National Diagnostic Protocol

Flavescence dorée phytoplasma

NDP 46 V1

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/initiatives/national-diagnostic-protocols/

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

Document status

This version of the National Diagnostic Protocol (NDP) for Flavescence dorée phytoplasma is current as at the date contained in the version control box below.

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Further information

Inquiries regarding technical matters relating to this protocol should be sent to: sphd@agriculture.gov.au

Contents

1 INTRODUCTION

The Flavescence dorée phytoplasma (FDp) is a member of the 16SrV taxonomic group. Three strain clusters of FDp have been recognised which differ in their geographic range and possible region of origin (Arnaud *et al.* 2007).

Grapevine (*Vitis vinifera*) is the primary host of FDp which is associated with Flavescence dorée (FD) disease. Whilst all *V. vinifera* cultivars can be infected some differ in their susceptibility/tolerance. Varieties such as Barbera, Cabernet Franc, Cabernet Sauvignon, Chardonnay, Pinot noir, Pinot Gris, Riesling, Sangiovese, Soave and Prosecco are sensitive; Nebbiolo, Merlot, Sauvignon Blanc and Syrah are more resistant (Pavan *et al.* 1997; Boudon-Padieu 1999; Belli *et al.* 2000, Kuzmanovic *et al.* 2003); Vercesi and Scattini 2000; Sancassani and Posenato 1995), *Vitis riparia*, *V. labrusca* and other rootstocks e.g. *420A* (*Vitis berlandieri* x *V. riparia*) and Millardet (*Vitis* interspecific hybrid) may also host this phytoplasma (Maixner and Pearson 1992; Borgo *et al.* 2007). Many rootstock varieties are symptomless hosts (Boudon-Padieu 1999).

All FDp strains are transmitted vegetatively in infected grapevine planting material or by insect vectors. They are naturally and persistently transmitted from grapevine to grapevine by *Scaphoideus titanus*. *S. titanus* was introduced to Europe from the USA in the early-mid 20th century, and has moved throughout Europe, most likely on infested planting material (Caudwell 1983; Bertin *et al.* 2007; Arnaud *et al.* 2007). *S. titanus* lives and feeds exclusively on grapevine in Europe. The rapid spread and resultant epidemics of FD occurs when *S. titanus* occurs in the presence of FDp (Schvester *et al.* 1969; Boudon-Padieu 2005; Bressan *et al.* 2006).

Clematis vitalba is an alternative host for the strain FD-3 and infected *C. vitalba* plants were found in regions where FDp infected grapevines have not been observed (Filippin *et al.* 2007). Phytoplasmas associated with Palatinate GY (PGYp) in Germany and alder yellows phytoplasma (AldYp) also belong to the 16SrV group and have a high sequence similarity with strains of FDp (Arnaud *et al*. 2007). It has been proposed that FDp, PGYp and AldYp have a common origin in Europe.

FD-3 strains have been detected in *Dictyophara europaea* and this leafhopper was shown to transmit FDp from clematis to grapevine in glasshouse conditions (Fillipin *et al.* 2007; Filippin *et al.* 2009). All three FDp strain clusters have been detected in the mosaic leafhopper *Orientus ishidae*. FD-1 and FD-3 strain clusters have also been detected in *Oncopsis alni* (Mehle *et al.* 2010; Mehle *et al.* 2011). The role of *O. ishidae* and *O. alni* as vectors is unknown.

2 TAXONOMIC INFORMATION

The taxonomic classification of the phytoplasma associated with Flavescence dorée is:

Bacteria; Firmicutes; Mollicutes; Acholeplasmatales; Acholeplasmataceae; *Candidatus* Phytoplasma; 16SrV (Elm Yellows group); Flavescence dorée phytoplasma.

The provisional name '*Candidatus* Phytoplasma vitis' has been suggested to denote the phytoplasma associated with Flavescence dorée disease in Europe, but the name has yet to be formally published.

