National Diagnostic Protocol for X-disease phytoplasma

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This version of the National Diagnostic Protocol (NDP) for X-disease phytoplasma 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/

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1 INTRODUCTION

X-disease phytoplasma is primarily associated with diseases in *Prunus avium* (sweet cherry), *P. cerasus* (sour cherry) and *P. persica* (peach), although it is occasionally associated with decline in *P. dulcis* (almond) and may infect plum species (Guerra and Eastwell, 2006). Four X-disease phytoplasma strains are reported: Green Valley (GVX), Napa Valley (NVX), Peach yellow leafroll 1 (WX/PYLR1) and the Seibe strain (Guerra and Eastwell, 2006). The GVX strain is most common. GVX and NVX strains are associated with slightly different symptoms in cherry (Nemeth, 1986).

1.1 Diseases associated with X-disease phytoplasma

X-disease phytoplasma is associated with Cherry X-disease, which is also known as cherry buckskin, in sweet cherry and sour cherry. Symptom development in cherry also depends on the cultivar and rootstock combinations. Cherry trees on Mahaleb rootstocks (*P. mahaleb,* black cherry) decline quickly and die due to a hypersensitive reaction by the rootstock in response to the presence of X-disease phytoplasma in the scion wood, which results in cell death at the graft union (Uyemoto, 1989). X-Disease develops slowly on other rootstocks such as Colt (*P. avium* x *P. pseudocerasus*), Mazzard *(P. avium)*, or Stockton Morello *(P. cerasus).* Trees on these rootstocks decline slowly and may survive for many years, however a severe reduction in yield occurs (Uyemoto, 1989; Kirkpatrick et al. 1995).

Peach X-disease is also associated with X-disease phytoplasma. The disease develops over several years and in the early stages of disease development, only a few branches are affected. However, the entire X-disease affected peach tree will show symptoms two to three years after initial infection. X-disease affected peach trees gradually decline and trees may die within 2-6 years (Nemeth, 1986; Kirkpatrick et al. 1995). The phytoplasma is also thought to be associated with several other diseases in peach including: Peach red suture, peach rosette, peach yellows (little peach) and peach yellow leafroll (Guerra and Eastwell, 2006).

Although symptoms may be indicative of X-disease phytoplasma infection its presence should be confirmed through diagnostic testing. X-disease phytoplasma 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 for the 16SrIII (X-disease) group may assist in narrowing down the identity of phytoplasma infecting stone fruit (Lee et al. 1994).

1.2 Alternative host plants

In the USA, two native *Prunus* sp., *P. virginiana* (wild chokecherry) and *P. emarginata* (bitter cherry), act as a reservoir of the phytoplasma for both peach and cherry plantings (Lukens et al. 1971). These *Prunus* sp are not reported in Australia. X-disease phytoplasma is associated with disease in chokecherry and symptoms often affect the entire shrubs. Leaves show premature bright yellow to red autumn coloration and shoots have shortened internodes. Diseased chokecherries often die within 1-3 years after first development of symptoms (Lukens et al. 1971).

Various weeds, which may be found in an around orchards such as burclover (*Medicago polymorpha*), clovers (*Trifolium* spp.), and dandelion (*Taraxacum officinale*) can also act as alternative hosts for the phytoplasma. These alternative host plants may also act as hosts for the vectors (Guerra and Eastwell 2006).

Japanese plum (*Prunus salicina)* and several wild plum species (*Prunus angustifolia, P.injucunda, P. mexicana* and *P. munsoniana)* are reported as alternative hosts for the X-disease phytoplasma strain associated with peach rosette disease.

Other X-disease phytoplasma hosts include almond, flowering almond, apricot, Asian plum, Damson plum, European plum, Mahaleb cherry, Manchu cherry, Mazzard cherry, sand cherry, sour cherry, western sand cherry, chokecherry and red maple.

2 TAXONOMIC INFORMATION

The taxonomic classification of the X-disease phytoplasma is:

> Bacteria; Firmicutes; Mollicutes; Acholeplasmatales; Acholeplasmataceae; Candidatus Phytoplasma; 16SrIII (Western X group).

X-disease phytoplasma is known by several names and these include:

> Western X (WX**)** mycoplasma-like organism (MLO), Western X phytoplasma, Peach –X phytoplasma, Cherry-X phytoplasma**,** Cherry buckskin MLO**,** Eastern peach X disease phytoplasma, Western peach-X phytoplasma, Green Valley X (GVX) phytoplasma, Napa Valley X (NVX) phytoplasma, Peach yellow leafroll 1 (WX/PYLR1) phytoplasma.

