Diagnostic protocol for European stone fruit yellows phytoplasma "*Candidatus* Phytoplasma prunorum"

PEST STATUS	Not present in Australia
PROTOCOL NUMBER	NDP 12
VERSION NUMBER	V1.2
PROTOCOL STATUS	Endorsed
ISSUE DATE	November 2011
REVIEW DATE	2016
ISSUED BY	SPHDS

Australian Government
Department of Agriculture

Prepared for the Subcommittee on Plant Health Diagnostic Standards (SPHDS)

This version of the National Diagnostic Protocol (NDP) for European stone fruit yellows phytoplasma "*Candidatus* Phytoplasma prunorum" is current as at the date contained in the version control box on the front of this document.

NDPs are updated every 5 years or before this time if required (i.e. when new techniques become available).

The most current version of this document is available from the SPHDS website: http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

Contents

1. INTRODUCTION

The species name *Candidatus* phytoplasma prunorum was proposed for ESFY phytoplasma in 2004 (Seemuller and Schneider, 2004). It is closely related to both *Candidatus* phytoplasma mali (apple proliferation phytoplasma) and *Candidatus* phytoplasma pyri (pear decline phytoplasma) and the three phytoplasmas share 98.5-99% sequence similarity across the 16S gene. All three phytoplasmas belong to the 16SrX (apple proliferation) phytoplasma group (Lee et al. 1998). However significant genetic differences are observed in other genes amongst the three phytoplasmas and they have distinct biological and epidemiological properties including host range and vectors.

1.1 Hosts

Ca. P. prunorum is associated with European stone fruit yellows (ESFY) disease, which includes diseases of apricot (*Prunus armeniaca)*, Japanese (flowering) cherry (*P. serrulata)*, black cherry *(P. mahaleb),* peach *(P. persica),* Japanese plum *(P. salicina),* European plum (*P.domestica),* cherry (myrobalan) plum (*P. cerasifera*) and almond (*P. dulcis* syn. *P. amygdalus* Batsch) (Lorenz et al. 1994; Seemüller and Foster 1995; Marcone et al. 1996; Sertkaya et al. 2005). Rootstocks can be infected by the phytoplasma including *P. marianna, P. domestica*, *P. cerasifera, P. domestica* x *P. cerasifera, P. salicina* x *P. spinosa, and P. persica* x *P. cerasifera* (Jarausch et al. 1998). The severity of symptom expression in *Prunus* sp. is dependent on the species and the variety (Jarausch et al. 2000).

1.1.1. Alternative host plants

Alternative hosts for ESFY include Hackberry *(Celtis australis),* Ash *(Fraxinus excelsior*), Dog rose *(Rosa canina),* Wild cherry *(Prunus avium) and* Blackthorn *(Prunus spinosa*). Non-*Prunus* species may be symptomless. These hosts are important in the epidemiology of the phytoplasma as they act as a source of phytoplasma inoculum for orchards and may also host insect vectors. Blackthorn is a preferred host of the vector.

1.2 Symptoms

General symptoms of ESFY disease in various stone fruit species include early flowering and shooting through winter and this early break in dormancy can increase the susceptibility of affected trees to frost, causing damage to the phloem. During the growing season leaves can be chlorotic and roll. Leaves can drop prematurely. Affected shoots are often stunted and bear smaller, deformed leaves. Shoots may die back. Fruit on affected branches develop poorly and may fall prematurely. Fruit yield can be reduced. Only a few branches are affected during the early stage of disease but as the disease progresses the whole tree may become affected. Many stone fruit tree species or varieties show decline (Nemeth, 1986; Seemüller and Foster 1995).

Specific symptoms in apricot (apricot chlorotic leafroll disease) include upward curling of leaves, which are chlorotic and early reddening, Sudden dieback can occur during the growing season. Small, wilted fruit and dried leaves may also persist during the autumn. In plum (plum leptonencrosis disease) the leaf margins roll upward and leaves may be chlorotic and /or smaller. In peach the midribs and

lateral veins of the leaves can become enlarged and corky tissue develops along the veins. The leaves become red and roll upward. In cherry the first symptom observed is slight chlorosis of leaves in summer. Flowers are malformed and fruit set is poor in the following year. Rosetting of leaves occurs on affected shoots and young shoots remain unlignified. (Nemeth, 1986; Seemüller and Foster 1995). In almond leaf rolling, reddening of the shoot bark and leaves, and sparse foliation may be observed.

Although symptoms are indicative of infection by *Ca.* P. prunorum, phytoplasma infection should be confirmed through diagnostic testing. *Ca.* P. prunorum can be detected using a PCR based on the 16S rRNA gene with universal primers for all known phytoplasmas and identified by RFLP analysis or sequencing (Seemüller et al. 1998). Specific primers may also be useful in identifying infection by *Ca.* P. prunorum.

Ca. P. prunorum can be detected in aerial parts of trees during dormancy (Jarausch et al. 1998) and the possibility exists that this phytoplasma might be transmitted through vegetative propagation of dormant budwood.

2. TAXONOMIC INFORMATION

The taxonomic classification of the phytoplasma associated with European stone fruit yellows is:

> Bacteria; Firmicutes; Mollicutes; Acholeplasmatales; Acholeplasmataceae; Candidatus Phytoplasma; 16SrX (Apple proliferation group). *Candidatus* Phytoplasma prunorum

Ca. P. prunorum is closely related to *Ca.* P. mali (apple proliferation phytoplasma) and *Ca.* P pyri (pear decline phytoplasma) and the three species share 98.6–99.1% sequence similarity. However, each species is transmitted by different vectors and has different hosts or induces different symptoms in the same host.