Three strain clusters of FDp have been recognised based on sequence analysis of the 16S rRNA, *secY, map* and *uvrB-degV* genes (Arnaud *et al*. 2007). These strain clusters include FD1 which comprises isolate FD70 (FD2000) and has low genetic variability, FD2, which comprises isolates FD92 and FD-D and has no genetic variability based on the three gene regions, and FD-3, which comprises isolate FD-C and has more genetic variability (Arnaud *et al.* 2007).

Several common names alternative names occur in the literature and include:

- Baco 22A disease (English)
- Flavescencia dorada (Spanish)
- Flavescenza dorata (Italian)
- Grapevine golden Flavescence.

3 DETECTION

The presence of symptoms may indicate FD disease; however, FD disease symptoms are identical to symptoms of grapevine yellows diseases associated with other phytoplasmas, including those that occur in Australia. Diagnosis of FD disease should be confirmed through PCR detection and sequencing of the 16S rRNA gene of FDp and specific detection and sequencing of other FDp genes including *secY*. Most symptoms, particularly if they are observed on their own, may be caused by other biotic and abiotic factors.

FDp is phloem-limited and can infect the phloem tissue of all parts of a grapevine, including roots, trunk, branches and shoots. Phytoplasmas can be unevenly distributed and be present in variable titre throughout woody hosts. Symptomatic tissue is therefore 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 in grapevines may be affected by seasonal changes and therefore the timing of sample collection for phytoplasma detection is important (Skoric *et al*. 1998; Constable *et al*. 2003). In Europe, the best time and tissue type for FDp detection is summer and symptomatic tissue is most reliable (Del Serrone and Barba 1996).

3.1 Symptoms

The following symptoms associated with FDp are often observed.

Vitis vinifera

- Irregular yellowing in white varieties or reddening in red varieties (Figures 1-4, 6-9)
- Yellow leaf tissue may become necrotic (Figure 4)
- Backward curling of the leaves (Figures 1-4, 6-9)
- Overlapping of leaves on affected shoots (Figure 6)
- Rows of black pustules can develop on the green bark of affected shoots
- Tips of affected shoots may die and shoots may die back (Figure 3)
- Affected shoots fail to harden off and remain rubbery
- Flowers on affected shoots may abort
- Berries may shrivel and whole clusters of berries can be subject to early drying (Figure 5)

It is important to note that symptoms associated with Flavescence dorée disease and FDp are difficult to distinguish from grapevine yellows diseases associated with other phytoplasmas, including those occurring in Australia. Therefore, an accurate diagnosis can only be made via the detection and identification of the specific phytoplasma that is present using molecular methods.

Symptomless infections can occur in grapevine.

Figure 1 Yellowing and leaf curling associated with Flavescence dorée disease in the cv. Prosecco, grown in Treviso, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 2 Yellowing and leaf curling associated with Flavescence dorée disease in the cv. Prosecco, grown in Treviso, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 3 Leaf curling and tip death associated with Flavescence dorée disease in the cv. Prosecco, grown in Treviso, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 4 Irregular yellowing, necrosis and leaf curling associated with Flavescence dorée disease in the cv. Prosecco, grown in Treviso, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 5 Berry shrivel associated with Flavescence dorée disease in the cv. Prosecco, grown in Bologna, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 6 Overlapping leaves with mild yellowing and leaf curling associated with Flavescence dorée disease in an unknown white variety, grown in Emilia-Romagna, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 7 Irregular reddening and leaf curling associated with Flavescence dorée disease in an unknown red variety grown in Emilia-Romagna, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 8 Irregular reddening and severe leaf curling associated with Flavescence dorée disease in an unknown red variety grown in Emilia-Romagna, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 9 Flavescence dorée affected wild grape in an unmanaged and overgrown area nearby a vineyard, Treviso Italy. The circled area shows a portion of the grapevine which is displaying grapevine yellows symptoms, including irregular yellowing and leaf curling. (Source: FE Constable, Agriculture Victoria Research)

3.2 Sampling

Vascular tissue from symptomatic plant material provides the best opportunity to detect phytoplasmas in grapevines. 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 less reliable for FDp detection.

