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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/62 (3)

Diagnostics

PM 7/62 (3) '*Candidatus* Phytoplasma mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum'

Specific scope

This Standard describes a diagnostic protocol for '*Candidatus* Phytoplasma mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum'.[1]

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

Specific approval and amendment

Approved as PM 7/62 *Candidatus* Phytoplasma mali and PM 7/63 *Ca*. P. pyri in 2006. First revision in 2017-02 as a single Standard as PM 7/62 (2) with the addition of '*Ca*. P. prunorum'. Second revision in 2019-06.

1. Introduction

Fruit trees of the family Rosaceae may be seriously affected by phytoplasmas of the Apple Proliferation group (AP 16SrX group). The AP group includes '*Candidatus* Phytoplasma mali', which causes apple proliferation (AP), '*Ca*. P. prunorum', associated with European stone fruit yellows (ESFY) and '*Ca*. P. pyri', associated with pear decline (PD) (Seemüller & Schneider, 2004; Marcone *et al*., 2010). Although '*Ca*. P. mali' infection occurs mainly in the genus *Malus*, it has also been occasionally identified in plants other than the typical host, for example stone fruits and both European pear (*Pyrus communis*) and Asian pear (*Pyrus pyrifolia*) (Lee *et al*., 1995; Del Serrone *et al*., 1998; Seemüller & Schneider, 2004; Mehle *et al*., 2007). '*Ca*. P. pyri' is mainly associated with the genus *Pyrus* (Seemüller & Schneider, 2004). '*Ca*. P. prunorum' causes economically important disorders in apricot (*Prunus armeniaca*), Japanese plum (*Prunus salicina*) and peach (*Prunus persica*) (Carraro & Osler, 2003). European plums (*Prunus domestica*) as well as some other wild *Prunus* species (*Prunus spinosa*, *Prunus cerasifera*, *Prunus insititia*) are susceptible to infection but generally do not show symptoms. Such species represent a hidden source of infection (Carraro *et al*., 1998a, 2004; Carraro & Osler, 2003). In contrast, *Prunus avium* has demonstrated a high level of resistance to '*Ca*. P. prunorum' (Jarausch *et al*., 1999). Phytoplasmas from the AP group have also been detected in hazel (*Corylus avellana*), ash (*Fraxinus excelsior*), dog rose (*Rosa canina*), hackberry (*Celtis australis*), hawthorn (*Crataegus monogyna*), oak (*Quercus robur* and *Quercus rubra*), hornbeam (*Carpinus betulus*) and bindweed (*Convolvulus arvensis*) (Seemüller & Schneider, 2004).

Psyllids seem to play a crucial role in the transmission of phytoplasmas from the AP group (Tedeschi & Alma, 2004). '*Ca*. P. prunorum' is transmitted to host plants of *Prunus* species by the vector *Cacopsylla pruni* (Carraro *et al*., 1998b). Additionally, the leafhopper *Asymmetrasca decedens* (synonym *Empoasca decedens*) has been suggested as a potential vector of this phytoplasma (Pastore *et al*., 2004). '*Ca*. P. pyri' is transmitted to the host plants by two vectors, *Cacopsylla pyricola* (Davies *et al*., 1992) and *Cacopsylla pyri* (Carraro *et al*., 1998c). Known psyllid vectors of '*Ca*. P. mali' are *Cacopsylla picta* (synonym *Cacopsylla costalis*) (Frisinghelli *et al*., 2000; Jarausch *et al*., 2003) and *Cacopsylla melanoneura* (Tedeschi & Alma, 2004). In addition to psyllids, some other insects have been reported as vectors of '*Ca*. P. mali', including the spittlebug *Philaenus spumarius*, the leafhopper *Artianus interstitialis* (Hegab & El-Zohairy, 1986) and possibly *Fieberiella florii* (Krczal *et al*., 1988).

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

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

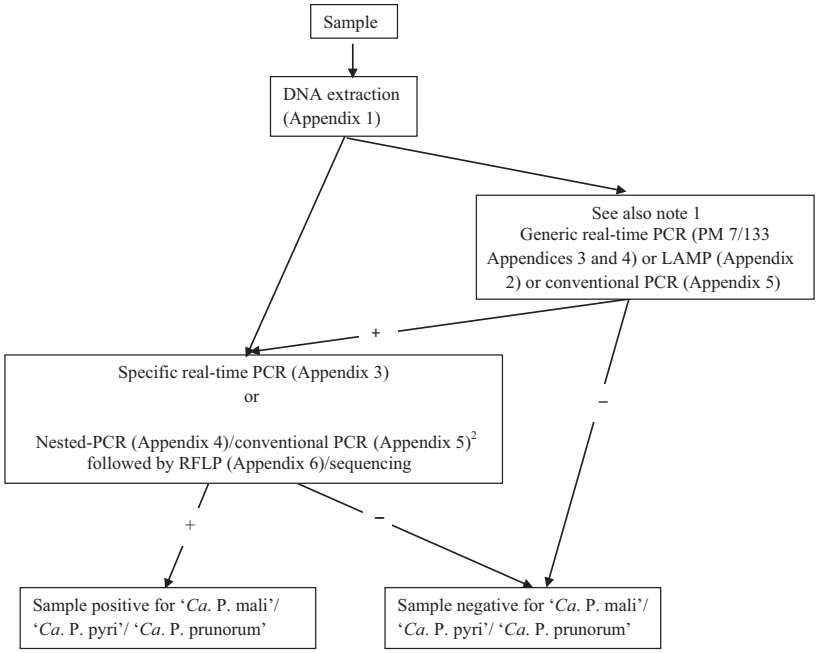

Fig. 1 Flow diagram for the detection and identification of '*Ca*. P. mali'/'*Ca*. P. pyri'/'*Ca*. P. prunorum'.[1]Depending on the circumstances of use (e.g. imported plant material versus plant material tested for a specific phytoplasma survey) it may be useful to perform a generic test which would then detect other phytoplasmas. [2]If the conventional PCR (Appendix 5) was performed as the first test, sequencing of the PCR product should be performed to identify the species.

