Subcommittee on Plant Health Diagnostics

National Diagnostic Protocol

Xylella species

NDP 6 V2

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Purpose

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

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

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs, unless it is shown that the NDP has improved procedures for Australian conditions. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

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

Process

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

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/work/subcommittee-on-plant-health-diagnostics/

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

Document status

This version of the National Diagnostic Protocol (NDP) for *Xylella* species is current as at the date contained in the version control box below.

PEST STATUS	Not present in Australia
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Further information

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

Endorsement and publication details

This National Diagnostic Protocol was endorsed by the Subcommittee on Plant Health Diagnostics in 2010. The initially endorsed version of the NDP covered *Xylella fastidiosa* only and did not include additional species and subspecies that are now identified around the world.

Review and update: First approved in 2010. Revisions approved in 2025.

Specific notes on the revision: The NDP has been revised to include updated diagnostic methodologies to cover a broader range of species and sub-species. The NDP has been reviewed and verified in Australia and New Zealand, except for the methods mentioned in the NDP for which the validation status (where applicable) has been provided.

Contents

1 INTRODUCTION

Xylella spp. are listed as Australia's number 1 National Priority Plant Pest (NPPP). *Xylella* spp. are Gram-negative fastidious bacterium that are xylem-limited and lives and replicates in both plant and insect hosts. Currently, there are two species of *Xylella*: *Xylella fastidiosa* and *X. taiwanensis*. *Xylella fastidiosa* is highly destructive to many important horticultural crops in Europe and America. It infects more than 700 plant species (https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7356), some of which are asymptomatic hosts. Symptomatic hosts have a reduced life span and reduced productivity. To date, *X. taiwanensis* has only been detected in Taiwan and is only known to cause disease in the Asian pear, *Pyrus pyrifolia*.

Currently, *X. fastidiosa* has three subspecies: *X. fastidiosa* subsp. *pauca* (clade I), *X. fastidiosa* subsp. *multiplex* (clade II) and *X. fastidiosa* subsp. *fastidiosa* (clade III, which also contains the unverified subspecies *X. fastidiosa* subsp. *morus* and *X. fastidiosa* subsp. *sandyi,* both now known as subsp. *fastidiosa sensu lato*) (Uceda-Campos et al., 2022). The subspecies vary in their host range, with subspecies *fastidiosa* having the greatest host range. The *X. fastidiosa* subspecies are further classified into sequence types (ST) through multilocus sequence typing (MLST) (Yuan et al., 2010) and there are more than 80 different STs across the three different subspecies (EPPO, 2018). ST can be used to determine whether there is a single strain or multiple strains involved in an infection.

Xylella is only known to be transmitted through xylem sap-feeding insects, of which the full range is not known, and tissue grafting. The bacterium can travel in both directions in the xylem and can form biofilms that block the transport of mineral nutrients and water, resulting in the slow death of the plant host.

The European and Mediterranean Plant Protection Organization (EPPO) and International Plant Protection Convention (IPPC) have developed protocols for *X. fastidiosa* (EPPO, 2019; IPPC, 2018) but do not incorporate the most recently developed new assays. While *X. fastidiosa* can be identified to subspecies and then ST, there is no specific diagnostic for *X. taiwanensis*. Identification must be done by 16S sequencing, MLST, or genome sequencing.

Molecular assays are essential for rapid *Xylella* identification; however, isolation should be attempted to confirm its presence and support the molecular diagnosis. Bacterial isolation enables the fulfillment of Koch's postulates to confirm the causal agent, and the isolated bacteria can be used in plant inoculation assays to determine the host range of new STs. *Xylella* spp. are slow growing (4-6 weeks) and the different *X. fastidiosa* subspecies have variable growth rates on different bacteriological media. The use of live positive controls is required for comparison, to facilitate identification and recognition of different species and subspecies. Isolation of *Xylella* spp. is possible only from fresh infected material (generally needs to be within 3 days of collection) that has a high enough bacterial titre. *Xylella fastidiosa* cannot always be isolated, particularly when in low titre and isolation is not recommended in asymptomatic hosts, therefore the molecular assays are the primary tools for detection and identification.

1.1 Host range

Primary hosts of *X. fastidiosa* include: *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).

A full list is available from the European Food Safety Authority.

(https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.2022.7356)

The primary hosts of *X. taiwanensis* is *P. pyrifolia* (Asian Pear).

Images and further information can be found at:

https://www.eppo.int/ACTIVITIES/plant_quarantine/shortnotes_qps/shortnotes_xylella

https://onlinelibrary.wiley.com/doi/full/10.1111/epp.12575

1.2 Insect Vectors

All sucking insects that feed on xylem sap are potential vectors for *X. fastidiosa*. Sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadellinae) were the first known vectors; however, the vector species list is increasing with the global spread of *Xylella* with Hemiptera Auchenorryncha species belonging to the superfamily Cercopoidea (froghoppers and spittlebugs) now recognised as a major vector of concern (Cornara et al., 2016). A separate NDP is currently under development on *Xylella* vectors, which can be referred to for further information on insect vectors in the future.

https://www.eppo.int/ACTIVITIES/plant_quarantine/shortnotes_qps/shortnotes_xylella

https://gd.eppo.int/download/standard/765/pm7-141-1-en.pdf

1.3 Modes of Transmission

Xylella fastidiosa can also be transmitted and spread by graft transmission. Propagative material is the pathway by which *X. fastidiosa* can spread (Smith et al., 1997). *Xylella fastidiosa* is not known to be transmitted by mechanical means such as contaminated pruning shears (Smith et al., 1997; Varela, 2000), however, seed transmission of *X. fastidiosa* is now under the spotlight with a recent publication claiming transmission in pecan seeds via detection with qPCR (Cervantes, 2022).

2 TAXONOMIC INFORMATION

Xylella fastidiosa:

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).

Subspecies: Recent studies have consolidated *X. fastidiosa* into three subspecies: *X. fastidiosa* subsp. *fastidiosa, X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *pauca.* It should be noted that within the *X. fastidiosa* subsp. *fastidiosa* group is *Xylella fastidiosa* subsp *fastidiosa sensu largo,* which contains the previous subspecies *sandyi* and *morus*.

Xylella taiwanensis:

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Xanthomonadales
Family: Xanthomonadaceae
Genus: *Xylella*
Species: *Xylella taiwanensis*

Scientific Name: *Xylella taiwanensis* (Su et al., 2016)

Common Names: Pear leaf scorch disease

3 DETECTION

Xylella fastidiosa is mostly confined to the xylem tissue of its host. The bacterium proliferates in the xylem of the infected host and systemically spreads to the shoots and root system. However, the detection of *Xylella* can be confounded by the presence of asymptomatic hosts and symptomatic hosts that show symptoms commonly associated with biotic and abiotic factors.

Another relevant point to consider is the latency and asymptomatic incubation periods, which can vary depending on the host species. Some examples are included in Table 1.

Table 1: Latency period of *Xylella* in infected hosts

Species	***Host***	***Latency***
X. fastidiosa subsp. *fastidiosa*	*Vitis vinifera*	2-3 months
X. fastidiosa subsp. *multiplex*	*Polygala myrtifolia*	3-4 months
X. fastidiosa subsp. *pauca*	*Polygala myrtifolia*	6-9 months
X. fastidiosa subsp. *pauca*	*Citrus* spp.	8-12 months
X. fastidiosa subsp. *pauca*	*Olea europaea*	9-14 months

The symptoms caused by *Xylella* infection vary between species and subspecies and the host infected. The IPPC protocol ISPM27 in Section 3.1 describes the symptoms of Pierce's disease of grapevines, Citrus variegated chlorosis, coffee leaf scorch, olive leaf scorching and quick decline, almond leaf scorch disease, bacterial leaf scorch of shade trees, bacterial leaf scorch of blueberry, Phony peach disease and plum scald, Alfalfa dwarf and recorded symptoms in other hosts. Pictures of symptoms on various hosts can be found at

https://gd.eppo.int/taxon/XYLEFA/photos.

Figure 1: Symptoms of *Xylella fastidiosa* on olives. Photos by Toni Chapman.

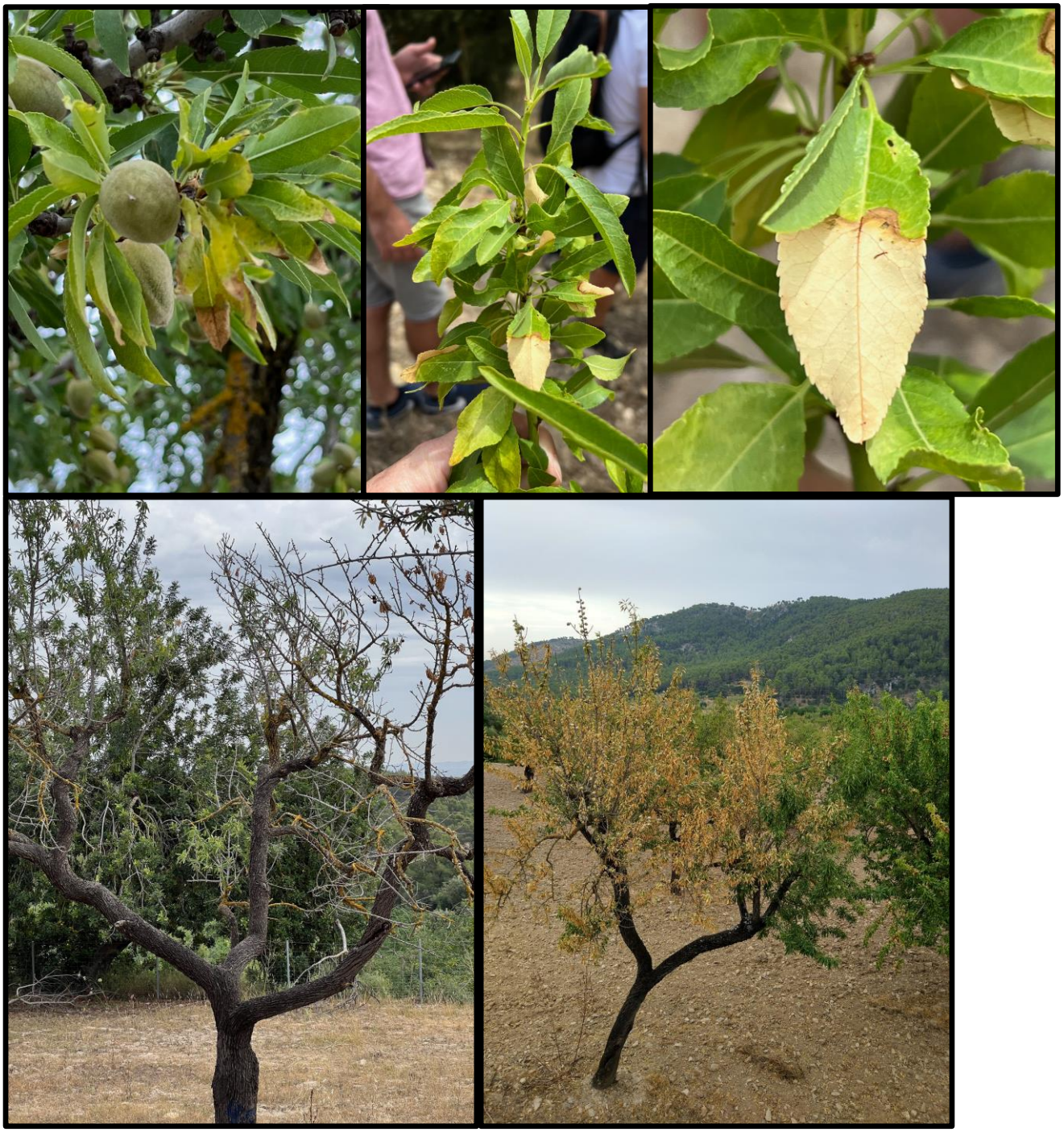

Figure 2: Symptoms of *Xylella fastidiosa* on almonds – Golden Death. Photos by Toni Chapman.

Figure 3: Symptoms of *Xylella fastidiosa* on grapevine: Chardonnay (left), Merlot (right)(Courtesy of Agriculture Service, Government of the Balearic Islands)

Figure 4: Symptoms of *Xylella fastidiosa* on grapevine, match sticks (left), affected grapes (middle), green symptoms on the cane (right)(Photos courtesy of Agriculture Service, Government of the Balearic Islands)

Figure 5: Symptoms of *Xylella fastidiosa* on *Polygala myrtifolia*. Photos by Toni Chapman.

Figure 6: Pear leaf scorch caused by *Xylella taiwanensis*. Photos by Chih-Horng Kuo

4 IDENTIFICATION

Identification of *Xylella* spp. can be obtained by two methods: culturing the bacterium on selective media or PCR (polymerase chain reaction). For confirmation, two methods of identification are needed. However, because the time required for isolation can take 4-6 weeks, confirmation for the presence of *Xylella* spp. can be achieved with two molecular methods.