The suggested scientific name of X-disease phytoplasma is *Candidatus* Phytoplasma pruni, although this name is not yet formally recognised (IRPCM Phytoplasma/ Spiroplasma Working Team – Phytoplasma taxonomy group 2004). This phytoplasma is a member of the 16SrIII (Western X) group of phytoplasmas (Lee et al. 1993). Strain variation of the X-disease phytoplasma is reported (Gundersen et al. 1996).

3 DETECTION

X- disease in stone fruit can be identified by the presence of characteristic symptoms, however diagnosis should be confirmed through PCR detection and sequencing of the 16S rRNA gene of the X-disease phytoplasma, particularly as other phytoplasmas may cause similar symptoms. Most symptoms, particularly if they are observed on their own, may also be caused by other biotic and abiotic factors.

X-disease phytoplasma is phloem limited, however it may infect the phloem tissue of all parts of a tree, including roots trunk, branches, shoots, leaf petioles and veins and fruit peduncles.

Phytoplasmas can be unevenly distributed and in uneven titre throughout woody hosts. Symptomatic tissue is usually 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 (Constable et al. 2003; Jarausch et al. 1999). Symptoms occur in spring and summer and symptomatic tissue is most likely to be infected by X-disease phytoplasma. During dormancy phloem tissue collapses in the branches and shoots and phytoplasmas are unlikely to persist in this tissue in high titre. However, some phytoplasma bodies may infect the buds of dormant shoots and may persist in the trunks of trees.

3.1 Symptoms of X-disease in cherry

In Cherry, symptom expression is dependent on the environment, the X-disease phytoplasma strain, the scion variety and the rootstock onto which the scion is grafted;

In cherry general symptoms include:

- The entire tree can become affected as the disease progresses over several years and the entire canopy is light green to yellow in colour (Figure 1, 3).
- Graft union failure is observed on Mahaleb rootstock due to a hypersensitive reaction of the rootstock resulting in necrosis of the phloem (Figure 2)
- The canopy becomes sparse
- Cherry trees decline and the shoots and branches die back
- Leaves on affected shoots can become chlorotic (Figure 3)
- Leaves can become bronze or rust coloured earlier than leaves on healthy trees (Figure 3)
- In sour cherries, dieback may affect older infected twigs and branches
- Symptoms may initially be restricted to one branch
- Rosetting of the leaves on the shoot tips
- Affected fruit is small and pale (Figure 4)
- Fruit fails to ripen and may have an astringent taste
- Affected fruits may be mixed on the same branch with unaffected fruit

Sweet cherry varieties infected with the NVX strain have normal shaped fruit with long stems whilst those infected with the GVX strain have pointed fruit with short stems (Figure 5).