2. Identity

Name: '*Candidatus* Phytoplasma mali'
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*
Provisional taxon: Phytoplasma Apple Proliferation (AP) group or 16SrX
EPPO Code: PHYPMA
Phytosanitary categorization: EPPO A2 List no. 87; EU Annex IV (RNQP)
Name: '*Candidatus* Phytoplasma pyri'
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*
Provisional taxon: Phytoplasma Apple Proliferation (AP) group or 16SrX
EPPO Code: PHYPPY
Phytosanitary categorization: EPPO A2 List no. 95; EU Annex IV (RNQP)
Name: '*Candidatus* Phytoplasma prunorum'
Taxonomic position: *Bacteria*, *Firmicutes*, *Mollicutes*, *Acholeplasmatales*, *Acholeplasmataceae*
Provisional taxon: Phytoplasma Apple Proliferation (AP) group or 16SrX
EPPO Code: PHYPPR
Phytosanitary categorization: EU Annex IV (RNQP)

3. Detection

3.1. Disease symptoms

The severity of the disease depends on a number of factors, including species, variety, rootstock and the age of the trees.

The distribution of phytoplasmas in the tree is uneven and is not constant over the year. It may vary from one year to the next, and in some years symptoms may not be observed. The distribution pattern in the tree is also dependent on temperature. In winter, the content of phytoplasmas in the aboveground part of the tree declines due to sieve-tube degeneration, and the phytoplasmas concentrate more in the roots. Phytoplasmas are detected in phloem tissues in shoots from mid-summer to the end of sap flow. Detection on roots is possible throughout the year, although uneven distribution also applies here (Schaper & Seemüller, 1982; Seemüller *et al*., 1984).

3.1.1. Apple proliferation

The most typical symptom caused by '*Ca*. P. mali' is witches' broom at the end of shoots (Fig. 2). On diseased trees, leaves roll downward and become brittle, they are finely and irregularly serrated and are smaller than normal, with unusually enlarged stipules (Fig. 3). Fruits are smaller

Fig. 2 Witches' broom caused by apple proliferation is particularly evident in wintertime. (Courtesy of F. Bondaz, Plant Protection Unit of Val d'Aosta Region, IT.)

Fig. 3 Leaves of apple proliferation infected trees (2 left) are smaller than normal ones (2 right) and have large stipules at the base of the stem. (Courtesy of F. Bondaz, Plant Protection Unit of Val d'Aosta Region, IT.)

and flattened (Fig. 4), and peduncles longer. Early leaf reddening is a good indication of the presence of phytoplasma. The presence of a fine hairy root system on nursery plants during winter may be another indication.

3.1.2. Pear decline

The most easily recognized symptoms occur in late summer with the development of premature autumn leaf colour on affected trees. Many cultivars develop a premature red colour (Figs 5 and 6), but some may develop a premature

Fig. 4 Apples cv. Jonagold from a healthy (left) and apple proliferation-infected tree (right). Infected fruits are undersized, misshapen and irregularly coloured. (Courtesy of F. Bondaz, Plant Protection Unit of Val d'Aosta Region, IT.)

Fig. 5 Reddening of the foliage with pear decline of young trees of *P. calleryana*. (Courtesy of ILVO.)

yellow colour. There may be some leaf cupping or curling and there is usually premature leaf drop. The following spring, affected trees suffer from weak growth and sparse pale foliage. The severity of the spring symptoms can vary from absence to death. There may be a line of necrotic tissue in the bark at the graft union between scion and rootstock.

3.1.3. European stone fruit yellows phytoplasma

Typical symptoms are reddening and curling of leaves (Fig. 7), and sometimes lines of necrotic tissue in the bark (Fig. 8).

Possible confusion. The premature autumn leaf colour symptoms associated with apple proliferation, pear decline and European stone fruit yellows may also have several other causes. Water logging, root damage, ring barking caused by feeding animals, some bacterial cankers, rootstock and variety incompatibility can all give rise to symptoms resembling those caused by phytoplasma infection.

Fig. 6 (A) and (B) Reddening of the laminar tissue of tree with pear decline. (Courtesy of EPPO Global Database).

3.2. Test sample requirements and sample preparation

3.2.1. Sampling of asymptomatic plants

There is limited experience in the EPPO region with testing on asymptomatic plants. In Slovenia, testing in nurseries is performed on small roots sampled from at least three different root areas of the tree. Root parts should each be 10 cm long. In the case of apricot trees in Switzerland, roots tissue collected in early winter has been shown to be more reliable for the detection of ESFY (Christen D. *pers. comm.*).

It should be noted that testing of roots for *Pyrus* species trees grafted on *Cydonia oblonga* is not recommended because this latter species shows different susceptibility to '*Ca*. P. pyri' (Poggi *et al*., 1995; Seemüller *et al*., 1986).

Fig. 7 Characteristic leafroll symptoms on apricot caused by European stone fruit yellows phytoplasma. (Courtesy of G. Morvan, INRA, Montfavet, FR.)

Testing of leaves as described in section 3.2.2 is recommended.

3.2.2. Sampling of symptomatic plants

Samples should be collected from shoots showing symptoms but in good condition (no necrotic areas) and not affected by other pests. Symptoms appear between June and October and the timing of appearance depends on the cultivar and the environment. The phytoplasmas may be unevenly distributed through the tree, requiring several different parts of the tree to be examined. It is advisable to examine the shoots from at least three different parts of the tree and collect a small branch from each part.

3.2.3. Sample preparation

Approximately 1–1.5 g of leaf mid-vein tissue and/or vascular tissue (phloem) from bark or roots should be randomly collected. For testing with real-time PCR, pooling of leaves, bark or roots collected from up to five plants is possible. There is little experience with pooling with conventional PCR.

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

Testing of vectors is only done for research purposes and is not described in this Standard.