4.1 Diagnostic flow chart

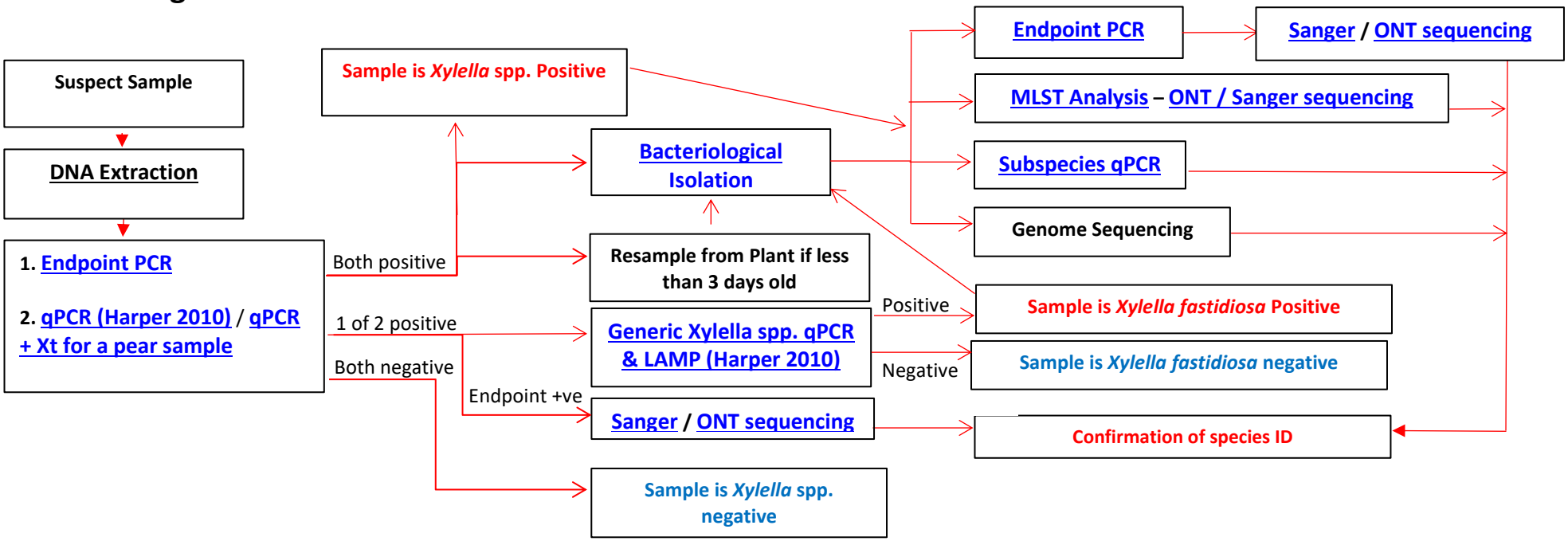

Figure 7: Diagnostic flow chart for the detection of *Xylella* spp.

4.2 Sampling Procedures

4.2.1 Sample collection for the detection of Xylella spp.

The distribution of *Xylella* spp. in a plant sample can vary. Spring (preferably mid), early summer and autumn are the best time to sample for *Xylella* infection when the plant is actively growing, and when the temperature is not too hot or cold. However, something to take into consideration is Australia's harsh summer conditions can result in similar symptomology of *Xylella* infection, making true symptoms hard to find. As *Xylella* cannot survive low temperatures and extreme highs, the bacteria retreats to the roots and lower extremities of the plant, making the collection of positive material harder in summer and winter. The samples should consist of symptomatic material preferentially actively growing. The stems sampled should not be the newest growth part of the plant as the bacterial load would be too low for sampling. It is essential the stem material is green and still viable. Testing has been successful in dormant hosts that were known positives, so this material can be used if necessary. However, extensive testing has not been completed at this point in time. The sample should consist of stems that have mature symptomatic leaves with petioles and woody twigs. Asymptomatic tissue sampling is not recommended given the uneven distribution of the pathogen in a host. Figure 8 depicts the type of material required for testing. It is recommended to collect three subsamples from the same host for testing. Note that the testing of stems is always the preferred sample type for both isolation of the bacteria and molecular assays. However, petioles of symptomatic *Vitis* spp. and *Citrus* spp. can be tested.

Figure 8: Example of the type of material required for testing. Photo by Toni Chapman.

Table 2: Number of leaves (including their petioles) or other plant material to be used and approximate weight of the laboratory sample. EPPO data (2018b) when testing individual plants. This table is taken from the IPPC protocol for guidance on sample numbers.

Type of sample	Host plants and type of tissue	Minimum number of leaves per laboratory sample	Approximate Weight of Laboratory Sample
Sample from an individual plant with leaves	Petioles of large-sized leaves (e.g. from *Vitis* spp., *Citrus* spp.) Noting this is only recommended with symptomatic hosts and stems are preferred.	5	0.5–1 g
	All plant species use stem material only	n/a	0.5–1 g
Dormant Plant or Cuttings	Xylem tissue *	n/a	0.5–1 g
Other Cuttings	stems	n/a	1 g

n/a, not applicable.

* The superficial bark should be removed and scraped from the active tissues (youngest external ring). Noting removal of bark should only be done in the testing laboratory.

- Note: Wrap the samples in dry paper towel or newspaper and place them inside a sealed plastic bag. It is important to shake the sample before bagging it to avoid spreading potential vectors of *Xylella* spp..

Send sample(s) to a diagnostic laboratory in your state immediately after the material is collected.

Please note: To isolate *Xylella* spp., symptomatic material, should be received in the laboratory within 3 days of collection and shipped at temperature not exceeding 35 °C. Do not freeze or refrigerate samples, room temperature is preferred.

NB. Negative test results do not necessarily mean that *Xylella* spp. is absent, as the bacteria can be unevenly distributed throughout the plant, or the titre may be very low, therefore it is important to sample symptomatic material.

4.2.2 Tissue Sampling for DNA Extractions and Bacterial Isolations

The optimal tissue to sample for the detection of *Xylella* spp. is the stem (Figure 8 as an example). The stem refers to:

- Preferentially a diameter around the size of a pencil, a bit larger or smaller is fine.
- Not the newest growth part of the plant, but the material still needs to be green
- Have mature symptomatic leaves with petioles and woody twigs
- Sections that contain both the dead and live stem and leaves is preferential

- Collected from the top third or quarter of the tree

If the sample is highly suspect, it is recommended to take tissue from three collection points from each host plant. *Xylella* spp. are typically in abundance in the top third or quarter of the tree. It is not recommended to sample asymptotic material based on overseas experience and given the variability in the titre. If Australia gets a positive detection this recommendation would change in delimitation surveys. Table 2 derives from the IPPC protocol for guidance on sampling.

4.3 Bacterial Isolation

Specialised media have been developed for isolating *Xylella* spp. for morphological diagnosis. Ideally, stems from symptomatic plants should be used for isolating *Xylella* spp. The stems need to be green, but not actively growing. Three media can be used for *Xylella* spp. growth: BCYE (Buffered Charcoal Yeast Extract), PWG (Periwinkle Media) and PD2 (Pierce's Disease Media). It is recommended to use all three media in the isolation process. However, if only one is possible the preference is for BCYE.

The optimal temperature for growth is 26-28 °C. When making media, care should be taken to use the nominated product brand to ensure optimal growth and consistent results. Note this media while enriched for *Xylella* growth, numerous other bacteria and fungi are capable of growth on them. Hence it is essential that aseptic techniques are used throughout the isolation process.

4.3.1 Isolation of Xylella spp. from infected plant material – Stem method

The stem method should be used to isolate *Xylella* spp. from olives. The method can be used with other woody plant species; however, the Xylem method (4.3.2) is the preferred method for other plant hosts.

1. Trim a 10 cm section of stem, wash with soap and water to remove any debris, dry with paper towel to remove any residual debris.
2. Sterilise in 10% bleach for 2 minutes (mins), transfer to 70% (v/v) ethanol for 2 mins x 2 washes, transfer to sterile water for 2 mins. Allow to air dry on a petri dish.
3. Cut the stem into two pieces, then use sterile pliers to squeeze the stem to force droplets of sap and bacteria from the cut end. Blot the stem on the BCYE media (Figure 9). Use one half of the stem to depress on half the agar plate, then discard and use the remaining stem piece to depress on the other half of the plate. Seal the plate with parafilm.
4. Incubate the plate at 28 ˚C for up to 4 weeks. Check the plate after 7 days under a stereomicroscope. If small colonies are present aseptically transfer the colony to a new BCYE plate for further incubation. If the plate is showing signs of non-*Xylella* growth, monitor plates for the full 4 weeks providing other bacteria do not overgrow the plate.

Figure 9: Demonstrating the cabinet setup with the sterilisation process and depressing the stem into the BCYE plate (left)., BCYE plate with 1-2 weeks of *Xylella* spp. growth (right) (Photos by Toni Chapman).

4.3.2 Isolation of Xylella spp. from infected plant material – Xylem method

Isolation from xylem tissue is the preferred methods for hosts other than olive, including for preparation of DNA extraction for all hosts.

1. Trim a 10 cm section of stem, wash with soap and water to remove any debris, dry with paper towel to remove any residual debris.
2. Sterilise in 10% bleach for 2 mins, transfer to 70% (v/v) ethanol for 2 mins x 2 washes, transfer to sterile water for 2 mins. Allow to air dry on a petri dish.
3. Debark the stem (Figure 10) then use a scalpel to remove xylem tissue.
4. Transfer 0.5-1 g of the xylem tissue to a homogeniser bag and break up with a mallet (Figure 11). Note the tissue can be sonicated at this point, this can release the bacteria and allow for easier isolation.
5. Add 3 mL of sterile water to the homogeniser bag, then homogenise with a Homex grinder or similar until the tissue is broken down.
6. Transfer 50 μl of macerate solution in triplicate onto each of the three types of agar plates. Tilt the plate to allow the drops to smear down the plate (Figure 12).
7. Incubate the plate at 28 ˚C for up to 4 weeks. Check the plate after 7 days under a stereomicroscope. If small colonies are present aseptically transfer single colonies to a new BCYE plate for further incubation. If the plate is showing signs of non-*Xylella* growth monitor plates for the full 4 weeks providing other bacteria do not overgrow the plate.

Figure 10: Left demonstrating debarking of almond stem, middle stem pieces for debarking, right collecting of xylem tissue (Photos by Toni Chapman).

Figure 11: Using a mallet to break down the xylem tissue, xylem tissue after using the mallet, using a homex grinder for homogenisation of the tissue in sterile water, tissue after maceration (Photos by Toni Chapman).

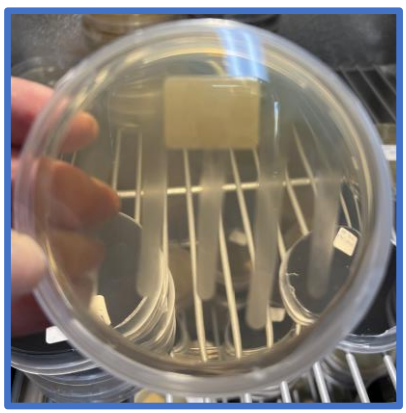

Figure 12: Agar plate showing the smearing of the *Xylella* spp. culture with four replicates (Photo by Toni Chapman).

The morphological and biochemical characteristics of *Xylella* spp. are as follows (Davis et al., 1978): Single flagellate straight rod, 0.25-0.35 x 0.9-3.5 µm, with filamentous strands under some cultural conditions (Figure 13). Colonies are of two types: convex to pulvinate smooth opalescent with entire margins and umbonate rough with finely undulated margins. Cells stain Gram-negative, nonmotile, oxidase negative and catalase positive. Strictly aerobic, non-fermentative, non-halophilic, and non-pigmented. Nutritionally fastidious, requiring a specialised medium. Hydrolyses gelatin and uses 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 optimal temperature for growth is 28 °C. The optimal pH is 6.5-6.9.

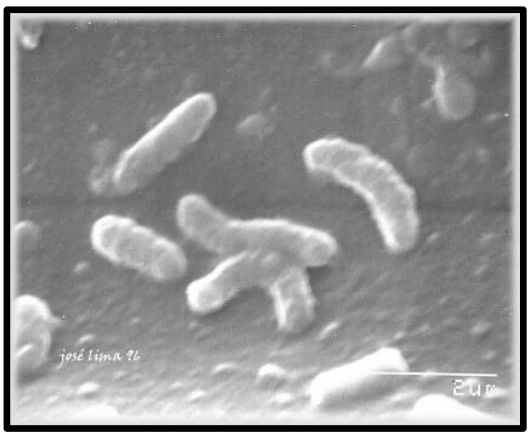

Figure 13: *Xylella fastidiosa* © Jose Lima (1996) Citrolima, Brazil

4.3.3 Bacteriological media for Xylella spp. *isolation*

Buffered Charcoal Yeast Extract (BCYE) media (ISPM 27 DP25; Wells et al., 1981; Table 3; Figure 14)

Table 3: BCYE Reagents

Reagents	**Per Litre**
ACES* buffer	10.0 g
Yeast extract	10.0 g
Activated charcoal	2.0 g
L-cysteine hydrochloride-1-hydrate	0.4 g
Ferric pyrophosphate	0.25 g
Phytagel® (Sigma P8169)	15.0 g
Distilled Water	1000 mL

*ACES - N-(2-Acetamido)-2-aminoethanesulfonic acid

Hydrate ACES buffer with 500 mL of distilled water at 50 °C. Mix buffer with approximately 40 mL of 1M KOH in 440 mL of distilled water and adjust pH to 6.9. The above solution is used to hydrate charcoal, yeast extract, and Phytagel®. Sterilise at 121 °C for 15 min. Cool medium to 60 °C (Phytagel® sets at 60-65 °C). Dissolve cysteine hydrochloride in 10 mL of distilled water. Filter sterilise through a 0.22 µm pore disc filter. Dissolve ferric pyrophosphate in 10 mL of distilled water and filter-sterilise as above. Add filter-sterilised ingredients to a cooled sterile medium.

