq Reaction buffer (Promega)	5×	5.0	1×
dNTPs (Promega)	10 mM	0.5	200 µM
inmpF2	10 µM	1.0	0.4 µM
inmpR2	10 µM	1.0	0.4 µM
GoTaq G2 DNA polymerase (Promega)	5 U µL^{-1}	0.125	0.625 U
Subtotal		23.0	
1:100 diluted inmpF1/ inpmR1 PCR product		2.0	
Total		25.0	

NA, Not applicable.

2.2.4. Nested PCR conditions: initial denaturation step at 94°C for 5 min; 35 cycles consisting of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; final extension at 72°C for 10 min.

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or, if not available, clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product[3]). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive control PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can be genes either present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample, for example the universal eukaryotic 28S rRNA gene primers of Werren et al. (1995).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: the same matrix spiked with nucleic acid from the target organism.

3.2. Interpretation of results:

Verification of the controls (after nested PCR)

- NIC and NAC: no band is visualized.
- PIC and PAC (and if relevant IC): a band of the 413 bp is visualized.

When these conditions are met

- A test will be considered positive if a band of 413 bp is visualized.
- A test will be considered negative if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

[3]Laboratories should take additional care to prevent risks of cross-contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons).

4. PERFORMANCE CHARACTERISTICS AVAILABLE

4.A. Validation data available from Quaglino et al., (2015)

4.A.1. Analytical sensitivity data

Not determined.

4.A.2. Analytical specificity data

Inclusivity: 100%

'*Ca*. P. phoenicium' was detected from 20 infected samples of plants of *Prunus dulcis*, *P. persica* and *P. persica* var. *nucipersica* collected from the field.

These tested samples were collected from three regions in Lebanon (in North and South Lebanon, and in Bekaa).

Exclusivity: 100%

No amplification was generated from plants infected by other phytoplasmas of the following 16Sr subgroups: 16SrI-B, 16SrV-A, 16SrIX-C and 16SrXII-A.

4.A.3. Selectivity

Not determined.

4.A.4. Repeatability and reproducibility

Not determined.

4.B. Validation data available from the test performance study in 2019 (Euphresco: DIPCAPP; 2017-F-234):

The six participating laboratories analysed a total of 12 blind DNA samples (six target, six non-target). Due to technical difficulties, the results from one of the laboratories were removed from the calculation. The samples consisted of total DNA extracted from one healthy peach and one healthy almond tree, from three samples infected by phytoplasmas of other groups ('*Ca*. P. mali' infected apple, '*Ca*. P. prunorum' infected peach and '*Ca*. P. solani' infected grapevine), from one sample of periwinkle infected with phytoplasma of subgroup 16SrIX-C, and from three '*Ca*. P. phoenicium' infected peaches and from three '*Ca*. P. phoenicium' infected almond trees. The concentration of '*Ca*. P. phoenicium' in these six DNA samples was medium.

4.B.1. Diagnostic sensitivity data

100%

4.B.2. Diagnostic specificity data

100%

4.B.3. Data on repeatability

Not available.

4.B.4. Data on reproducibility

Reproducibility 100%.

Calculated as concordance.

APPENDIX 4 – REAL-TIME PCR FOR SPECIFIC DETECTION OF '*CA*. P. PHOENICIUM' (JAWHARI ET AL., 2015)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. GENERAL INFORMATION

1.1. The real-time PCR protocol is performed for the detection and identification of '*Ca*. P. phoenicium'.

1.2. The test is based on primers and probe published by Jawhari et al. (2015).

1.3. Primers amplify a specific fragment of 132 bp spanning the hypervariable intergenic spacer (ITS) region and the 23SrRNA region (position 1674–1805 for Genbank accession no. AF390136).

1.4. Oligonucleotides:

	Primers/ probe	Sequence	Amplicon size
Forward primer	AWsF	5′-AGGCCCACCA AACGTCTTAA-3′	132 bp
Reverse primer	AWsR	5′-CCTTCATCGG CTCTTAGTGC-3′	
Probe	AW23plus	5′-FAM-ACAAGAGA ACAGCGACCTTT ATTA-BHQplus-3′	

1.5. The test has been successfully performed using the iQ Supermix (Bio-Rad), qPCRmix-HS (Evrogen) and the TaqMan Universal PCR Master Mix (Applied Biosystems) on a range of different real-time PCR systems including the ABI 7900HT Fast (Applied Biosystem), CFX96 Touch thermal cycler (Bio-Rad) and Agilent AriaMx Real-Time PCR system (Agilent). Note: The procedure described below is for the iQ Supermix (Bio-Rad). If other enzymes are used the master mix and/or real-time PCR conditions might need to be adapted.