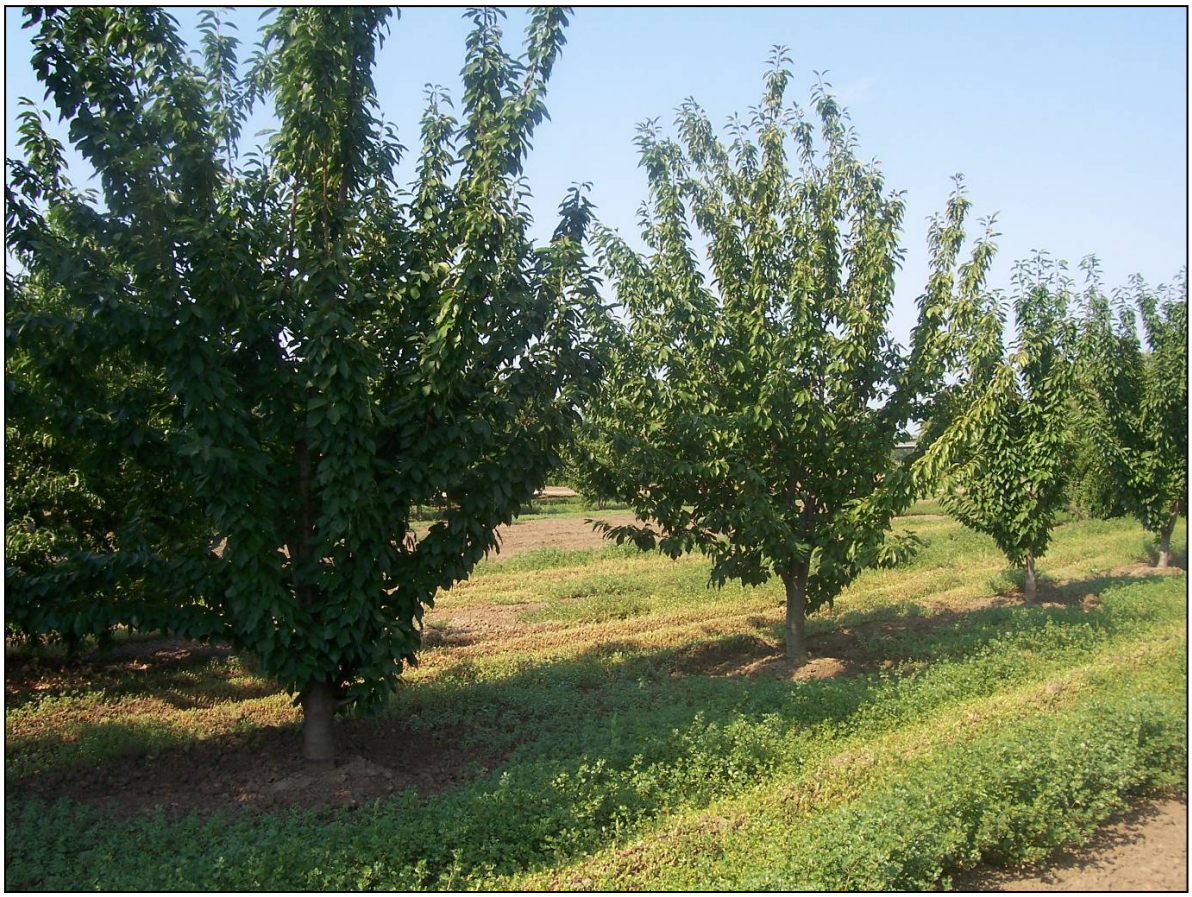

Figure 1. An unaffected cherry tree cv. Bing (Left) compared to an X-disease affected cherry tree cv. Bing infected with the Napa valley X-disease phytoplasma strain (right) (© F. Constable).

Figure 2. A hypersensitive reaction to X-disease at the graft union of sweet cherry on the *Prunus* mahaleb rootstock (Image courtesy of A. H. Purcell University of California).

Figure 3. Shoot from an X-disease affected cherry tree (left) compared to a shoot from an unaffected tree (right). Note the smaller chlorotic leaves, which are starting to exhibit some bronzing on the affected shoot. The affected shoot is stunted compared to the unaffected shoot (© F. Constable).

Figure 4. Cherry fruit from an X-disease affected tree (Top) compared to fruit from an unaffected tree (bottom) (© F. Constable).

Figure 5. Cherry (cv Bing) fruit unaffected (left) or associated with Napa Valley strain (middle) and the Green Valley strain (right) of X-disease phytoplasma (© F. Constable).

3.2 Symptoms of X-disease in peach:

X-disease phytoplasma in peach is associated with several diseases.

3.2.1 Peach X disease

The GVX and NVX strains are associated with Peach X disease and symptoms include:

- Dieback of branches early in the growing season
- Affected leaves may be smaller, curl inward and develop irregular yellow to reddish-purple spots
- The leaf spots become dry and brittle and drop out leaving tattered leaves with a "shot hole" appearance (Figure 6)
- Leaves on affected branches fall prematurely, starting at the base of the branch, and often leave a cluster of leaves at the tip of the branches
- Yield and quality of the fruit are affected
- Fruit can drop prematurely
- Fruit can colour and ripen prematurely and have a bitter taste
- In the early stages of disease development, only a few branches are affected
- After 2-3 years the entire canopy of an X-disease affected peach tree will show symptoms
- Peach trees gradually decline and trees may die within 2-6 years.

The GVX and NVX strains may be symptomless in some varieties.