Note: Phytagel® is a substitute for agar which can inhibit *Xylella* growth. Phytagel® cannot be microwaved or reheated and will set at 60 °C. The media must be poured immediately.

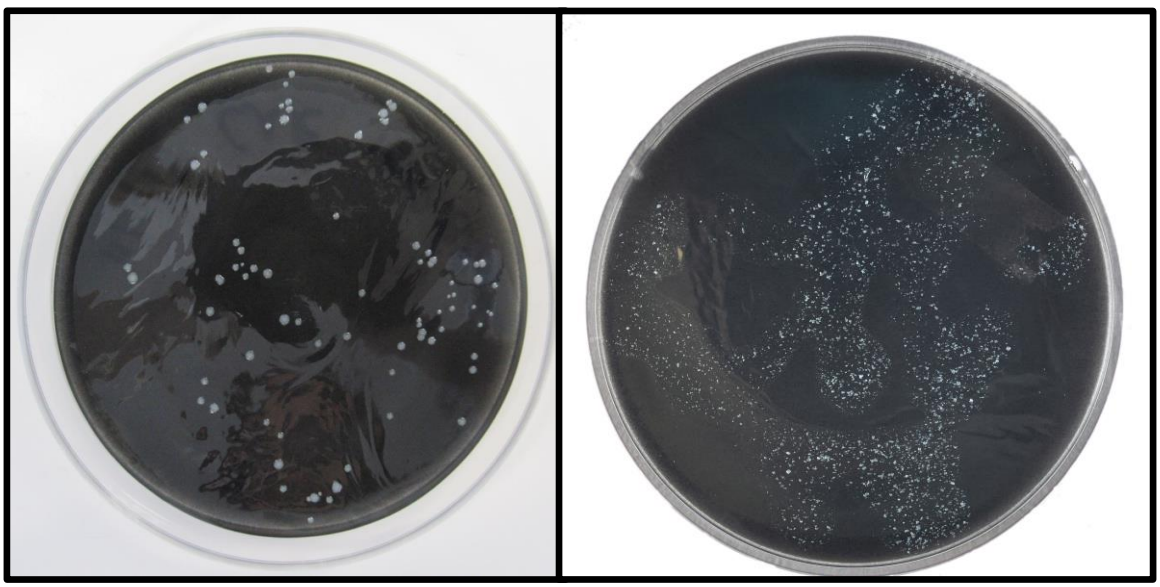

Figure 14: *Xylella fastidiosa* on BCYE media (Photos by Luciano Rigano).

PD2 Medium (Taken from IPPC 27 DP25; Table 4) (This method has not been validated in Australian laboratories due to the lack of access to live isolates. However, it is used globally for *Xylella* spp. isolation)**.**

Table 4: Pierces Disease (PD2) medium

Reagents	**Per Litre**
Phytone peptone (BD BBL)	2.0 g
Bacto tryptone (Oxoid)	4.0 g
Trisodium citrate	1.0 g
Disodium succinate	1.0 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH)	10 mL
BSA (20% w/v) (Sigma)	10 mL
$MgSO_4 \cdot 7H_2O$	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Bacto agar (e.g. BD Difco)	15.0 g
Distilled water	1000 mL

All components, except BSA (bovine serum albumin) and hemin chloride stock solution, are added to 980 mL of distilled water before autoclaving. The pH is adjusted to 7.0 after dissolving the agar. After autoclaving, BSA (dissolved in distilled water) and the hemin chloride stock solution are filter sterilised (0.2 µm membrane) and added to the sterile basal medium cooled (45-50 °C).

Modified PWG medium (Taken from IPPC 27; Table 5; Figure 15) (This method has not been validated in Australian laboratories due to the lack of access to live isolates. However, it is used globally for *Xylella* spp. isolation).

Table 5: Modified PWG Medium (based on Hill and Purcell, 1995) and information provided in EPPO (2018b)

Reagents	**Per Litre**
Gelrite gellan gum (Sigma)	9.0 g
Phytone peptone (e.g. BD BBL)	4.0 g
Bacto tryptone (e.g. Oxoid)	1.0 g
Phenol red stock solution (0.2%)	10 mL
L-glutamine (Sigma)[1]	4 g
Hemin chloride stock solution (0.1% in 0.05 N NaOH)	10 mL
BSA (Sigma)[1]	3.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
K_2HPO_4	1.5 g
KH_2PO_4	1.0 g
Distilled water	1000 mL

All constituents except L-glutamine, hemin chloride stock solution, and BSA are added before autoclaving. BSA (3 g) is dissolved in 15 mL of distilled water, and 4 g of L-glutamine is dissolved in 50 mL of distilled water over low heat (50 °C). The hemin chloride stock is 0.1% bovine hemin chloride dissolved in 0.05 N NaOH. These three solutions are sterilised by filter sterilisation (0.2 µm membrane) and added to the cooled sterile basal medium.

Figure 15: *Xyllela fastidiosa* in PWG medium (Photo by Luciano Rigano).

PD3 medium for isolation of bacteria from Grapevine's petioles (Xff), USA method (Table 6)

Table 6: Pierces Disease (PD3) medium

Reagents	**Per Litre**
Bacto Soytone - BD Biosciences (Cat# 243620)	2.0 g
Bacto Tryptone - BD Biosciences (Cat# 211705)	4.0 g
Potato Starch - Sigma Aldrich (Cat# 52004-500G)	2.0 g
Potassium phosphate monobasic - EMD (Cat# 1562-5)	1.5 g
Potassium phosphate dibasic - Fisher Scientific (Cat# P288-500)	1.0 g
Magnesium sulfate heptahydrate - Sigma Aldrich (Cat# 230391-500G)	1.0 g
Sodium succinate hexahydrate - Fisher Scientific (Cat# S413-500)	1.0
Sodium citrate dihydrate - Calbiochem (Cat# 7810)	1.0
Hemin - Calbiochem (Cat# 3741-5GM) ** Solution made by dissolving Hemin in 0.05 N NaOH **	10 mL
Difco Granulated Agar - BD Biosciences (Cat# 214530)	15 g
Distilled water	1000 mL

Add all ingredients to the water, autoclave and pour plates. Store at 4 °C.

4.4 Molecular Methods

The molecular methods described in this protocol have been tested in five different diagnostic laboratories with different reagents and equipment. Diagnostic PCRs for *Xylella* spp. within this protocol are designed so that results can be obtained within 24 hours from plant extraction. These tests can be used on boiled preparations from bacterial colonies, bacterial DNA extracts, plant tissue DNA extracts, and insect tissue DNA extracts. The molecular procedure should be carried out as per the diagnostic flow chart (Figure 7). The generic *Xylella* endpoint PCR and Harper qPCR (Xf/Xt qPCR for pear samples) should be run as the primary screening assays. The likelihood of a false positive result occurring is low with these two assays, 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 can be overcome by running controls such as 16S rDNA, 18S rDNA or COI.

Note all PCR and qPCR assays should be run with a positive and negative control.

- The negative control should be sterilised water in place of DNA.
- Positive controls should include *Xylella fastidiosa* (any subspecies) and *Xylella taiwanensis* DNA samples.
- DNA can be requested from the NSW Department of Primary Industries and Regional Development (NSW, DPIRD), Plant Health Diagnostic Service or Microbial Services, Pests and Diseases, Agriculture Victoria Research Department of Energy, Environment and Climate Action.
- It is recommended that each sample be run in triplicate in each of the primary molecular assays.

4.4.1 DNA extraction

Several DNA extraction protocols can be used for bacterial isolates, plant tissue, and insect tissue. The following are the extraction protocols that were used in the testing of the molecular assays presented in this NDP.

Bacterial culture

1. Add 1 mL of sterile buffered phosphate saline solution (PBS) to a microcentrifuge tube.
2. Using a sterile inoculation loop, transfer three loopfuls of bacterial culture into sterile water.
3. Vortex the bacterial suspension for 30 sec to mix, then centrifuge at 8000 g for 10 min at ambient temperature.
4. Discard the supernatant without disturbing the bacterial pellet. The bacterial pellet is then ready for DNA extraction using the DNeasy Blood and Tissue Mini Kit (Qiagen) following the manufacturer's instructions or using the CTAB method.

Plant tissue

DNA extraction from xylem tissue is the preferred method for the detection of *Xylella* spp. from plant hosts.

1. Trim multiple 10 cm sections from stem samples. Wash with soap and water to remove any debris, dry with paper towel to remove any residual debris.
2. Debark the stem then use a scalpel to remove xylem tissue (Figure 10).
3. Transfer ~0.5 g of tissue to a homogeniser bag (Figure 11) and break up with a mallet.
4. Add 2.5 mL of sterile water or CTAB solution to the homogeniser bag, then homogenise with Homex grinder or similar until the tissue is broken down (Figure 11).
5. The recommendation is to use either the Qiagen Plant Tissue Kit as per the manufacturer's instructions or the CTAB extraction method (**See below**).

Insects

1. If the insect samples were stored in ethanol, dry the insect samples with a clean paper towel.
2. Homogenise insect samples by grinding with a micropestle in liquid nitrogen.
3. Complete DNA extraction using the DNeasy Blood and Tissue Mini Kit (Qiagen) following the manufacturer's instructions.

CTAB DNA Extraction Procedure (Table 7), (Method taken from EPPO PM7/24 *Xylella fastidiosa*)

1. Transfer 1 mL of homogenised plant tissue to a 1.5 mL microcentrifuge tube, heat at 65 ˚C for 30 mins then centrifuge at 16,000 g for 5 min.
2. Transfer as much supernatant as possible to a new 2 mL microcentrifuge tube, add 1 mL of chloroform: isoamyl alcohol (24:1), mix via shaking.
3. Centrifuge at 16,000 g for 5 mins then transfer 700 µl to a 1.5 mL microcentrifuge tube, then add 490 µL of cold 2-propanol. Mix by inversion, twice.
4. Transfer to -20º C for 20 mins. Centrifuge at 16,000 g for 20 mins.
5. Remove supernatant and wash with 1 mL 70% (v/v) ethanol, centrifuge at 16,000 g for 10 mins. Repeat.
6. Decant the ethanol with a pipette, air dry.
7. Resuspend the pellet with TE buffer or RNase and DNase free water.

Table 7: CTAB Reagents (sterilise by autoclaving before use)

Reagent	Amount required for 1 L	Final Concentration
Celtyltrimethylammonium bromide (CTAB)	25 g	2.5%
Sodium Chloride	56 g	1.4 M
1M Tris-HCL, pH 8.0 (Sterile)	100 mL	100 mM
0.5M EDTA, pH 8.0 (Sterile)	40 mL	20 mM
Polyvinylpyrrolidone (PVP-40)	10 g	1%

4.4.2 PCR detection

For each suspect sample, it is recommended to run both the generic endpoint PCR for *Xylella* (Table 9) and the Harper 2010 qPCR (Table 10) or Harper qPCR/Xt for a pear sample (Table 11) according to the diagnostic flow diagram (Figure 7). Please note that random amplification artifacts have been seen when using non-Hot Start Taq (not kept chilled), it is recommended that only Hot-Start Taq be used with this assay. In addition to running these assays, it is recommended that an internal control be run that could be either 16S, 18S or CO1 for plant and insect material to ensure DNA has been extracted from the sample without any issues. Note there have been non-amplification issues with CO1 with certain plant material particularly almond samples.

It is recommended that each DNA extract is tested in triplicate with each of the two assays, *Xylella* generic endpoint PCR and the Harper qPCR (or Harper qPCR/Xt for pear samples). It is also recommended that *X. fastidiosa* (any subspecies) DNA be run as the positive control for all samples, and both *X. fastidiosa* (any subspecies) and *X. taiwanensis* for pear samples. Water should be used as the negative control.

Note that the *Xylella* generic endpoint PCR product can be sequenced, and that the sequence can provide a subspecies identification.

Table 8 (a-c) provides endpoint PCR conditions for the amplification of a *Xylella* specific product of 650 bp. Also included are the assay conditions for running this product on a nanopore sequencing device (Oxford Nanopore Technology).

Xylella generic endpoint PCR (X-ComEC PCR)

Table 8a: Primer sets used for the detection of *Xylella* spp. with the generic endpoint PCR assay as described by Wong-Bajracharya et al. (2024).

