National Diagnostic Protocol for Pierce’s Disease,

*Xylella fastidiosa*

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<tr>
<td>PROTOCOL NUMBER</td>
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</tr>
<tr>
<td>VERSION NUMBER</td>
<td>V1.1</td>
</tr>
<tr>
<td>PROTOCOL STATUS</td>
<td>Endorsed</td>
</tr>
<tr>
<td>ISSUE DATE</td>
<td>18 February 2010</td>
</tr>
<tr>
<td>REVIEW DATE</td>
<td>December 2012 (Under Review)</td>
</tr>
<tr>
<td>ISSUED BY</td>
<td>SPHDS</td>
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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/protocols/national-diagnostic-protocols/
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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 © Dr. Doug Cook UC Davis](image)

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

1.1.2 Secondary host range


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: Xylella 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 raising, 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](https://example.com/xylella-fastidiosa.png) © Dr. Doug Cook UC Davis.

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 Symptons 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).
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 Grapevine sample collection

3.1, 3.2 Symptom recognition

3.5.2 Tissue sampling for DNA extractions and bacterial isolations

4.2.4 Bacterial Isolation

4.2.8 PCR on suspect bacterial colonies

4.2.1 DNA extraction from grapevine

4.2.2 PCR detection using grapevine DNA extract

4.2.9 rDNA sequencing (by suitable laboratory)

Preliminary Identification (24 hours)

Confirmation of results (14-21 days)
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, H₂S, 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).
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°C
### 4.2.1.2 Reagents

#### Modified SCP

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<tr>
<th>Component</th>
<th>For 500 ml</th>
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<tr>
<td>Disodium succinate $C_4H_4Na_2O_7$ (Sigma S2378)</td>
<td>0.5 g</td>
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<tr>
<td>Trisodium citrate $C_6H_5Na_3O_7$ (Sigma S4641)</td>
<td>0.5 g</td>
<td>1 g</td>
</tr>
<tr>
<td>$K_2HPO_4$ (Ajax A2221-500g)</td>
<td>0.75 g</td>
<td>1.5 g</td>
</tr>
<tr>
<td>$KH_2PO_4$ (Ajax A391-500g)</td>
<td>0.5 g</td>
<td>1 g</td>
</tr>
<tr>
<td>PVP40 (Sigma PVP-40)</td>
<td>25 g</td>
<td>50 g</td>
</tr>
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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 (-20°C) 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**

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<td>$KH_2PO_4$</td>
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<tr>
<td>$Na_2HPO_4$ (Ajax 478 or 621)</td>
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<tr>
<td>KCl (Ajax 382-500g)</td>
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Autoclave. Store at room temperature.

**b) PBS/BSA**

1x PBS plus 0.2% BSA. Store at 4°C.

#### CTAB buffer + 0.2% mercaptoethanol

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<td>1M Tris, pH 7.5 $H_2NC(CH_2OH)_3$ (Amresco 0234)</td>
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<tr>
<td>5M NaCl</td>
<td>28 ml</td>
</tr>
<tr>
<td>500mM EDTA, pH 8.0 $[CH_2.N(CH_2.COOH).CH_2.COONa]_2.2H_2O$</td>
<td>4 ml</td>
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<tr>
<td>CTAB C$<em>{19}H</em>{42}NBr$ (Sigma H6269)</td>
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<tr>
<td>β-Mercaptoethanol (Sigma M3148)</td>
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Mix and make up to 100 ml with dH$_2$O. Store at room temperature.

#### Chloroform:isoamyl alcohol

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

#### Isopropanol

100% isopropanol stored at 4°C.

#### Ethanol

80% ethanol. Store at room temperature.

#### Water

Sterile dH$_2$O.

### 4.2.1.3 Method

1. Place CTAB buffer + 0.2% mercaptoethanol in 60°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 dH₂O

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

Primer Name

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<th>Target Gene</th>
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<td>XF6-R</td>
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<td>16S rDNA</td>
<td>Firrao &amp; Bazzi, 1994</td>
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<td>16S rDNA</td>
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<tr>
<td>RP1</td>
<td>ACGGTTACCTGTGTTACGACTT</td>
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<td>Weisburg et al., 1991</td>
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PCR Master Mix

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<th>Component</th>
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<td>Sterile dH₂O</td>
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<td>1 mM dNTPs</td>
<td>2.5 μl</td>
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<tr>
<td>10 x concentration buffer</td>
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<tr>
<td>25 mM MgCl₂</td>
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<td>RST31</td>
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<tr>
<td>RP1</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>FD2</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>DNA template, undiluted</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>RedHotTaq 5U/ml</td>
<td>0.15 μl</td>
</tr>
</tbody>
</table>

RedHot Taq (ABgene AB-0406/A). Kits with MgCl₂ 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