3.3. Screening tests

3.3.1. Molecular methods

Different molecular methods for phytoplasma detection are available. The tests recommended in this diagnostic protocol are:

- Two real-time PCR tests developed by Christensen *et al*. (2004) and Hodgetts *et al*. (2009). These PCR tests are described in PM 7/133 *Generic detection of phytoplasmas* Appendices 3 and 4 respectively (EPPO, 2018)

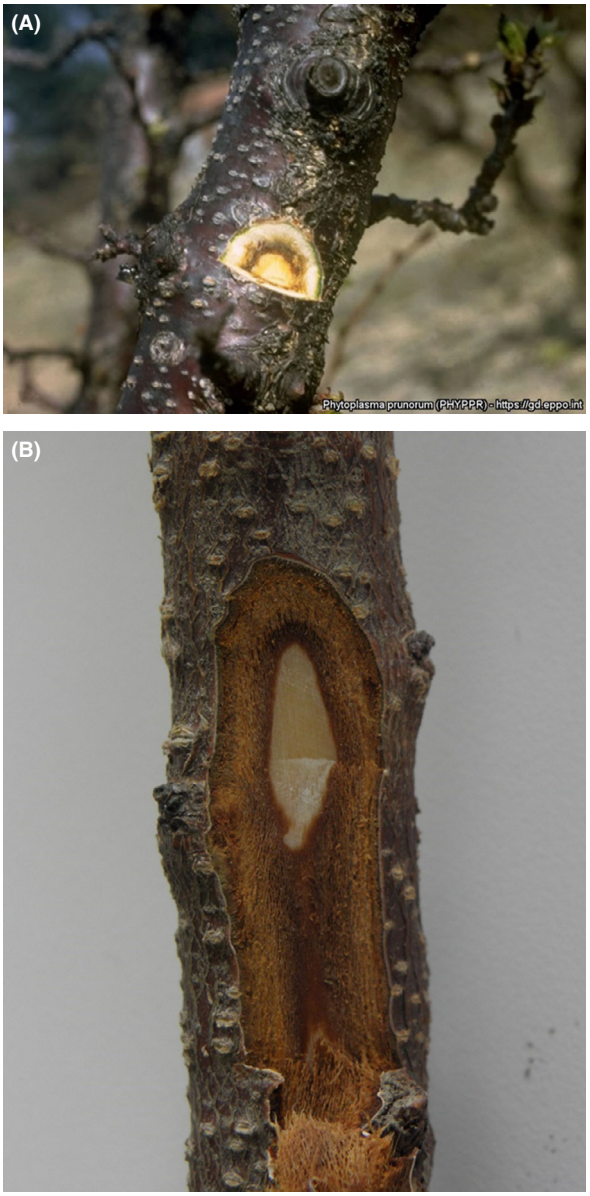

Fig. 8 (A) and (B) Browning and necrosis of the middle layer of apricot bark (phloem) of tree caused by European stone fruit yellows phytoplasma after a severe winter (Courtesy of G. Morvan, INRA, Montfavet, FR.)

- A LAMP test (De Jonghe *et al*., 2017), described in Appendix 2.
- A conventional PCR test with primers fU5/rU3 (Lorenz *et al*.,1995) described in Appendix 5.

Phytoplasmas may occasionally be identified infecting 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 phytoplasma detection survey), it may be useful to perform a generic test (PM 7/133 *Generic detection of phytoplasmas* Appendices 3 and 4 respectively, and Appendix 5) which would then detect other phytoplasmas.

3.3.2. Other tests

Testing on woody indicators and microscopic examination, using 4′,6-diamidino-2-phenylindole (DAPI) staining, are methods recommended in PM 4/27 *Pathogen-tested material of* Malus, Pyrus (EPPO, 1999) *and* Cydonia and PM 4/30 *Certification scheme for almond, apricot, peach and plum* (EPPO, 2001). Such tests are mainly used in the framework of the production of certified material, not for routine testing.

4. Identification

4.1. Molecular methods

4.1.1. Molecular tests

Molecular tests recommended for the identification of species are:

- Specific real-time PCR test for the detection of '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' (Nikolić et al., 2010; Mehle *et al*., 2013b) (Appendix 3).
- AP group-specific nested PCR, with the primer pairs P1/P7 (Deng & Hiruki, 1991; Schneider *et al*., 1995), followed by group-specific PCR with f01/r01 (Lorenz *et al*., 1995) (Appendix 4). In the case of positive results, this should be followed by restriction fragment length polymorphism (RFLP) (Appendix 6) or sequencing to identify the phytoplasma.
- Conventional PCR test with primers fU5/rU3 (Lorenz *et al*., 1995) (Appendix 5). In the case of positive results, this should be followed by RFLP (Appendix 6) or sequencing to identify the phytoplasma.

The real-time PCR and the nested PCR were included in the test performance study in 2011 (EUPHRESCO FruitPhytoInterlab Group, 2011). No relevant differences regarding analytical sensitivity were observed between the two tests. The primers f01/r01 may also be used directly in a conventional PCR test format, but analytical sensitivity is reduced.

A conventional PCR test with primers fU5/rU3 was not evaluated during the EUPHRESCO FruitPhytoInterlab project. Its main advantage is to avoid the risks of cross-contamination that may occur with a nested PCR test, although it is known that analytical sensitivity with conventional PCR is reduced compared with nested PCR.

4.1.2. DNA barcoding

General procedures for DNA barcoding of phytoplasmas are described in the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* including procedures for '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum' (EPPO, 2016).

5. Reference material

Institut national de la recherche agronomique, UMR GDPP Bordeaux, BP 81, 33883 Villenave d'Ornon Cedex, France.

http://www6.bordeaux-aquitaine.inra.fr/bfp_eng/Resources/Phytoplasmas-collection.

Phytobacteriology Laboratory, Plant Pathology, DiSTA – *Alma Mater Studiorum*, University of Bologna, Italy (assunta.bertaccini@unibo.it). Phytoplasma Collection. International Phytoplasmologists Working Group. http://www.ipwgnet.org/index.php?option=com_content&view=article&xml:id=29&Itemxml:id=5.

Sequences for different strains are available in Q-bank (http://qbank.eppo.int/phytoplasmas/).

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 are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended that this database is consulted 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:

Ms Bertacini, Phytobacteriology Laboratory, Plant Pathology, DiSTA – *Alma Mater Studiorum*, University of Bologna, (IT); email: assunta.bertaccini@unibo.it.

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Mr Jarausch, Institut für Molekulare und Angewandte Pflanzenforschung Rheinland-Pfalz, RLP AgroScience GmbH, Breitenweg 71, 67435 Neustadt-an-der-Weinstrasse (DE); email: wolfgang.jarausch@agroscience.rlp.de

9. Feedback on this Diagnostic Protocol

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

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols 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.