Ca. P prunorum has several common names and acronyms depending on the disease and host and these include:

> European stone fruit yellows (ESFY) mycoplasma-like organism, European stone fruit yellows phytoplasma, Apricot chlorotic leaf roll (ACLR) phytoplasma, plum leptonecrosis (PLN) phytoplasma

3. DETECTION

European stone fruit yellows disease can be identified by the presence of symptoms, however diagnosis should be confirmed through PCR detection and sequencing of the 16S rRNA gene of *Ca*. P. prunorum, particularly as other phytoplasmas may cause similar symptoms. Most symptoms, particularly if they are observed on their own, may be caused by other biotic and abiotic factors.

Symptomless infections can occur and if this is suspected it is important to thoroughly sample different phloem tissue from different shoots and branches of the one plant for phytoplasma isolation.

Ca. P. prunorum is phloem-limited, however it may infect the phloem tissue of all parts of a tree, including roots trunk, branches and shoots.

Phytoplasmas can be unevenly distributed and in uneven titre throughout woody hosts, and symptomatic tissue is optimal for phytoplasma detection (Berges et al. 2000; Christensen et al. 2004; Constable et al. 2003; Necas and Krska, 2006).

The location and titre of phytoplasmas may be affected by seasonal changes and therefore the timing of sample collection for phytoplasma detection is important (Jarausch et al. 1999). In Europe the best time and tissue type for *Ca.* P. prunorum detection is June (early summer) for phloem samples from woody shoots and September (summer) from petiole samples (Necas et al. 2008). *Ca.* P. prunorum can persist and be detected in the phloem of aerial parts of trees during the dormant season (Seemuller et al. 1998).

Vascular tissue from symptomatic plant material provides the best opportunity to detect phytoplasmas in stone fruit trees. Leaf petioles, mid veins from symptomatic leaves and bark scrapings from shoots and branches can be used from actively growing plant hosts. If the plant is dormant, buds and bark scrapings from branches, trunk and roots can be used, although these are likely to be less reliable. If using bark scrapings from woody material remove the dead outer bark layer, to reveal the green inner vascular tissue.

3.1 The signs or symptoms associated with infection

ESFY disease may be suspected if the following symptoms described for each stone fruit species are observed.

Trees flower and shoot in winter

- Chlorosis of the leaves later in the growing season.
- Premature leaf drop
- Stunted shoots bearing smaller, deformed leaves
- Die back of shoots
- Necrosis of the phloem.
- Fruit on affected branches develop poorly and may fall prematurely
- Yield is reduced

Many stone fruit tree species or varieties show decline (Nemeth, 1986; Seemüller and Foster 1995). During the early stage of disease often only a few branches are affected but the whole tree may become affected as the disease progresses. Symptom expression can differ in severity amongst different cultivars of the one stone fruit species.

Specific symptoms in apricot (apricot chlorotic leafroll) include (Figures 1-4):

- upward curling of leaves
- chlorotic leaves
- early reddening
- Sudden dieback can occur during the growing season.
- Small, wilted fruit and dried leaves may also persist during the autumn.

Specific symptoms in peach include (Figure 5-9):

- midribs and lateral veins of the leaves can become enlarged and corky tissue develops along the veins (Figure 9)
- leaves become red and roll upward (Figure 7 and 8).

Specific symptoms in Japanese plum (plum leptonencrosis) include:

- leaf margins roll upward
- leaves may be chlorotic
- small leaves
- necrosis of the vascular tissue (Figure 10)

Specific symptoms in cherry include:

- slight chlorosis of leaves in summer
- flowers are malformed and fruit set is poor in the following year.
- rosetting of leaves occurs on affected shoots
- young shoots remain unlignified (Nemeth, 1986; Seemüller and Foster 1995).

Specific symptoms in almond include:

- leaf rolling
- reddening of the shoot bark and leaves
- sparse foliation may be observed.

Figure 1. Apricot tree partially affected by ESFY disease, symptoms include chlorosis and rolling of leaves on the affected braches at the front. (Source: F. Constable)

Figure 2. ESFY affected apricot tree (front) with severe decline compared to an unaffected tree (rear) (Source: F. Constable).

Figure 3. Severe chlorosis and leaf rolling of an ESFY affected apricot tree (Source: F. Constable).

Figure 4. Rolling and chlorosis of leaves on an ESFY affected apricot tree (Source: F. Constable).

Figure 5. Peach tree partially affected by ESFY disease (front) showing chlorosis and leaf rolling compared with an unaffected part of the same tree (back) (Source: F. Constable).

Figure 6. Peach tree affected by ESFY disease exhibiting decline, sparse foliation, chlorosis and smaller leaves (Source: F. Constable).

Figure 7. Chlorosis, some reddening and rolling of peach leaves on a shoot affected by ESFY disease (Source: F. Constable).

Figure 8. Rolling and reddening of peach leaves on a shoot affected by ESFY disease (Source: F. Constable).

Figure 9. Development of corky tissue along a lateral vein of a peach leaf affected by ESFY disease (Image courtesy of Dr B. Schneider Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany).

Figure 10. Necrosis of the vascular tissue of an ESFY affected Prunus tree (Image courtesy of Dr B. Schneider Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany).

4. IDENTIFICATION

The most reliable method for confirmation of *Ca.* P. prunorum is polymerase chain reaction (PCR), which is used to detect the DNA of the phytoplasma. The efficiency of this test is dependent on appropriate sampling of plant tissue and reliable nucleic acid extraction methods.