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris H$_2$NC(CH$_2$OH)$_3$</td>
<td>54.0 g</td>
</tr>
<tr>
<td>Boric acid H$_3$BO$_3$</td>
<td>27.5 g</td>
</tr>
<tr>
<td>0.5 M EDTA [CH$_2$.N(CH$_2$.COOH).CH$_2$.COON$_3$]$_2$.H$_2$.O</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl pH 8.0</td>
<td>50 mL</td>
</tr>
<tr>
<td>0.5M EDTA pH 8.0</td>
<td>20 mL</td>
</tr>
<tr>
<td>dH$_2$.O</td>
<td>30 mL</td>
</tr>
</tbody>
</table>

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$_2$.O to the negative control tube, 2 μl test template to each tube, and 2 μl grapevine DNA infected with *X. fastidiosa* 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. Electrophoreose 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).

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA molecular weight</td>
</tr>
<tr>
<td>2-16</td>
<td>Australian grapevine</td>
</tr>
<tr>
<td>14</td>
<td>negative control</td>
</tr>
<tr>
<td>15</td>
<td>positive control</td>
</tr>
</tbody>
</table>

**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 dH2O. Store at room temperature.

Hypochlorite
1% hypochlorite, diluted with dH2O. 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 sdH2O. Mix periodically to dissolve.

ii)  Dissolve 4 g L-glutamine in 100 mL sdH2O by heating on a hot plate at low heat. Do not let boil.

iii)  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)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/L</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone (Oxoid LP0042)</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Soytone (Difco 243620)</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Trisodium citrate (Sigma S4641)</td>
<td>1.0</td>
<td>3.9 mM</td>
</tr>
<tr>
<td>Disodium succinate (Sigma S2378)</td>
<td>1.0</td>
<td>3.7 mM</td>
</tr>
<tr>
<td>Hemin chloride (0.1% in 0.05N NaOH) (Aldrich H2250)</td>
<td>10 ml</td>
<td></td>
</tr>
<tr>
<td>Potato starch (soluble) (Mallinckrodt #8188)</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>MgSO(_4) (7)H(_2)O (Ajax 302)</td>
<td>1.0</td>
<td>4.06 mM</td>
</tr>
<tr>
<td>K(_2)HPO(_4) (Ajax A2221-500g)</td>
<td>1.5</td>
<td>8.6 mM</td>
</tr>
<tr>
<td>KH(_2)PO(_4) (Ajax A391-500g)</td>
<td>1.0</td>
<td>7.3 mM</td>
</tr>
<tr>
<td>Adjust the pH to 6.8, add agar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar (Oxoid LP0013)</td>
<td>15.0</td>
<td></td>
</tr>
</tbody>
</table>

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\(_2\)O
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.

![Xylella fastidiosa colonies on PW media (20 day old culture) © Jo Luck, DPI 2002.](image)
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**
d\textsubscript{2}O
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\textsubscript{2}O. Store at room temperature.

4.2.5.2 Method
1. Put a droplet of dH\textsubscript{2}O on a slide
2. Using a flamed loop transfer a small amount of the fresh suspect culture to the drop of dH\textsubscript{2}O. Mix the bacteria into the dH\textsubscript{2}O 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% H₂O₂
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% H₂O₂
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 $C_4H_8Na_2O_7$ | 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 (-20°C) for up to 6 months.

**PBS/BSA**

a) 10X PBS

For 1000 ml

| | 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°C.

**CTAB buffer + 0.2% mercaptoethanol**

For 100 ml

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

Mix and make up to 100 ml with dH2O. Store at room temperature.

**Choloroform:isoamyl alcohol**

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

**Isopropanol**

100% isopropanol stored at 4°C.

**Ethanol**

80 % ethanol. Store at room temperature.

**Water**

Sterile dH2O.

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 H2O (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
- Forward and Reverse primers
- Sterile dH₂O

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Name</th>
<th>Sequence (5'→3')</th>
<th>Size</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>RST 31</td>
<td>GCG TTA ATT TTC GAA GTG ATT CGA</td>
<td>24 nt</td>
<td>Unique EcoR1 fragment</td>
</tr>
<tr>
<td>Reverse</td>
<td>RST 33</td>
<td>CAC CAT TCG TAT CCC GGT G</td>
<td>19 nt</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>FD2</td>
<td>AGA GTT TGA TCA TGG CTC AG</td>
<td>20 nt</td>
<td>Unique 16S rDNA fragment</td>
</tr>
<tr>
<td>Reverse</td>
<td>RP1</td>
<td>ACG GTT ACC TTG TTA CGA CTT</td>
<td>21 nt</td>
<td></td>
</tr>
</tbody>
</table>