Acknowledgements

The protocol for '*Ca*. P. mali' was originally drafted by F. Costard, Ministère de l'Agriculture, Service de la Protection des Végétaux, Unité de virologie des ligneux, Villenave d'Ornon (FR). The protocol for '*Ca*. P. pyri' was originally drafted by D.L. Davies, Horticulture Research International, East Malling (GB). Revision 2 (common protocol and addition of '*Ca*. P. prunorum') was prepared by N. Mehle, National Institute of Biology, Večna pot 111, 1000 Ljubljana (SI) (natasa.mehle@nib.si) and M. Loiseau, ANSES-LSV Plant Health Laboratory, 49044 Angers (FR) (marianne.loiseau@anses.fr). Revision 3 (addition of the LAMP test) was prepared by K. De Jonghe, ILVO 9820 Merelbeke (BE) (Kris.DeJonghe@ilvo.vlaanderen.be).

The revision was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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Appendix 1 – DNA extraction from plant material

CTAB procedure (modified from Doyle & Doyle, *1990)*

Several methods have been developed and compared (Palmano, 2001). The method described below is an optimization of a method described by Doyle & Doyle (1990) for extraction of DNA from woody plants.

Nucleic acids can be extracted from fresh or frozen (−20 or −80°C) tissues [leaf veins, vascular tissue (phloem) from bark or roots].

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, 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 10 000 *g* for 10 min. Recover the aqueous phase 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.

Alternative method

Another DNA extraction method applicable to a large number of plant samples combines a simple and quick homogenization step of crude extracts with DNA extraction based on the binding of DNA to magnetic beads. This extraction method has been validated in combination with the real-time PCR test Christensen *et al*. (2004) described in PM 7/133 *Generic detection of phytoplasmas* (Appendix 3 of that protocol) and the real-time PCR (Appendix 3 current protocol). It has also been used with other molecular tests (nested PCR and loop-mediated isothermal amplification, LAMP) and performed well, but validation data have not yet been published (Mehle, pers. comm., 2016).

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 tissue homogenizer (e.g. FastPrep®-24 with TN 12 × 15-TeenPrep™ Adapter, MP Biochemicals). Alternative grinding procedures include with 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*., 2013a).

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 tenfold diluted DNA is suitable for testing.

Extracted total DNA can be kept at −20°C.

Other extraction methods may be used but should be validated in combination with the PCR test to be used.

Appendix 2 – Loop-mediated isothermal amplification (LAMP) (De Jonghe *et al*., 2017)

1. General information

1.1 The following LAMP test is performed for the detection of 16SrX phytoplasmas including the important fruit tree phytoplasmas AP, PD and ESFY.

1.2 The test was developed by De Jonghe *et al*. (2017) and is suitable for on-site detection as well as for screening in laboratories. The test can be performed on DNA extracts and on crude homogenates, without DNA extraction. However, it is recommended to prepare a 10-fold dilution from the crude extract (see section 4.1). With crude extracts, the test can be completed in 1 h.

1.3 The test was designed within the 16S rRNA gene.

1.4 The test has been successfully performed on-site using a Genie®III (OptiGene Ltd) portable device and in the laboratory using a Genie®II and III or ABI 7900HT real-time PCR instrument (Applied Biosystems). Alternatively, the test also produced consistent results using either the GeneAmp PCR system 9700 machine (Applied Biosystems) or the thermomixer comfort heat block (Eppendorf, Hamburg, Germany) for the incubation step. All endpoint products showed a ladder-like band pattern, visualized on agarose gels or by means of capillary gel electrophoresis (QIAxcel Advanced with ScreenGel v.1.2.0. software; Qiagen).

1.5 The complementary Genie Explorer software (OptiGene Ltd) for the Genie instruments and SDS v2.4.1 software for the real-time PCR instrument 7900HT were used to assess the threshold values (C_t), detecting the fluorescence of the DNA intercalator in a time-dependent manner and melting temperature (T_m) of the amplicons in a subsequent dissociation step.

1.6 The melting curves and T_m were obtained by increasing the temperature after amplification to 95°C at a 2% speed of the ABI7900HT standard ramp rate and measured on the FAM channel of the instrument. All AP, PD and ESFY strains generated a peak corresponding to a T_m of approximately 86°C.

1.7 Oligonucleotides

Primer ID (description)	Length (nt)	Sequence (5′–3′)
F3 (External forward primer)	20	CCTGCCTCTTAGACGAGGAT
B3 (External reverse primer)	19	CAATGTGGCCGTTCAACCT
FIP (Hybrid inner forward primer (F1c + TTTT + F2))	46	AGCATACCCTTGCGGGTC TTTTTTTTACAGTTGGAA ACGACTGCTA
BIP (Hybrid inner reverse primer (B1c + TTTT + B2))	44	AAGAGATGGGCTTGCGGC ACTTTTCTCAGTCCAGCTACA CATCA

2. Methods

2.1 Nucleic acid extraction and purificationSee Appendix 1. For the preparation of the plant lysis method, without extraction, follow the instructions for the Plant Material Lysis Kit (OptiGene Ltd, Cat. No. EXT-001L, Batch No1).

2.2 LAMP

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Isothermal master mix (OptiGene)	1x	15	1x
Primers			
F3	10 µM	0.5	200 nM
B3	10 µM	0.5	200 nM
FIP	10 µM	2.5	1 µM
BIP	10 µM	2.5	1 µM
Subtotal		21	
Ten-fold diluted DNA extract or plant homogenate		4	
Total		25	

2.2.2 LAMP amplification conditions: 65°C for 30 min. When the incubation is performed in an ABI7900HT real-time PCR machine (Applied Biosystems), amplification curves, melting curves and T_m can be obtained by increasing the temperature after amplification to 95°C at a 2% speed of the ABI7900HT standard ramp rate measured on the FAM channel of the instrument. When using the portable Genie (II or III) instrument and complementary Genie Explorer software (OptiGene), similar fluorescent signals and curves are produced, allowing real-time assessment of the LAMP results.

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 the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- 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 nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA 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 controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can be genes either present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls 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).
- 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/plant homogenate. Same matrix spiked with nucleic acid from the target organism

3.2 Interpretation of results

Verification of the controls:

- NAC (and if relevant NIC) should produce no fluorescence.
- The PAC (and if relevant PIC, IC) amplification curve should be exponential. The T_m (melting temperature) should be between 84.0 and 85.0°C for GenieII and

[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).

around 85.5°C for the Genie instrument or 86°C for the ABI7900HT instrument. A similar T_m range is expected when analysed on any other device but this needs to be verified.