Symptomless infections can occur and if this is suspected it is important to thoroughly sample different shoots of the one plant for phytoplasma detection.

4 IDENTIFICATION

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

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 recommended extraction procedure of Boudon-Padieu *et al* (2003) or Green *et al* (1999; Appendix 1) it may be useful to use a phytoplasma enrichment procedure (Appendix 1) to improve detection from symptomless material or from material collected outside the optimum time frame for detection. The enrichment procedure uses a preliminary differential centrifugation procedure to clarify the homogenate and concentrate the phytoplasmas by pelleting.

If a phytoplasma infection is suspected but the identity of the phytoplasma is uncertain the universal nested PCR test should be used for detection. If a positive result is obtained the product must be sequenced to identify which phytoplasma is present. If FDp infection is suspected the specific nested test can be used.

4.1 PCR detection of Flavescence dorée phytoplasma.

The quality control PCR, using the FD2 and RP1 primers (Table 2) and the components and concentrations listed in Table 3, 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 5. Electrophoretic analysis of the resulting PCR products is performed as described below. The quality control 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 as described in Appendix 2 or the sample should be re-extracted and a quality check PCR conducted on these extracts. If the quality control PCR is successful the PCR reactions for phytoplasma detection can be done.

For FDp detection a nested PCR using the specific primers FD9f/FD9r and FD9f3b/FD9r2 (Table 2) should be conducted. These primers amplify the *secY* gene of FDp and several other phytoplasma species in the 16SrV (Elm yellows) phytoplasma group. It is possible that these primers will also detect *Candidatus* Phytoplasma ulmi, which is associated with Palatinate grapevine yellows in Germany. Therefore sequencing of the PCR product is required to determine the identity of the phytoplasma detected with these primers. Table 3 lists the reagents required and their final concentration for each PCR reaction for FDp detection in single and nested PCR. Table 5 lists the cycling conditions for each PCR. If a positive result is obtained the PCR product should be sequenced to identify the organism that is detected.

To confirm the positive result obtained with the specific primers, or if a negative result is obtained with the FD9f/FD9r and FD9f3b/FD9r2 primers but a phytoplasma infection is suspected, a universal phytoplasma nested PCR should be conducted using the primers P1/P7 and R16F2n/R16R2 primers (Table 2) to determine if other grapevine yellows associated phytoplasma species are present. The expected sizes of the PCR products are listed in Table 2. The recommended primers are universal and were developed to amplify all known phytoplasmas. Table 4 lists the reagents required and their final

concentration for each PCR reaction for universal phytoplasma detection in single and nested PCR. If a positive result is obtained the PCR product should be sequenced to identify the organism that is detected.

A multiplex real-time PCR method is available for simultaneous detection of FDp, *Candidatus* Phytoplasma solani phytoplasmas and an internal endogenous gene of grapevines in a single sample (Pelletier *et al.* 2009; Appendix 3). This method uses dual labelled hydrolysis ("Taqman") minor groove binder (MGB) probes that are each labelled with a different fluorescent reporter dye for each target. This method is as sensitive as nested PCR and could be used for high throughput detection of FDp and *Ca.* P. solani phytoplasmas in grapevines during surveys if an incursion occurs. It is possible to use the real-time primers and probe for FDp separately in a simplex real-time PCR assay or with the endogenous gene primers and probe in a duplex real-time PCR assay for detection of FDp.

The conventional nested PCR using the specific primers FD9f/FD9r and FD9f3b/FDr2 (Table 2) or the multiplex real-time PCR can be used for detection of FDp in grapevines during surveys if an incursion occurs. During the survey the universal phytoplasma nested PCR may not be required.

