National Diagnostic Protocol for Pierce's Disease, *Xylella fastidiosa*

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This version of the National Diagnostic Protocol (NDP) for *Xylella fastidiosa* is current as at the date contained in the version control box on the front of this document.

NDPs are updated every 3 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

Pierce's disease is a lethal grapevine disease caused by the bacterium *Xylella fastidiosa* which infects the xylem tissue of grapevine. Bacterial aggregates and plant tyloses and gums, produced in response to infection, are thought to block the vessels which conduct water through the plant.

Xylella fastidiosa is a gram-negative bacterium confined to the xylem vessels of its host. The organism, designated *Xylella fastidiosa* was first described by Wells et al. (1987), and is the sole species belonging to this genus. *X. fastidiosa* has not been recorded in Australia.

Infections of the bacteria form dense aggregates within the xylem vessels (Figure 1). These aggregates, along with gums and tyloses produced by the grapevine restrict vascular flow of the xylem (Goheen and Hopkins, 1988). A phytotoxin produced by *X. fastidiosa* may also play a role in the development of the disease (Goheen and Hopkins, 1988). Symptoms appear when a significant amount of xylem is blocked (Varela, 2000).

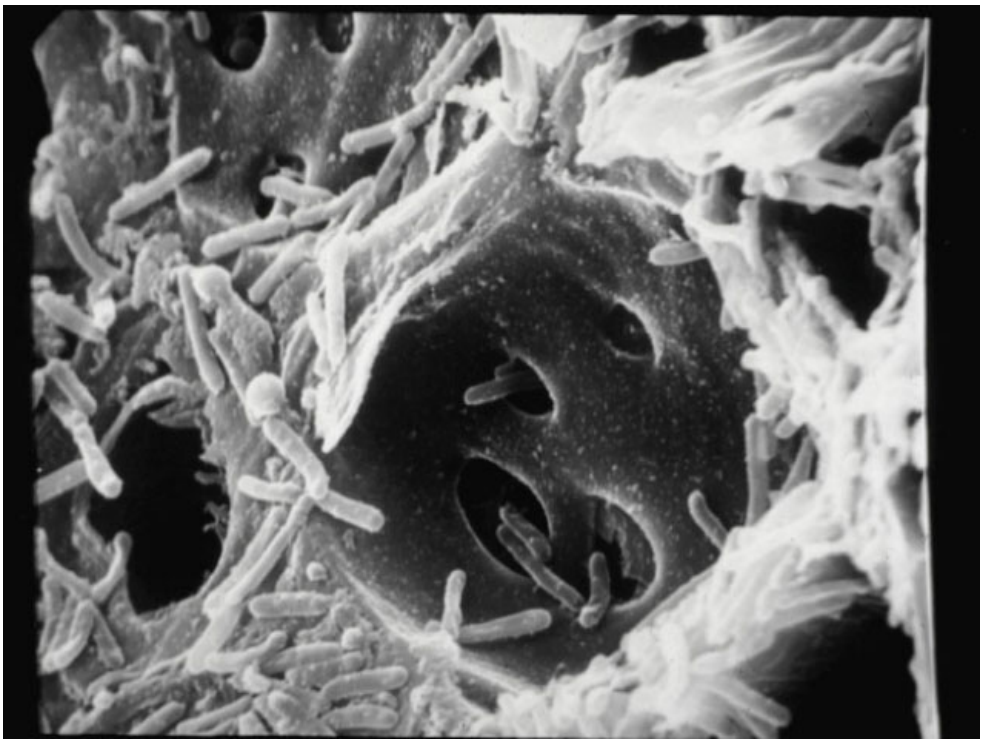

Figure 1. Electron micrographs of *Xylella fastidiosa* in xylem vessels of grapevine
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Pierce's disease kills grapevines outright by blocking the plant's water transporting tissue - the xylem. The plant can die within 1 - 2 years of the initial infection date. The disease and the vector persist all year round, although the longer the time between initial infection and the onset of winter, the greater the chance of the disease persisting over winter and the faster the disease will progress.

1.1 Host range

1.1.1 Primary host range

Vitis vinifera (grapevine), *Prunus persica* (peach), *Prunus salicina* (Japanese plum), *Vitis labrusca* (fox grape), *Prunus dulcis* (almond), *Vitis rupestris* (sand-grape), *Acer* (maple), *Acer saccharum* (sugar maple), *Citrus latifolia* (tahiti lime), *Citrus reticulata* (mandarin), *Citrus reticulata x paradisi* (tangelo), *Citrus sinensis* (navel orange), *Liquidambar styraciflua* (American red gum), *Medicago sativa* (lucerne), *Morus alba* (mora), *Platanus occidentalis* (eastern sycamore), *Prunus angustifolia* (Chickasaw plum tree), *Pyrus* (pear), *Ulmus* (elm)

1.1.2 Secondary host range

Poaceae (cereal), *Brachiaria* (signal grass), *Conium maculatum* (poison hemlock), *Cynodon* (quick grass), *Cyperus* (flatsedge), *Digitaria*, *Echinochloa frumentacea* (Japanese millet), *Fragaria vesca* (European strawberry), *Lolium* (ryegrass), *Medicago* (medic), *Paspalum*, *Paspalum dilatatum* (dallis grass), *Rubus* (blackberry, raspberry), *Sambucus* (elderberry), *Salix* (willow), *Taraxacum officinale* (dandelion), *Trifolium* (clovers), *Vinca minor* (common periwinkle), *Coffea* (coffee).

Alternative *Xylella fastidiosa* hosts are detailed in the Appendix.

1.2 Effect on hosts

The main symptoms include scorched leaf margins, leaf abscission with petiole retention, irregular cane maturation, fruit raisining and delayed spring growth. Some of the symptoms of Pierce's disease can be confused with other syndromes such as salt toxicity, boron, copper or phosphorus deficiency and other diseases e.g. Eutypa.

1.3 Vectors

All sucking insects that feed on xylem sap are potential vectors of *X. fastidiosa*, but all known vectors are limited to the Homoptera suborder (Purcell, 1999c). Vectors acquire the bacterium by feeding on infected plants. The bacteria adhere to the insect's foregut where they multiply and are then transmitted to healthy plants. Vectors remain infective indefinitely after acquiring the bacteria with the exception of nymphs which cannot transmit bacteria after they shed their external skeleton. After moulting, insects must feed again on an infected plant before they can acquire and transmit the bacterium (Purcell, 1999c). Insects currently known to be capable of transmitting *X. fastidiosa* all belong to the spittlebug/froghopper family (Cercopidae) and the 'sharpshooter' subfamily in the leafhopper family (Cicadellidae, subfamily Cicadellinae). . None of these genera have been reported in Australia. Of the 14 species of Cicadellidae in Australia, none have been recorded on Vitaceae. Within the Americas many genera of sharpshooters and spittlebugs serve as vectors of the bacterium (Goheen and Hopkins, 1988). However, in California, the major vectors are the blue-green sharpshooter (*Graphocephala atropunctata*), glassy-winged sharpshooter (*Homalodisca coagulata*), green sharpshooter (*Draeculacephala minerva*), and the red-headed sharpshooter (*Carneocephala fulgida*) (Gubler et al., 1999; Purcell, 1999b; Varela, 2000). Spittlebug vectors of Pierce's disease have been recorded in California (Delong and Severin, 1950), but none have been found on grapevines in California (Severin, 1950). Other sucking insects such as grape leafhoppers, are not vectors in California (Gubler et al., 1999). Cicadas (family Cicadidae) are also xylem feeders but there are no published reports of their being tested as vectors.

Prior to the introduction of *H. coagulata*, plants infected shortly before winter by other species of sharpshooter have recovered and been free of the bacteria in the following spring. This is partly because very cold winter weather helps cure vines of the bacterium and because other sharpshooters feed on and infect the tips of younger shoots, which are pruned during the summer. As *H. coagulata* feed much lower on the cane than other sharpshooters, late season infections are not removed by pruning and may survive the winter to cause chronic Pierce's disease the following season. This enables vine-to-vine spread of the disease rather than linear spread, as has been the case in the past.

Xylella fastidiosa can also be transmitted and dispersed by graft transmission. Propagative material is the pathway by which *X. fastidiosa* may spread (Smith et al., 1997). *Xylella fastidiosa* is not transmitted via contaminated pruning shears or by seed transmission (Smith et al., 1997; Varela, 2000).

Australia has no record of *X. fastidiosa* or sharpshooters.

2 Taxonomic Information

Kingdom: Bacteria

Phylum: Proteobacteria

Class:	Gammaproteobacteria
Order:	Xanthomonadales
Family:	Xanthomonadaceae
Genus:	*Xylella*
Species:	*Xylella fastidiosa*

Scientific Name: *Xyllela fastidiosa* (Wells et al 1987)

Common Names: Pierce's disease, California vine disease, Anaheim disease (grapevine), leaf scorch (almond, coffee, elm, maple, mulberry, oak, oleander, sycamore), variegated chlorosis (citrus), phony peach disease (peach), leaf scald (plum), dwarf (lucerne), wilt (periwinkle).

3 Detection

Xylella fastidiosa is mostly confined to the xylem tissue of its hosts (Figure 2). The major symptoms of Pierce's disease include; leaf necrosis in concentric rings or in sections, leaf abscission with petiole retention, "green islands" on canes, fruit raisining, dieback, delayed growth in spring, and decline in vigour leading to death. The first evidence of Pierce's disease infection usually is a drying or "scorching" of leaves. The best time to observe symptoms of Pierce's disease is late summer through to autumn.

It takes about four-five months for the symptoms to appear, with only one or two canes showing symptoms in the first season. However, in young vines the symptoms may appear over the entire vine in a single season (Varela et al, 2001). In chronically infected vines new growth may be delayed by two weeks with interveinal chlorosis in the first four to eight leaves which may be small or distorted. The internodes are often shortened or zig-zagged. Delayed budbreak or bud failure may also occur (Varela et al, 2001).