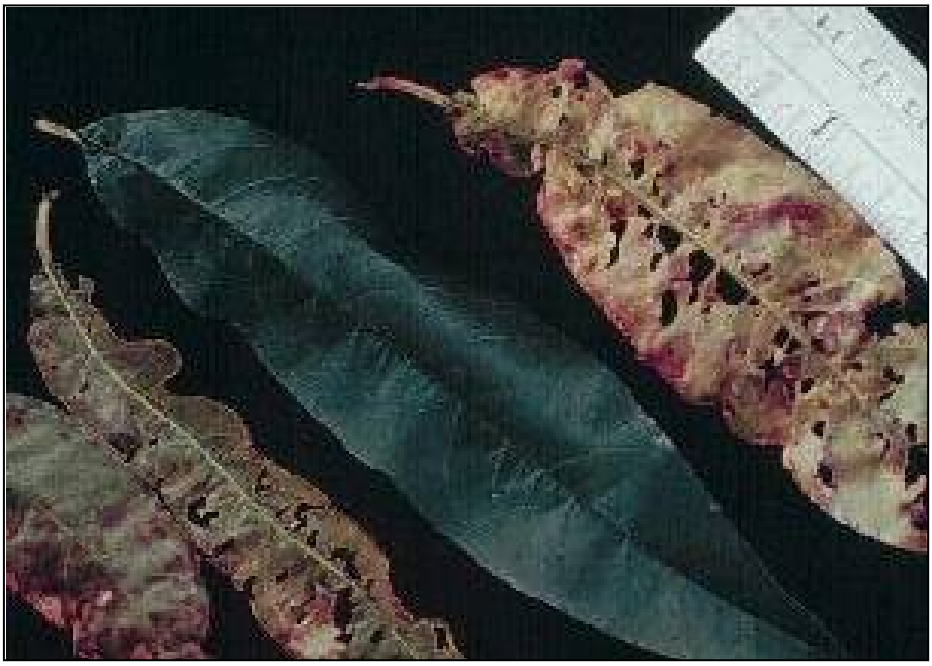

Figure 6. An unaffected peach leaf (middle) compared to X-disease affected peach leaves with shothole symptoms (left and right). (Image courtesy of B. Howell Washington State University).

3.2.2 Peach yellow leafroll disease

The WX/PYLR1 strain is associated with peach yellow leafroll disease and specific symptoms include:

- Leaves are of normal size
- Leaf chlorosis
- Leaf rolling
- Early leaf fall
- Swollen veins on leaves
- Trees show reduced vigour

3.2.3 Peach yellows, Peach rosette and peach red suture.

Peach yellows is also known as peach little leaf. Peach Yellows, peach rosette and peach red suture are also associated with strains of X-disease phytoplasma. The 16SrRNA genes of these strains and the X-disease phytoplasma have 99% sequence similarity but they each have a different biology, indicating that the associated strains are distinct. The Plum leafhopper (*Macropsis trimaculata* Fitch) is a known vector for the X-disease phytoplasma strain associated with peach yellows strain but is not reported as a vector of X-disease phytoplasma strains associated with other stone fruit diseases.

Peach yellows symptoms include:

- Leaf chlorosis
- Upward rolled leaves
- In severe infection shoots are slender with small, narrow and yellow leaves
- Small fruit which may be deformed
- Fruit ripens early
- Red fruits become highly coloured
- Trees decline and may dies within 2-3years after infection

Peach rosette symptoms include:

- Early bud burst
- Sparse flowering
- Leaves roll inward
- Leaves form compact rosettes
- Most fruit drops
- Trees die within one year

Mahaleb cherry does not exhibit a hypersensitive reaction to the peach rosette strain, indicating that it is distinct to other X-disease phytoplasma strains.

Peach red suture symptoms include:

- Leaves become yellowish-green or bronze colour after petal fall.
- Autumn colouring develops early

- On two-year-old branches, buds may grow into spur-like outgrowths.
- Fruit matures and colours earlier than normal on their suture sides, while the opposite side remains green and hard (Figure 7)
- The suture side may become swollen and the flesh, coarse and watery (Figure 8)
- Fruit may develop an uneven shape (Figure 7)

Figure 7. Red suture disease in peach. (Photo courtesy of Holly Thornton, University of Georgia Plant Pathology Archive, University of Georgia, Bugwood.org)