4.1.1 Tissue sampling

Vascular tissue from leaf petioles, mid veins from symptomatic leaves and bark scrapings from shoots and branches of symptomatic plant material should be used for detection by PCR methods.

If the plant is dormant, buds and bark scrapings from branches, trunk and roots can be used, although these are less reliable for FDp detection. 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 phloem tissue from leaf petioles, mid veins from leaves and bark scrapings from shoots and branches different shoots of the one plant for phytoplasma detection

4.1.2 Nucleic acid extraction method for phytoplasmas in grapevines

From Boudon-Padieu *et al*. 2003.

This DNA extraction method has been validated for detection of *Ca.* P. solani and FDp using conventional and real time PCR assays (Boudon-Padieu *et al*. 2003; Pelletier *et al*. 2009). An alternative and extensively validated DNA extraction method for detection of phytoplasmas in many hosts, including grapevine, uses the Qiagen DNeasy Plant kit (Green *et al*. 1999; Appendix 1).

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 65°C
- Latex or nitrile gloves
- Buffers:
    - 3% CTAB buffer (Table 1)
    - Chloroform:iso-amyl alcohol (24:1 v/v)
    - 70% (v/v) ethanol
    - Sterile distilled water
    - Ice-cold isopropanol

Table 1. 3% cetyltrimethylammonium bromide (CTAB) buffer for DNA purification

Reagent	Final concentration	Amount needed for 1 L
CTAB (cetyltrimethylammonium bromide)	3%	30 g
Sodium chloride	1.4 M	56 g
1 M Tris-HCl, pH 8.0 (sterile)	100 mM	100 mL
0.5 M EDTA, pH 8.0 (sterile)	20 mM	40 mL
Polyvinylpyrrolidone (PVP-40)	1%	10 g

Method

Perform all operations on ice unless otherwise specified.

1. Grind 1.0 g of plant material in 10 mL of CTAB extraction buffer containing 0.2% β – mercaptoethanol.
2. Transfer 1.5 mL of extract to a 2 mL microfuge tube, close the tube. Centrifuge in a microfuge at room temperature for 10 minutes at 1000 x g.
3. Transfer 1 mL of the clarified extract to a 2 mL microfuge tube, close the tube and incubate at 65°C for 20 min, mixing gently several times.
4. Add an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and mix thoroughly but gently for 5 minutes. Centrifuge in a microfuge at room temperature for 10 minutes at 10000 x g.
5. Transfer the 750 µL of the epiphase into a new 2 mL centrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately. Centrifuge for 15 minutes at 10000 x g. Discard the supernatant and wash the pellet twice with 70% ethanol.
6. Dry the pellet under vacuum or air dry and resuspend in 100 µL of water.

4.1.3 Conventional 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 laboratory 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 must be used as well as 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 2)
- 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

Table 2. PCR primers used for phytoplasma detection and quality check primers during conventional PCR

PCR test	Primer name (direction)	Primer sequence (5´-3´)	Primer annealing temperature	Product size (bp)	Reference
Phytoplasma					
Group specific primers for 16SrV EY (Flavescence dorée) single or nested first stage PCR	FD9f (forward) FD9r (reverse)	GAATTAGAACTGTTTGAAGACG TTTGCTTTCATATCTTGTATCG	55°C	1,300	Angelini *et al.* (2001), Clair *et al.* (2003), Daire *et al.* (1997)
Group specific primers for 16SrV EY (Flavescence dorée) nested second stage PCR	FD9f3b (forward) FD9r2 (reverse)	TAATAAGGTAGTTTTATATGACAAG GACTAGTCCCGCCAAAAG	56°C	1,150	Angelini *et al.* (2001) Clair *et al.* (2003), Daire *et al.* (1997)
Universal phytoplasma – single or nested first stage PCR	P1 (forward) P7 (reverse)	AAGAGTTTGATCCTGGCTCAGGATT CGTCCTTCATCGGCTCTT	55°C	1,784	Deng and Hiruki (1991) Schneider *et al*. (1995)
Universal phytoplasma – single PCR or nested second stage PCR	R16F2n (forward) R16R2 (reverse)	GAAACGACTGCTAAGACTGG TGACGGGCGGTGTGTACAAACCCCG	55°C	1,248	Lee *et al.* (1993)
Quality check					
16S bacterial and plant chromosomal	FD2 RP1	AGAGTTTGATCATGGCTCAG ACGGTTACCTTGTTACGACTT	55ºC	approx. 1,400-1,500	Weisberg *et al.* (1991)