When these conditions are met:

- A test will be considered positive if it produces a positive reaction as defined for PAC (see above).
- A test will be considered negative if it produces no fluorescence.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data available from the Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Merelbeke, Belgium.

4.1 Analytical sensitivity data

The relative limit of detection (LOD) was assessed on 10-fold serial dilutions of infected AP, PD and ESFY host tissue with variable concentration of phytoplasmas.

All tested AP, PD and ESFY infected samples tested positive for the 10^1 concentration (DNA in demineralized water), which is recommended for routine detection in this method.

Depending on the initial concentration of the phytoplasma in the host tissue, and the visualizing method of the result, the phytoplasmas could be detected to a dilution of 10^4 of the original sample. All three visualization methods (gel electrophoresis, and real-time detection in the ABI7900HT and Genie® instruments) proved to produce stable and easily interpretable results under the standard recommended conditions, and no clear differences in sensitivity can be seen between the methods.

Similar results were obtained for PD and ESFY within infected *Pyrus* and *Prunus* material, respectively.

4.2 Analytical specificity data

The LAMP method is specific to the AP group (16SrX). *In silico* analysis indicated no significant sequence homology with non-targets.

- Inclusivity

Number of group X strains used in the validation: 7 (2 AP, 4 ESFY and 1 PD). In addition, a wide range (>50) of AP and PD isolates/infected samples from a local survey were tested.

The reference sequences for *Spartium* witches' broom (SpaWB), Buckthorn witches' broom (BWB) and *Allocasuarina* yellows disease (AlloY), the other group X phytoplasmas, were also checked. Zero and 3 mismatches were noted in the primers for SpaWB and BWB, respectively. The AlloY 16S region was only partially available and an *in silico* blast against the developed group X LAMP primers was not possible. SpaWB, BWB and AlloY strains were not available and not included in the validation experiments, therefore their reaction in the LAMP protocol is not known. However, all three phytoplasmas are not known to occur in the targeted host plants (*Malus*, *Pyrus* and *Prunus*).

- Exclusivity

Tested isolates from other phytoplasma belonged to RFLP groups 16SrI-A, 16SrIIB, 16SrIII-A, 16SrIII-F, 16SrIII-H, 16SrV-C, 16SrVII-A, 16SrXI, 16SrXII-A and 16SrXII-B. None of the isolates gave a positive result in the LAMP.

Other non-phytoplasma bacterial targets from collections and isolated from apple rhizosphere and phyllo sphere did not result in positive reactions.

4.3 Diagnostic sensitivity

The optimized LAMP procedure was compared to the generic phytoplasma real-time PCR test (Christensen *et al*., 2004). A stable detection of the pathogen was obtained up to a dilution of 10^4 for the two LAMP tests (in the Genie instrument and the ABI7900HT instrument). Phytoplasma detection on the same dilution series by this test allowed a stable detection up to 10^5, i.e. ten times more sensitive than the LAMP test.

4.4 Diagnostic specificity

100 %

4.5 Repeatability and reproducibility

None of the (7) isolates included in the validation trials showed problems with repeatability and reproducibility in the laboratory.

Appendix 3 – Real-time PCR for specific detection of '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri' (Nikolić *et al*., 2010; Mehle *et al*., 2013b)

1. General information

1.1 The following real-time PCR protocol is performed for the detection and identification of '*Ca*. P. mali', '*Ca*. P. prunorum' and '*Ca*. P. pyri'.

1.2 The test was developed by Nikolić *et al*. (2010) and the detailed description of that test was published by Mehle *et al*. (2013b).

1.3 Primers and probes were designed within a variable region of the intergenic spacer region (IGS) between 16S and 23S rDNA. To design the specific sets of primers/probes the following nucleotide sequences with accession numbers were used: for '*Ca*. P. mali' (AF248958, AJ430067, AJ542541, AJ542542, APU54985, AY598319, CU469464, EF392654, EF392655, EF392656, EU168781, X68375), for '*Ca*. P. prunorum' (AJ542544, AJ542545, AJ575105, AJ575106, AJ575107, AM933142, AY029540, EF560638, EF560639, EF560640, EF560641, EF560642, EF560643, EF560644, EF560645, EF560646, ESU54988, EU168783, Y11933) and for '*Ca*. P. pyri' (AJ542543, AJ964959, DQ011588, PDU54989).

1.4 The AP amplicon covers a 147-bp region of the IGS of '*Ca*. P. mali', corresponding to nucleotides 1608–1754 in the isolate with accession number AJ542541. The ESFY amplicon covers a 147-bp region of the IGS of '*Ca*. P. prunorum', corresponding to nucleotides 1608–1754 in the isolate with accession number AJ542544. The PD amplicon covers a 146-bp region of the IGS of '*Ca*. P. pyri', corresponding to nucleotides 1609–1754 in the isolate with accession number AJ542543.

1.5 Oligonucleotides

Primer pairs are identical for all three species-specific tests:

Forward primer	5′-TGGTTAGAGCACACGCCTGAT-3′
Reverse primer	5′-TCCACTGTGCGCCCTTAATT-3′
AP-specific probe	5′-FAM-CAAAGTATTTATCTTAAGAAAACA AGC T-MGB-3′
ESFY-specific probe	5′-FAM-CAAAATATTTATTTTAAAAAAACA AGCTC-MGB-3′
PD-specific probe	5′-FAM-AATATTTATTTTAAAAAA AAGCT CTTTG-MGB-3′

1.6 The test has been successfully performed using the Maxima Probe qPCR master mix (Fermentas) reagent and on a range of different real-time PCR systems including ABI (7900, 7900HT Fast, ViiA™7).

1.7 Validation data have been generated using software (e.g. SDS 2.4, Applied Biosystems) for fluorescence acquisition and calculation of threshold cycles (C_t).

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)[a]	Final concentration
Molecular-grade water[b]	NA	0.84	NA
Maxima™ qPCR master mix (Fermentas) containing UNG[c]	2×	5.0	1×
Forward primer	10 µM	0.9	0.9 µM
Reverse primer	10 µM	0.9	0.9 µM
AP-, ESFY- or PD-specific probe	2.5 µM	0.36	0.09 µM
Subtotal		8.0	
DNA dilution		2.0	
Total		10.0	

[a]If a 25-µL reaction volume is used, multiply each component by 2.5.
[b]Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free.
[c]UNG or UDG (uracil-DNA glycosylase).
NA, not applicable.