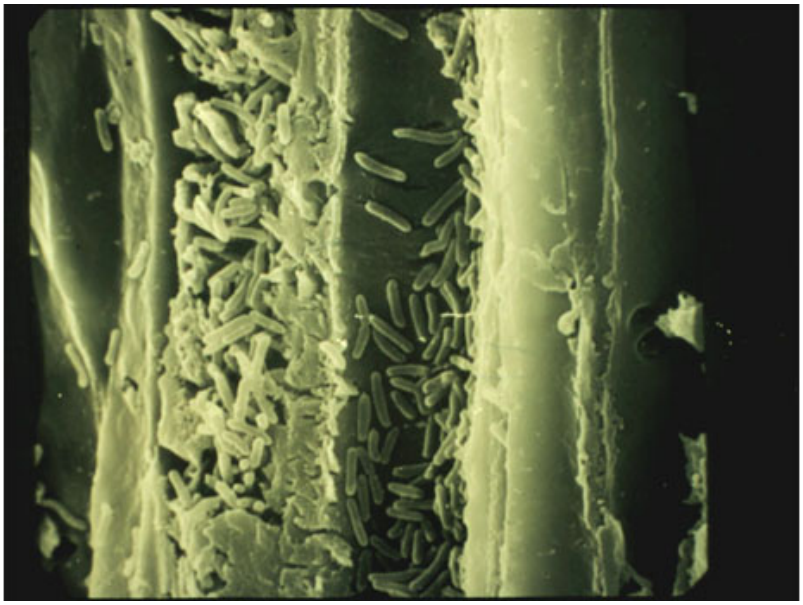

Figure 2. Electron micrographs of *Xylella fastidiosa* in xylem vessels of grapevine
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3.1 Leaf symptoms

The leaves become slightly chlorotic along the margins before drying inwards, or the outer leaf may dry suddenly while still green. The leaf dries progressively over a period of days to weeks, leaving a series of concentric zones of discoloured and dead tissue.

On white varieties, a yellow chlorotic zone appears between the necrotic margin and the green interior of the leaf (Figure 3). The scorching develops inward from the margin and is continuous. On red varieties a dark-reddish to purple band appears between the green and necrotic tissue (Figure 4, Figure 5). There is a wide range of leaf symptoms ranging from highly regular, concentric zones of chlorosis followed by necrosis to discolouration and necrosis occurring in sectors of the leaf only (Varela et al, 2001).

Symptoms vary with the species and cultivar that is affected. Symptoms in muscadine and other native American grapes from the south eastern United States are milder than those in *V. vinifera*. Symptoms are usually more pronounced in vines that are stressed by high temperatures or drought conditions (Goheen and Hopkins, 1988).

The most characteristic symptom of *X. fastidiosa* infection is leaf scorch. An early sign is sudden drying of part of a green leaf, which then turns brown while adjacent tissue turns yellow or red. The desiccation spreads and the whole leaf may shrivel and drop, leaving only the petiole attached (Figure 6).

Leaf symptoms vary among grape varieties (Gubler et al., 1999). Grape varieties such as Pinot Noir and Cabernet Sauvignon have highly regular zones of progressive marginal discolouration and drying on blades. In the varieties Thompson seedless, Sylvaner, and Chenin Blanc (Figure 10), the discolouration and scorching may occur in sectors of the leaf rather than along the margins. Climatic differences between regions can affect the timing and severity of symptoms, but not the type of symptoms (Gubler et al., 1999). Hot climates accelerate symptom development, as moisture stress is more severe even with adequate soil moisture.

In later years, infected plants develop late and produce stunted chlorotic shoots. Highly susceptible cultivars rarely survive more than 2-3 years, despite any signs of recovery early in the growing season. Young vines succumb more quickly than older vines. More tolerant cultivars may survive chronic infection for more than 5 years.

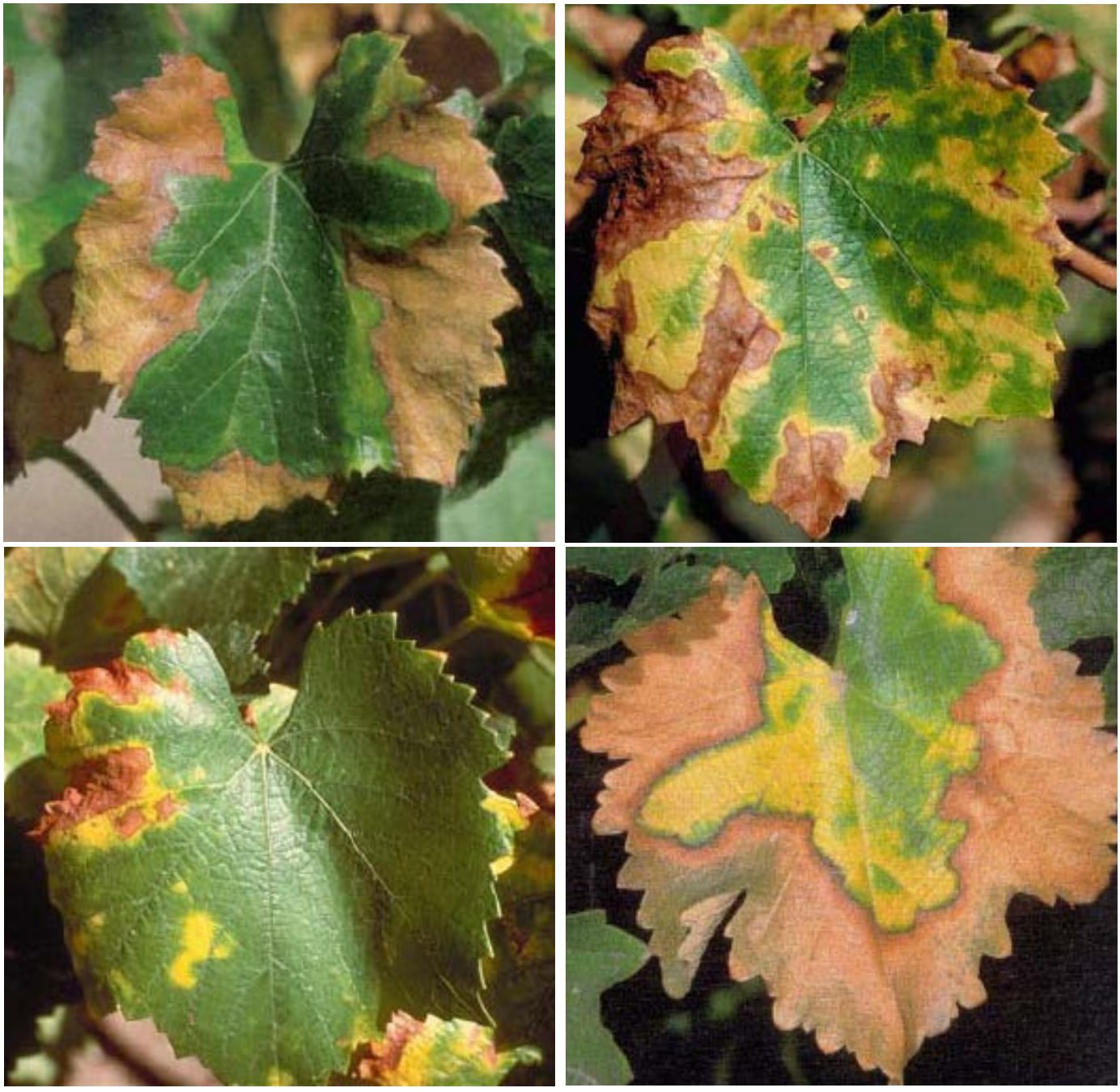


Figure 3 Symptoms of *X. fastidiosa on* Chardonnay (© Regents, University of California 1999).