Region	Primer Name	Sequence (5'-3')	Product size
ComEC / Rec2 DNA internalisation-related competence protein	X-ComEC-F	AGTCATGCTGATAGTGATCACGT	650 bp
	X-ComEC-R	CAGCATGTCTCGTTTCTCCGA	

Table 8b: Reaction mixture for the generic endpoint PCR assay for *Xylella* spp. as described by Wong-Bajracharya et al. (2024).

Reagents	Each	Final Concentration
dH_2O	6 µL	N/A
2x PCR buffer (Bioline MyTaq HS)*	10 µL	1x (w/ dNTPs and $MgCl_2$)
Forward primer X-ComEC-F (10 mM)	1 µL	500 nM
Reverse primer X-ComEC-R (10 mM)	1 µL	500 nM
DNA template	2 µL	N/A

* The reaction has been validated for use with various PCR reagents, including MyTaq RedMix, GoTaq green mastermix, GoTaq G2 green mastermix and MangoTaq DNA polymerase, using the same concentration of primers and the same quantity of DNA template.

Table 8c: Reaction conditions for the generic endpoint PCR assay for *Xylella* spp. as described by Wong-Bajracharya et al. (2024).

Step	Temperature	Duration	Number of cycles
Initial Denaturation	95°C	2 mins	1
Denaturation	95°C	30 secs	x 35*
Annealing	60°C	30 secs	
Extension	72°C	30 secs	
Final Extension	72°C	5 mins	1

* 40 cycles are recommended to increase the sensitivity of *Xylella* detection in plant metagenomic samples

After completion of the PCR reaction, perform gel electrophoresis to visualise the PCR amplification products using a 2% agarose gel in TBE running for 40 min at 100V. If a product is amplified for the endpoint PCR, sequencing of this product is the next step, either by Sanger sequencing or nanopore sequencing.

PCR purification and sanger sequencing of endpoint PCR product

1. Purify PCR products using purification kits, such as the ISOLATE II PCR and Gel Kit (Bioline), following the manufacturer's instructions.
2. Sequence the purified PCR products with Sanger sequencing technology in both directions.

3. After obtaining the Sanger sequencing data, inspect the raw chromatogram trace file (file extension = .ab1) using appropriate analysis software such as Geneious, SnapGene, or UGENE. Align the forward sequence with the reverse sequence to produce a consensus sequence.
4. Search for the consensus sequence against the nucleotide collection in NCBI using BLAST to determine the species identity. Alternatively, a customised blast database with the amplicon sequences for different species/subspecies of *Xylella* can be used (all amplicon sequences are listed here in Appendix 8.1).

Oxford Nanopore Technologies (ONT) Sequencing of the endpoint PCR product

1. Purify PCR products using purification kits, such as the ISOLATE II PCR and Gel Kit (Bioline), following the manufacturer's instructions.
2. After PCR purification, quantify the product using a NanoDrop spectrophotometer and Qubit fluorometers. Adjust the sample concentration according to the ONT library preparation requirement. A no-template control should also be included for library preparation as a negative control of ONT sequencing.
3. Prepare the ONT library using the Ligation Sequencing Kit or Rapid Barcoding Kit according to the manufacturer's instructions (this protocol has been tested to work with the ligation sequencing kit SQK-LSK109, the rapid barcoding kits SQK-RBK110.96 and SQK-RBK004 kits in R9 flowcells with kit 10 chemistry). For the wash step, use the short fragment buffer (SFB).
4. Load the library into the MinION flow cell or Flongle flow cell according to the manufacturer's instructions. Enough data should be generated within 2 hours.
5. Perform base-calling and barcode removal to generate fastq files. This step can be performed in real-time or after execution on the MinKNOW platform or using the command line-based tool 'guppy' (Version 6.1.5) using the high accuracy mode.

After base-calling and demultiplexing, the resulting .fastq file can be analysed using Graphical User Interface-based tools such as Geneious (Option A) or using command line-based tools (Appendix 8.2; Option B) on a Linux environment. Described below are examples of workflows for nanopore sequencing data analysis by using Geneious Prime.

<u>Option A: Using Geneious Prime</u>

1. Import .fastq files into the Geneious program. Sort all reads by length by right-clicking on the sequence name and selecting 'Sort > By length' (see Figure 16 below).

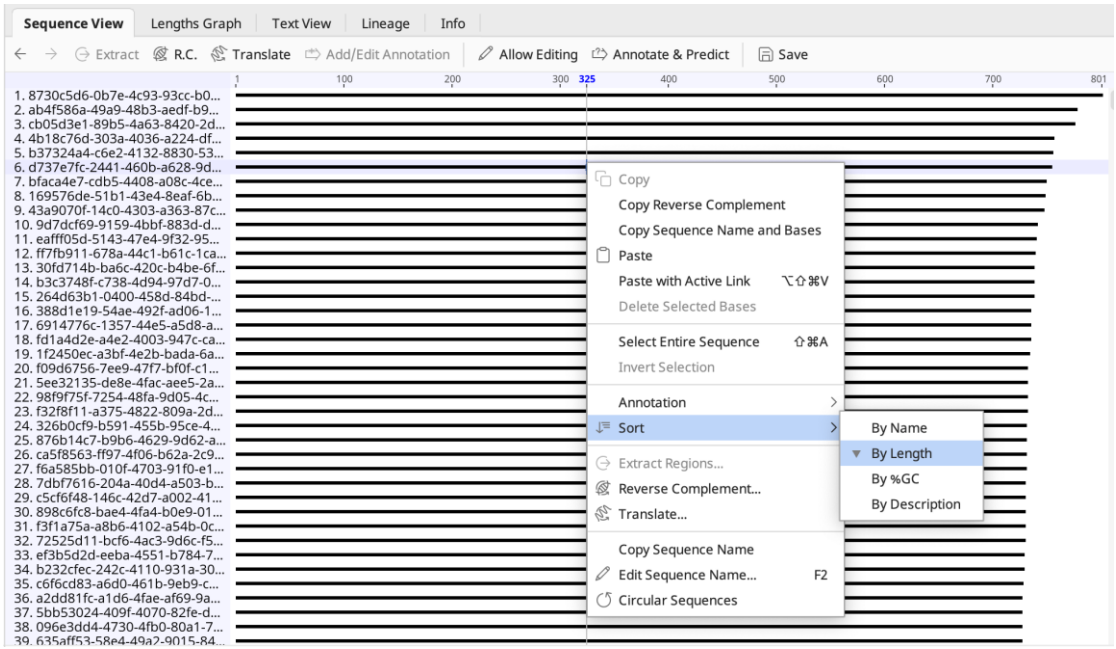

Figure 16: Using Geneious Prime software: the image demonstrates sorting all reads by length by right-clicking on the sequence name and selecting 'Sort > By length' function.

2. Subsample approximately 500 - 1000 sequences in 650±100 bp region and select 'Extract Regions'
3. Align the extracted sequences using MAFFT Alignment with enabled 'Automatically Determine the direction of the sequences'.
4. Go to the sequence alignment file, highlight the region in the consensus panel, and select 'Extract' to generate a consensus sequence (see Figure 17 below).

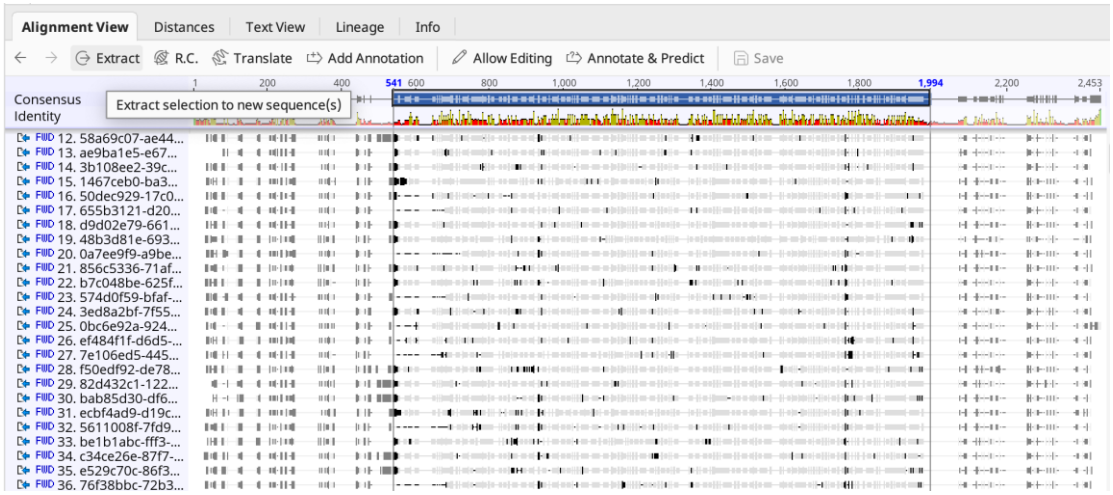

Figure 17: Using Geneious Prime software: the image demonstrates generating a consensus sequence by clicking on the sequence alignment file, highlighting the region in the consensus panel, and then selecting 'Extract'.

6. Search for the consensus sequence against the nucleotide collection in NCBI using BLAST to determine the species identity. Alternatively, a customised blast database with the amplicon sequences for different species/subspecies of *Xylella* can be used (all amplicon sequences are listed in Appendix 8.1).

7. Once the alignment has been completed, you can generate a tree to visualise the species as subspecies groups as per Figure 18.

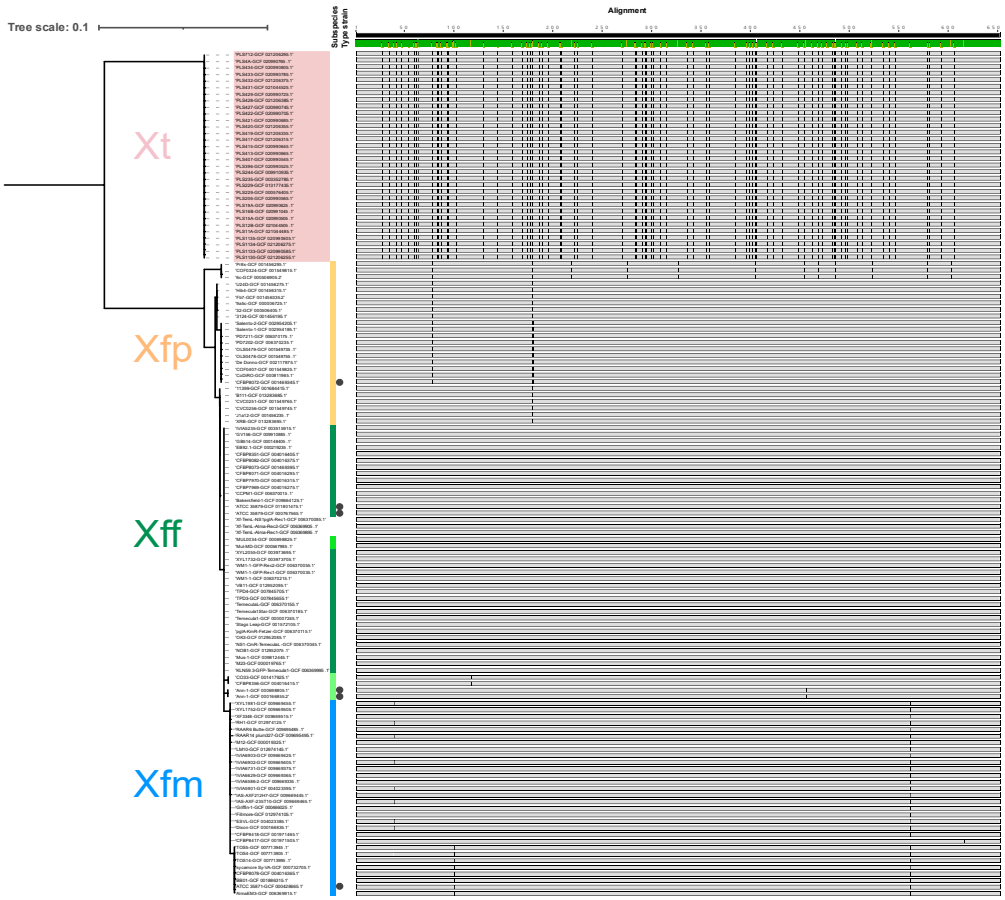

Figure 18: Geneious phylogenetic tree output depicting the *Xylella* spp. alignment. The colour of the text labels represents the different *Xylella* species/subspecies. Xt: *Xylella taiwanensis*, Xfp: *X. fastidiosa* subsp. *pauca*; Xff: *X. fastidiosa* subsp. *fastidiosa*; Xfm: *X. fastidiosa* subsp. *multiplex*.

Interpretation of Results

Positive detection of *Xylella* results in the amplification of a product of 650 bp. This product can be sequenced by Sanger sequencing or nanopore sequencing for the confirmation of *X. fastidiosa* or *X. taiwanensis*. Alignments of the fragment with reference *Xylella* controls will provide identification of species and subspecies. Note that the reference alignments are available in Appendix 8.1. The PCR is only considered valid if both the positive and negative controls have worked as expected in the reaction. It is also recommended that the sample is run in triplicate.