PCR Reagent mix - Tamaki

<table>
<thead>
<tr>
<th>PCR Reagent mix*</th>
<th>volume reaction (20µl)</th>
<th>Cycling parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled H₂O</td>
<td>10.8 µl</td>
<td>94°C 3 min x 1</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.0 µl</td>
<td>94°C 45 sec</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>0.6 µl</td>
<td>55°C 30 sec x 30-35</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.4 µl</td>
<td>72°C 30 sec</td>
</tr>
<tr>
<td>5µM RST 31 primer</td>
<td>1.0 µl</td>
<td>72°C 10 min x 1</td>
</tr>
<tr>
<td>5µM RST 33 primer</td>
<td>1.0 µl</td>
<td></td>
</tr>
<tr>
<td>5µM FD2 primer</td>
<td>1.0 µl</td>
<td></td>
</tr>
<tr>
<td>5µM RP1 primer</td>
<td>1.0 µl</td>
<td></td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.2 µl</td>
<td></td>
</tr>
<tr>
<td>DNA template</td>
<td>2.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

* Master mix for Invitrogen PCR reagents. If an alternative supplier is used reagent concentrations may need modification.

PCR controls

<table>
<thead>
<tr>
<th>Description</th>
<th>Buffer (choose one)</th>
<th>Predicted size amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>X. fastidiosa DNA spiked citrus/hydrangea/grape extract.</td>
<td>1X TAE or 1X TBE</td>
</tr>
<tr>
<td>X. fastidiosa DNA . (Plasmid DNA - 1 x 10⁻⁴)</td>
<td></td>
<td>X. fastidiosa = 733 bp</td>
</tr>
<tr>
<td>Negative</td>
<td>Healthy plant tissue.</td>
<td></td>
</tr>
<tr>
<td>Reagent</td>
<td>Water</td>
<td>MW marker = 100 bp</td>
</tr>
</tbody>
</table>

Electrophoresis

<table>
<thead>
<tr>
<th>% Agarose gel</th>
<th>Buffer (choose one)</th>
<th>Predicted size amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5-2%</td>
<td>1X TAE or 1X TBE</td>
<td>X. fastidiosa = 733 bp</td>
</tr>
</tbody>
</table>

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)
<table>
<thead>
<tr>
<th>Suppliers</th>
<th>Contact Information</th>
</tr>
</thead>
</table>
| 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  
| Crown scientific          | PO Box 2450  
Rowville  
Vic 3178  
Tel: (03) 9764 4722  
Toll Free: 1800 134 175  
Fax: (03) 9764 4733  
Email: crownvic@crownsci.com.au  
| Micromon                  | Monash University  
Microbiology Department  
PO Box 53  
Victoria 3800  
Tel: (03) 9905 4803  
Fax: (03) 9905 4811  
Email: oligo@med.monash.edu.au  
sequence@med.monash.edu.au  
http://www.med.monash.edu.au/microbiology/services |
| 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  
| 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 |
6 Contact points for further information

6.1 Australia
Jo Luck
Plant Pathologist Exotic Diseases
Institute for Horticultural Development
Department of Natural Resources and Environment
Private Mail Bag 15
Ferntree Gully Delivery Centre
Victoria
Phone: 03 9210 9222
jo.luck@nre.vic.gov.au

6.2 United States
Bruce Kirkpatrick-(Biology, genetics and detection of Xylella fastidiosa)
Plant Pathology
University of California
452 Hutchison Hall
Phone: (530) 752-2831
bckirkpatrick@ucdavis.edu

Donald Hopkins-Xylella fastidiosa
Central Florida Research and Education Centre
University of Florida
PO Box 111578 Gainesville FL 32611-1578
Phone: (352) 360-6686
dhop@gnv.ifas.ufl.edu

Alexander (Sandy) Purcell-Sharpshooters
Division of Insect Biology
University of California
Berkeley, California 94720-3112
Phone: purcell@nature.Berkeley.EDU

Matthew Blua (PD and GWSS, biology and ecology)
Entomologist
UC Riverside
Phone (909) 787-6301
matthew.blua@ucr.edu

Douglas Cook (vector/pathogen relations, plant genomics)
Plant Pathologist
UC Davis
Phone (530) 754-6561;
drcook@ucdavis.edu

7 Acknowledgements
The information in this document was sourced from PaDIL (www.padil.gov.au), Pierce’s Disease Draft Diagnostic Manual (Luck, J., Mann, R., Van Rijswijk, R., Moran, J. and Merriman, P. (2006)) and the Pierce’s Disease Pest Risk Review (2004). These documents were kindly provided by Office of the Chief Plant Protection Officer and Plant Health Australia.