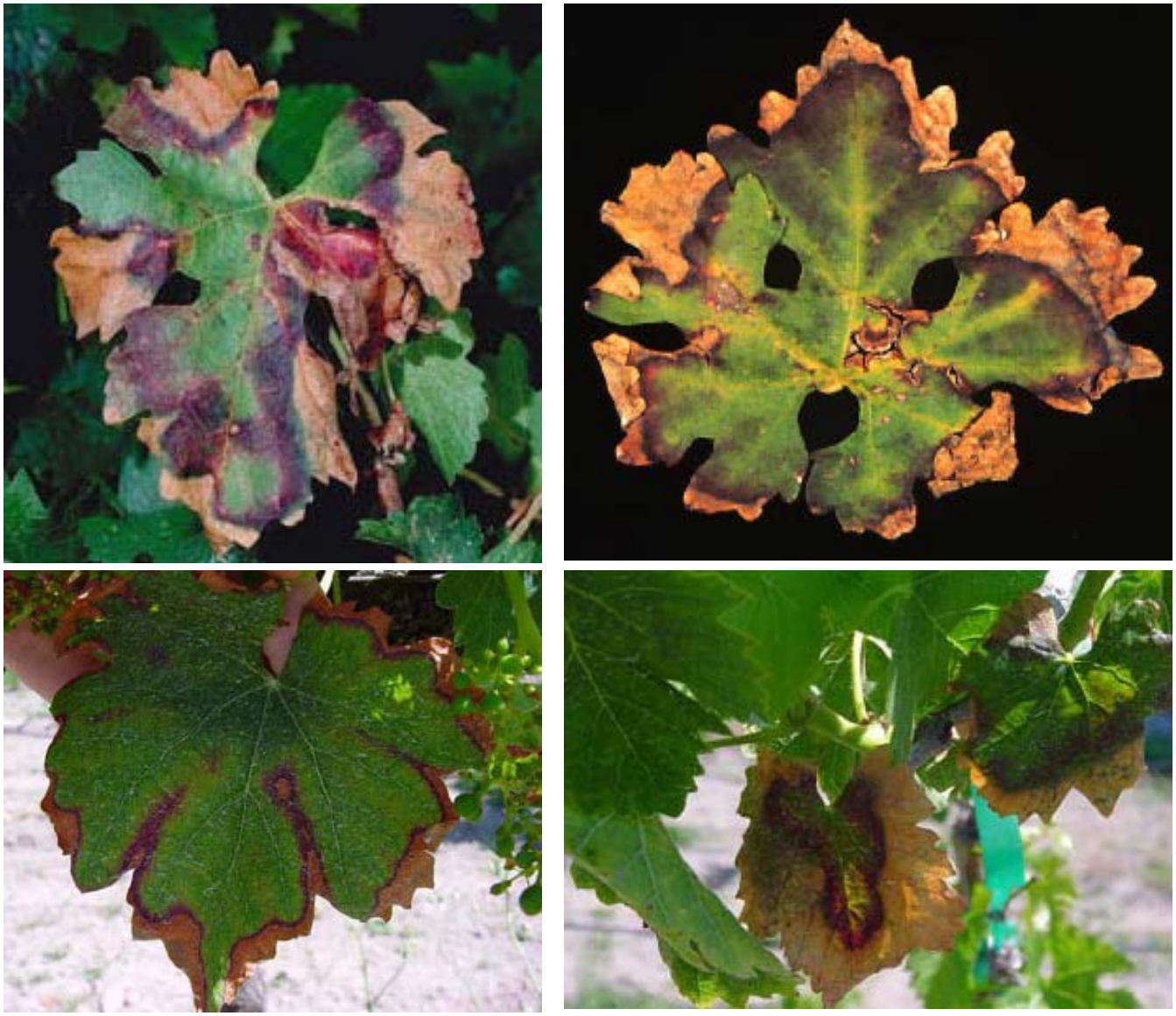


Figure 4 Symptoms of *X. fastidiosa* on Cabernet sauvignon (top © Regents, University of California 1999; bottom © Jo Luck DPI, 2002).

Figure 5 Symptoms of *X. fastidiosa* on red varieties (© Regents, University of California 1999).

Figure 6 Leaf abscission is another characteristic symptom of Pierce's disease. (© Regents, University of California 1999).

3.2 Cane, vine and fruit symptoms

Usually only one or two canes will show Pierce's disease symptoms late in the first season of infection (Gubler et al., 1999). Diseased stems often mature irregularly, with patches of brown and green tissue. These are known as "green islands" (Figure 7).

Symptoms gradually spread along the cane from the point of infection out towards the apex and more slowly towards the base (Figure 8). By mid-season some or all fruit clusters on the infected cane may wilt and dry (Gubler et al., 1999)(Figure 9). Flower clusters on infected vines may set berries, but these usually dry up (Goheen and Hopkins, 1988). Tips of canes may die back, and roots may also die back. Vines deteriorate rapidly after appearance of symptoms. Shoot growth of infected plants becomes progressively weaker as symptoms become more pronounced.

In the following year, some canes or spurs may fail to bud out. New leaves become chlorotic (yellow) between leaf veins and scorching appears on older leaves. From late April through summer infected vines may grow at a normal rate, but the total new growth is less than that of healthy vines (Gubler et al., 1999). In late summer leaf burning symptoms reappear.

Figure 7 Regions of irregular wood maturation on canes, designated green islands are a characteristic symptom of Pierce's disease (© Regents, University of California 1999).

Figure 8 Symptoms spread along the cane out towards the tip and more slowly towards the base and the tips of canes may die back (L). Chronically infected vines had restricted spring growth and stunted shoot growth (R) (© Regents, University of California 1999).

Figure 9 Fruit bunches may shrivel or raisin (© Regents, University of California 1999).

Figure 10 Symptoms of *X. fastidiosa* on Chenin blanc (© Regents, University of California 1999).

3.3 Impact of climatic conditions and seasonality

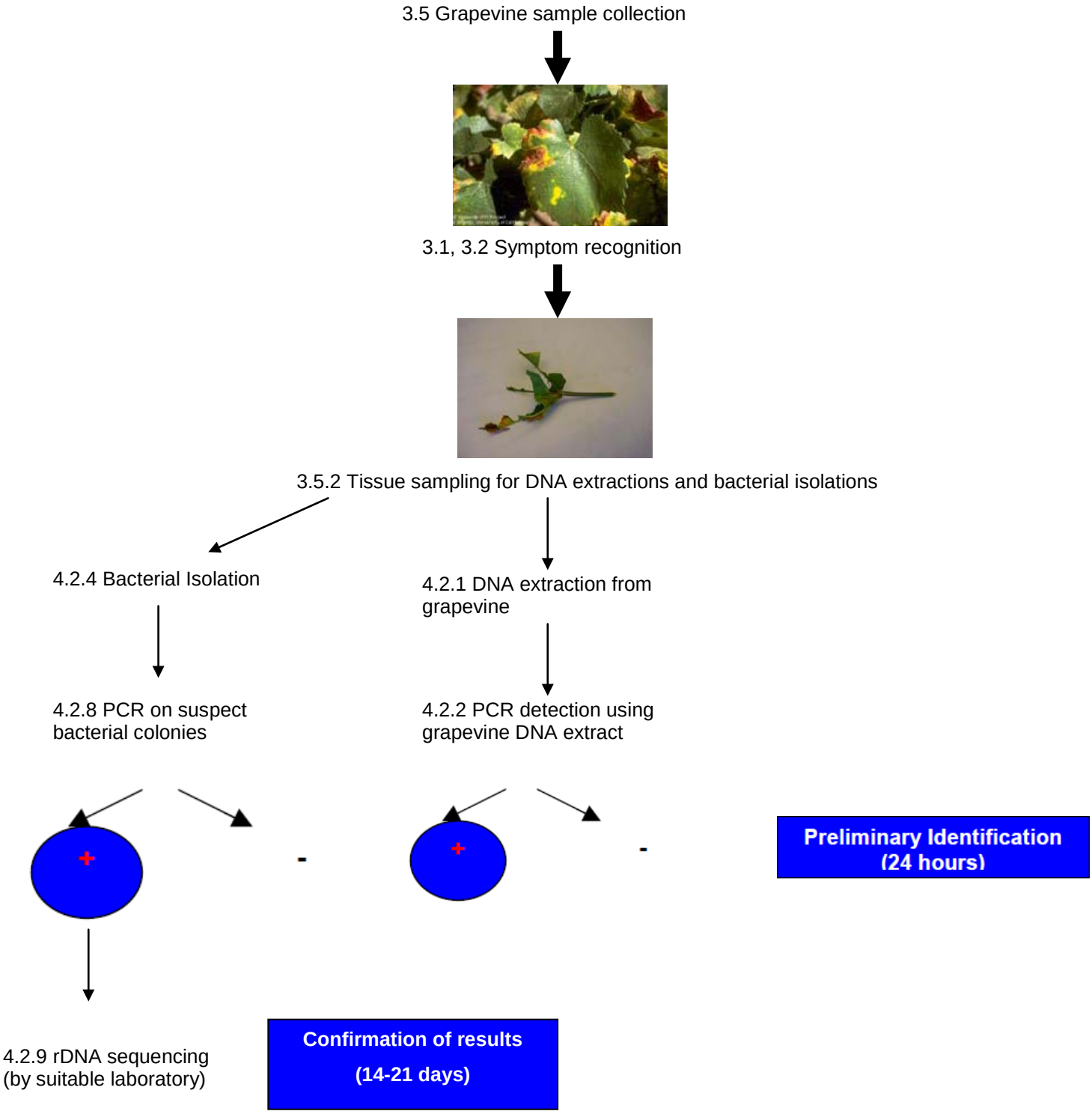
Physiological changes in the vines induced by cold weather can cause death of the bacteria. The longer the time between initial infection and the onset of winter, the greater the chance of the disease persisting over winter and the faster the disease will progress. Plants infected shortly before winter have recovered and been free of the bacteria in the following spring. Laboratory observations from Purcell and Saunders (1995) work on harvested grape clusters as inoculum for Pierce's disease showed that the number of viable *X. fastidiosa* decreased with time spent in cold storage and declined sharply after cold storage at 4°C. The bacterium was not recovered from infected grapes after 21 days of storage at this temperature. This data supports the observations made by Varela (2000). Further, experimental cold therapy of diseased grapevines suggests that freezing temperatures can eliminate the bacterium directly from plants (Purcell, 1980).

Winter weather conditions in Australia are not as severe as those experienced in the USA and in many areas vines are not considered to go dormant over the winter non-growing period. The effects of winter are not likely to affect survival of the bacterium in Australia.

Some vines infected during the season appear to recover from Pierce's disease the first winter following infection (Varela, 2000). Recovery from Pierce's disease depends on the grape variety. In Cabernet, recovery is high while in Barbera, Chardonnay and Pinot Noir it is low. In more tolerant cultivars, the bacterium spreads more slowly within the plant than in more susceptible cultivars (Varela, 2000). Once the vine has been infected for over a year (i.e. bacteria survive the first winter) recovery is much less likely (Varela, 2000). Young vines are more susceptible than mature vines, possibly because the bacteria can move more quickly through younger vines than through older vines. Rootstock species and hybrids vary greatly in susceptibility. Testing of rootstock plants show that *V. riparia* is rather susceptible; *V. rupestris* (St George) and 420A are very tolerant.

Rootstock does not confer resistance to susceptible *V. vinifera* varieties grafted on to it. Climate, variety and age determine how long a vine with Pierce's disease can survive (Varela, 2000). One-year old Pinot Noir or Chardonnay can die the year they become infected, whereas chronically infected 10-year-old Chenin Blanc or Ruby Cabernet can live for more than five years. Long before that, however, these chronically infested vines will cease to bear a crop (Varela, 2000).