Controls

To consider a test result as reliable, the following PCR controls are required as a minimum: quality check nucleic acid control, a phytoplasma positive nucleic acid control and a negative amplification control (no template control).

- **Positive control: DNA of known good quality**: This control is required for the quality check PCR. This control is used to monitor the efficiency of quality check PCR.
- **Positive control: DNA of FDp**: This control is used to monitor the efficiency of the phytoplasma PCR. DNA extracted from a FDp infected host plant or insect, amplified whole genome DNA, or a synthetic control (e.g. a cloned PCR product) may be used
- **No template control: PCR grade water:** This control is necessary for conventional and real-time PCR to ensure that false positive results due to due to contamination during preparation of the reaction mixture have not occurred.

Table 3. Conventional PCR reaction master mix for the specific detection of Flavescence dorée phytoplasma using the primers FD9f/FD9r in the first round and FD9f3b/FDr2 in the nested PCR.

Reagent	Volume per reaction	Final concentration
Sterile (RNase, DNase free) water	16.9 µL	
10 X reaction buffer	2.5 µL	1 X
50 mM $MgCl_2$	1.25 µL	2.5 mM
10 mM dNTP mixture	0.63 µL	0.25 mM
10 µM Forward primer†	1.25 µL	0.5 µM
10 µM Reverse primer†	1.25 µL	0.5 µM
5 units/µL Platinum® *Taq* DNA polymerase (Invitrogen 10966-026)‡	0.2 µL	1 unit
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.

† FD9f/FD9r are used in the first round and FD9f3b/FDr2 are used for nested PCR (Table 2)

‡Other polymerases can be used – where possible these should be validated in advance.

*Up to 5 µL DNA template may be added, reducing water accordingly, as target DNA may be in low concentration. For the nested PCR only 1 µL of the first round PCR product is required, increase the volume of water accordingly.

Non-acetylated molecular biology grade bovine serum albumin (BSA) can be added to the master mix at 0.5 mg/mL to reduce the effect of inhibitors on the PCR.

Table 4. Conventional PCR reaction master mix for universal detection of phytoplasmas using the primers P1/P7 in the first round and R16F2n/R16R2 in the nested PCR

Reagent	Volume per reaction	Final concentration
Sterile (RNase, DNase free) water	18.05 µL	
10 X reaction buffer	2.5 µL	1 X
50 mM $MgCl_2$	0.75 µL	1.5 mM
10 mM dNTP mixture	0.5 µL	0.2 mM
10 µM Forward primer†	1 µL	0.4 µM
10 µM Reverse primer†	1 µL	0.4 µM
5 units/µl Platinum® *Taq* DNA polymerase (Invitrogen 10966-026)	0.2 µL	1 unit
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.

† P1/P7 are used in the first round and R16F2n/R16R2 are used for nested PCR (Table 2)

*Up to 5 µL DNA template may be added, reducing water accordingly, as target DNA may be in low concentration. For the nested PCR only 1 µL of the first round PCR product is required, increase the volume of water accordingly.

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 5. PCR cycling conditions

	Quality check primers			Phytoplasma universal and FDp 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		55ºC or 56ºC*	1 min	
Elongation	72°C	1 min 30s		72°C	1 min 30s	
Final elongation	72°C	10 min	1	72°C	10 min	1

*The Primers FD9f3b/FD9r2 used in the nested PCR for specific detection of FDp require a 56°C annealing temperature

4.1.4 Electrophoresis

Separate PCR products (5-20 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.