2.2.2 Real-time PCR conditions: UNG pre-treatment step at 50°C for 2 min; initial denaturation at 95°C for 10 min; 45 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).[2] 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.

[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).

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 produces no exponential 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

For amplicon names used in this section see section 1.4.

4.A Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab)

The 12 (AP and PD amplicon)/13 (ESFY amplicon) participating laboratories analysed a total of 30 blind samples. The samples consisted of 9 healthy fruit trees, 6 closely related bacteria, 5 samples infected by '*Ca*. P. mali', 5 samples infected by '*Ca*. P. prunorum' and 5 samples infected by '*Ca*. P. pyri'. In three (for '*Ca*. P. mali')/four (for '*Ca*. P. pyri') participating laboratories, analytical sensitivity was also tested using a serial dilution of a cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' at concentration of 10^7 to 10^1.

4.A.1 Analytical sensitivity data

AP amplicon: 10^1

PD amplicon: 10^1

4.A.2 Diagnostic sensitivity

AP amplicon: 100%

ESFY amplicon: 100%

PD amplicon: 100%

4.A.3 Diagnostic specificity

AP amplicon: 98.7%

ESFY amplicon: 93.8%

PD amplicon: 99.7%

4.A.4 Data on repeatability

Not available

4.A.5 Data on reproducibility

Agreement between laboratories – measured by calculation of the Kappa coefficient.[3] (Fleiss *et al*., 2003):

AP amplicon: 0.924

ESFY amplicon: 0.84

PD amplicon: 0.98

4.B Validation data available from the National Institute of Biology (SI) (for details see http://dc.eppo.int/validationlist.php)

4.B.1 Analytical sensitivity data

Analytical sensitivity is represented as C_t values with 95% probability of detection (the dilution of sample DNA is given in parentheses):

AP amplicon: 34.1 (between $10^4$and 10^5)

ESFY amplicon: 33.2 (between 10^3 and 10^4)

PD amplicon: 37.8 (between 10^4 and 10^5)

4.B.2 Diagnostic sensitivity

AP amplicon: 100%

ESFY amplicon: 100%

PD amplicon: 100%

4.B.3 Analytical specificity data

Percentage of accurate results:

AP amplicon: 100%

ESFY amplicon: 100%

PD amplicon: 100%

4.B.4 Diagnostic specificity:

AP amplicon: 100%

ESFY amplicon: 87.5%

PD amplicon: 97.8%

4.B.5 Data on repeatability

- High and medium target phytoplasma concentration:
  AP amplicon): 100% positive repeats
  ESFY amplicon: 100% positive repeats
  PD amplicon: 100% positive repeats
- Low target phytoplasma concentration:
  AP amplicon: 100% positive repeats
  ESFY amplicon: 100% positive repeats
  PD amplicon: 95% positive repeats

4.B.6 Data on reproducibility

- Sample with medium target phytoplasma concentration:
  AP amplicon: 100%
  ESFY amplicon: 100%
  PD amplicon: 100%
- Sample with low target phytoplasma concentration:
  AP amplicon: 100%
  ESFY amplicon: 100%
  PD amplicon: 89%

4.B.7 Other performance criteria available

The full validation data and report on the critical points in the diagnostic process and relating to uncertainty of measurement are available from the National Institute of Biology (SI) (see validation data deposited with the EPPO database on Diagnostic Expertise: http://dc.eppo.int/validationlist.php).

Appendix 4 – AP group-specific nested PCR

1. General information

1.1 The following nested PCR protocol is performed for the detection and identification of AP group (16SrX) phytoplasmas.

1.2 Two sets of primers are used: P1/P7 primers (Deng & Hiruki, 1991; Schneider *et al*., 1995) for first PCR and f01/r01 (Lorenz *et al*., 1995) for second PCR (nested PCR). The first step confirms the presence of

[3]Interpretation of Kappa values: <0, poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement (Landis & Koch, 1977)

a phytoplasma while the second step (nested PCR) is specific for 16SrX group phytoplasmas.

1.3 The P1/P7 primers amplify the whole length of 16S and intergenic 16S–23S and a small part of 23S rRNA gene (1850 bp).

1.4 The amplicon size of nested PCR (f01/r01) is around 1100 bp.

1.5 Oligonucleotides

P1	5′-AAGAGTTTGATCCTGGCTCAGGATT-3′
P7	5′-CGTCCTTCATCGGCTCTT-3′
f01	5′-CGGAAACTTTTAGTTTCAGT-3′
r01	5′-AAGTGCCCAACTAAATGAT-3′

1.6. The test performance study (Euphresco: FruitPhytoInterlab) was performed with a GoTaq DNA polymerase from Promega.

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[a]	NA	16.375	NA
Green GoTaq Reaction buffer (Promega)	5×	5	1×
dNTPs (Promega)	10 mM	0.5	0.2 mM
Forward primer (P1)	10 µM	1	0.4 µM
Reverse primer (P7)	10 µM	1	0.4 µM
GoTaq DNA polymerase (Promega)	5 U μL^{-1}	0.125	0.625 U
Subtotal		24	
Genomic DNA extract		1	
Total		25	

[a]Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free.
NA, not applicable.

2.2.2 PCR conditions: initial denaturation step at 94°C for 2 min; 36 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 8 min.

2.2.3 Master mix for nested PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water[a]	NA	16.375	NA
Green GoTaq Reaction buffer (Promega)	5×	5	1×
dNTPs (Promega)	10 mM	0.5	0.2 mM
Forward primer (f01)	10 µM	1	0.4 µM
Reverse primer (r01)	10 µM	1	0.4 µM
GoTaq DNA polymerase (Promega)	5 U μL^{-1}	0.125	0.625 U
Subtotal		24	
1/30 diluted P1/P7 PCR product		1	
Total		25	

[a]Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free.
NA, not applicable.

2.2.4 Nested PCR conditions: initial denaturation step at 94°C for 2 min; 38 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 8 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).[4] 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.

[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).

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 a plant cytochrome oxidase gene (e.g. Weller *et al*. 2000; Papayiannis *et al*. 2011) or eukaryotic 18S rDNA(AB kit cat no. 4319413E)
- 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 should produce no amplicons.
- PIC and PAC (and if relevant IC) should produce amplicons of the expected size (depending on whether the target, endogenous or exogenous nucleic acid is used).