3.4 Diagnostic flow chart



3.5 Sampling procedures critical for the detection methods and diagnostic procedures

3.5.1 Grapevine sample collection for detection of *X. fastidiosa*

1. Late-summer to autumn is the best time to sample for Pierce's disease. In chronically infected vines, bacteria do not move into the new season's growth until the middle of summer. Leaves attached to the cane generally give the most reliable result.
2. Collect leaf material which is showing symptoms characteristic of *Xylella fastidiosa* infection, and which is still attached to the cane
3. Collect 4-5 canes from the suspect plant.
4. Wrap the cane samples in damp newspaper and place inside a sealed plastic bag.
5. Ship to a diagnostic laboratory (for details see below) immediately after the material is collected.

NB. Negative test results, do not mean that *Xylella fastidiosa* is absent as the bacteria may be unevenly distributed through the vine. It is important to sample symptomatic material.

3.5.2 Tissue Sampling for DNA Extractions and Bacterial Isolations

The most optimum tissue to sample for the detection of *X. fastidiosa* is the mid-rib and petiole from symptomatic leaves. Select five leaves from affected canes and treat as one sample. Replicate sampling.

Further detection and identification methods are outlined in Section 4.

4 Identification

Positive identification of *X. fastidiosa* can be obtained by three methods: culturing the bacterium on selective media, serological test such as ELISA (enzyme linked immunosorbent assay) or PCR (polymerase chain reaction) (Varela, 2000).

4.1 Morphological methods

For cultural diagnosis a specialised media (section 4.2.4.2) has been developed for isolating and growing the Pierce's disease bacterium. Petioles are used to isolate the bacteria. Using this technique, 100 bacterial cells per gram of plant tissue are able to be detected (Hill and Purcell, 1995). The disadvantages are that it is time consuming, colonies may require 32 days to develop, microbial contaminants cloud or obscure results and the bacteria can only be isolated from petioles during the summer and early fall (Varela, 2000). Colonies of *X. fastidiosa* on most selective media are convex, smooth, entire or rough with finely undulate margins (Bradbury 1991).

The morphological and biochemical characteristics of *X. fastidiosa* are as follows (Davis et al 1978):

Single aflagellate straight rods, 0.25-0.35 X 0.9-3.5 µm, with filamentous strands under some cultural conditions. Colonies are of two types: convex to pulvinate smooth opalescent with entire margins and umbonate rough with finely undulated margins. Cells stain Gram negative. Non-motile. Oxidase negative and catalase positive. Strictly aerobic, non-fermentative, non-halophilic, non-pigmented. Nutritionally fastidious, requiring a specialised medium such as BC-YE containing charcoal or glutamine-peptone medium (PW) containing serum albumin. Optimal temperature for growth is 26-28°C. Optimum pH is 6.5-6.9. Habitat is exclusively in the xylem of plant tissue.

Hydrolyses gelatin and utilises hippurate. Most strains produce β-lactamase. Glucose is not fermented. Negative in tests for indole, H_2S, β-galactosidase, lipase, amylase, coagulase, and phosphatase. The species has been isolated as a phytopathogen from tissues of a number of host plants. The type strain was isolated from grapevine with Pierce's disease (Wells et al 1987).

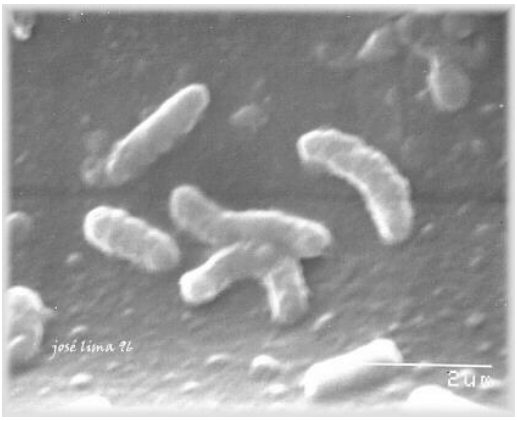


Figure 11 *Xylella fastidiosa* © Jose Lima (1996) Citrolima, Brazil

4.2 Molecular methods

PCR enzymatically amplifies specific parts of the bacterium's DNA. This is the most sensitive technique to detect small numbers of bacteria in plants. It is specific for *X. fastidiosa* but has the disadvantages that it is expensive, cannot determine if the bacteria are dead or alive or how many bacteria are present in the sample (Varela, 2000). The *X. fastidiosa* diagnostic PCR is rapid, with a result within 24 hours using plant DNA extracts from suspected hosts, whether the host is symptomatic or asymptomatic. This test can also be used on boiled preparations from bacterial colonies, bacterial DNA extracts and plant tissue extract. The likelihood of a false positive result occurring is low, providing the correct internal controls are used. There is however, a possibility of getting a false negative result due to extremely low bacterial numbers. The possibility of a false negative result occurring due to template inhibition is eliminated by including an additional set of PCR primers that amplify the 16S ribosomal DNA gene from a wide range of bacteria. If this fails then the template contains inhibitors and should be re-extracted.

4.2.1 DNA extraction from grapevine

The following protocol utilises a fume hood (for handling chloroform:isoamyl alcohol) and as such DNA extraction kits such as the Qiagen Plant Tissue Mini Kit, which do not require a hood, may be easier to use for some laboratories.

4.2.1.1 Equipment

1. 2 ml centrifuge tubes
2. 20-200 µL and 200-1000 µL pipettes and tips
3. Autoclave
4. Autoclaved mortar and pestles
5. Balance
6. Centrifuge
7. Distilled water unit
8. Ice machine or freezer
9. Sterile cheesecloth
10. Sterile sand
11. Sterile scalpel blades
12. Vortex
13. Water bath at 60^{o}C

4.2.1.2 Reagents

Modified SCP	***For 500 ml***	***For 1000 ml***
Disodium succinate $C_4H_4Na_2O_7$ (Sigma S2378)	0.5 g	1 g
Trisodium citrate $C_6H_5Na_3O_7$ (Sigma S4641)	0.5 g	1 g
K_2HPO_4 (Ajax A2221-500g)	0.75 g	1.5 g
KH_2PO_4 (Ajax A391-500g)	0.5 g	1 g
PVP40 (Sigma PVP-40)	25 g	50 g

Autoclave. Add ascorbic acid (0.02M final concentration) and adjust to pH 7 just prior to use. The stock buffer (without ascorbic acid) can be stored frozen (-20C) for up to 6 months. The buffer with ascorbic acid shouldn't be frozen once mixed but should be used immediately.

PBS/BSA

a) 10X PBS	***For 1000 ml***
NaCl (BDH Analar #10241.AP)	80 g
KH_2PO_4	2 g
Na_2HPO_4 (Ajax 478 or 621)	11.5 g
KCl (Ajax 382-500g)	2 g

Autoclave. Store at room temperature.

b) PBS/BSA

1x PBS plus 0.2% BSA. Store at 4^{o}C.

CTAB buffer + 0.2% mercaptoethanol	***For 100 ml***
1M Tris, pH 7.5 $H_2NC(CH_2OH)_3$ (Amresco 0234)	20 ml
5M NaCl	28 ml
500mM EDTA, pH 8.0 $[CH_2.N(CH_2.COOH).CH_2COON_9]_2.2H_2O$	4 ml
CTAB $C_{19}H_{42}NBr$ (Sigma H6269)	2 g
β-Mercaptoethanol (Sigma M3148)	200 µl

Mix and make up to 100 ml with dH_2O. Store at room temperature.

Choloroform:isoamyl alcohol
24:1 mix of choloroform (BDH 152835F) to isoamyl alcohol (Sigma I9392). Store at room temperature.

Isopropanol
100% isopropanol stored at 4^{o}C.

Ethanol
80% ethanol. Store at room temperature.

Water
Sterile dH_2O.

4.2.1.3 Method

1. Place CTAB buffer + 0.2% mercaptoethanol in 60^{o}C water bath
2. Select 5 symptomatic grapevine leaves from sample (repeat for duplication of test)
3. Weigh approximately 700 mg midrib and petiole tissue (combined from all 5 leaves)

4. Homogenise in 5 ml of modified SCP grinding buffer with autoclaved mortar and pestle, and using approximately 0.1 g sterile sand
5. Strain homogenate through sterile cheesecloth and transfer 500 µl to a sterile 2 ml centrifuge tube, or trim pipette tip with sterile scalpel blade and transfer 500 µl to a 2 ml sterile centrifuge tube
6. Centrifuge at 12000 RPM (~17,000 xg) for 5 minutes
7. Discard supernatent and re-suspend the pellet in 500 µl of PBS/BSA with pipette (temperature of the PBS/BSA is not significant)
8. Immediately add 800 µl pre-warmed (60°C) CTAB buffer + 0.2% mercaptoethanol
9. Vortex and incubate the centrifuge tube at 60°C for 20 minutes, with occasional mixing (2-3 second vortex every 5 minutes)
10. Add 600 µl chloroform:isoamyl alcohol (24:1) and vortex vigorously
11. Centrifuge at 12000 RPM (~17,000 xg) for 5 minutes
12. Transfer supernatant to a sterile 2 ml centrifuge tube
13. Add equal volume of cold isopropanol, mix well and leave on ice (or in freezer, 0°C or -20°C) for 10 minutes
14. Centrifuge at 12000 RPM for 10 minutes.
15. Rinse pellet with 500 µl 80% ethanol
16. Centrifuge at 12000 RPM for 5 minutes and remove all ethanol with pipette
17. Air dry pellet by placing tube on its side. The minimum time to air dry is the time required to evaporate the residual water and ethanol. This will vary depending on ambient temperature and humidity.
18. Re-suspend pellet in 200 µl sterile dH_2O

Please note that other DNA extraction methods may be used, as long as when the DNA template is used in PCR that the internal controls (primer pair rP1 and fD2) amplify the correct size amplicon (~1.5 kb). If no amplification occurs, the DNA will need to be re-extracted.