4.1.5 Interpretation of results

Failure of the samples to amplify with the quality 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:

a) the quality control primers amplify the expected PCR product
b) the positive control produces the correct size product as indicated in Table 2; and
c) no bands are produced in the negative control (if used) and the no template control.

Confirmation of the specific phytoplasma species can only be determined through sequence analysis.

4.2 Sequencing

DNA sequencing is used to confirm the detection of a pathogen by PCR methods. Commercially available PCR or gel purification kits can be used to purify PCR products directly if a single amplicon is present or from agarose if multiple amplicons are observed after gel electrophoresis PCR products should be sequenced either directly or by first cloning them into a PCR cloning vector according to the manufacturer's instructions. Prepare the purified PCR products or plasmids containing the PCR product for sequencing according the guidelines provided by the sequencing facility to which the products will be sent for sequencing.

Sequence data can be analysed using the Basic Local Alignment Search Tool, BLASTN, available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

To be considered FDp the sequence of the nested PCR product generated using the FD9f/FD9r and FD9f3b/FD9r2 primers should have greatest similarity (92-100%) with FDp isolate FD92 FD9 GenBank accession AF458384.

If the sequence of the nested PCR products generated by the P1/P7 and R16F2n/R16R2 primer pairs shares greatest similarity (97.5-100%) with GenBank accession X76560 (Mycoplasma (MLO;FD) transmitted from *V.vinifera* to *V.faba*, 16SrRNA gene) then the phytoplasma detected is FDp. If the 16S rRNA gene shares greater than 97.5% nucleotide sequence similarity with another phytoplasma species then the phytoplasma that was detected with the universal PCR test is likely to be a strain of that species. If the 16S rRNA gene shares 97.5% or less nucleotide sequence identity with other phytoplasmas, then it may be a separate '*Candidatus* Phytoplasma' species. this case, the entire 16S rRNA gene should be sequenced and phylogenetic analysis performed.

Sequencing a separate region of the genome such as the 16S/23S rRNA spacer region, ribosomal protein genes or the tuf gene may assist in identifying the '*Candidatus* Phytoplasma' species that was detected

5 CONTACTS FOR FURTHER INFORMATION

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8 APPENDICES

8.1 Alternative extraction methods

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

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 6.1) and sodium (Table 6.2) 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/50 mL phytoplasma isolation buffer.

      Adjust pH to 7.6 after adding ascorbic acid and BSA.

    - 3% CTAB grinding buffer (Table 6.3)
    - Chloroform:iso-amyl alcohol (24:1 v/v)
    - 70% (v/v) ethanol
    - Sterile distilled water
    - Ice-cold isopropanol

Table 6.1 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 solution

Table 6.2 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 solution

Table 6.3 3% cetyltrimethylammonium bromide (CTAB) buffer for DNA purification

Reagent.	Final concentration	Amount needed for 1 L
CTAB (cetyltrimethylammonium bromide)	3%	30 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

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 mL of the ground sample to a cold 2 mL micro centrifuge tube and centrifuge at 4ºC for 5 min at 4,500 rpm.
3. Transfer supernatant into a new 2 L micro centrifuge 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 micro centrifuge 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.

References:

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. Phytopathol 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

8.1.2 DNA extraction procedure using 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:
  - 3% CTAB grinding buffer (see Table 6.3)
  - Absolute ethanol

Method

1. Grind 0.5 g of plant tissue in 5 mL of CTAB extraction buffer (room temperature) containing 0.2% β – mercaptoethanol.
2. 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.
3. Add 130 µL of QIAGEN buffer AP2 to extract. Invert 3 times to mix and place on ice for 5 minutes.
4. Apply lysate onto a Qiashredder column and centrifuge at 20,000 x g (14,000 rpm or maximum speed) for 2 minutes.
5. 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.
6. Transfer 650 µL of extract onto a DNeasy column and spin at 6,000 x g (8000 rpm) for 1 minute
7. Discard flow-through and add the rest of the sample to the column and spin at 10000 rpm for 1 minute
8. 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.
9. Discard flowthrough and add another 500 µL of QIAGEN buffer AW and spin at maximum speed for 2 minutes.
10. 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 labelled 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.