4.1 Recommended phytoplasma detection method

1. Extract total DNA using the method described by Green et al. (1999), which uses a CTAB extraction buffer and the DNeasy® Plant Mini Kit (Qiagen Cat. No. 69104)
2. Perform a housekeeping PCR with the rP1/fD2 primers. The rP1/fD2 primers amplify the 16S rRNA gene from most prokaryotes as well as from chloroplasts. If this test is negative then there is no DNA present or there are DNA polymerase inhibitors co-extracted with the nucleic acid. In this situation, try cleaning the nucleic acid (Appendix 1) or repeat the extraction using a different procedure (Appendix 2).
3. Perform PCR using the following procedure:

   Use a nested PCR on the purified DNA using the universal phytoplasma primer pair, P1/P7 for the first-stage PCR followed by the R16F2n/R16R2 primer pair for the second-stage PCR (Table 4).
4. Analyse the PCR products by agarose gel electrophoresis.
5. To determine phytoplasma identity, direct sequence the nested PCR product. If direct sequencing is problematic, the PCR product can be cloned and then sequenced using standard cloning and sequencing procedures. Sequence data can be analysed using the Basic Local Alignment Search Tool (BLAST) available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi. If sequencing facilities are unavailable, a single PCR using the *Ca.* P prunorum specific primers ECA1 and ECA2 can be used to determine the identity of the phytoplasma that was detected. However this is a single PCR and may not detect phytoplasmas associated with low tire infections. A nested PCR using PCR product for the first stage (P1/P7) PCR product and 16SrX group specific primers (Table 4) can be used to identify the phytoplasma to the group level, however this will not determine which 16SrX phytoplasma species is present.

4.1.1. DNA extraction procedure using the QIAGEN DNeasy® Plant mini kit (Green et al. 1999)

Materials and equipment

1. QIAGEN DNeasy® Plant mini kit
2. 1.5 ml centrifuge tubes
3. 20-200 µl and 200-1000 µl pipettes
4. 20-200 µl and 200-1000 µl sterile filter pipette tips
5. Autoclave
6. Balance

7. Bench top centrifuge
8. Distilled water
9. Ice machine
10. Freezer
11. Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)
    - If using mortar and pestles, ensure they are thoroughly cleaned prior to use to prevent cross-contamination from previous extractions. To clean thoroughly, soak mortars and pestles in 2% bleach for 1 hour. Rinse with tap water then soak in 0.2 M HCl or 0.4 M NaOH for 1 hour. Rinse thoroughly with distilled water.
12. Scalpel handle
13. Sterile scalpel blades
14. Vortex
15. Water bath or heating block at 55-65°C
16. Latex or nitrile gloves
17. Buffers:
    - CTAB grinding buffer (Table 1)
    - Absolute ethanol

The 2% cetylmethylammonium bromide (CTAB) buffer (Table 1) is required for all extraction procedures:

Table 1. 2.5% cetyltrimethylammonium bromide (CTAB) buffer for DNA purification

Reagent	**Final concentration**	**Amount needed for 1 L**
CTAB (cetyltrimethylammonium bromide bromide)	2.5%	25 g
Sodium chloride	1.4 M	56 g
1 M Tris-HCl, pH 8.0 (sterile)	100 mM	100 ml
0.5 M EDTA, pH8.0 (sterile)	20 mM	40 ml
Polyvinylpyrrolidone (PVP-40)	1%	10 g

Make up to volume with sterile distilled water. Store at room temperature. Just before use, add 0.2% 2-mercaptoethanol (v/v) to the required volume of buffer.

If a fume hood is unavailable β – mercaptoethanol can be omitted but the quality of the extract from some plant species may be affected.

Method

- Grind 0.5 g of plant tissue in 5 ml of CTAB extraction buffer (room temperature) containing 0.2% ß – mercaptoethanol.
- Transfer 500 µl of extract to a 1.5 ml microfuge tube and add 4 µl of RNase A (Supplied with the DNeasy kit), cap tube and incubate at 65°C for 25-35 min, mixing gently several times.
- Add 130 µl of QIAGEN buffer AP2 to extract. Invert 3 times to mix and place on ice for 5 minutes.
- Apply lysate onto a Qiashredder column and centrifuge at 20,000 x g (14,000 rpm or maximum speed) for 2 minutes.
- Transfer 450 µl of flowthrough from QIAshredder™ column to a 1.5 ml centrifuge tube containing 675 µl QIAGEN buffer AP3/E. Mix by pipetting.
- Transfer 650 µl of extract onto a DNeasy column and spin at 6,000 × g (8000 rpm) for 1 minute
- Discard flow-through and add the rest of the sample to the column and spin at 10000 rpm for 1 minute
- Place DNeasy column in a new 2 ml collection tube and add 500 µl of QIAGEN buffer AW (wash buffer) and spin at 10000 rpm for one minute.
- Discard flowthrough and add another 500 µl of QIAGEN buffer AW and spin at maximum speed for 2 minutes.
- Discard flowthrough and collection tube. Ensure that the base of the column is dry (blot on tissue if it is not) and place in an appropriately labeled microfuge tube. Add 100 µl of pre-warmed 65°C AE buffer directly to the filter (don't apply down the side of the tube) and spin at 10000 rpm for 1 minute. Discard column and store DNA in Freezer.

The reliability of the PCR test is affected by phytoplasma titre in the plant host (Marzachì et al. 2004) and low titres can lead to false negative results. If a phytoplasma infection is suspected but phytoplasmas have not been detected using the extraction procedure of Green et al. (1999) it may be useful to use a phytoplasma enrichment procedure (Appendix 2) to improve detection from symptomless material or from material collected outside the optimum time frame for detection.

4.1.2. PCR

Laboratory requirements

To reduce the risk of contamination and possible false positive results, particularly when nested PCR is used for phytoplasma detection, it is desirable to set up PCR reactions in a different lab to where nucleic acid extractions have been done. It is also desirable to handle PCR reagent stocks and to set up PCR reactions in a clean room or bio-safety cabinet with dedicated pipettes, PCR tubes and tips that have not been exposed to nucleic acid extracts. Use a separate pipette for the addition of nucleic acids to the PCR reactions. Do not add nucleic acid to reactions in the same clean room or bio-safety cabinet in which PCR stocks are handled.