4.2.2 PCR detection using grapevine DNA extract

4.2.2.1 Equipment

1. 0-2 µl, 2-20 µl, 20-200 µl, and 200-1000 µl pipettes and tips
2. 0.2 or 0.5 ml PCR tubes
3. 1.5 or 2 ml centrifuge tubes to store reagents
4. Bulb spinner or centrifuge
5. Freezer
6. Gel tanks, rigs and racks
7. Ice machine
8. Latex, and leather gloves
9. Microwave
10. Power pack
11. Thermocycler
12. UV transilluminator with camera

4.2.2.2 Reagents

Primers

To detect *Xylella fastidiosa*, three specific primers sets can be used. For the Pierce's disease strain of *Xylella fastidiosa* the RST primers should be used (Minesavage *et al*, 1994). For strains not occurring in grapevine the XF primers can be used. It is important to use housekeeping genes such as ribosomal DNA to ensure the DNA template does not contain PCR inhibitors. This eliminates the possibility of a false negative result. All primers were used at a concentration of 100 ng/µl.

Primer Name	***Sequence (5'-3')***	***Target Gene***	***Reference***
RST31	GCGTTAATTTTCGAAGTGATTCGA	Unknown fragment	Minesavage *et al*.,1994
RST33	CACCATTCGTATCCCGGTG		Minesavage *et al*.,1994
XF1-F	CAGCACATTGGTAGTAATAC	16S rDNA	Firrao & Bazzi, 1994
XF6-R	ACTAGGTATTAACCAATTGC		Firrao & Bazzi, 1994
FD2	AGAGTTTGATCATGGCTCAG	16S rDNA	Weisburg *et al.*, 1991
RP1	ACGGTTACCTTGTTACGACTT		Weisburg *et al.*, 1991

PCR Master Mix	***25 µl reaction***
Sterile dH_2O	15.35
1 mM dNTPs	2.5
10 x concentration buffer	2.5
25 mM $MgCl_2$	1.5
RST31	0.5
RST33	0.5
RP1	0.5
FD2	0.5
DNA template, undiluted	1.0
RedHotTaq 5U/ml	0.15

RedHot Taq (ABgene AB-0406/A). Kits with $MgCl_2$ in the buffer can also be used but the master mix should be modified accordingly.

PCR Controls

1. Positive control=Total nucleic acid extraction from Malbec vine infected with *X. fastidiosa* using the above method. Alternatively, healthy grapevine nucleic acid spiked with *X.fastidiosa* DNA can be

used where *X. fastidiosa* infected material cannot be maintained in the laboratory.
2. Negative control is the master mix (24 µl) with 1.0 of RNAase/DNAase free water instead of DNA template.

5 x TBE	***1L***
Tris $H_2NC(CH_2OH)_3$	54.0 g
Boric acid H_3BO_3	27.5 g
0.5 M EDTA $[CH_2.N(CH_2.COOH).CH_2COON_9]_2.2H_2O$	20 ml

Store at room temperature.

1% Agarose gel with SYBR Safe stain

1. Agarose gel is 1 g DNA grade agarose per 100 ml 1 x TBE.
2. Melt in the microwave.
3. Use SYBR Safe stain as per the manufacturers instructions.

Store at room temperature.

100 x TE solution	***100 ml***
Tris-Cl pH 8.0	50 mL
0.5M EDTA pH 8.0	20 mL
dH_2O	30 mL

Store at room temperature.

Loading dye

Loading dye should be purchased rather than made to ensure consistency. One suitable option is QIAGEN GelPilot Loading Dye 5x (239901).

4.2.2.3 Method

1. Label sterile 100 µl centrifuge tubes
2. Prepare "master mix" in sterile 1 ml centrifuge as described above
3. Add 2 µl sdH_2O to the negative control tube, 2 µl test template to each tube, and 2 µl grapevine DNA infected with *X. fastidosa* into positive control tube.
4. Cycle the tubes with the following PCR conditions:1 cycle 95°C 1 min, 30 cycles (94°C for 45 secs, 55°C for 30 secs, 72°C for 30 secs), 1 cycle 72°C, 10 mins and 1 cycle 25°C, 1 min. (The PCR conditions were adapted for duplex PCR using conditions described in Minesavage *et al*., 1994, Firrao and Bazzi, 1994 and Weisburg *et al.*, 1991)
5. Mix 10 µl each PCR sample with 5 µl running dye
6. Load samples onto a 1% agarose gel containing SYBR Safe stain as per manufacturer's instructions.
7. Electrophorese in 1 X TBE at 100V for approximately 40 minutes
8. Visualise and photograph gel on UV transilluminator.

4.2.3 Examples of PCR for *X. fastidiosa* on Australian grown hosts

X. fastidiosa primers [RST31/RST33 or XF1/XF6 (both will specifically amplify *X. fastidiosa* strains, although the University of California labs uses RST31/RST33)] were combined with universal bacterial primers (RP1/FD2) in duplex PCRs to test various plant DNA extracts (Figure 12, Figure 13).

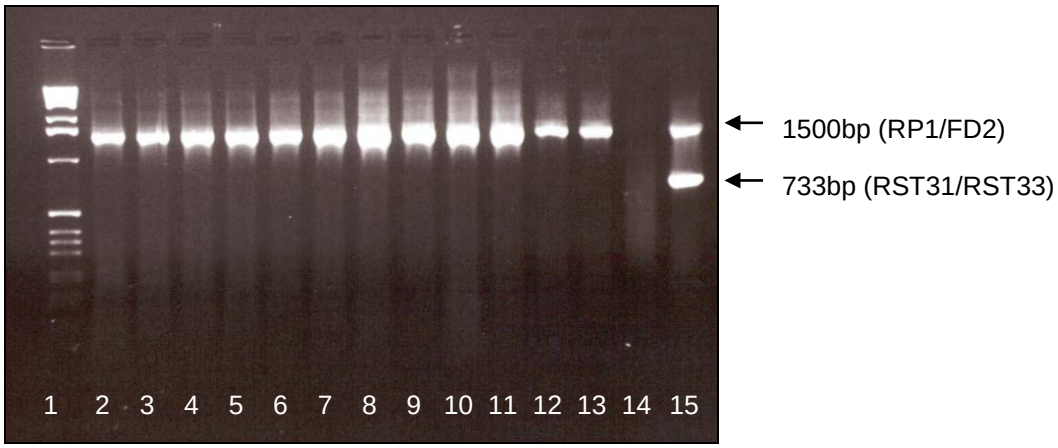


Figure 12 Electrophoresis gel showing PCR products generated from grapevine samples with the primer pairs (XF1/XF6 and RP1/FD2). DNA molecular weight marker X, 0.07-12.2 kb (lane 1) (Roche™), Australian grapevine samples (lanes 2-13), negative control (lane 14), positive control (lane 15).

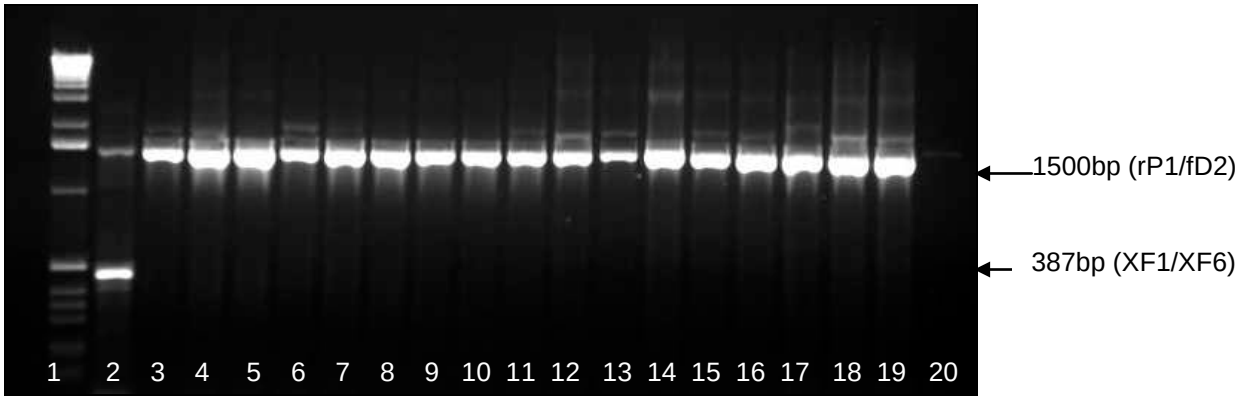


Figure 13 Electrophoresis gel showing PCR products generated from Australian-occurring alternative hosts for *X. fastidiosa* with the primer pairs (XF1/XF6 and RP1/FD2). DNA molecular weight marker X, 0.07-12.2 kb (lane 1) (Roche™), positive control (lane 2), Mexican Tea - *Chenopodium ambrosioides* (lane 3), Citrus 'Meyer' lemon - *Citrus limon* (lane 4), Lemon Balm - *Melissa officinalis* (lane 5), Paspalum - *Paspalum dilatatum* (lane 6), Winter Grass - *Poa annua* (lane 7), Common Purslane - *Portulaca oleracea* (lane 8), Rosemary - *Rosmarinus officinalis* (lane 9), Snowberry - *Symphoricarpos albus* (lane 10), Scrub Cherry - *Syzygium australe* (lane 11), Oleander - *Nerium oleander* (lane 12), Pear - *Pyrus* sp. (lane 13), Mulberry - *Morus rubra* (lane 14), Hydrangea - *Hydrangea* sp. (lane 15), Lilac - *Syringa vulgaris* (lane 16), *Parthenocissus* sp. (lane 17), Box Elder - *Acer negundo* (lane 18), English Ivy - *Hedera helix* (lane 19), negative control (lane 20).