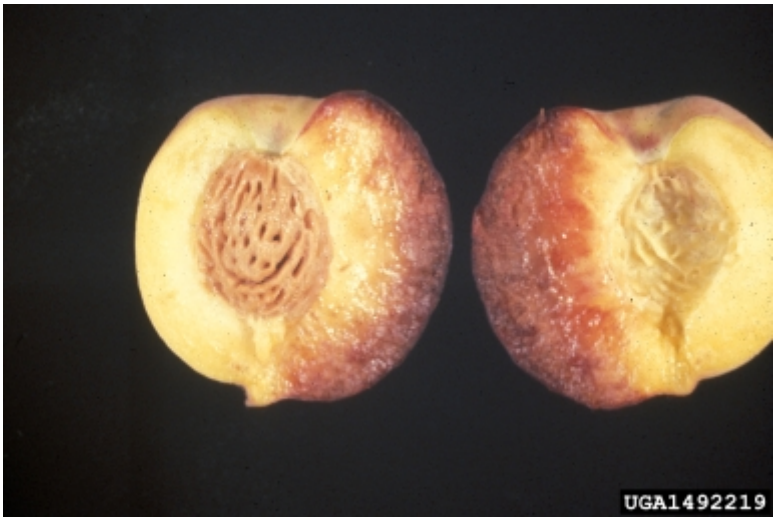

Figure 8 Red suture disease in peach. (Photo courtesy of Holly Thornton, University of Georgia Plant Pathology Archive, University of Georgia, Bugwood.org)

3.3 Symptoms of X-disease in Almond and Japanese plum

The X-disease phytoplasma can infect both almond (*Prunus dulcis*) and Japanese plum (*P. salicina*) but the symptoms observed may be strain dependent and some varieties are symptomless

In almond symptoms include:

- Small leaves that are chlorotic and have a tattered appearance
- Early leaf fall
- Trees may decline (Figure 9,10)

In Japanese plum symptoms include:

- Mild defoliation
- Chlorotic leaves that can become red
- Leaves may occasionally be tattered

Figure 9. X-disease phytoplasma infect almond tree cv. Padre (front) compared to an uninfected tree (back) and PD/PYLRV2 (pear decline phytoplasma, 16Srgroup X) infected tree (right) (© F. Constable).

Figure 10. Uninfected almond tree cv. Padre left, X-disease phytoplasma infect almond tree (middle) and PD/PYLRV2 (pear decline phytoplasma, 16Srgroup X) infected almond tree (right) (© F. Constable).

3.4 Phytoplasma detection

3.4.1 Sample selection

Phytoplasmas are phloem limited and vascular tissue should be used for successful PCR detection. Leaf petioles, mid veins from symptomatic leaves and bark scrapings from shoots and branches can be used from actively growing plant hosts. The peduncles of cherry fruit may be used and in almond the bottom pointed portion of the shell while it is still soft can be used (Lauri Guerra Pers. Comm.). 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.

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.

3.4.2 Recommended phytoplasma detection method

- 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).

- Perform a internal control 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).

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).
- Analyse the PCR products by agarose gel electrophoresis.

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 nested PCR using PCR product for the first stage (P1/P7) PCR product and 16SrIII group specific primers (Table 4) can be used to identify the phytoplasma to the group level, however this will not determine which 16SrIII phytoplasma species is present.

3.4.3 DNA extraction procedure

This extraction procedure uses the QIAGEN DNeasy® Plant mini kit (Green et al. 1999)

Materials and equipment

- QIAGEN DNeasy® Plant mini kit
- 1.5 ml centrifuge tubes
- 20-200 μl and 200-1000 μl pipettes
- 20-200 μl and 200-1000 μl sterile filter pipette tips
- Autoclave
- Balance
- Bench top centrifuge
- Distilled water
- Ice machine
- Freezer
- 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.

- Scalpel handle
- Sterile scalpel blades
- Vortex
- Water bath or heating block at 55-65°C
- Latex or nitrile gloves
- 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	Amount needed for 1
CTAB (cetyltrimethylammonium	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.

3.4.4 Polymerase Chain Reaction

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

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

Polymerase Chain Reaction

The internal control PCR, using the components and concentrations listed in Table 5 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 internal control PCR conducted on these extracts. If the internal control 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 4. 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 16SrIII group specific primers (Table 4).

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

Table 4. 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) Schneider et al. (1995)
	P7 (reverse)	CGTCCTTCATCGGCTCTT			
Universal phytoplasma – single PCR or nested second stage PCR	R16F2n (forward)	GAAACGACTGCTAAGACTGG	55°C	1,248	Lee et al. (1993)
	R16R2 (reverse)	TGACGGGCGGTGTGTACAAACCCCG			
16SrIII group specific single PCR or nested second stage PCR with P1/P7 primers used for the first stage PCR*	R16(III)F2 (forward)	AAGAGTGGAAAAACTCCC	50°C	836	Lee et al. (1994)
	R16(III)R1(reverse)	TCCGAACTGAGATTGA			
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 16SrIII group specific primer pairs can be used in single PCR for X-disease phytoplasma detection, however single PCR is less sensitive thna 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.