PCR materials and equipment

1. PCR reagents of choice
2. Primers (Table 2)
3. PCR grade water
4. 0-2 μl, 2-20 μl, 20-200 μl and 200-1000 μl pipettes
5. 0-2 μl, 2-20 μl, 20-200 μl and 200-1000 μl sterile filter pipette tips
6. 1.5 ml centrifuge tubes to store reagents
7. PCR tubes (volume depends on thermocycler)
8. Bench top centrifuge – with adapters for small tubes
9. Freezer
10. Ice machine
11. Latex or nitrile gloves
12. Thermocycler
13. DNA molecular weight marker

Table 2. PCR primers used for phytoplasma detection and internal control primers

PCR test†	Primer name (direction)	Primer sequence (5´-3´)	Tm	Product size (bp)	Reference
Phytoplasmas					
Universal phytoplasma – single or nested first stage PCR	P1 (forward)	AAGAGTTTGATCCTGGCTCAGGATT	55°C	1,784	Deng and Hiruki (1991)
	P7 (reverse)	CGTCCTTCATCGGCTCTT			Schneider *et al*. (1995)
Universal phytoplasma – single PCR or nested second stage PCR	R16F2n (forward)	GAAACGACTGCTAAGACTGG	55°C	1,248	Lee *et al*. (1993)
	R16R2 (reverse)	TGACGGGCGGTGTGTACAAACCCCG			
16SrX group specific single PCR or nested PCR with P1/P7 primers used for the first PCR*	fO1 (forward)	CGGAAACTTTTAGTTTCAGT	55°C	1071	Lorenz *et al*. (1995)
	rO1(reverse)	AAGTGCCCAACTAAATGAT			
Ca. P prunorum specific single PCR	ECA1	AATAATCAAGAACAAGAAGT	55ºC	237bp	Jarausch et al. (1998)
	ECA2	GTTTATAAAAATTAATGACTC			
Internal control					
16S bacterial and plant chromosomal	FD2	AGAGTTTGATCATGGCTCAG	55ºC	approx. 1400-1500 bp.	Weisberg et al. (1991)
	RP1	ACG GTT ACC TTG TTA CGA CTT			

† Both the R16F2n/R16R2 and 16SrX group specific primer pairs can be used in single PCR for X-disease phytoplasma detection, however single PCR is less sensitive than nested PCR.

* If sequencing facilities are unavailable these can be used to indicate if the phytoplasma is likely to belong to the X-disease (16SrIII group) phytoplasmas. These primers do not identify the phytoplasm to species or strain level.

Polymerase Chain Reaction

The housekeeping PCR, using the components and concentrations listed in Table 3 below, is done prior to conducting the phytoplasma PCR, to determine if the nucleic extract is of sufficient quality for phytoplasma detection. The cycling times are listed in Table 6. Run the PCR products on a gel as described below. The house keeping PCR is successful if a product of the expected size is observed, indicating the presence of quality DNA in the nucleic acid extract. If no product is observed the nucleic acid extract should be cleaned up or the sample should be re-extracted and a housekeeping PCR conducted on these extracts. If the housekeeping PCR is successful the universal phytoplasma PCR reactions can be done.

For universal phytoplasma detection the primers and the expected size of the PCR product are listed in Table 2. The recommended primers are universal and were developed to amplify all known phytoplasmas.

For nested PCR, the first-stage PCR products, generated by the P1 and P7 primers are diluted 1:25 (v/v) in water prior to re-amplification using the second-stage PCR primers using the R16F2n and R16R2 primers.

If a positive result is obtained the PCR product should be sequenced to determine the identity of the organism that is detected. If sequencing facilities are unavailable a nested PCR can be done using the first-stage PCR products, generated by the P1 and P7 primers in a second-stage PCR primers using the 16SrX group specific primers (Table 2) to determine phytoplasma identity. It is also possible to determine if the phytoplasma is *Ca.* P. prunorum by conducting a single PCR using the specific primers ECA1 and ECA2 (Table 2).

When establishing the test initially, it is advised that a negative control (DNA extracted from healthy plant tissue) is included.

Controls

Positive control: DNA of known good quality (internal control PCR)

DNA extracted from any phytoplasma-infected tissue (phytoplasma PCR)

No template control: Sterile distilled water

Table 3. Conventional PCR reaction master mix

Reagent	***Volume per reaction***
Sterile (RNase, DNase free) water	18.05 µl
10 × reaction buffer	2.5 µl
50 mM $MgCl_2$	0.75 µl
10 mM dNTP mixture	0.5 µl
10 µM Forward primer	1 µl
10 µM Reverse primer	1 µl
5 units/µl Platinum® *Taq* DNA polymerase (Invitrogen 10966-026)	0.2 µl
DNA template or control*	1 µl
Total reaction volume	**25 µl**

Pipette 24 μl of reaction mix into each tube then add 1 μl of DNA template.

*Up to 5μl DNA template may be added, reducing water accordingly, as target DNA may be in low concentration.
Non-acetylated molecular biology grade bovine serum albumin (BSA) can be added to the master mix at 0.5mg/ml to reduce the effect of inhibitors on the PCR.

Table 4. PCR cycling conditions

	Housekeeping primers			**Phytoplasma universal and 16SrIII group primers**		
Step	**Temperature**	**Time**	**No. of cycles**	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	94°C	2 min	1	94°C	2 min	1
Denaturation	94°C	45 s	35	94°C	1 min	35
Annealing	55ºC	45 s		55 °C	1 min	
Elongation	72°C	1 min 30 s		72°C	1 min 30 s	
Final elongation	72°C	10 min	1	72°C	10 min	1

Electrophoresis

Electrophorese PCR products (5-10 μl) on a 1% agarose gel containing ethidium bromide or SybR-Safe and visualise using an UV transilluminator (ethidium bromide staining) or blue light box (SybR-Safe staining). Use a DNA molecular weight marker to determine the size of the products. Table 2 lists the expected PCR product size for each primer pair.