2. METHODS

2.1. Nucleic acid extraction and purification.

2.1.1. DNA extraction methods that are described in Appendix 1 may be used.

2.2. Real-time PCR.

2.2.1. Master mix.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	1.6	NA
iQ Supermix (Bio-Rad)	2×	5.0	1×
AWsF	10 µM	0.3	0.3 µM
AWsR	10 µM	0.3	0.3 µM
AW23plus (probe)	2.5 µM	0.8	0.2 µM
Subtotal		8.0	
DNA extract		2.0	
Total		10.0	

NA, Not applicable.

2.2.2. Real-time PCR conditions: initial denaturation at 95°C for 10 min; 40 cycles consisting of 15 s at 95°C and 1 min at 60°C

3. ESSENTIAL PROCEDURAL INFORMATION

3.1. Controls

For a reliable test result to be obtained the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product[4]). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the external positive controls (PIC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPCs can either be genes present in the matrix DNA or added to the DNA solutions.

Alternative IPCs can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA).
- Amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation to the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

Other possible controls

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract: the same matrix spiked with nucleic acid from the target organism.

[4]Laboratories should take additional care to prevent risks of cross-contamination when using high concentration positive controls (e.g. cloned products, gBlocks and whole genome amplicons).

3.2. Interpretation of results:

Verification of the controls

- The PIC and PAC (as well as IC and IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. PERFORMANCE CHARACTERISTICS AVAILABLE

4.A. Validation data available from Jawhari et al. (2015):

4.A.1. Analytical sensitivity data

Aliquots of a 10-fold serial dilution from DNA extracts of a severely infected almond tree were tested. The test allowed detection of '*Ca*. P. phoenicium' in DNA extracts up to a dilution of 10^{-5}.

4.A.2. Analytical specificity data

In the BLAST analysis of the designed primers and probe, no other organism than '*Ca*. P. phoenicium' was detected. The specificity resides in both the primer AWsF and the probe. BLAST analyses and sequence alignments showed that the primers and probe can detect all '*Ca*. P. phoenicium' isolates reported in GenBank at the time when the test was designed. These reported sequences were from the three *Prunus* species: almond, peach and nectarine.

Inclusivity: 100%

Ca. P. Phoenicium was detected in 59 infected samples of *Prunus dulcis*, *P. persica* var. *persica* and *P. persica* var. *nucipersica* collected in the field.

The tested samples were collected from various regions in North and South Lebanon.

Exclusivity:

- DNA extracted from *Catharanthus roseus* infected with phytoplasmas belonging to groups/ subgroups 16SrI-B, 16SrI-C, 16SrII-A, 16SrIII, 16SrIV-D, 16SrV-A, 16SrVI-A, 16SrVII-A, 16SrIX-C, 16SrX, 16SrX-B, 16SrX-C, 16SrXII-A and 16SrXIV.

4.A.3. Selectivity

100% evaluated with 38 DNA extracts from field collected asymptomatic (healthy) plants of *Prunus dulcis*, *P. persica* var. *persica* and *P. persica* var. *nucipersica*.

4.A.4. Repeatability and reproducibility

Not determined.

4.A.5. Other information

Using this test '*Ca*. P. phoenicium' was detected in 80 samples of *Asymmetrasca decedens* (an insect vector of '*Ca*. P. phoenicium') collected in the field.

4.B. Validation data available from the test performance study in 2019 (Euphresco: DIPCAPP; 2017-F-234):
The six participating laboratories analysed a total of 12 blind DNA samples (six target, six non-target). Due to technical difficulties, the results from one of the laboratories were removed from the calculation of the performance characteristics. The samples consisted of total DNA extracted from one healthy peach and one healthy almond tree, from three samples infected by phytoplasmas of other groups ('*Ca*. P. mali' infected apple, '*Ca*. P. prunorum' infected peach and '*Ca*. P. solani' infected grapevine), from one sample of periwinkle infected with phytoplasma of subgroup 16SrIX-C, and from three '*Ca*. P. phoenicium' infected peaches and from three '*Ca*. P. phoenicium' infected almond trees. The estimated concentration of '*Ca*. P. phoenicium' in all these six analysed DNA samples were medium.

4.B.1. Diagnostic sensitivity data

100%

4.B.2. Diagnostic specificity data

100%

4.B.3. Data on repeatability

Not available.

4.B.4. Data on reproducibility

Reproducibility: 100%.

Calculated as concordance.