If nanopore sequencing was used, the following criteria should be taken into consideration to validate the result:

1. A positive amplification at 650 bp should be detectable using gel electrophoresis.
2. Both the positive and negative controls have worked as expected in the reaction.
3. At least 50% of all reads should map to any of the amplicon sequence variants (see Appendix 8.1).
4. BLAST search result should have a nucleotide identity percentage ≥ 90% and a query coverage ≥ 70% to the amplicon sequence.

A valid *Xylella* species/subspecies identification should meet all of the above criteria. If the BLAST search produced a match to the generic endpoint PCR amplicons but the sample does not meet all four criteria, it is categorised as an indeterminate result. If the BLAST search did not produce any matches to the PCR amplicon sequences, *Xylella* detection is considered negative.

***Harper qPCR* (Tables 9 a-c)**

Table 9a: Primer sets used for the detection of *Xylella fastidiosa* with the qPCR assay described by Harper et al., (2010)

Region	Primer Name	Sequence (5'-3')	Product size
16S ribosomal RNA processing protein *rimM*	XF-F	CACGGCTGGTAACGGAAGA	70 bp
	XF-R	GGGTTGCGTGGTGAAATCAAG	
	XF-P	6'FAM-TCG CAT CCC GTG GCT CAG TCC-BHQ1	

Table 9b: Reaction mixture for the qPCR assay described by Harper et al., (2010)

Reagents	Each	Final Concentration
dH_2O	5.3 µL	N/A
Forward primer XF-F (10 mM)	0.3 µL	300 nM
Reverse primer XF-R (10 mM)	0.3 µL	300 nM
Probe XF-P (10 mM)	0.1 µL	100 nM
10x Immobuffer (Bioline)*	1 µL	1X
dNTPs (10 mM)	1 µL	1 mM
MgCl2 (50 mM)	0.8 µL	4 mM
Immolase 5U/µL (Bioline)*	0.2 µL	N/A
DNA Template	1 µL	N/A

* The reaction has been validated for use with qPCR mastermix, including QuantaBio Perfecta Toughmix, GoTaq, and QuantiNova, using the same concentration of primers and probes.

Table 9c: Reaction condition for the qPCR assay described by Harper et al., (2010)

Step	Temperature	Duration	Number of Cycles
Preincubation	50 °C	2 min	1
Initial Denaturation	95 °C	2 – 5 mins	1
Denaturation	95 °C	10 secs	x 40
Annealing	62 °C	40 secs	

Interpretation of Results

Positive detection of *Xylella* results in the amplification with a CT value of 37 or less. This is required for 2 of the 3 triplicate reactions. The PCR is only considered positive if both the positive and negative controls

have worked as expected.

Harper qPCR including 18S rDNA Internal Control **(Tables 10 a-c)**

Table 10a: Primer sets used for the detection of *Xylella fastidiosa* with the qPCR assay described by Harper et al. (2010) and Ioos et al. (2009). The 18S internal control is recommended in this assay and has been substituted for the COI region, due to amplification issues identified with the COI region in almonds in Europe. This internal control has been validated at the Plant Health Diagnostic Laboratory of NSW DPIRD and by the Molecular Bacteriology research team.

Region	Primer Name	Sequence (5'-3')	Product size
Eukaryote 18S Ribosomal DNA	18S Uni-F	GCAAGGCTGAAACTTAAAGGAA	150 bp
	18S Uni-R	CCACCACCCATAGAATCAAGA	
	18S Uni-P	Cy5-ACGGAAGGGCACCACCAGGAGT-BHQ-2	
16S ribosomal RNA processing protein *rimM*	XF-F	CACGGCTGGTAACGGAAGA	70 bp
	XF-R	GGGTTGCGTGGTGAAATCAAG	
	XF-P	6'FAM-TCG CAT CCC GTG GCT CAG TCC-BHQ1	

Table 10b: Reaction mixture for the qPCR and 18S duplex assay described in the *Xylella fastidiosa* EPPO protocol.

Reagents	Working concentration	Each	Final Concentration
dH_2O	NA	4.49 µL	N/A
Forward primer XF-F	10 µM	0.3 µL	0.3 µM
Reverse primer XF-R	10 µM	0.3 µL	0.3 µM
Probe XF-P	10 µM	0.1 µL	0.1 µM
Forward primer 18S Uni-F	10 µM	0.15 µM	0.15 µM
Reverse primer 18S Uni-R	10 µM	0.15 µM	0.15 µM
Probe 18S Uni-P	10 µM	0.05 µM	0.05 µM
Molecular-grade BSA (non-acetylated)	50 mg/mL	0.06	0.30 µg μL^{-1}
10x Immobuffer (Bioline)*		1 µL	1X
dNTPs (10 mM)		1 µL	1 mM
MgCl2 (50 mM)		1.2 µL	6 mM
Immolase 5U/µL (Bioline)*		0.2 µL	
DNA Template		1 µL	N/A

* The reaction has been validated for use with qPCR mastermix, including QuantaBio Perfecta Toughmix, GoTaq, and QuantiNova, using the same concentration of primers and probes.

Table 10c: Reaction condition for the qPCR assay described by Harper et al. (2010)

Step	Temperature	Duration	Number of Cycles

Preincubation	50 °C	2 min	1
Initial Denaturation	95 °C	2 – 5 mins	1
Denaturation	95 °C	10 secs	x 40
Annealing	62 °C	40 secs	

Interpretation of Results

Positive detection of *Xylella* results in amplification with a CT value less than 37 or less in triplicate. CT values over 38 or in only one or two replicate reactions, will require confirmation with an additional assay. The qPCR is only considered valid if both the positive and negative controls have worked as expected. It is also recommended that the sample is run in triplicate.

4.4.3 Confirmation of Xylella spp.

Upon receipt of a suspect *Xylella* infected plant sample, given the importance of this pathogen, it is recommended that the *Xylella* generic endpoint PCR and Harper et al., 2010 qPCR (or Harper qPCR/Xt assay for pear samples, Table 11) are used, and that the sample is tested in triplicate. If the *Xylella* generic endpoint PCR generates a positive product, this product then requires sequencing either using Sanger sequencing or nanopore sequencing (described in section 4.4.2). Confirmation of a positive Xf result would also require a positive result with the Harper et al. (2010) qPCR. The PCR is only considered positive if both the positive and negative controls have worked as expected.

If only one of the assays generates a positive result, the following interpretations and steps are recommended:

- If a positive result occurs with the *Xylella* generic endpoint PCR and not Harper et al. (2010) qPCR, the sample could be a *Xylella* species other than *X. fastidiosa* or a false positive result. In this case, it is recommended that the generic *Xylella* qPCR (Table 13a-c) and Harper LAMP assay (Table 16a-c) are run, and the *Xylella* generic endpoint PCR product should also be sequenced.
- If Harper et al., 2010 qPCR generates a positive result and not the endpoint PCR, this could either be titre issue as the Harper assay is more sensitive than the endpoint PCR (note: digital PCR has again increased sensitivity of both the endpoint and qPCR assays), or it could also be a false positive. Then it is recommended to perform the Harper LAMP assay (Table 16) and preferentially the generic *Xylella* qPCR (Table 13) as the other assays (Section 4.4.4) are less sensitive. For the qPCR a CT of 37 or is considered a positive, anything greater than 38 requires re-extracting and rerunning. If the same occurs, then further testing with another qPCR (or dPCR if available) is necessary.

***Duplex Harper Xf/Xt qPCR* (Tables 11 a-c; Figure 19)**

Table 11a: Primer sets used for duplex detection of *Xylella fastidiosa* (XF primer set) and *X. taiwanensis* (Xt-ComEC qPCR primer set).

Assay	Target Region	Primer Name	Sequence (5'-3')	Product size
Harper et al. (2010)	16S ribosomal RNA processing protein rimM	XF-F	CACGGCTGGTAACGGAAGA	70 bp
		XF-R	GGGTTGCGTGGTGAAATCAAG	

		XF-P	6'FAM-TCG CAT CCC GTG GCT CAG TCC-BHQ1	
This study	ComEC / Rec2 DNA internalization-related competence protein	X-ComEC-F	AGTCATGCTGATAGTGATCACGT	156 bp
		qPCR-Xgen-ComEC-R	GAAACGAAACTGSACTCCATCC	
		qPCR-Xt-ComEC-P	Cy5-CGGGCGCGCCACTTAACGTTGAT-BHQ3	

Table 11b: Reaction mixture for the duplex Xf/Xt qPCR assay

Reagents	Each (µL)	Final Concentration
dH2O	4 µL	N/A
10X Immobuffer (Bioline)*	1 µL	1X
dNTPs (10 µM)	1 µL	1 µM
MgCl2 (50 µM)	1.2 µL	6 µM
XF-F (10 µM)	0.3 µL	300 nM
XF-R (10 µM)	0.3 µL	300 nM
XF-P (10 µM)	0.2 µL	200 nM
X-ComEC-F (10 µM)	0.3 µL	300 nM
qPCR-Xgen-ComEC-R (10 µM)	0.3 µL	300 nM
Xt-ComEC-P (10 µM)	0.2 µL	200 nM
Immolase 5U/µL (Bioline)*	0.2 µL	
DNA Template	1 µL	N/A

*The reaction has been validated for use with qPCR mastermix, including SsoAdvanced, QuantaBio Perfecta Toughmix, GoTaq, and QuantiNova, using the same concentration of primers and probes.

Table 11c: Reaction condition for the duplex Xf/Xt qPCR assay

Step	Temperature	Duration	Number of Cycles
Preincubation	50°C	2 min	1
Initial Denaturation	95°C	2 – 5 mins	1
Denaturation	95°C	10 secs	x 40
Annealing	62°C	40 secs	

Detection with the Cy5 channel for the Xt probe and the FAM channel for the Xf probe.

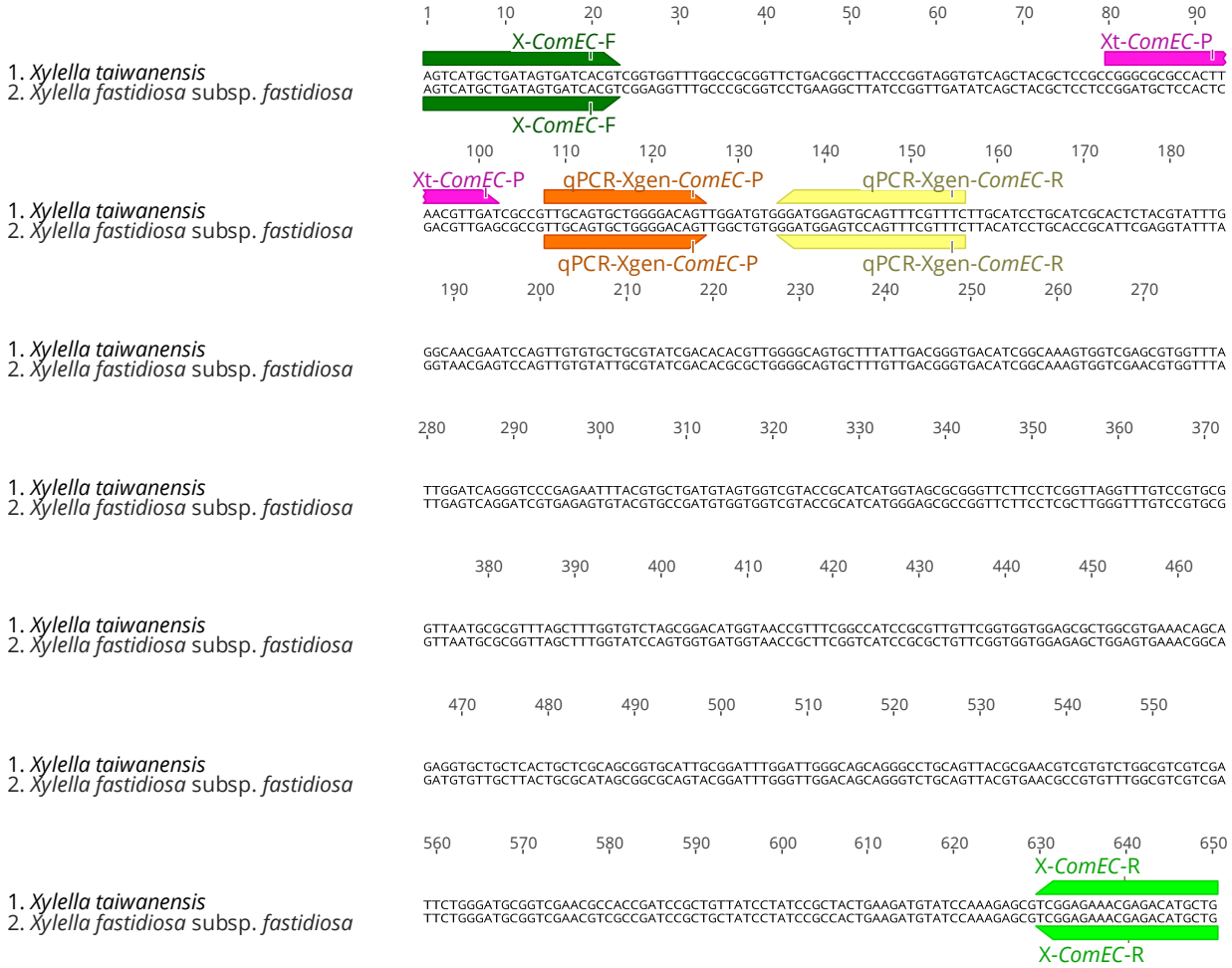

Figure 19: Primer and probe binding sites of *Xylella* X-ComEC PCR and qPCR on *X. fastidiosa* and *X. taiwanensis*

Interpretation of results

The Harper et al. (2010) Xf qPCR assay targets *X. fastidiosa* specifically, whereas Xt-ComEC qPCR targets *X. taiwanensis* specifically. Xf detection will result in an increase in fluorescence detected with the FAM channel, while Xt detection will result in fluorescence signals on the Cy5 channel. A mixed detection of Xt and Xf will result in amplification in both channels. A positive result is only applicable when both the positive control for both Xf and Xt are positive, and the negative control is negative. A CT value of 37 or lower signifies a positive result.