4.2 Interpretation of results

Failure of the samples to amplify with the housekeeping primers suggests that the DNA extraction has failed, compounds inhibitory to PCR are present in the DNA extract or the DNA has degraded.

The phytoplasma universal and specific PCR tests will only be considered valid if:

(a) the positive control produces the correct size product as indicated in Table 2; and

(b) No bands are produced in the negative control (if used) and the no template control.

Confirmation of the specific phytoplasma species infecting the tree can only be determined through sequence analysis. As sequence similarity of 97.5% or above indicates that the phytoplasma detected is most likely to be a strain of *Ca.* P. prunorum.

5. CONTACT POINTS FOR FURTHER INFORMATION

Dr. Fiona Constable

Department of Primary Industries - Knoxfield

Private Bag 15

Ferntree Gully Delivery Centre

Victoria 3156

AUSTRALIA

Ph + 61 3 92109222

6. ACKNOWLEDGEMENTS

This protocol was written and complied by Dr. Fiona Constable, Department of Primary Industries – Knoxfield, Private Bag 15 , Ferntree Gully Delivery Centre, Victoria 3156, AUSTRALIA, Ph + 61 3 92109222

Many thanks to the following researchers for providing information, advice and images for this protocol: Wolfgang Jarausch and Barbara Jarausch AlPlanta, Nuestadt and der Weinstrasse, Germany, Bernd Schneider. Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Dossenheim, Germany

Much of this phytoplasma detection protocol is based on the advice and experience of various researchers who brought together and developed a protocol for the International Sanitary and Phytosanitary Measures (ISPM) for phytoplasma detection for the International Plant Protection Convention (IPPC). The researchers included:

- Fiona Constable DPI, Victoria Australia
- Wilhelm Jelkmann' Institute for Plant Protection in Fruit Crops and Viticulture Dossenheim Germany,
- Lia Liefting, MAF, New Zealand
- Phil Jones, Rothamsted Research Harpenden Hertfordshire
- Esther Torres, Laboratori de Sanitat Vegetal, Departament d'Agricultura Ramaderia i Pesca, Barcelona
- Jacobus Verhoeven' Plant Protection Service, Wageningen, Netherlands

This protocol was reviewed by New Zealand Ministry of Agriculture and Forestry, MAF Biosecurity.

7. REFERENCES

Berges R, Rott M and Seemüller E. 2000. Range of phytoplasma concentration in various hosts as determined by competitive polymerase chain reaction, *Phytopathology* **90,** 1145–1152.

Christensen NM, Nicolaisen M, Hansen M, and Schulz A. 2004. Distribution of phytoplasmas in infected plants as revealed by Real-Time PCR and bioimaging, *Molecular Plant Microbe Interactions* **17** , 1175–1184.

Constable FE, Gibb KS and Symons RH, 2003. Seasonal distribution of phytoplasmas in Australian grapevines, *Plant Pathology* **52**, 267–276.

Deng SJ and Hiruki C 1991 Amplification of 16S rRNA genes from culturable and non-culturable Mollicutes. *Journal of Microbiological Methods* **14**, 53-61

Green MJ, Thompson DA and MacKenzie DJ. 1999. Easy and efficient DNA extraction from woody plants for the detection of phytoplasmas by polymerase chain reaction. *Plant Disease* **83**, 482-485.

The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group (2004) 'Candidatus Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects International *Journal of Systematic and Evolutionary Microbiology* 54, 1243–1255

Jarausch W, Lancas M and Dosba F. 1999. Seasonal colonization pattern of European stone fruit yellows phytoplasmas in different prunus species detected by specific PCR, *Journal of Phytopathology* **147**, 47–54.

Jarausch W, Lansac M, Saillard C, Broquaire JM and Dosba F. 1998 PCR assay for specific detection of European stone fruit yellows phytoplasmas and its use for epidemiological studies in France. *European Journal of Plant Pathology*. 104, 17-27.

Jarausch, W., Eyquard, J.P., Lansac, M., Mohns, M. and Dosba, F. 2000. Susceptibility and tolerance of new French *Prunus domestica* cultivars to European stone fruit yellows phytoplasmas. *Journal of Phytopathology* **148,** 489-493.

Lee I-M, Hammond RW, Davis RE and Gundersen, DE. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* **83**, 834-842

Lee I-M, Gundersen DE, Hammond RW and Davis RE, 1994. Use of mycoplasma like organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology* **84,** 559-566

Lee I-M, Gundersen-Rindal DE, Davis RE and Bartoszyk IM, 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology* **48,** 1153-1169.

Lorenz KH, Dosba F, Poggi Pollini C, Llacer G, Seemüller E. 1994. Phytoplasma diseases of *Prunus* species in Europe are caused by

genetically similar organisms. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* **101**, 567-575.

Lorenz, K-H, Schneider, B., Ahrens, U. and Seemuller, E. 1995. Detection of the apple proliferation and pear decline phytoplasmas of ribosomal and non ribosomal DNA. *Phytopathology* **85**, 771-776.

Marcone C, Ragazzino A, Seemüller E. 1996. European stone fruit yellows phytoplasma as the cause of peach vein enlargement and other decline diseases of stone fruits in southern Italy. *Journal of Phytopathology* **144**, 559-564.

Necas T and Krska B. 2006. Selection of woody indicators and the optimum plant material and sampling time for phytoplasma ESFY detection. *Acta Horticulturae* **717**, 101-105.

Necas T; Maskova V and Krska B. 2008. The possibility of ESFY phytoplasma transmission: through flowers and seeds. *Acta Horticulturae*. 781, 443-447.