References:

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 Dis 83, 482-485.

Marzachi C, Saracco P and Bosco D (2004) Multiplication and movement of Chrysanthemum yellows phytoplasma in the host plant *Chrysanthemum carinatum* J Plant Pathol 86, 296-296.

8.2 Nucleic acid clean-up

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

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 1.5 mL microcentrifuge tube and add an equal volume of isopropanol (stored at -20°C). Mix immediately by inversion. Centrifuge for 15 minutes at 13000 rpm.

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.

8.3 Alternative PCR detection methods

A multiplex real-time PCR method is available for simultaneous detection of Flavesence dorée phytoplamsa (FDp), (*Ca.* P. solani) and an endogenous gene of grapevines (quality check) in a single grapevine sample (Pelletier *et al*. 2009). This method uses dual labelled hydrolysis ("Taqman") minor groove binder (MGB) probes that are each labelled with a different fluorescent reporter dye for each target. This method has the same sensitivity as nested PCR and could be used for high throughput detection of FDp and *Ca.* P. solani in grapevines if an incursion occurs. It is possible to run each of the assays for detection of FDp, *Ca.* P. solani and the endogenous gene of grapevines independently if required.

Pelletier C, Salar P, Gillet J, Cloquemin G, Very P, Foissac X and Malembic-Maher S (2009) Triplex realtime PCR assay for sensitive and simultaneous detection of grapevine phytoplasmas of the 16SrV and 16SrXII-A groups with an endogenous analytical control. Vitis 48, 87-95.

Table 6.4 The primers and probes used for detection of FDp, *Ca.* P. solani and the *Vitis* endogenous gene by real time PCR

Target	Primer or probe name	Primer or probe sequence	Reference
Phytoplasmas of group 16SrV (Flavescence dorée)	mapFD-F	5'-TCA AGG CTT CGG BGG TTA TA-3'	Pelletier *et al*. 2009
	mapFD-R :	5'-TTG TTT TAG AAG GTA ATC CGT GAA CTA C-3'	
	Probe: mapFD-FAM	FAM- TTG TAT TTC AGT GAA TGA AG –MGB	
Phytoplasmas of group 16SrXII (Bois Noir)	mapBN-F :	5'-ATT TGA TGA AAC ACG CTG GAT TAA-3'	
	mapBN-R :	5'-TCC CTG GAA CAA TAA AAG TYG CA-3'	
	Probe : mapBN-VIC :	VIC- AAA CCC ACA AAA TGC –MGB1	
Edogenous - *Vitis* gene (quality check)	VITIS-F	5'-AAA TTC AGG GAA ACC CTG GAA-3'	
	VITIS-R	5'-CCC TTG GTT GTT TTC GGA AA-3'	
	Probe: *VITIS*-Cy5	Cy5- CTG AGC CAA ATC C –BHQ	

Controls

Positive control: Grapevine DNA of known good quality (quality check PCR)

Positive control: DNA extracted from *Ca.* P. solani and FDp (if needed) infected tissue

No template control: Sterile distilled water

Table 6.5 Real-time PCR reaction master mix using the QuantiTect® Multiplex Kit RT-PCR (Qiagen†) for the simultaneous detection of FDp, *Ca.* P. solani and the *Vitis* endogenous gene (quality check) using dual-labelled MGB hydrolysis probes