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 5. Conventional PCR reaction master mix

Reagent	Volume per
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	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

Table 6. PCR cycling conditions

	Internal control 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 Annealing Elongation	94°C 55°C 72°C	45 s 45 s 1 min 30 s	35	94°C 55 °C* 72°C	1 min 1 min 1 min 30 s	35
Final elongation	72°C	10 min	1	72°C	10 min	1

*NB if using the 16SrIII grioup specific primers an annealing temperature of 50°C is required.

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 to determine the size of the products. Table 5 lists the expected PCR product size for each primer pair.

3.5 Interpretation of results

Failure of the samples to amplify with the internal control 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:

- the positive control produces the correct size product as indicated in Table 5; and
- 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 the X-disease phytoplasma

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5 ACKNOWLEDGEMENTS

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- Fiona Constable DPI, Victoria Australia
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- Esther Torres, Laboratori de Sanitat Vegetal, Departament d'Agricultura Ramaderia i Pesca, Barcelona
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This protocol was reviewed and verified by New Zealand Ministry of Agriculture and Forestry, MAF Biosecurity.

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7 APPENDICES

7.1 Appendix 1: Nucleic acid cleanup

Materials and equipment

- 1.5 ml centrifuge tubes
- 20-200 µl and 200-1000 µl pipettes
- 20-200 µl and 200-1000 µl sterile filter pipette tips
- Autoclave
- Balance
- Bench top centrifuge
- Distilled water
- Freezer
- Vortex
- Latex or nitrile gloves
- Reference:
- 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

- Add an additional 100-200 µl of sterile water or TE to the nucleic extract to assist ease of handling.
- 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.
- 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.
- Discard the supernatant and wash the pellet once with 70% ethanol.
- 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.

7.2 Appendix 2: Alternative extraction methods

7.2.1 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

- 2 ml centrifuge tubes
- 20-200 µl and 200-1000 µl pipettes
- 20-200 µl and 200-1000 µl sterile filter pipette tips
- Autoclave
- Balance
- Bench top centrifuge
- Distilled water
- Ice
- Freezer
- Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)
- Scalpel handle
- Sterile scalpel blades
- Vortex
- Water bath or heating block at 55-65°C
- Latex or nitrile gloves
- Buffers:
  - Phytoplasma isolation buffer - The potassium (Table 2) and sodium (Table 3) 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 2. Potassium phosphate phytoplasma isolation buffer

Reagent	Final concentration	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

Table 3. Sodium phosphate phytoplasma isolation buffer

Reagent	Final concentration	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

Method

- 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
- 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.
- Transfer supernatant into a new 2 ml microcentrifuge tube and centrifuge at 4ºC for 15 min at 13,000 rpm.
- Discard the supernatant.
- Resuspend the pellet in 750 µl hot (55-65°C) CTAB buffer.
- Incubate at 55-65°C for 30 min with intermittent shaking then cool on ice for 30 seconds.
- 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.
- Carefully remove upper aqueous layer into a new 1.5 ml microcentrifuge tube.
- 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.
- 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.
- Dry DNA pellet in a DNA concentrator or air-dry.
- Resuspend in 20 µl sterile distilled water. Incubating the tubes at 55°C for 10 min can aid DNA resuspension.
- Store DNA at -20ºC for short term storage or -80ºC for long term storage.

7.2.2 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

- 2 ml centrifuge tubes
- 20-200 µl and 200-1000 µl pipettes
- 20-200 µl and 200-1000 µl sterile filter pipette tips
- Autoclave
- Balance
- Bench top centrifuge
- Distilled water
- Freezer
- Sterile mortars and pestles or "Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba)
- Scalpel handle
- Sterile scalpel blades
- Vortex
- Water bath or heating block at 55-65°C
- Latex or nitrile gloves
- 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.
- Grind 0.5 g of plant material in 5 ml of CTAB extraction buffer containing 0.2% ß – mercaptoethanol.
- 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.
- 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.
- 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.
- Dry the pellet under vacuum or air dry and resuspend in 50 µl of water.

7.3 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 their morphology and ultrastructure 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 suggested scientific name of X-disease phytoplasma is *Candidatus* Phytoplasma pruni, although this name is not yet formally recognised (IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group 2004). This phytoplasma is a member of the 16SrIII (Western X) group of phytoplasmas (Lee et al. 1993). Strain variation of the X-disease phytoplasma is reported (Gundersen et al. 1996).