Nemeth M. 1986. Virus, Mycoplasma and Rickettsia Diseases of Fruit Trees**,** Martinus Nijhoff Publishers, Dordrecht, The Netherlands

Seemüller E, Foster JA, 1995. European stone fruit yellows. In: Ogawa JM, Zehr EI, Bird GW, Ritchie DF, Uriu K, Uyemoto JK, eds. Compendium of stone fruit diseases. St. Paul, MN, USA: American Phytopathological Society, 59-60.

Seemüller E, Schneider B. 2004. Taxonomic description of '*Candidatus* Phytoplasma mali' sp. nov., '*Candidatus* Phytoplasma pyri' sp. nov. and '*Candidatus* Phytoplasma prunorum' sp. nov., the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *International Journal of Systematic and Evolutionary Microbiology* **54**, 1217-1226.

Seemüller E, Marcone E, Lauer U, Ragozzino A, Göschl M, 1998. Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology* **80,** 3-26.

Sertkaya G, Martini M, Ermacora P, Musetti R and OslerR. 2005 Detection and characterization of phytoplasmas in diseased stone fruits and pear by PCR-RFLP analysis in Turkey. *Phytoparasitica* **33**, 380-390.

Weisberg, WG, Barns, S, Pelletier, DA and Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**, 697-703

7.1 Useful references

Carraro L, Osler R, Loi N, Ermacora P, Refatti E. 1998. Transmission of European stone fruit yellows phytoplasma by *Cacopsylla pruni*. *Journal of Plant Pathology* **80**, 233-239.

Carraro L, Osler R, Loi N, Ermacora P and Refatti E. 2001. Fruit tree phytoplasma diseases diffused in nature by psyllids. *Acta Horticulturae*. **550**, 345-350.

Carraro L; Ferrini F; Ermacora P and Loi N. 2004a. Transmission of European stone fruit yellows phytoplasma to *Prunus* species by using vector and graft transmission. *Acta Horticulturae*. **657**, 449-453.

Carraro L; Ferrini F; Labonne G, Ermacora P and Loi, N. 2004b. Seasonal infectivity of *Cacopsylla pruni*, vector of European stone fruit yellows phytoplasma. *Annals of Applied Biology.* **144**, 191-195.

Conci C, Rapisarda C and Tamanini L. 1992. Annotated catalogue of Italian *Psylloideae*. *Attidell Accademia Roveretana degli Agiati II-B (ser. VII)*, *Rovereto, Italy,* pp. 104-107.

Firraro G, Smart GD and Kirkpatrick BC, 1996. Physical map of the Western X-Disease phytoplasma chromosome. *Journal of Bacteriology* **178,** 3985-3988.

Gibb KS, Padovan AC and Mogen BD, 1995. Studies on sweet potato little-leaf phytoplasma detected in sweet potato and other plant species growing in Northern Australia. *Phytopathology* **85,** 169-174.

Kirkpatrick BC. 1991. Mycoplasma like organisms – plant and invertebrate pathogens, Chapter 229, p. 4050-4067. In: Balows A, Truper HG, Dworkin M, Harder W and Schleifer KH (eds.)The prokaryotes Volume IV, 2nd Ed.. Springer-Verlag, New York.

Kollar A and Seemüller E. 1989. Base composition of the DNA of mycoplasma-like organisms associated with various plant diseases. *Journal of Phytopathology* **127,** 177-186.

Lim PO and Sears BB. 1989. 16S rRNA sequence indicates that plant-pathogenic mycoplasma like organisms are evolutionarily distinct from animal mycoplasmas. *Journal of Bacteriology* 171, 5901–5906.

McCoy RE. 1984. Mycoplasma-like organisms of Plants and invertebrates. In: Krieg NR and Holt JG (eds). Bergey's manual of systematic bacteriology volume 1 pp792 – 793,. William and Wilkins, Baltimore/London.

Marcone C, Ragozzino A, Cousin MT, Berges R and Seemuller E. 1999. Phytoplasma diseases of trees and shrubs of urban areas in Europe. Acta Horticulturae. 496, 69-75;

Marzachi C. 2004. Molecular diagnosis of phytoplasmas *Phytopathologia Mediterranea*. **43**, 228-231.

Neimark H and Kirkpatrick BC. 1993. Isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic Mycoplasma-like organisms. *Molecular Microbiology* **7,** 21-28.

Oshima K, Shiomi T, Kuboyama T, Sawayanagi T, Nishigawa H, Kakizawa S, Miyata S, Ugaki M and Namba S. 2001. Isolation and characterisation of derivative lines of the onion yellows phytoplasma that do not cause stunting or phloem hyperplasia. *Phytopathology* **91,** 1024-1029.

Ossiannilsson F, 1992. *The Psylloidea (Homoptera) of Fennoscandia and Denmark. Fauna Entomologica Scandinava*, **Vol. 26**. Leiden, the Netherlands: E. J. Brill.

Poggi Pollini C, Zelger R, Wolf M, Bissani R, Giunchedi L, 2002. Indagine sulla presenza di psillidi infetti dal fitoplasma degli scopazzi del melo (AP = apple proliferation) in provincia di Bolzano. *ATTI Giornate Fitopatologiche* **2**, 607–12.

Padovan AC, Firrao G, Schneider B and Gibb KS. 2000. Chromosome mapping of the sweet potato little leaf phytoplasma reveals genome heterogeneity within the phytoplasmas. *Microbiology Reading* **146,** 893-902.

Razin S and Freundt EA, 1984. The mycoplasmas. In Bergey's Manual of Systematic Bacteriology, Volume 1. Pp740-792. Noel R. Krieg and John G. Holt Eds. William and Wilkins, Baltimore/London.

Sears BB, Lim P-O, Holland N, Kirkpatrick BC and Klomparens KL. 1989. Isolation and characterization of DNA from a mycoplasmalike organism. *Molecular Plant Microbe Interactions* **2,** 175-180.