When these conditions are met:

- A test will be considered positive if amplicons of nested PCR around 1100 bp are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

In the case of positive results, RFLP analysis (Appendix 6) or sequencing of PCR product should be performed to identify the phytoplasma.

4. Performance characteristics available

Validation data available from the test performance study in 2011 (Euphresco: FruitPhytoInterlab), where the 20 participating laboratories analysed a total of 30 blind samples. In particular: 9 healthy fruit trees, 6 bacteria (*Bacillus subtilis*, *Erwinia chrysanthemi*, *Fructobacillus fructosus*, *Paenibacillus alvei*, *Pseudomonas syringae* pv. *cersicola*, *Ralstonia solanacearum*), 5 samples infected by '*Ca*. P. mali', 5 samples infected by '*Ca*. P. prunorum' and 5 samples infected by '*Ca*. P. pyri'. In two participating laboratories, analytical sensitivity was also tested using a serial dilution of cloned P1/P7 fragments from '*Ca*. P. mali' and '*Ca*. P. pyri' at concentrations from 10^7 to 10^1 copy numbers per mL

4.1. Analytical sensitivity

Analytical sensitivity for '*Ca*. P. mali': 10^1–10^3

Analytical sensitivity for '*Ca*. P. pyri': 10^1

4.2. Diagnostic sensitivity

Diagnostic sensitivity – an estimation of the ability of the method to detect the target: 99.3%

4.3. Diagnostic specificity

Diagnostic specificity – an estimation of the ability of the method not to detect the non-target: 97.7%

4.4. Data on repeatability

Not available

4.5. Data on reproducibility

Agreement between laboratories – measured by calculation of the Kappa coefficient.[5](Fleiss *et al*., 2003): 0.94.

Appendix 5 – Conventional PCR for the generic detection of phytoplasmas (Lorenz *et al*., 1995)

1. General information

1.1 The following PCR protocol is performed for the detection of phytoplasmas.

1.2 The test was developed by Lorenz *et al*. (1995).

1.3 The fU5/rU3 primers amplify a part of the 16S rRNA gene.

1.4 The amplicon size of PCR is around 870 bp.

1.5 Oligonucleotides:

fU5	5′-CGGCAATGGAGGAAACT-3′
rU3	5′-TTCAGCTACTCTTTGTAACA-3′

1.6 The performance study was performed with Amplitaq DNA polymerase from Applied Biosystems.

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[a]	NA	29.94	NA
Reaction buffer (Applied Biosystems)	10×	4	1×
dNTPs	20 mM	0.5	0.25 mM
BSA	50 mg mL^{-1}	0.04	0.05 mg mL^{-1}
Forward primer (fU5)	100 μM	0.2	0.5 μM
Reverse primer (rU3)	100 μM	0.2	0.5 μM
Amplitaq DNA polymerase (Applied Biosystems)	5 U μL^{-1}	0.12	0.6 U
Subtotal		35	
Genomic DNA extract		5	
Total[b]		40	

[a]Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-μm filtered) and nuclease-free.

[b]Total reaction volume is recommended for proceeding with RFLP analysis or sequencing.

NA, not applicable.

[5]Interpretation of Kappa values: <0, poor agreement; 0.00–0.20, slight agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; 0.81–1.00, almost perfect agreement (Landis & Koch, 1977).

2.2.2. PCR conditions: initial denaturation step at 94°C for 2 min; 40 cycles consisting of 20 s at 94°C, 20 s at 55°C and 1 min at 72°C; final extension at 72°C for 4 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).[6] 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 a plant cytochrome oxidase gene (e.g. Weller *et al*. 2000, Papayiannis *et al*. 2011) or eukaryotic 18S rDNA(AB Kit cat. no. 4319413E)
- 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).

[6]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:

- NIC and NAC should produce no amplicons.
- PIC and PAC (and, if relevant, IC) should produce amplicons of the expected size (depending on whether the target, endogenous or exogenous nucleic acid is used).

When these conditions are met:

- A test will be considered positive if amplicons of PCR around 870 bp are produced.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

In the case of positive results, RFLP analysis (Appendix 6) or sequencing of PCR product should be performed to identify the phytoplasma.

4. Performance characteristics available

Validation data available from the Plant Heath Laboratory of ANSES (FR).

NB: This validation data was not obtained in the framework of the Euphresco project FruitPhytoInterlab, consequently the sensitivity data for this test cannot be compared with those presented for the other tests. For phytoplasmas, relative quantification is not possible when samples are not analysed together.

4.1 Sensitivity data

Diagnostic sensitivity – an estimation of the ability of the method to detect the target: 98.55%

Last level at 100% positive results: 10^{-4} to 10^{-5} (levels tested between 10^{-1} and 10^{-8} for 3 different positive DNA extracts diluted in healthy DNA extract; one PD, one ESFY and one AP).

4.2 Specificity data

Diagnostic specificity – an estimation of the ability of the method not to detect the non-target: 88.46%.

4.3 Data on repeatability

97.56%

4.4 Data on reproducibility

Not available.

Appendix 6 – RFLP

1. General information

1.1 The amplification products of AP group-specific nested PCR (Appendix 4) or of conventional PCR (Appendix 5) may be digested by the restriction enzymes for differentiation of '*Ca*. P. mali'/'*Ca*. P. pyri'/'*Ca*. P. prunorum'.

1.2 The protocol was published by Schneider *et al*. (1995).

1.3 For differentiation of AP group phytoplasmas the endonucleases *Ssp*I and *Bsa*AI or *Rsa*I proved to be useful.

1.4 Different profiles are obtained with each enzyme and allow the identification of '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum'.