Duplex Harper Xf/Xt digital PCR Optional Running Procedure

The Duplex Harper Xf/Xt qPCR is compatible to be run on a digital PCR System to enhance sensitivity and accuracy of quantification on low-titre samples. Detailed below is the protocol to perform the Duplex Harper Xf/Xt digital PCR on the Qiagen QIAcuity system using the primer and probe sets as listed in **Table 12a**. This method has been validated at the Plant Health Diagnostic Laboratory of NSW DPIRD.

1. Prepare reaction mixture as described in **Table 12a.**
2. Vortex the reaction mixture before dispensing appropriate volumes of the reaction mix into the wells of a standard PCR plate. Add DNA template.

3. Carefully transfer the content of each well from a standard PCR plate to the wells of the nanoplate without introducing bubbles. Seal the nanoplate with QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.
4. Programme the QIAcuity according to **Table 12b**. Set detection with the Cy5 (Maroon) channel for the Xt probe and the FAM (Green) channel for the Xf probe.
5. Run digital PCR and imaging with default setting. The whole program should be completed in approximately 1.5 hour.

Table 12a: Reaction mixture for the duplex Xf/Xt digital PCR assay (using the 26k Nanoplate)

Reagents	Each (µL)	Final Concentration
4X Probe PCR master mix (QIAcuity Probe PCR kit; Qiagen)	10 µL	N/A
XF-F (10 µM)	3.2 µL	800 nM
XF-R (10 µM)	3.2 µL	800 nM
XF-P (10 µM)	1.6 µL	400 nM
X-ComEC-F (10 µM)	3.2 µL	800 nM
qPCR-Xgen-ComEC-R (10 µM)	3.2 µL	800 nM
Xt-ComEC-P (10 µM)	1.6 µL	400 nM
DNase-free water	9 µL	
DNA Template	5 µL	N/A

Table 12b: Reaction condition for the duplex Xf/Xt digital PCR assay

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95 °C	2 mins	1
Denaturation	95 °C	15 secs	x 40
Annealing	62 °C	30 secs	

Interpretation of resultss

The Harper Xf qPCR probe targets *X. fastidiosa* specifically, whereas XT-ComEC qPCR probe targets *X. taiwanensis* specifically. Xf detection will result in an increase in fluorescence detected with the FAM channel, while Xt detection will result in fluorescence signals on the Cy5 channel. A mixed detection of Xt and Xf will result in amplification in both channels.

4.4.4 Alternative molecular methods for the detection of X. fastidiosa if additional confirmation methods are required

***Generic Xylella spp. qPCR* (Tables 13 a-c)**

Table 13a: Primer sets used for the detection of *Xylella* species with generic *Xylella* spp. qPCR assay

Region	Primer Name	Sequence (5'-3')	Product size

ComEC / Rec2 DNA internalization-related competence protein	Xgen-ComEC-F	AGTCATGCTGATAGTGATCACGT	156 bp
	qPCR-Xgen-ComEC-156-R	GAAACGAAACTGSACTCCATCC	
	qPCR-Xgen-ComEC-P	6'FAM-TTGCAGTGCTGGGGACAGT-iBHQ*	

*iBHQ is Iowa Black flurophore quencher it is from IDT. This protocol has not been tested with the standard BHQ.

Table 13b: Reaction mixture for the detection of *Xylella* species with generic *Xylella* spp. qPCR assay

Reagents	Each	Final Conc.
dH_2O	4.8 µL	N/A
Forward primer Xgen-ComEC-F (10 mM)	0.3 µL	300 nM
Reverse primer qPCR-Xgen-ComEC-156-R (10 mM)	0.3 µL	300 nM
Probe qPCR-Xgen-ComEC-P (10 mM)	0.2 µL	200 nM
10x Immobuffer (Bioline)*	1 µL	1X
dNTPs (10 mM)	1 µL	1 mM
MgCl2 (50 mM)	1.2 µL	6 mM
Immolase 5U/µL (Bioline)*	0.2 µL	N/A
DNA Template	1 µL	N/A

*The reaction has been validated for use with qPCR mastermix, including QuantaBio Perfecta Toughmix, GoTaq, and QuantiNova, using the same concentration of primers and probes.

Table 13c: Reaction conditions for the detection of *Xylella* species with generic *Xylella* spp. qPCR assay

Step	Temperature	Duration	Number of Cycles
Preincubation	50°C	2 min	1
Initial denaturation	95°C	2 – 5 mins	1
Denaturation	95°C	30 secs	x 40
Annealing	64°C	30 secs	

Ouyang qPCR **(**Tables 14 a-c)

Table 14a: Primer sets used for the detection of *Xylella fastidiosa* with the qPCR assay described by Ouyang et al. (2013)

Region	Primer Name	Sequence (5'-3')	Product size
Cobalamin synthesis protein	Xf.Csp6F	CCCATTACGCTTCAACCATT	93 bp
	Xf.Csp6R	CCCAATCCATACGACTTGCT	
	Xf.Csp6P	6'FAM-GGTGTGATTCGCAGCAAGGGC-BFQ1	

Table 14b: Reaction mixture for the qPCR assay described by Ouyang et al. (2013)

Reagents	Each	Final Concentration
dH_2O	4.9 µL	N/A
Forward primer Xf.Csp6F (10 mM)	0.3 µL	300 nM
Reverse primer Xf.Csp6R (10 mM)	0.3 µL	300 nM
Probe Xf.Csp6P (10 mM)	0.1 µL	100 nM
10x Immobuffer (Bioline)*	1 µL	1X
dNTPs (10 mM)	1 µL	1 mM
MgCl2 (50 mM)	1.2 µL	6 mM
Immolase 5U/mL (Bioline)*	0.2 µL	N/A
DNA Template	1 µL	N/A

*The reaction has been validated for use with qPCR mastermix, including QuantaBio Perfecta Toughmix, GoTaq, and QuantiNova, using the same concentration of primers and probes.

Table 14c: Reaction condition for the qPCR assay described by Ouyang et al. (2013)

Step	Temperature	Duration	Number of Cycles
Preincubation	50°C	2 mins	1
Initial denaturation	95°C	2 – 5 mins	1
Denaturation	95°C	15 secs	x 40
Annealing	60°C	60 secs	

Dupas qPCR (XF probe only) **(Tables 15 a-c)**

Table 15a: Primer sets used for the detection of *Xylella fastidiosa* and its subspecies with the multiplex qPCR assay described by Dupas et al. (2019)

Region	Primer Name	Sequence (5'-3')	Product Size
Ketol-acid reductoisomerase (*X. fastidiosa*)	Dupas-XF-F	AACCTGCGTGACTCTGGTTT	118
	Dupgas-XF-R	CATGTTTCGCTGCTTGGTCC	
	Dupas-XF-P	6'FAM-GCTCAGGCTGACGGTTTCACAGTGCA-BHQ1	

Table 15b: Reaction mixture for the multiplex qPCR assay described by Dupas et al. (2019)

Reagents	Each	Final Conccentration
dH_2O	4.08 µL	N/A
Forward primer Dupas-XF-F (10 mM)	0.6 µL	600 nM
Reverse primer Dupas-XF-R (10 mM)	0.6 µL	600 nM
Probe Dupas-XF-P (10 mM)	0.2 µL	200 nM
10x Immobuffer (Bioline)*	1 µL	1X
dNTPs (10 mM)	1 µL	1 mM
MgCl2 (50 mM)	1.2 µL	6 mM

Immolase 5U/µL (Bioline)*	0.2 µL	N/A
Bovine serum albumin (50 mg/mL	0.12 µL	600 ng/µL
DNA Template	1 µL	N/A

* The reaction has been validated for use with qPCR mastermix, including QuantaBio Perfecta Toughmix, GoTaq, and QuantiNova, using the same concentration of primers and probes.

Table 15c: Reaction condition for the multiplex qPCR assay described by Dupas et al. (2019)

Step	Temperature	Duration	Number of Cycles
Preincubation	50°C	2 mins	1
Initial denaturation	95°C	3 mins	1
Denaturation	95°C	15 secs	x 40
Annealing	60°C	30 secs	

LAMP assays for X. fastidiosa

A loop-mediated isothermal amplification (LAMP) assay has been developed for the detection of *X. fastidiosa* (Harper et al., 2010). The method is rapid, more sensitive than conventional PCR, and only 10 times less sensitive than Harper et al. (2010) qPCR. LAMP can be used for low-throughput field-based surveillance due to the portability of instruments that can perform LAMP such as a Genie (OptiGene) (see Section 9). Furthermore, due to its ease of use and sensitivity, LAMP can also be run as high throughput on a qPCR machine as a laboratory test. Based on European experience it is not recommended to use the LAMP assay with asymptomatic material, only symptomatic material should be tested.

The LAMP assay can be used with plant DNA extracted using the DNeasy Plant Mini Kit (Qiagen) in the laboratory and the Plant Lysis Kit (Optigene) in the field, according to the manufacturer's instructions. The LAMP assay can be performed on insect DNA extracted using the DNeasy blood and tissue kit or QuickExtract DNA solution according to the manufacturer's instructions.

Specific LAMP detection of *X. fastidiosa*

Real-time detection of *X. fastidiosa* by LAMP is performed using the primers designed by Harper et al. (2010) that target the *rimM* gene (Table 9a standard PCR, Table 16a LAMP primers). These primers are designed to be species-specific for *X. fastidiosa.* The internal control Cox primers are given in Table 16c. The LAMP reaction setup is outlined in Table 16e.

In the testing, a positive and a negative amplification control should be included and samples run in triplicate.

Internal amplification control

It is recommended that a LAMP test targeting a universal housekeeping gene from the host (where practical and available) be run on all samples to ensure that the DNA extraction has been successful and that no inhibitors from the insect or plant host tissues (e.g. lignins, polyphenols, pigments, fats, proteins) prevent DNA amplification during the LAMP reaction.

The Cox LAMP test is designed to amplify the plant cytochrome oxidase gene sequence (Tomlinson et. al., 2010, Tables 16 a-e). The Cox gene is highly conserved among plants, and amplification of the Cox gene serves as an internal/extraction control for use with plant samples.

Failure to produce amplification with the housekeeping gene primers may suggest the following:

- Extraction has failed and re-extraction is required; or
- The inhibitor concentration in the sample is too high and interferes with the enzyme activity. Dilution of the extraction may resolve the issue; or
- The concentration of the DNA in the extraction is too low, and re-extraction is required.

Table 16a: Primer sequences for the *Xylella fastidiosa*-specific LAMP assay described by Harper et al. (2010)

Primer Name	Sequence (5'-3')
LAMP-XF-F3 (external primer)	CCGTTGGAAAACAGATGGGA
LAMP-XF-B3 (external primer)	GAGACTGGCAAGCGTTTGA
LAMP-XF-FIP (internal primer)	ACCCCGACGAGTATTACTGGGTTTTTCGCTACCGAGAACCACAC
LAMP-XF-BIP (internal primer)	CGCTGCGTGGCACATAGATTTTTGCAACCTTTCCTGGCATCAA
LAMP-XF-LF (loop primer)	TGCAAGTACACACCCTTGAAG
LAMP-XF-LB (loop primer)	TTCCGTACCACAGATCGCT

Table 16b: LAMP Primer mix concentrations and compositions for the *Xylella fastidiosa*-specific LAMP assay described by Harper et al. (2010)

LAMP test for *X. fastidiosa*	**Primer**	**Stock concentration (µM)**	**Amount to add for primer mix (µL)**	**Concentration in primer mix (µM)**	**Final concentration in reaction (µM)**
Ratios			1:8:4		
X. fastidiosa-specific LAMP	B3	10	10	1	0.2
	F3	10	10	1	0.2
	BIP	100	8	8	1.6
	FIP	100	8	8	1.6
	LF	100	4	4	0.8
	BF	100	4	4	0.8
Subtotal (µL)			44		
H2O (µL)			56		
Total (µL)			100		

Table 16c: Primers sequences for internal amplification control Cox LAMP assay (Tomlinson et al., 2013).