4.2.4 Bacterial isolation

4.2.4.1 Equipment

1. 20-200 µl and 200-1000 µl pipettes and tips
2. Autoclave
3. Autoclaved mortar and pestles
4. Balance
5. Bunsen burner
6. 1.5 - 2.0 ml Centrifuge tubes
7. Fridge
8. Glass spreaders
9. Incubator at 28°C
10. Laminar flow
11. Petri dishes
12. Sterile bottles
13. Sterile cheesecloth
14. Sterile sand
15. Sterile scalpel blades
16. Syringe with 0.2 µm filter (Sartorius Minisart)
17. Tweezers
18. Water bath at 55°C
19. Hot plate

4.2.4.2 Reagents

Ethanol
95% ethanol, diluted with dH_2O. Store at room temperature.

Hypochlorite
1% hypochlorite, diluted with dH_2O. Store at 4°C temperature.

PW (Periwinkle Wilt with Gelrite) Media (Davis et al., 1983)

i) Float 3 g bovine serum albumin on top of 15 mL sdH_2O. Mix periodically to dissolve.

ii) Dissolve 4 g L-glutamine in 100 mL sdH_2O by heating on a hot plate at low heat. Do not let boil.

iii)

	g/L	***Final concentration***
Phytone peptone (BD (BBL) #211906)	4.0	
Trypticase peptone (BD (BBL) #211921)	1.0	
$MgSO_4$ (Ajax 302)	0.4	3.3mM
K_2HPO_4 (Ajax A2221-500g)	1.2	6.9mM
KH_2PO_4 (Ajax A391-500g)	1.0	7.3mM
Phenol red stock (0.2% in sdH_2O) (Ajax A2300 -5g)	10 m	
Hemin chloride (0.1% in 0.05N NaOH) (Aldrich H2250)	10 ml	
Gelrite gellan gum (Sigma G1910)	8	

iv) Autoclave. Let the mixture cool to 55°C in water bath, then add the bovine albumin serum solution and L-glutamine solution using a syringe with a 0.2 m filter attached. Pour into petri dishes. Store plates at room temperature.

PD3 Media (Hopkins and Adlerz, 1988)	***g/L***	***Final concentration***
Tryptone (Oxoid LP0042)	4.0	
Soytone (Difco 243620)	2.0	
Trisodium citrate (Sigma S4641)	1.0	3.9 mM
Disodium succinate (Sigma S2378)	1.0	3.7 mM
Hemin chloride (0.1% in 0.05N NaOH) (Aldrich H2250)	10 ml	
Potato starch (soluble) (Mallinckrodt #8188)	2.0	
$MgSO_4$. $7H_2O$ (Ajax 302)	1.0	4.06 mM
K_2HPO_4 (Ajax A2221-500g)	1.5	8.6 mM
KH_2PO_4 (Ajax A391-500g)	1.0	7.3 mM
Adjust the pH to 6.8, add agar		
Agar (Oxoid LP0013)	15.0	

Autoclave. Pour into petri dishes. Store plates at room temperature.

4.2.4.3 Methods

1. Weigh approximately 100 mg midrib and petiole tissue combined from 5 symptomatic grapevine leaves
2. Surface sterilise material as follows: 1 min in 95% ethanol, 2 mins in 1% hypochlorite and rinse 3 times in sterile water
3. The ex-plant is aseptically cut into 1 mm pieces
4. Homogenise using a mortar and pestle with approximately 0.1 g of sterile sand in 2 ml of sterile distilled H_2O
5. Filter through sterile cheesecloth
6. Prepare serial dilutions to 10^{-4} by adding 100 ul to 900 ul sterile distilled water in sterile eppendorf tubes
7. Spread plate 100 µl of undiluted, 1:10 and 1:100, 1:1000 and 1:10000 dilutions onto Periwinkle Wilt (PW) media or PD media.
8. Incubate at 28°C for a minimum of 3 weeks. Colonies are <1 mm entire and colourless, turning opaque with time. Colonies on PW are circular with entire margins, convex, opalescent-white, reaching 0.7-1.0 mm diameter after 2-3 weeks.

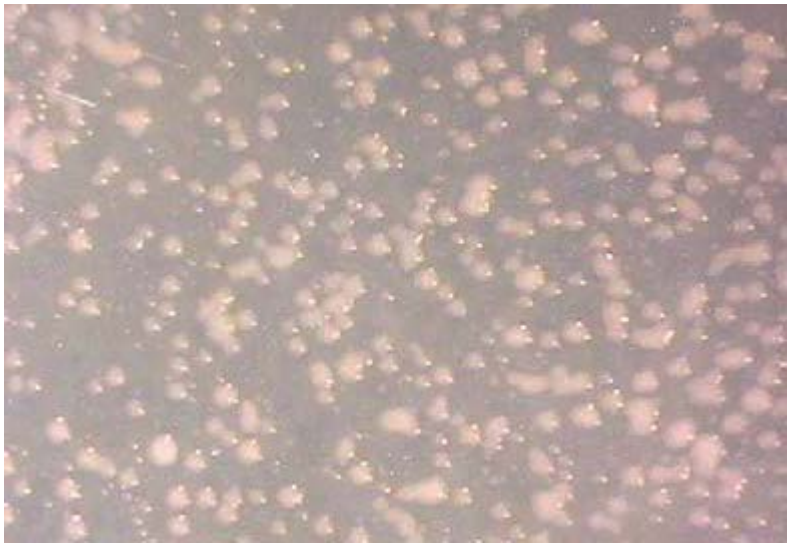


Figure 14 *Xylella fastidiosa* colonies on PW media (20 day old culture) © Jo Luck, DPI 2002.

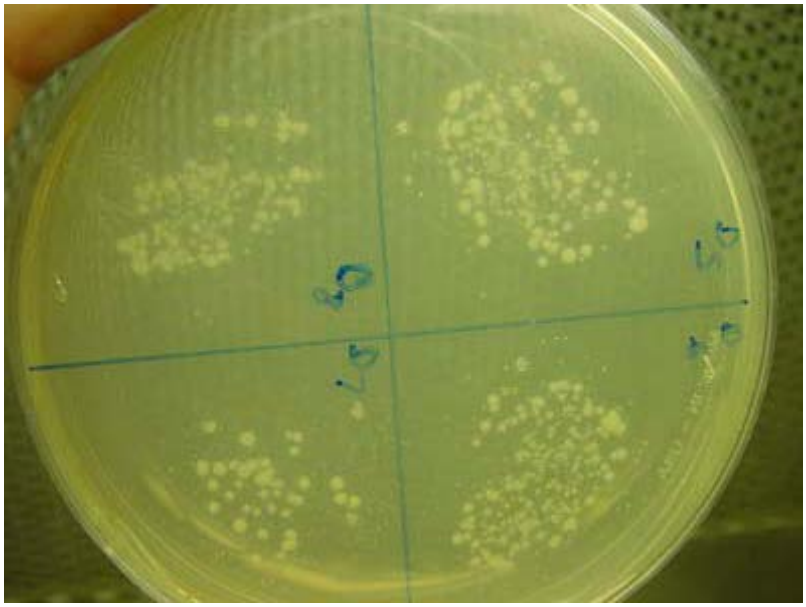


Figure 15 *Xylella fastidiosa* colonies on PW media © Jo Luck, DPI 2002.

4.2.5 Suspect colony gram stain

4.2.5.1 Equipment

1. Bunsen burner
2. Compound microscope
3. Loop
4. Microscope slides
5. Suspect bacterial colonies on PW or PD3 media

Materials

dH_2O
Immersion oil

Stains

1. Crystal violet solution
2. Gram's iodine solution
3. Safranin solution

It is recommended that these solutions are purchased in solution due to their toxicity.
Store at room temperature.

Ethanol

95% ethanol, diluted with dH_2O. Store at room temperature.

4.2.5.2 Method

1. Put a droplet of dH_2O on a slide
2. Using a flamed loop transfer a small amount of the fresh suspect culture to the drop of dH_2O. Mix the bacteria into the dH_2O droplet to create a slightly turbid solution
3. Allow the suspension to air dry
4. Pass the slide two or three times through the bunsen burner to fix the bacterial cells
5. Flood the slide with crystal violet solution
6. After 30 s pour off the stain
7. Flood the slide with Gram's iodine solution
8. After 30 s pour off the solution
9. Rinse immediately under a gentle stream of water
10. Decolourise the stained area by washing the slide for 10-15 s with 95% ethanol
11. Flood the slide with safranin solution
12. After 90 s pour off the stain and rinse the slide with water
13. Allow the slide to dry
14. Using immersion oil view the slide with the 100 x magnification lens on the compound microscope

4.2.6 Oxidase test

4.2.6.1 Materials and equipment

1. Oxidase identification stick impregnated with a solution of N,N-dimethyl-phenylenediamine oxalate, ascorbic acid and α-napthol (Oxoid) (stored at 4°C).
2. Suspect bacterial colonies on PW or PD3 media

4.2.6.2 Method

1. Remove the container from the refrigerator and allow it to stand for five min at room temperature
2. Choose a well separated representative colony on the primary isolation medium
3. Remove one stick (colour coded red) from the container and holding it by the coloured end, touch the colony with the impregnated end of the stick and rotate the stick, picking off a small mass of cells
4. Place the stick between the lid and the base of the inverted plate
5. Examine the impregnated stick after 30 s. If no colour change has occurred examine again after 3 min.
6. A positive reaction is shown by the development of a blue-purple colour. No colour change is observed with organisms that are oxidase negative (Oxoid, 2002).

4.2.7 Catalase test

4.2.7.1 Materials and equipment

1. Hydrogen peroxide, 3% H_2O_2
2. Loop
3. Microscope slides
4. Suspect bacterial colonies on PW or PD3 media

4.2.7.2 Method

1. Put a sterile smear of cells onto a microscope slide
2. Add a drop of 3% H_2O_2
3. The release of bubbles indicate the bacteria is catalase positive.