2. Methods

2.1 PCR

2.1.1 Nested PCR and conventional PCR are described in Appendices 4 and 5

2.1.2 PCR/nested PCR product can be kept at −20°C

2.1.3 RFLP reaction

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water[a]	NA	10.7 (or 5.7)[b]	NA
Restriction enzyme buffer (Promega/Fermentas)	10×	2.0	1×
BSA (Promega)	100×	0.2	1×
Restriction enzyme SspI (Promega/Fermentas)	10 U µL^{-1}	0.1	1 U
Subtotal		13 (or 8)	
Nested PCR product		7 (or 12)[b]	
Total		20	
Molecular-grade water[a]	NA	10.9 (or 5.9)[b]	NA
Restriction enzyme buffer (Biolabs/Fermentas)	10×	2.0	1×
Restriction enzyme BsaAI (Biolabs/Fermentas)	10 U µL^{-1}	0.1	1 U
Subtotal		13 (or 8)	
Nested PCR product		7 (or 12)[b]	
Total		20	
Molecular-grade water[a]	NA	10.9 (or 5.9)[b]	NA
Restriction enzyme buffer (Promega[c]/Fermentas)	10×	2.0	1×
Restriction enzyme *Rsa*I (Promega[c]/Fermentas)	10 U µL^{-1}	0.1	1 U
Subtotal		13 (or 8)	
Nested PCR product		7 (or 12)[b]	
Total		20	

[a]Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.22-µm filtered) and nuclease-free.
[b]Depends on the nested PCR product: strong or weak.
[c]Validation data for *Rsa*I obtained with restriction enzymes from Promega with amplicon of PCR using fU5/rU3 primers (Appendix 5).
NA, not applicable.

2.1.3.1 Reaction incubation: 37°C for 4 h. Digested PCR products are subject to electrophoresis on 2% agarose gel along with a DNA ladder to size fragments.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, NAC and PAC controls from nested PCR (see Appendix 4) should be included. Three different PACs (PAC for '*Ca*. P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum') are recommended.

3.2 Interpretation of results:

Verification of the controls:

- NAC should produce no profiles.
- PACs should produce expected profiles (see below).

When these conditions are met:

- A test will be considered positive for '*Ca*. P. mali' if *Ssp*I digests the amplicons and *Rsa*I and/or *Bsa*AI digest the amplicons with the profiles described in the table below. Generally, two fragments are visible on the electrophoresis gel for this phytoplasma.
- A test will be considered positive for '*Ca*. P. prunorum' if amplicons are not digested by *Ssp*I, and *Rsa*I and/or *Bsa*AI digest the amplicons with the profiles described in the table below.
- A test will be considered positive for '*Ca*. P. pyri' if amplicons are not digested by *Ssp*I, and *Rsa*I and/or *Bsa*AI digest the amplicons with the profiles described in the table below.
- Tests should be repeated if any contradictory or unclear results are obtained.

Expected RFLP electrophoretic profiles (size of the fragments) obtained with the two different PCRs followed by RFLP with *Rsa*I, *Bsa*AI and *SspI* (virtual RFLP analysis with http://tools.neb.com/REBsites/index.php (Roberts *et al*., 2010).

Identified phytoplasma		'*Ca*. P. mali' 16SrX	'*Ca*. P. prunorum' 16SrX	'*Ca*. P. pyri' 16SrX
The fragment length expected from sequences obtained after nested PCR – Appendix 4 (bp)	*Rsa*I	744		744
			392	
			351	
		249	249	249
		44[a]	44[a]	44[a]
		16[a]	16[a]	16[a]
		4[a]	4[a]	4[a]
	*Bsa*AI	766		766
			452	
		251	251	251
		40[a]	40[a]	40[a]
	*Ssp*I		1056	1056
		713		
		344		
The fragment length expected from sequences obtained after amplification with primers fU5/rU3 – Appendix 5 (bp)	*Rsa*I	449		449
			392[b]	
		363	363[b]	363
			58[a]	
		44[a]	44[a]	44[a]
		16[a]	16[a]	16[a]
		4[a]	4[a]	4[a]
	*Bsa*AI	511		511
			452	
		365	365	365
			60	
	*Ssp*I		876	876
		827		
		49[a]		

[a]Bands are not always visible on electrophoresis gel at these lengths.
[b]See Fig 9.

4. Performance characteristics available

Not available for nested PCR followed by RFLP.

Validation data available from the Plant Heath Laboratory of ANSES (FR). Here, performance characteristics are

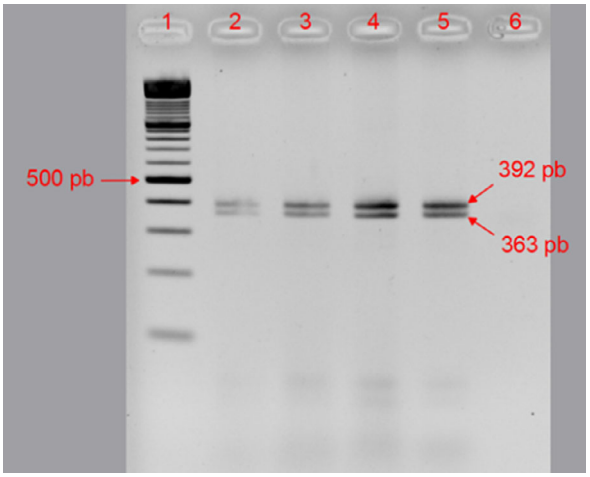

Fig. 9 Picture of a gel from a RFLP test with *Rsa*I after digestion of amplicons (fU5, rU3 primers, Appendix 5) showing two distinct bands. 1, Molecular marker 100 bp; 2 and 3, sample in duplicate; 4 and 5, positive amplification control in duplicate, DNA extract positive for '*Candidatus* Phytoplasma prunorum'; 6, negative amplification control, DNase-free water. [Colour figure can be viewed at wileyonlinelibrary.com]

a result of generic PCR described in Appendix 5 followed RFLP described in this appendix.

4.1 Analytical sensitivity data

Last level at 100% positive results (levels tested between 1×10^{-1} and 1×10^{-8} for 3 different positive DNA extracts diluted in healthy DNA extract; one PD, one ESFY and one AP):

For '*Ca*. P. mali': 1×10^{-4}

For '*Ca*. P. prunorum': 1×10^{-4}

For '*Ca*. P. pyri': 1×10^{-5}

Diagnostic sensitivity – an estimation of the ability of the method to detect the target:

For '*Ca*. P. mali': 97.4%

For '*Ca*. P. prunorum': 100%

For '*Ca*. P. pyri': 96.7%

4.2 Analytical specificity data

Diagnostic specificity – an estimation of the ability of the method not to detect the non-target

For '*Ca*. P. mali': 92.3%

For '*Ca*. P. prunorum': 91.7%

For '*Ca*. P. pyri': 100%

4.3 Data on repeatability

For '*Ca*. P. mali': 98.3%

For '*Ca*. P. prunorum': 100%

For '*Ca*. P. pyri': 97.8%

4.4 Data on reproducibility

Not available