Schneider B, Seemüller E, Smart CD and Kirkpatrick BC, 1995. Phylogenetic classification of plant pathogenic Mycoplasma like organisms or phytoplasmas. In: Razin S and TullyJG (eds). Molecular and Diagnostic Procedures in Mycoplasmology, Vol. 1, pp369-380, Academic press, San Diego.

Tedeschi R, Bosco D, Alma A, 2002. Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation in northwestern Italy. *Journal of Economic Entomology* **95**, 544–51.

Schaub L, Monneron A, 2003. Phénologie de *Cacopsylla pruni* vecteur de l'enroulement chlorotique de l'arbricotier. *Revue Suisse de Viticulture, Arboriculturae, Horticulturae* **35**, 123–6.

Thebaud G., 2005.Etude du développement spatio-temporel d'une maladie transmise par vecteur en intégrant modélisation statistique et expérimentation: cas de l'ESFY (European stone fruit yellows). 176 p., *PhD Thesis*, SupAgro, Montpellier, France.

Zreik L, Carle P, Bove JM and Garnier M. 1995. Characterization of the mycoplasma like organism associated with witches'-broom disease of lime and proposition of a Candidatus taxon for the organism, "*Candidatus* Phytoplasma aurantifolia". *International Journal of Systematic Bacteriology* **45,** 449-453.

8. APPENDICES

8.1 Appendix 1: Nucleic acid cleanup

Materials and equipment

1. 1.5 ml centrifuge tubes
2. 20-200 µl and 200-1000 µl pipettes
3. 20-200 µl and 200-1000 µl sterile filter pipette tips
4. Autoclave
5. Balance
6. Bench top centrifuge
7. Distilled water
8. Freezer
9. Vortex
10. Latex or nitrile gloves
11. Reference:
12. Buffers/solutions:
    - Chloroform:iso-amyl alcohol (24:1 v/v)
    - Ice-cold isopropanol
    - 70% (v/v) ethanol
    - Sterile distilled water
    - TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 or 8.0)

Method

1. Add an additional 100-200 µl of sterile water or TE to the nucleic extract to assist ease of handling.
2. Add an equal volume of chloroform:isoamyl alcohol (24:1) and mix thoroughly by vortexing. Centrifuge in a microfuge at room temperature for 15 minutes at 13000 rpm.
3. Transfer the epiphase into a new 1.5ml microcentrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately by inversion. Centrifuge for 15 minutes at 13000rpm.
4. Discard the supernatant and wash the pellet once with 70% ethanol.
5. Air dry the pellet and resuspend in 20-50 µl of water.

Alternatively the DNA may be purified through a MicroSpin™ S-300 HR column (GE Healthcare Cat. No 27-5130-01) according to the manufacturer's instructions.

8.2 Appendix 2: Alternative extraction methods

8.2.1. Phytoplasma enrichment extraction method (Kirkpatrick et al. 1987 and modified by Ahrens and Seemüller, 1992)

Ahrens U and Seemüller E. 1992. Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the16S rRNA gene. *Phytopathology* **82,** 828-832

Kirkpatrick BC, Stenger DC, Morris TJ and Purcell AH. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplamsa-like organism. *Science* **238**, 197-199

Materials and equipment

1. 2 ml centrifuge tubes
2. 20-200 µl and 200-1000 µl pipettes
3. 20-200 µl and 200-1000 µl sterile filter pipette tips
4. Autoclave
5. Balance
6. Bench top centrifuge
7. Distilled water
8. Ice
9. Freezer
10. Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)
11. Scalpel handle
12. Sterile scalpel blades
13. Vortex
14. Water bath or heating block at 55-65°C
15. Latex or nitrile gloves
16. Buffers:

- Phytoplasma isolation buffer - The potassium (Table 5) and sodium (Table 6) isolation buffers are interchangeable.

  To make the isolation buffer use sterile distilled water or filter sterilise. The phytoplasma isolation buffer can be stored in 50 ml aliquots at -20°C and defrosted for use. Just before use add 0.15% [w/v] bovine serum albumin and 1 mM ascorbic acid.

  Make up 100 mM stocks of ascorbic acid (0.176 g/ml water) and store in 500 µl aliquots at -20°C for up to two weeks. Just before using the grinding buffer, add ascorbic acid at 500 µl/50ml phytoplasma isolation buffer. Adjust pH to 7.6 after adding ascorbic acid and BSA.

- CTAB grinding buffer (Table 1)
- Chloroform:iso-amyl alcohol (24:1 v/v)
- 70% (v/v) ethanol
- Sterile distilled water
- Ice-cold isopropanol

Table 5. Potassium phosphate phytoplasma isolation buffer

Reagent	Final concentratio	Amount needed for 1 L
K_2HPO_4-$3H_2O$	0.1 M	21.7 g
KH_2PO_4	0.03 M	4.1 g
Sucrose	10%	100 g
Polyvinylpyrrolidone (PVP-40)	2%	20 g
EDTA, pH 7.6	10 mM	20 ml of a 0.5 M solution

Table 6. Sodium phosphate phytoplasma isolation buffer

Reagent	Final concentratio n	Amount needed for 1 L
Na_2HPO_4	0.1 M	14.2 g
NaH_2PO_4	0.03 M	3.6 g
Sucrose	10%	100 g
Polyvinylpyrrolidone (PVP-40)	2%	20 g
EDTA, pH 7.6	10 mM	20 ml of a 0.5 M solution

Method

1. Grind 0.3 g leaf petioles and mid-veins or buds and bark scrapings in 3 ml (1/10; w/v) in ice-cold isolation buffer
2. Transfer 1.5-2 ml of the ground sample to a cold 2 ml microcentrifuge tube and centrifuge at 4ºC for 5 min at 4,500 rpm.
3. Transfer supernatant into a new 2 ml microcentrifuge tube and centrifuge at 4ºC for 15 min at 13,000 rpm.
4. Discard the supernatant.
5. Resuspend the pellet in 750 µl hot (55-65°C) CTAB buffer.
6. Incubate at 55-65°C for 30 min with intermittent shaking then cool on ice for 30 seconds.