4.2.8 PCR on bacterial colonies

4.2.8.1 Equipment

1. 0-2 mL, 2-20 mL, 20-200 mL, and 200-1000 mL pipettes and tips
2. 0.2 or 0.5 mL PCR tubes
3. 1.5 or 2 mL centrifuge tubes to store reagents
4. Bulb spinner or centrifuge
5. Freezer
6. Gel tanks, rigs and racks
7. Ice
8. Latex, and leather gloves
9. Microwave
10. Power pack
11. Thermocycler
12. UV transilluminator with camera
13. Bunsen burner
14. Centrifuge tubes
15. Kettle
16. Loop
17. Suspect bacterial colonies on PW or PD3 media

4.2.8.2 Reagents

Modified SCP	***For 500 ml***	***For 1000 ml***
Disodium succinate $C_4H_4Na_2O_7$	0.5 g	1 g
Trisodium citrate $C_6H_5Na_3O_7$	0.5 g	1 g
K_2HPO_4	0.75 g	1.5 g
KH_2PO_4	0.5 g	1 g
PVP40	25 g	50 g

Autoclave. Add ascorbic acid (0.02M) and adjust to pH 7 just prior to use. The stock buffer (without ascorbic acid) can be stored frozen (-20C) for up to 6 months.

PBS/BSA

a) 10X PBS	**For 1000 ml**
NaCl	80 g
KH_2PO_4	2 g
Na_2HPO_4	11.5 g
KCl	2 g

b) PBS/BSA
1x PBS plus 0.2% BSA. Store at 4^{o}C.

CTAB buffer + 0.2% mercaptoethanol	***For 100 ml***
1M Tris, pH 7.5 $H_2NC(CH_2OH)_3$	20 ml
5M NaCl	28 ml
500mM EDTA, pH 8.0 $[CH_2.N(CH_2.COOH).CH_2COON_9]_2.2H_2O$	4 ml
CTAB $C_{19}H_{42}NBr$	2 g
β-Mercaptoethanol	200 µl

Mix and make up to 100 ml with dH_2O. Store at room temperature.

Choloroform:isoamyl alcohol
24:1 mix of choloroform to isoamyl alcohol. Store at room temperature.

Isopropanol
100% isopropanol stored at 4^{o}C.

Ethanol
80 % ethanol. Store at room temperature.

Water
Sterile dH_2O.

4.2.8.3 Method

As per section 4.2.2, but rather than using plant DNA extracts as template, boiled preparations are used, which are a loopful of bacteria from a suspect bacterial colony boiled for 5 mins in 100µL of sterile dsH2O. If a suspect colony is found to be positive by PCR, sequencing of the PCR product must be done to confirm if it is *X. fastidiosa*.

4.2.8.4 PCR controls

(i) PCR *Xylella fastidiosa* DNA (positive control)
(ii) PCR H_20 (negative control)
(iii) rP1 and fD2 primers (template internal control)
To detect *Xylella fastisiosa*, three specific primers sets are used in conjunction with a generic set (which target the bacterial 16S rDNA gene). PCR primers and protocol as per previous section.

4.2.9 rDNA sequencing

4.2.9.1 Equipment

1. 0-2 µl, 2-20 µl, 20-200 µl, and 200-1000 µl pipettes and tips
2. 0.2 or 0.5 ml PCR tubes
3. 1.5 or 2 ml centrifuge tubes to store reagents
4. Bulb spinner or centrifuge
5. Freezer
6. Ice machine
7. Latex gloves
8. PC with internet access
9. Thermocycler
10. UV illuminator

4.2.9.2 Reagents

- QIAQuick PCR Purification Kit - Available from Qiagen, Catalogue Number 28104
- ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits **-** Available from Applied Biosystems www.appliedbiosystems.com
- Forward and Reverse primers
- Sterile dH_2O

4.2.9.3 Method

PCR products are cleaned using the QIAquick Spin kit (Qiagen) as per manufacturer's instructions. The cleaned PCR products are prepared for sequencing with ABI Big Dye (Roche), as per the manufacturer's instructions. Sequencing is outsourced. The raw sequences are compared against all sequences posted on the GenBank database using the program BlastN (Altschul *et al*., 1997), to determine if the sequence is *X. fastidiosa*, and which strain. Please note: GenBank data is not always reliable and should not be used as a diagnostic method alone.

Test name: *Xylella fastidiosa* (Pierce's disease) PCR - Tamaki

Test courtesy of Dr. Brett Alexander, Team Manager Mycology and Bacteriology, MAF Biosecurity New Zealand.

Nucleic acid extraction
DNA

Primer	**Name**	**Sequence (5'-3')**	**Size**	**Target**
Forward	**RST 31**	**GCG TTA ATT TTC GAA GTG ATT CGA**	**24 nt**	**Unique *Eco*R1 fragment**
Reverse	**RST 33**	**CAC CAT TCG TAT CCC GGT G**	**19 nt**	
Forward	**FD2**	**AGA GTT TGA TCA TGG CTC AG**	**20 nt**	**Unique 16S rDNA fragment**
Reverse	**RP1**	**ACG GTT ACC TTG TTA CGA CTT**	**21 nt**	

PCR Reagent mix - Tamaki

PCR Reagent mix*	**volume reaction (20µl)**	**Cycling parameters**
Sterile distilled H_2O	**10.8 µl**	**94°C 3 min x 1**
10X PCR buffer	**2.0 µl**	**94°C 45 sec**
50 mM $MgCl_2$	**0.6 µl**	**55°C 30 sec x 30-35**
10 mM dNTPs	**0.4 µl**	**72°C 30 sec**
5µM RST 31 primer	**1.0 µl**	**72°C 10 min x 1**
5µM RST 33 primer	**1.0 µl**	
5µM FD2 primer	**1.0 µl**	
5µM RP1 primer	**1.0 µl**	
***Taq* polymerase**	**0.2 µl**	
DNA template	**2.0 µl**	
*** Master mix for Invitrogen PCR reagents. If an alternative supplier is used reagent concentrations may need modification.**		

PCR controls	**Description**
Positive	***X. fastidiosa* DNA spiked citrus/hydrangea/grape extract.** ***X. fastidiosa* DNA . (Plasmid DNA - 1 x 10^{-4})**
Negative	**Healthy plant tissue.**
Reagent	**Water**

Electrophoresis	**Description**	**Buffer (choose one)**	**Predicted size amplicon (bp)**
% Agarose gel	**1.5-2%**	**1X TAE or 1X TBE**	***X. fastidiosa* = 733 bp**
MW marker	**100 bp**		**Internal Control = 1500bp**

Location of reagents
Primers: and Freezer E (Machinery room, Virology Laboratory, Tamaki)
DNA controls: Freezer 597 (PC2 Facility, Lincoln) and -80°C Freezer B (Machinery room, Virology laboratory, Tamaki). P07-F2 - P07-F4.
Reagents: Freezer 597 (PC2 Facility, Lincoln) and Freezer C (Machinery room, Virology Laboratory, Tamaki)

5 Suppliers

Agdia C/O TasAg ELISA and Pathogen Testing Service 13 St John's Ave New Town Tas 7008 Inquiries: Peter Cross Phone: (03) 6233 6845 Fax: (03) 6278 2716 Email: Peter.Cross@dpiwe.tas.gov.au	**Applied biosystems** Head Office (Melbourne) 52 Rocco Drive Scoresby VIC 3179 Melbourne Office Free call: 1800 033 747 Tel: (03) 9730 8600 Fax: (03) 9730 8799 Orders hotline: 1800 801 644 Orders fax: (03) 9730 8798 Email: abozsupport@appliedbiosystems.com abozorders@appliedbiosystems.com http://www.appliedbiosystems.com.au/index.asp
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Oxoid Australia Pty Ltd 104 Northern Road West Heidelberg Melbourne Victoria 3081 Tel: (03) 9458 1311 Fax: (03) 9458 4759 Email: info@oxoid.com.au http://www.oxoid.com/uk/index.asp?mpage=isubs	**Promega** ABN 84 003 696 151 PO Box 168 Annandale NSW 2038 Tel: (02) 9565 1100 Freecall: 1800 225 123 Fax: (02) 9550 4454 Freefax: 1800 626 017 http://www.promega.com/au/default.htm
QIAGEN Pty Ltd ABN 75 072 382 944 PO Box 25 Clifton Hill Victoria 3068 Orders: (03) 9489 3666 Fax: (03) 9489 3888 Technical: 1800 243 066 http://www.qiagen.com/	**Roche Diagnostics Australia Pty. Ltd.** 31 Victoria Avenue Castle Hill NSW 2154 Tel: (02) 9899 7999 Fax: (02) 9899 7893 http://www.tib-molbiol.de/oligos/AdressenKontakte/Comp_Address_ROCHE_e.htm
Sigma-Aldrich Pty Ltd Sydney, Australia Tel: (02) 9841 0555 Fax: (02) 9841 0500 Email: ausmail@sial.com http://www.sigmaaldrich.com/cgi-bin/hsrun/Distributed/HahtShop/HahtShop.htx;start=HS_FramesetMain	

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New Zealand http://www.biosecurity.govt.nz/about-us/structure/phel

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Appendix: Alternative *Xylella fastidiosa* hosts