Authors:
J. Luck, R. Mann, B. van Rijswijk, Jane Moran and Peter Merriman
8 References


Day and Fletcher, 1994 (from WA ag PDS no reference)


## Appendix: Alternative *Xylella fastidiosa* hosts

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Common Name</th>
<th>References</th>
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<tbody>
<tr>
<td>1. <em>Acacia longifolia</em></td>
<td>Sydney golden wattle</td>
<td>Freitag, 1951</td>
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<td>2. <em>Acer macrophyllum</em></td>
<td>big leaf maple</td>
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<td>3. <em>Acer negundo</em></td>
<td>box elder</td>
<td>McElrone <em>et al</em>., 1999; Sherald <em>et al</em>., 1987; Purcell &amp; Saunders, 1999</td>
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<td>4. <em>Acer sp.</em></td>
<td>maple</td>
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<td>5. <em>Aesculus californica</em></td>
<td>Californian buckeye</td>
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<td>7. <em>Amsinckia douglasiana</em></td>
<td>buckthorn weed</td>
<td>Freitag, 1951</td>
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<td>8. <em>Artemisia vulgaris</em></td>
<td>mugwort</td>
<td>Hill &amp; Purcell, 1995</td>
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<td>9. <em>Artemisia vulgaris var. heterophylla</em></td>
<td>Californian mugwort</td>
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<td>10. <em>Avena fatula</em></td>
<td>wild oat</td>
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<td>11. <em>Baccharis halimifolia</em></td>
<td>eastern baccharis</td>
<td>Hopkins, 1988</td>
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<td>12. <em>Baccharis pilularis</em></td>
<td>coyote brush</td>
<td>Purcell &amp; Saunders, 1999</td>
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<td>13. <em>Baccharis salicifolia</em></td>
<td>mule fat</td>
<td>Purcell &amp; Saunders, 1999</td>
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<td>16. <em>Bromus catharticus</em></td>
<td>rescue grass</td>
<td>Freitag, 1951</td>
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<td>17. <em>Bromus rigidus</em></td>
<td>ripgut grass</td>
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<td>18. <em>Bromus sp.</em></td>
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<td>20. <em>Callistephus chinensis</em></td>
<td>China aster</td>
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<td>21. <em>Canna sp.</em></td>
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<td>Freitag, 1951</td>
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<td>22. <em>Castanea sp.</em></td>
<td>chestnut</td>
<td>Purcell &amp; Saunders, 1999</td>
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<td>23. <em>Celastrus orbiculatus</em></td>
<td>oriental bittersweet</td>
<td>Purcell &amp; Saunders, 1999</td>
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<td>24. <em>Chenopodium ambrodioides</em></td>
<td>Mexican tea</td>
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<td>25. <em>Conium maculatum</em></td>
<td>poison hemlock</td>
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<td>26. <em>Coprosma baueri</em></td>
<td>maddock family</td>
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<td>27. <em>Cornus florida</em></td>
<td>flowering dogwood</td>
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<td>28. <em>Cotoneaster rotundifolia var. lanata</em></td>
<td>cotoneaster</td>
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<td>29. <em>Cynodon dactylon</em></td>
<td>Bermuda grass</td>
<td>Hill &amp; Purcell, 1995</td>
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<td>30. <em>Cyperus eragrostis</em></td>
<td>nutgrass, tall umbrella plant, umbrella sedge</td>
<td>Purcell &amp; Saunders, 1999; Raju <em>et al</em>., 1980; Hopkins, 1989</td>
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<td>31. <em>Cyperus esculentus</em></td>
<td>yellow nutgrass</td>
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<td>32. <em>Cytisus scoparius</em></td>
<td>Scotch broom</td>
<td>Freitag, 1951</td>
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</tbody>
</table>
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. *Melilotus alba* white meliot Freitag, 1951
71. *Melilotus alba var. annua* hubum clover Freitag, 1951
72. *Melilotus indica*  annual yellow sweet clover  Freitag, 1951
73. *Melilotus officinalis*  yellow sweet clover  Freitag, 1951
74. *Melilotus sp.*  sweet clover  Freitag, 1951
75. *Melissa officinalis*  garden balm  Freitag, 1951
76. *Mentha sp.*  mint  Freitag, 1951
77. *Merremia glabra*  Freitag, 1951
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*  Hernandez & Ochoa, 1994
97. *Polygonum convolvulus*  black bindweed  Freitag, 1951
98. *Polygonum persicaria*  lady's thumb  Freitag, 1951
99. *Populus fremonti*  fremont cottonwood  Purcell & Saunders, 1999
100. *Prunus serotina*  blackcherry  Purcell & Saunders, 1999
101. *Reseda odorata*  common mignonette  Freitag, 1951
102. *Rhamnus californica*  cofeeberry  Purcell & Saunders, 1999
103. *Rheum raphanicum*  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
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<td>Vicia monanthus</td>
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* natural host