7. Add 750 µl chloroform:isoamyl alcohol (24:1 v/v), vortex thoroughly and centrifuge at 4°C or at room temperature for 4 min at 13,000 rpm.
8. Carefully remove upper aqueous layer into a new 1.5 ml microcentrifuge tube.
9. Add 1 volume ice-cold isopropanol, vortex thoroughly and incubate on ice for 4 min. Centrifuge at 4°C or at room temperature for 10 min at 13,000 rpm. Discard supernatant.
10. Wash DNA pellet with 500 µl ice-cold 70% (v/v) ethanol, centrifuge at 4°C or at room temperature for 10 min at 13,000 rpm.
11. Dry DNA pellet in a DNA concentrator or air-dry.
12. Resuspend in 20 µl sterile distilled water. Incubating the tubes at 55°C for 10 min can aid DNA resuspension.
13. Store DNA at -20ºC for short term storage or -80ºC for long term storage.

8.2.2. Quick nucleic acid extraction methods for phytoplasmas in plants (Maixner et al. 1995)

Maixner M, Ahrens U and Seemüller E. 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology* **101,** 241-250.

Materials and equipment

1. 2 ml centrifuge tubes
2. 20-200 µl and 200-1000 µl pipettes
3. 20-200 µl and 200-1000 µl sterile filter pipette tips
4. Autoclave
5. Balance
6. Bench top centrifuge
7. Distilled water
8. Freezer
9. Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)
10. Scalpel handle
11. Sterile scalpel blades
12. Vortex
13. Water bath or heating block at 55-65°C
14. Latex or nitrile gloves
15. Buffers:

- CTAB buffer (Table 2)
- Chloroform:iso-amyl alcohol (24:1 v/v) – these two solutions are interchangeable
- 70% (v/v) ethanol
- Sterile distilled water
- Ice-cold isopropanol

Method

Perform all operations on ice unless otherwise specified.

1. Grind 0.5 g of plant material in 5 ml of CTAB extraction buffer containing 0.2% β – mercaptoethanol.
2. Transfer 500 µl of extract to a 1.5 ml microfuge tube, close the tube and incubate at 55-65°C for 25-35 min, mixing gently several times.
3. Add 0.8-1 ml of chloroform:isoamyl alcohol (24:1 v/v) and mix thoroughly but gently. Centrifuge in a microfuge at room temperature for 5 minutes at 13000 rpm.
4. Transfer the epiphase into a new 2 ml centrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately. Centrifuge for 5 minute at 13000 rpm. Discard the supernatant and wash the pellet twice with 70% ethanol.
5. Dry the pellet under vacuum or air dry and resuspend in 50 µl of water.

8.3 Appendix 3: Phytoplasmas

Phytoplasmas are obligate intracellular parasites, principally restricted to the phloem cells of infected plant hosts or the salivary glands of their insect vectors (McCoy, 1984). Phytoplasmas have not been successfully cultured *in vitro* (Kirkpatrick, 1991). Phytoplasmas were originally referred to as mycoplasma–like organisms (MLO) since morphologically and ultrastructurally they resemble animal mycoplasmas (*Mycoplasma sp*.), which belong in the class *Mollicutes* (common name: mollicutes) of the kingdom *Prokaryotae*. Like mycoplasmas, phytoplasmas lack a rigid cell wall, have a double membrane and are pleiomorphic. Phytoplasmas are susceptible to antibiotics such as oxy-tetracycline but they are resistant to penicillin because they lack a cell wall (Razin and Freundt 1984). The genome sizes of phytoplasmas are amongst the smallest known for cellular organisms and range between 530 kilobases (kb) to 1350kb (Firrao et al. 1996; Gibb et al. 1995; Marcone et al. 1999; Neimark and Kirkpatrick 1993; Oshima et al. 2001; Padovan et al. 2000; Zriek et al. 1995). Like other members of the mollicutes, phytoplasma genomes have a low mole percent guanine plus cytosine content (mol % G+C) value compared to other organisms (Kollar and Seemüller, 1989; Sears et al. 1989).

Analysis of the 16S ribosomal RNA (rRNA) gene of phytoplasmas showed that these organisms were distinguishable from mycoplasmas and more closely related to acholeplasmas (Lim and Sears 1989). In 1992 at the 9th Congress of the International Organization of Mycoplasmology, the Phytoplasma Working Team of the International Research Project for Comparative Mycoplasmology (IRPCM) assigned these simple bacteria the trivial name 'phytoplasma' to acknowledge that they formed a large, distinct, monophyletic group within the class Mollicutes.

Most phytoplasmas were originally named for the symptoms with which they were associated, e.g. European stone fruit yellows (ESFY) phytoplasma. In 2004 the IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma Taxonomy Group published guidelines for the description of a '*Candidatus* Phytoplasma' genus. The '*Candidatus*' status is used for phytoplasmas because they cannot be cultured nor characterised using many traditional methods for the classification of bacteria, which are based on morphological, biochemical and physiological properties, antigenicity and pathogenicity.

The species name *Candidatus* phytoplasma prunorum was proposed for ESFY phytoplasma in 2004 (Seemuller and Schneider, 2004). It is closely related to both *Candidatus* phytoplasma mali (apple proliferation phytoplasma) and *Candidatus* phytoplasma pyri (pear decline phytoplasma) and the three phytoplasmas share 98.5-99% sequence similarity across the 16S gene. All three phytoplasmas belong to the 16SrX (apple proliferation) phytoplasma group (Lee et al. 1998). However significant genetic differences are observed in other genes amongst the three phytoplasmas and they have distinct biological and epidemiological properties including host range and vectors.