Primer Name	Sequence (5'-3')
LAMP-Cox-F3 (external primer)	TATGGGAGCCGTTTTTGC
LAMP-Cox-B3 (external primer)	AACTGCTAAGRGCATTCC
LAMP-Cox-FIP (internal primer)	ATGGATTTGRCCTAAAGTTTCAGGGCAGGATTTCACTATTGGGT
LAMP-Cox-BIP (internal primer)	TGCATTTCTTAGGGCTTTCGGATCCRGCGTAAGCATCTG
LAMP-Cox-LF (loop primer)	ATGTCCGACCAAAGATTTTACC
LAMP-Cox-LB (loop primer)	GTATGCCACGTCGCATTCC

Table 16d: LAMP Primer mix concentrations and compositions for internal amplification control Cox LAMP assay (Tomlinson et al., 2013).

LAMP test for Cox	Primer	Stock concentration (µM)	Amount to add for primer mix (µL)	Concentration in primer mix (µM)	Final concentration in reaction (µM)
Ratios			1:10:5		
Cox housekeeping LAMP*	B3	10	10	1	0.2
	F3	10	10	1	0.2
	BIP	100	10	10	2
	FIP	100	10	10	2
	LF	100	5	5	1
	BF	100	5	5	1
Subtotal (µL)			50		
H2O (µL)			50		
Total (µL)			100		

* For plant tissues only

Table 16e: Reaction setup for LAMP assays for detection of *Xylella fastidiosa* and for Cox internal control

Components	Per reaction (25 µL)
Sterile water	3 µL
Isothermal Mix (Optigene ISO-DR004)	15 µL
LAMP Primer Mix	5 µL
Template (extraction)	2 µL*
Total reaction	25 µL

*Template volume can be increased to up to 5 µL, in this case, the water volume needs to be adjusted accordingly.

1. Make master mix and thoroughly mix by slowly pipetting 10 times. Aliquot 23 µL of master mix in each tube of LAMP strip tubes.
2. Add 2 µL of sterile water to the negative control tube and close the lid.
3. Add 2 µL of the template (DNA extraction) to the reaction tube and close the lid.
4. Add 2 µL of positive control DNA to the positive control tube and close the lid.
5. Load the LAMP strip tube into the Genie III and run the below protocol, this protocol can also be saved as a template in the machine.

LAMP reaction conditions:

- On a Genie II or III instrument (Optigene) run at 65 °C for 30 mins, followed by an annealing step of 98 °C to 80 °C (ramp rate of 0.05 °C/sec).
- On a qPCR machine using the SYBR chemistry setting run at 65 °C for 15 sec for 120 cycles (30 mins equivalent with imaging intervals), followed by a melt curve stage from 80 °C to 98 °C (ramp rate of 0.05 °C/sec).

Result Interpretation

If target DNA is present, it will be amplified and an increase in fluorescence from the dsDNA intercalating dye will be detected on the instrument. A positive amplification plot will display as a sigmoid curve and have an anneal derivative. A negative result will not display an amplification curve or anneal derivative.

As confirmation of amplification of the correct target, an anneal step is performed after the isothermal step. Each target will have its own signature anneal temperature. Positive amplification followed by an anneal temperature in the expected range is a confirmation of a true positive in a sample.

The LAMP test will be considered valid only if ALL the following criteria are met:

- A positive result has a time to positive of 25:00 mins or less, anything >25:00 mins is considered indeterminate, and further tests are required.
- The positive control produces a sigmoidal amplification curve with the *X. fastidiosa*-specific primers.
- The negative control must be negative.
- The positive control must be positive.
- Positive samples have the same annealing temperature within 1 °C of the positive control (+/-).

4.4.5 Further identification of X. fastidiosa at the subspecies level

Further identification of positive samples may be required on a cultured bacterium or an extracted DNA sample from the host tissue.

Subspecies qPCR Assay

The generic *Xylella* endpoint PCR can determine subspecies; however, it should be combined with the subspecies multiplex qPCR for secondary confirmation.

The subspecies multiplex qPCR is performed using the primers designed by Dupas et al. (2019), which includes six different primer and probe sets targeting different subspecies of *X. fastidiosa.* The subspecies multiplex qPCR is run as two separate assays that are described as Module 1 and Module 2, the primer sets are detailed in Table 17a and their combinations and concentrations in Table 17b. Module 1 includes four primer sets targeting *X. fastidiosa*, *X. fastidiosa* subsp*. fastidiosa*, *X. fastidiosa* subsp. *multiplex* and *X. fastidiosa* subsp. *morus.* Module 2 includes four primer sets targeting *X. fastidiosa*, *X. fastidiosa* subsp. *fastidiosa sensu largo*, *X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *multiplex.*

Tables 17 b-d provide qPCR conditions for the multiplex assay amplification of *Xylella* spp. and subspecies differentiation using the Dupas et al. (2019) protocol. In the testing, a positive control per each testing *X. fastidiosa* subspecies should be included. To run this test, follow the following steps:

1. Make forward primer mix, reverse primer mix and probe mix based on the composition detailed in Table 17b.
2. Using the primer mixes, prepare the reaction mix for module 1 and module 2 separately based on the reaction mix detailed in Table 17c.
3. Run the multiplex qPCR using the reaction condition detailed in Table 17d.

An additional method for running the Dupas subspecies PCR is to include the Harper qPCR primers instead of the Dupas Xf primers. Then select the additional primers for the assay. Conditions for this modified qPCR are outlined in Tables 17e-g.

Table 17a: Primer sets used for the detection of *Xylella fastidiosa* and its subspecies with the multiplex qPCR assay described by Dupas et al. (2019). Primer sets used for the detection of *X. fastidiosa* and its subspecies, listed below, have been tested individually, and have not been tested in a multiplex qPCR format. It is up to the individual laboratories to decide the format of qPCR (individual/multiplex) that they would like to run.

Region (target sp./subsp.)	**Primer Name**	**Sequence (5'-3')**	**Product size**	**Module**
Ketol-acid reductoisomerase (*X. fastidiosa*)	Dupas-XF-F	AACCTGCGTGACTCTGGTTT	118	1 & 2
	Dupas-XF-R	CATGTTTCGCTGCTTGGTCC		
	Dupas-XF-P	FAM-GCTCAGGCTGACGGTTTCACAGTGCA-BHQ1		
Restriction Modification System (*X. fastidiosa* subsp. *fastidiosa*)	XFF-F	TTACATCGTTTTCGCGCACG	100	1
	XFF-R	TCGGTTGATCGCAATACCCA		
	XFF-P	HEX-CCCGACTCGGCGCGGTTCCA-BHQ1		

Unknown (*X. fastidiosa* subsp. *fastidiosa sensu largo*)	XFFSL-F	TAGTATGCGTGCGAGCGAC	75	2
	XFFSL-R	CGCAATGCACACCTAAGCAA		
	XFFSL-P	HEX-CGCGTACCCACTCACGCCGC-BHQ1		
Unknown and DNA adenine methylase (*X. fastidiosa* subsp. *multiplex*)	XFM-F	ACGATGTTTGAGCCGTTTGC	88	1 & 2
	XFM-R	TGTCACCCACTACGAAACGG		
	XFM-P	ROX-ACGCAGCCCACCACGATTTAGCCG-BHQ2		
Peptidase S24 (*X. fastidiosa* subsp. *morus*)	XFMO-F	TAACGCTATCGGCAGGTAGC	123	1
	XFMO-R	GCATCAGCTTCACGTCTCCT		
	XFMO-P	CY5-GGTTCCGCACCTCACATATCCGCCC-BHQ2		
Histidine Kinase and ABC Transporter Substrate Binding (*X. fastidiosa* subsp. *pauca*)	XFP-F	TGCGTTTTCCTAGGTGGCAT	154	2
	XFP-R	GTTGGAACCTTGAATGCGCA		
	XFP-P	CY5-CCAAAGGGCGGCCACCTCGC-BHQ2		

Table 17b: Primer and probe concentration for the detection of *Xylella fastidiosa* and its subspecies with the multiplex qPCR assay described by Dupas et al. (2019)

Primer mix	Primer	Stock Concentration	Target Concentration in Primer mix	Volume to add for making a 100 µL primer mix at 10 µM
Module 1 forward primer mix	Dupas-XF-F	100 µM	10 µM	10 µL
	XFF-F	100 µM	10 µM	10 µL
	XFM-F	100 µM	10 µM	10 µL
	XFMO-F	100 µM	10 µM	10 µL
Module 1 reverse primer mix	Dupas-XF-R	100 µM	10 µM	10 µL
	XFF-R	100 µM	10 µM	10 µL
	XFM-R	100 µM	10 µM	10 µL
	XFMO-R	100 µM	10 µM	10 µL
Module 1 probe mix	Dupas-XF-P	100 µM	10 µM	10 µL
	XFF-P	100 µM	10 µM	10 µL
	XFM-P	100 µM	10 µM	10 µL

	XFMO-P	100 µM	10 µM	10 µL
Module 2 forward primer mix	Dupas-XF-F	100 µM	10 µM	10 µL
	XFFSL-F	100 µM	10 µM	10 µL
	XFM-F	100 µM	10 µM	10 µL
	XFP-F	100 µM	10 µM	10 µL
Module 2 reverse primer mix	Dupas-XF-R	100 µM	10 µM	10 µL
	XFFSL-R	100 µM	10 µM	10 µL
	XFM-R	100 µM	10 µM	10 µL
	XFP-R	100 µM	10 µM	10 µL
Module 2 probe mix	Dupas-XF-P	100 µM	10 µM	10 µL
	XFFSL-P	100 µM	10 µM	10 µL
	XFM-P	100 µM	10 µM	10 µL
	XFP-P	100 µM	10 µM	10 µL

Table 17c: Reaction mixture for the multiplex qPCR assay described by Dupas et al. (2019)

Reagents	Each	Final Concentration
Water	4.7 mL	N/A
2x TaqMan qPCR buffer (SsoAdvanced universal probe supermix)	10 mL	1x (w/ dNTPs and $MgCl_2$)
Forward primer mix from Module 1 or 2 (10 mM per primer)	1.15 mL	575 nM per primer
Reverse primer mix from Module 1 or 2 (10 mM per primer)	1.15 mL	575 nM per primer
Probe mix from Module 1 or 2 (10 mM per primer)	0.4 mL	200 nM per probe
Bovine serum albumin 20 mg/mL stock	0.6 mL	600 ng/mL
DNA Template	2 mL	N/A

Table 17d: Reaction condition for the multiplex qPCR assay described by Dupas et al. (2019)

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95°C	3 mins	1
Denaturation	95°C	15 secs	x 40
Annealing	60°C	30 secs	

Table 17e: Primer and probe concentration for the detection of *Xylella fastidiosa* and its subspecies with the multiplex qPCR assay described by Dupas et al. (2019) with the Harper qPCR inclusion modification.

Primer/probe1			volume (µl) taken from 100 µM stock	final concentration (µM)
X. fastidiosa (harper)	XF-F	CACGGCTGGTAACGGAAGA	5.8	5.75
	XF-R	GGGTTGCGTGGTGAAATCAAG	5.8	5.75
	XF-P	FAM-TCG CAT CCC GTG GCT CAG TCC-BHQ1	2	2
Primer/probe2				
X. fastidiosa subsp. *Multiplex*	XFM-F	ACGATGTTTGAGCCGTTTGC	5.8	5.75
	XFM-R	TGTCACCCACTACGAAACGG	5.8	5.75
	XFM-P	ROX-ACGCAGCCCACCACGATTTAGCCG-BHQ2	2	2
Primer/probe3				
X. fastidiosa subsp. *fastidiosa sensu largo*	XFFSL-F	TAGTATGCGTGCGAGCGAC	5.8	5.75
	XFFSL-R	CGCAATGCACACCTAAGCAA	5.8	5.75
	XFFSL-P	HEX-CGCGTACCCACTCACGCCGC-BHQ1	2	2
Primer/probe4				
X. fastidiosa subsp *pauca*	XFP-F	TGCGTTTTCCTAGGTGGCAT	5.8	5.75
	XFP-R	GTTGGAACCTTGAATGCGCA	5.8	5.75
	XFP-P	CY5-CCAAAGGGCGGCCACCTCGC-BHQ2	2	2
Water			46	
Total volume			100	

Table 17f: Reaction mixture for the multiplex qPCR assay described by Dupas et al. (2019) with the Harper qPCR inclusion modification.

Reaction master mix	Volume per reaction		Final concentration
Ultra pure water	3.48		
10X immobuffer	1		1X

Bovine Serum Albumin (50 µg/µL)	0.12		600 ng/µL
dNTPs (10 µM)	1		1 µM
MgCl2 (50 µM)	1.2		6 µM
Immolase 5U/µL	0.2		
10X primer probe mix	1		
Template	2		
total vol	10		

Table 17g: Reaction condition for the multiplex qPCR assay described by Dupas et al. (2019) with the Harper qPCR inclusion modification.

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95°C	3 mins	1
Denaturation	95°C	15 secs	x 40
Annealing	60°C	30 secs	

4.4.6 Multilocus Sequence Typing (MLST) of X. fastidiosa

Determining the sequence type of *X. fastidiosa* is important for diagnosis and biosecurity activities because it provides information on host range, origin and potentially pathway of entry. The primers and PCR recipe for the MLST are in Tables 18a and 18b. MLST involves the sequencing and analysis of seven housekeeping genes (*cysG, gltT, holC, leuA, malF, nuoL* and *petC*) described by Yuan et al., 2010. More details can be found on the MLST website of *X. fastidiosa* (http://pubmlst.org/xfastidiosa/).