Reagent *	Volume per reaction	Final concentration‡
Sterile (RNase, DNase free) water	3 µL	
2xQTreaction mix	12.5 µL	1 X
10 µM MapFD-F primer*	0.5 µL	0.2 µM
10 µM MapFD-R primer *	0.5 µL	0.2 µM
10 µM MapFD-FAM probe*	0.5 µL	0.2 µM
10 µM MapBN-F primer	0.5 µL	0.2 µM
10 µM MapBN-R primer	0.5 µL	0.2 µM
10 µM MapBN-VIC probe	0.5 µL	0.2 µM
10 µM *VITIS*-F primer	0.5 µL	0.2 µM
10 µM *VITIS*-R primer	0.5 µL	0.2 µM
10 µM *VITIS*-Cy5 probe	0.5 µL	0.2 µM
DNA template or control*	5 µL	
Total reaction volume	25 µL	

†Other real time PCR reaction mixes may be used after verification

‡The primers and probes for the multiplex assay are listed at the concentration recommended by Pelletier *et al* (2009). It may be necessary to optimize the concentration of primers and probe for each assay prior to use.

*DNA extracted from the sample, positive control DNA or a no template control (e.g. water).

The primers and probe for Ca. P. solani are not required if the presence of Ca. P. solani is not suspected – adjust the volume of water accordingly.

Table 6.6 PCR cycling conditions for detection of FDp, *Ca.* P. solani and the *Vitis* quality (internal) control by real time PCR

Step	Temperature	Time	No. of cycles
Initial activation and denaturation	95°C	15 min	1
Denaturation	94°C	1 min	45
Annealing, elongation and fluorescence acquisition	59°C	1 min 30 s	

Interpretation of the results of real-time PCR

The real-time PCR test is valid if:

- The PCR no template control gives no fluorescence for any of the probes;
- The PCR positive controls (FDp + *Ca.* P. solani + *Vitis* DNA) show fluorescence above the threshold line and less than a Ct of 40 (threshold cycle) for each of the probes.

The following decision scheme is recommended to determine when a sample is positive for FDp, *Ca.* P. solani or both using the multiplex real-time PCR assay (Pelletier *et al*. 2009).

	Ct *Vitis* endogenous gene (quality check) PCR < 25	Ct *Vitis* endogenous gene (quality check) PCR ≥ 25 or not detected
FDp or *Ca.* P. solani Ct Values < 40 positive	positive	positive
FD-P or BN-P Ct Values ≥ 40	negative	not interpretable

8.4 Alternative hosts of Flavescence dorée phytoplasma

8.4.1 Alternative host plants

Alternative hosts of Flavescence dorée phytoplasma include in the climbing shrub *Clematis vitalba* ("old man's beard"), in which only the FD-3 strain cluster has been detected (Angelini *et al.* 2001; 2004; Filippin *et al.* 2009; Filippin *et al.* 2007). All three FDp strain clusters have also been detected in alder trees *Alnus glutinosa* and *A. incana* (Cvrković *et al*. 2008; Mehle *et al.* 2011) and strain FD-3 has been detected in *Ailanthus altissima* ("tree of heaven") (Fillipin *et al.* 2011).

Symptoms

Clematis vitalba

- Yellowing and reddening and rolling of leaves (Figure 10-11)
- May be symptomless

***Alder glutinosa* (common alder)**

- Yellowing, small leaves and reduced foliage
- May be symptomless

***Alder incana* (grey alder)**

- No symptoms reported

***Ailanthus altissima* (Tree-of-Heaven)**

- Yellowing of the leaves may occur, but no clear association as some plants may be symptomless

Figure 10 Leaves of *Clematis vitalba* with yellowing and leaf curling that is associated with Flavescence dorée phytoplasma infection, occurring in Treviso, Italy (Source: FE Constable, Agriculture Victoria Research)

Figure 11 Leaves of *Clematis vitalba* with reddening, yellowing and leaf curling that is associated with Flavescence dorée phytoplasma infection, occurring in Treviso, Italy (Source: FE Constable, Agriculture Victoria Research)