Host Species	Common Name	References
*1. Acacia longifolia**	Sydney golden wattle	Freitag, 1951
2. Acer macrophyllum	big leaf maple	Purcell & Saunders, 1999
3. Acer negundo	box elder	McElrone *et al*., 1999
4. Acer sp.	maple	Sherald *et al*., 1987; Purcell & Saunders, 1999
5. Aesulus californica	Californian buckeye	Purcell & Saunders, 1999
*6. Ampelopsis arborea**	peppervine	Hopkins & Adlerz, 1988
7. Amsinckia douglasiana	buckthorn weed	Freitag, 1951
8. Artemisia vulgaris	mugwort	Hill & Purcell, 1995
*9. Artemisia vulgaris var. heterophylla**	Californian mugwort	Purcell & Saunders, 1999
*10. Avena fatula**	wild oat	Freitag, 1951
*11. Baccharis halimifolia**	eastern baccharis	Hopkins, 1988
*12. Baccharis pilularis**	coyote brush	Purcell &Saunders, 1999
13. Baccharis salicifolia	mule fat	Purcell &Saunders, 1999
14. Baccharis sp.	Eastern baccharis	Hopkins, 1989
15. Boerhavia diffusa		Hernandez & Ochoa, 1994
16. Bromus catharticus	rescue grass	Freitag, 1951
*17. Bromus rigidus**	ripgut grass	Freitag, 1951
18. Bromus sp.	Russian brome grass	Freitag, 1951
*19. Callicarpa americana**	American beautiberry	Hopkins, 1989
20. Callistephus chinensis	China aster	Freitag, 1951
21. Canna sp.	canna lily	Freitag, 1951
22. Castanea sp.	chestnut	Purcell & Saunders, 1999
23. Celastrus orbiculatus	oriental bittersweet	Purcell & Saunders, 1999
*24. Chenopodium ambrodioides**	Mexican tea	Freitag, 1951
*25. Conium maculatum**	poison hemlock	Purcell & Saunders, 1999
26. Coprosma baueri	maddock family	Freitag, 1951
27. Cornus florida	flowering dogwood	Purcell & Saunders, 1999
28. Cotoneaster rotundifolia var. lanata	cotoneaster	Freitag, 1951
*29. Cynodon dactylon**	Bermuda grass	Hill & Purcell, 1995
*30. Cyperus eragrostis**	nutgrass, tall umbrella plant, umbrella sedge	Purcell & Saunders, 1999; Raju *et al*., 1980; Hopkins, 1989
31. Cyperus esculentus	yellow nutgrass	Freitag, 1951
*32. Cytisus scoparius**	Scotch broom	Freitag, 1951

33. Daucus carota var. sativa	short white carrot	Freitag, 1951
*34. Digitaria sanguinalis**	hairy crabgrass	Freitag, 1951
*35. Duranta repens**	pigeon-berry	Freitag, 1951
*36. Echinochloa crusgalli**	water grass	Hill & Purcell, 1995
37. Epilobium californicum	willow herb	Freitag, 1951
38. Epilobium panniculatum	panicled willow herb	Freitag, 1951
39. Eragrostis diffusa	diffuse love grass	Freitag, 1951
40. Erodium cicutarium	red-stem filaree	Freitag, 1951
*41. Escallonia montevidensis**	saxifrage family	Raju *et al*., 1980
*42. Eugenia myrtifolia**	Australian bushberry	Freitag, 1951
43. Festuca megalura	foxtail fescue	Freitag, 1951
44. Fragaria californica	wild strawberry	Raju *et al*., 1980
*45. Fragaria vesca**	wild strawberry	Purcell & Saunders, 1999
46. Franseria acanthicarpa	annual burr weed	Freitag, 1951
*47. Fraxinus dipetala**	foothill ash	Freitag, 1951
48. Fraxinus latifolia	Oregan ash	Purcell and Saunders, 1999
*49. Fuschia magellanica**	Fuschia	Freitag, 1951
50. Genista monspessulanus	French broom	Purcell and Saunders, 1999
51. Godetia grandiflora	Godetia	Freitag, 1951
52. Hedera helix	ivy	Freitag, 1951
53. Heliotropium fruticosum		Hernandez & Ochoa, 1994
54. Heliotropium indicum		Hernandez & Ochoa, 1994
55. Holcus sudanensis	Sudan grass	Freitag, 1951
56. Hordeum murinum	common foxtail	Freitag, 1951
57. Hordeum vulgare	barley	Freitag, 1951
*58. Hydrangea panniculata**	hydrangea	Freitag, 1951
59. Ipomoea crassicaulis		Hernandez & Ochoa, 1994
60. Juglans nigra	black walnut	Purcell & Saunders, 1999
61. Lactuca scariola	prickly lettuce	Freitag, 1951
62. Lathyrus ciceria	pea family	Freitag, 1951
63. Lathyrus clymenium	pea family	Freitag, 1951
64. Lathyrus sativa	grass pea	Freitag, 1951
*65. Lolium multiflorum**	Italian ryegrass	Freitag, 1951
66. Lolium temulentum	darnel	Freitag, 1951
67. Lonicera japonica	Japanese honeysuckle	Freitag, 1951
*68. Marjorana hortensis**	sweet marjoram	Freitag, 1951
*69. Medicago hispida**	bur clover	Freitag, 1951
70. Meliotus alba	white meilot	Freitag, 1951
71. Meliotus alba var. annua	hubum clover	Freitag, 1951

72. Meliotus indica	annual yellow sweet clover	Freitag, 1951
73. Meliotus officinalis	yellow sweet clover	Freitag, 1951
*74. Meliotus sp.**	sweet clover	Freitag, 1951
*75. Melissa officinalis**	garden balm	Freitag, 1951
76. Mentha sp.	mint	Freitag, 1951
77. Merremia glabra		Hernandez & Ochoa, 1994
*78. Montia linearis**	miner's lettuce	Raju *et al*., 1980
79. Nicotiana tabaccum	tobacco	Lopes *et al*., 2000
80. Oenanthe sarmetosa	water parsley	Freitag, 1951
81. Oenothera hookeri	evening primrose	Freitag, 1951
*82. Parthenocissus quinquefolia**	Virginia creeper	Hopkins, 1988
*83. Parthenocissus tricuspidata**	ivy	Freitag, 1951
*84. Paspalum dilatum**	dallis grass	Hopkins, 1989
85. Passiflora foetida		Hernandez & Ochoa, 1994
86. Pelargonium hortorum	fish geranium	Freitag, 1951
87. Pennisetum clandestimum	ikuyu grass	Freitag, 1951
88. Phalaris minor	Mediterranean Canary grass	Freitag, 1951
89. Phalaris paradoxa	gnawed Canary grass	Freitag, 1951
90. Phleum pretense	timothy	Freitag, 1951
91. Photinia arbutifolia	oyon/christmas berry	Freitag, 1951
92. Pittosporum crassifolium	karo	Freitag, 1951
93. Plantago lanceolata	ribgrass	Purcell & Saunders, 1999
94. Platanus occidentalis	sycamore	Hartman *et al*., 1992
*95. Poa annua**	annual bluegrass	Freitag, 1951
96. Portulaca oleracea		Hernnandez & Ochoa, 1994
97. Ploygonum convolvulus	black bindweed	Freitag, 1951
*98. Polygonum persicaria**	lady's thumb	Freitag, 1951
99. Populus fremonti	freemont cottonwood	Purcell & Saunders, 1999
100.Prunus serotina	blackcherry	Purcell & Saunders, 1999
101.Reseda odorata	common mignonette	Freitag, 1951
102.Rhamnus californica	coffeeberry	Purcell & Saunders, 1999
103.Rheum rhaponicum	rhubarb	Freitag, 1951
*104.Rhus diversiloba**	poison oak	Purcell & Saunders, 1999
*105.Rhus sp.**	sumac	Hopkins, 1988
*106.Rosa californica**	Californian rose	Purcell & Saunders, 1999
*107.Rosemarinus officinalis**	rosemary	Freitag, 1951
108.Rubus discolor	Himalayan blackberry	Purcell & Saunders, 1999

*109.Rubus procerus**	blackberry	Hopkins, 1989
110.Rubus ursinus	California blackberry	Purcell & Saunders, 1999
*111.Rubus vitifolius**	Californian blackberry	Freitag, 1951
*112.Rumex crispus**	curly dock	Freitag, 1951
*113.Salix sp.**	willow	Freitag, 1951; Purcell & Saunders, 1999
*114.Sambucus caerulea**	blue elder	Freitag, 1951
*115.Sambucus canadensis**	American elder	Hopkins, 1989
116.Sambucus mexicana	blue elderberry	Purcell & Saunders, 1999
117.Setaria lutescens	yellow bristle grass	Freitag, 1951
*118.Solidago fistulosa**	goldenrod	Hopkins, 1989
119.Sonchus asper	prickly sawthistle	Freitag, 1951
*120.Sorghum halepense**	Johnson grass	Yonce & Chang, 1987
121.Symphoricarpos albus	snowberry	Purcell & Saunders, 1999
122.Syringa vulgaris	lilac	Freitag, 1951
123.Toxicodendron diversilobum	poison oak	Purcell & Saunders, 1999
124.Trifolium fragerum	Strawberry clover	Freitag, 1951
125.Trifolium hybridum	alsike clover	Freitag, 1951
126.Trifolium incarnatum	Crimson clover	Freitag, 1951
127.Trifolium pratense	red clover	Freitag, 1951
*128.Trifolium repens**	white clover	Freitag, 1951
*129.Trifolium repens var. latum**	Ladino clover	Freitag, 1951
130.Ulnus sp	elm	Sherald, 1993
131.Umbellularia californica	bay laurel	Purcell & Saunders, 1999
132.Urtica dioica	Stinging nettle	Purcell & Saunders, 1999
*133.Urtica gracilis**	creek nettle	Freitag, 1951
*134.Veronica sp.**	Speedwell	Freitag, 1951
135.Vicia monanthus	Vetch	Freitag, 1951
*136.Vinca major**	greater periwinkle	Purcell & Saunders, 1999
*137.Vinca minor**	lesser periwinkle	Purcell & Saunders, 1999
*138.Vitis californica**	wild grape	Freitag, 1951
*139.Vitis munsoniana**	wild grape	Hopkins, 1989
140.Vitis riparia	wild grape	Hopkins, 1989
141.Xanthium canadense	Cocklebur	Freitag, 1951

* natural host