MLST amplicons can be generated from bacterial cultures and or infected plant tissues. If erratic amplification occurs, the following PCR parameters can be adjusted: dilution of the DNA extract (to limit inhibition) or increase of DNA input, use of a different Taq polymerase/master mix, a decrease in annealing temperature from 65 °C to 60 °C or 58 °C or increase of primer concentration from 0.3 to 0.5 µM (recommendation from EPPO, 2018). The PCR amplification protocol has been adopted from EPPO 2018 and Tables 18a-c provide PCR conditions for amplification of the MLST genes. Run the PCR reactions as follows:

1. Make a reaction mix based on the composition detailed in Table 18b, one reaction mix for each primer pair (Table 18a; total of seven reaction mixes).
2. Run the PCR assays using the reaction conditions detailed in Table 18c.
3. After completion of the PCR reactions confirm the presence of amplicons of the correct size for sequencing by visualising on a 2% agarose gel using gel electrophoresis in TBE running for 40 min at 100V.

Table 18a: Primer sets used for Multi-Locus Sequence Typing (MLST) of *Xylella fastidiosa* described by Yuan et al. (2010)

MLST gene	Primer Name	Sequence (5'-3')	Product size
holC	holC-F	ATGGC ACGCG CCGAC TTCT	379
	holC-R	ATGTC GTGTT TGTTC ATGTG CAGG	
nuoL	nuoL-F	TAGCG ACTTA CGGTT ACTGG GC	557
	nuoL-R	ACCAC CGATC CACAA CGCAT	
gltT	gltT-F	TCATG ATCCA AATCA CTCGC TT	654
	gltT-R	ACTGG ACGCT GCCTC GTAAA CC	
cysG	cysG-F	GCCGA AGCAG TGCTG GAAG	600
	cysG-R	GCCAT TTTCG ATCAG TGCAA AAG	
petC	petC-F	GCTGC CATTC GTTGA AGTAC CT	533
	petC-R	GCACG TCCTC CCAAT AAGCC T	
leuA	leuA-F	GGTGC ACGCC AAATC GAATG	708
	leuA-R	GTATC GTTGT GGCGT ACACT G	
malF	malF-F	TTGCT GGTCC TGCGG TGTTG	730
	malF-R	GACAG CAGAA GCACG TCCCA GAT	

Table 18b: Reaction mixture for MLST PCR of *Xylella fastidiosa* described by Yuan et al. (2010)

Reagents	Stock Conc	Final Concentration	Volume
dH_2O			adjust
Taq DNA polymerase buffer	X10	1x	2.5 mL
MgCl2	50 mM	1.5 mM	0.75 mL
dNTPs	10 mM	200 µM	0.5 mL
Forward primer	10 mM	0.3 µM	0.75 mL
Reverse primer	10 mM	0.3 µM	0.75 mL
Taq polymerase	1 U/mL	0.2U	0.2 mL
DNA Template			adjust
Total			25 mL

Table 18c: Reaction condition for the multiplex qPCR assay described by Yuan et al. (2010).

Step	Temperature	Duration	Number of Cycles
Initial Denaturation	95°C	2 mins	1
Denaturation	95°C	30 secs	x 35*
Annealing	65°C	30 secs	
Extension	72°C	1 min	
Final extension	72°C	10 mins	

*Note that increasing the cycle number to 40 cycles may increase the detection of low-titre infections in host tissue.

The PCR products amplified from the MLST genes can be sequenced using either Sanger sequencing or nanopore sequencing devices such as Flongle or MinION. Unpublished validation work using the MinION device has demonstrated that this technique is 10 times more sensitive than the Harper et al., 2010 qPCR.

4.4.7 Multilocus Sequence Typing (MLST) of X. fastidiosa using Nanopore sequencing

The amplicon size of each of the MLST PCRs are confirmed by visualisation with agarose gel electrophoresis as described above (PCR conditions contained in Table 18 a to c) and then sequenced either on an Oxford Nanopore Technologies (ONT) Flongle or MinION device. Below the MinION method is described.

1. All seven PCR products for each sample are pooled, purified, and quantified using a NanoDrop spectrophotometer and Qubit fluorometers. The average amount of DNA needed for the library prep is 100-400 ng depending on the kit used for the library preparation.
2. Library preparation is done using either Ligation or rapid kit that enables barcoding for multiple samples. The products and protocols that require third party items are available at https://store.nanoporetech.com. The method described here is using Ligation kit SQK-109, and flowcell R9.4.1 but new chemistry is available to use Kit 14 and R10 flowcells for sequencing.
3. The sequence calling is done using the software for MinION, MinION Release 22.10.7 or above which can be downloaded from https://community.nanoporetech.com/downloads
4. The data requirement is up to 50-100 Mbp. With the optimum DNA quantity loaded on the device, a sequencing run of 2 hr is sufficient to obtain the recommended minimum of 2000 reads per run. The data requirement can be increased or decreased based on the library load, flow cell health and kit used for the library preparation.
5. Perform base-calling and barcode removal on the Fast5 files generated after sequencing. This step can be performed in real-time or after execution on the MinKNOW/.pod5 platform or using the command line-based tool 'guppy' (Version 6.1.)/dorado using the high accuracy mode https://community.nanoporetech.com/downloads#gns[searchValue]=guppy to get .fastq files.

The generated .fastq files are analysed using command line-based tools in a Linux environment. Conda or Miniconda needs to be installed before running most of these scripts. Refer to this link on how to install Conda or Miniconda on your computer. Basic knowledge of bash commands is also required for the protocols.

The detailed setup, dependencies, and usage of relevant analysis tools could be found on the following GitHub page: https://github.com/Pragya2019/Xylella_MLST_ONT. It also has an automated script to run analysis.

Two protocols are used to confirm results and can be found in Appendix 8.3. *Whilst a nanopore (HTS) method has been included in the NDP, please note that SPHD are currently in the process of defining guidelines for HTS for inclusion in NDPs. The NDP will be updated once these guidelines have been developed.*

5 CONTACTS FOR FURTHER INFORMATION

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The original protocol for *X. fastidiosa* was developed from the Pierce's Disease Draft Diagnostic Manual (Luck, J., Mann, R., Van Rijswijk, R., Moran, J. and Merriman, P. (2006)) and Pierce's Disease Pest Risk Review (2004), which was then updated by Fiona Constable, Agriculture Victoria Research, Department of Energy, Environment and Climate Action and from EPPO and IPPC documents. This new *Xylella* spp. NDP was adapted from the original *X. fastidiosa* NDP.

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8 APPENDICES

8.1 Generic *Xylella* endpoint PCR Sequences

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>Xylella_fastidiosa_fastidiosa-XComEC-amplicon_2

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>Xylella_fastidiosa_pauca-XComEC-amplicon_1

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CAGCATGACT

8.2 Using command-line-based tools for analysing .fastq file

The nanopore sequencing data can be analysed with three different methods as described by Wong-Bajracharya et al. (2024), briefly described below. The detailed setup, dependencies, and usage of relevant analysis tools could be found on the following GitHub page: https://github.com/chewbeckie/Xylella_pcrONT

***De novo* sequence clustering method**

1. Using .fastq files as input, tools such as NGSpeciesID, isONclust, or ONTrack can be used to generate *de novo* sequence cluster. These tools generate one or more consensus sequences based on sequence similarity.
2. Search for the consensus sequence against the nucleotide collection in NCBI using BLAST to determine the species identity. Alternatively, a customised blast database with the amplicon sequences for different species/subspecies *Xylella* can be used (all amplicon sequences are listed in Appendix 8.1)

Reference-guided consensus method

1. Using .fastq files as input and one of the known amplicon sequences (all amplicon sequences are listed in Appendix 8.1) as the reference, generate and polish the consensus sequence with medaka with the 'medaka_consensus' function.
2. Search for the consensus sequence against the nucleotide collection in NCBI using BLAST to determine the species identity. Alternatively, a customised blast database with the amplicon sequences for different species/subspecies *Xylella* can be used.

Alignment and Variant Calling Method

1. A variable calling tool, such as 'medaka_haploid_variant', can be used for species or subspecies identification using the amplicon sequence of each species or subspecies as a reference (all amplicon sequences are listed in Appendix 8.1).
2. The resulting sequence should be perfectly aligned with the reference without mismatch.

8.3 Protocols to confirm stringMLST results

PROTOCOL 1: analysis using Conda

StringMLST **https://github.com/jordanlab** is an assembly- and alignment-free, lightweight, platform-independent program capable of rapidly typing bacterial isolates directly from raw sequence reads. It works well with low read counts. It is the fastest and easiest tool to determine ST if the reads are accurate. Therefore, the requirement is to use the high accuracy model for base calling and demultiplexing. It will require installation with Conda..

Installation with conda
conda create --name stringMLST
conda activate stringMLST
conda install stringMLST

to run it requires a database to be built using –buildDB
download both alleles and MLST profiles to build it. Make a txt file as given below and run –buildDB

[loci]

leuA path_to_fasta/leuA.fa

petC path_to_fasta/petC.fa

malF path_to_fasta/malF.fa

cysG path_to_fasta/cysG.fa

holC path_to_fasta/holC.fa

nuoL path_to_fasta/nuoL.fa

glT path_to_fastap/glT.fa

[profile]

profile Path_to_profile.txt

stringMLST.py --buildDB -c Path_to_file/config.txt -k 35 -P NM

or download the MLST scheme directly from **https://github.com/jordanlab**

usage

stringMLST.py --predict -1 /path_to_fasta_or_fastq -s --prefix /path_to_StringMLSTdatabase -o /sampleST.txt

PROTOCOL 2: analysis using BLASTn

BLASTn- This method utilised two steps. First, make a database of MLST genes downloaded from https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadAlleles_using the script:

makeblastdb -in /path_to_fasta_file -dbtype nucl -title name_db -out name_db

Secondly, convert the fastq file to fasta:

seqkit fq2fa reads.fq.gz -o reads.fa.gz

and do blast with -max_hsps 1:

blastn -db /Path_to_/MLSTdatabase -num_threads 8 -task megablast -outfmt 6 -max_hsps 1 -max_target_seqs 1 -query /path_to_fasta | grep "malF_" | awk '{print $2}' | sort | uniq -c | sort -nk1 -r > malF_blast.txt

Sort and count the occurrence of malF for all genes and visualise output files for each gene to identify the most abundant allele. The correct allele should be in abundance. Once all the alleles are known, sequence type is determined from the *Xylella fastidiosa* MLST profiles present on the PubMLST website: https://pubmlst.org/bigsdb?db=pubmlst_xfastidiosa_seqdef&page=downloadProfiles&scheme_id=1

9 DIAGNOSTIC PROCEDURES TO SUPPORT SURVEILLANCE

9.1 Introduction

Xylella fastidiosa and Xylella taiwanensis are fastidious bacterial pathogens that primarily reside in the xylem tissue of plants. *Xylella fastidiosa* can infect over 700 host plants in the Americas and Europe and is reported to be expanding. *Xylella taiwanensis* has only been detected in Taiwan and is only known to cause disease in the Asian pear, *Pyrus pyrifolia.* Typical symptoms of *X. fastidiosa* include leaf scorch, defoliation, leaf wilt, and a general decline in vigour. Symptoms of *X. fastidiosa* are not diagnostic for many host plants, as they can resemble water stress, physiological damage, and symptoms of other diseases. Detection of *X. fastidiosa* is further complicated by the pathogen having a long latent period, surviving in plant xylem at low titre, and not all plant hosts that exhibit symptoms. *Xylella taiwanensis* causes symptoms of leaf scorch in pear leaves similar to those caused by *X. fastidiosa* (Su et al., 2016).

9.2 General Sampling Procedures

The concentration of *X. fastidiosa* in a plant depends on environmental factors, strain, and host plant species or cultivar. To maximise the likelihood of detection, sampling should be performed during the active growth period of the plant. *Xylella fastidiosa* is restricted to xylem tissue and therefore stem is the most reliable source for consistent detection. The section of the plant sampled, and the timing of sampling can also influence the successful detection of *X. fastidiosa*. Branch or cane samples with attached leaves that include mature leaves generally provide the most reliable results, and it is important to avoid the newest growing shoots. General guidelines are presented below:

- Each sample should include at least 8 x 15-20 cm long stem sections per tree
- Shake samples before bagging them to avoid spreading potential vectors of *X. fastidiosa*.
- Seal collected samples in a bag labelled with an identification code, which links to required surveillance data fields such as host, geographical coordinates, date, and presence or absence of symptoms, collector name.
- Disinfect pruning tools with 80% (v/v) ethanol before taking a sample from a new plant. While *Xylella* spp. has not been demonstrated to be transferrable on pruning tools, it is good practice in case other diseases are present.
- Complete sampling operations on site; transfer bags with individual samples into a larger bag labelled with sampling date, site, and team (or with a unique identifier that links back to required data fields).
- For optimum results, samples should be processed as soon after collection as possible. Checking capacity of the receiving laboratory is critical as samples need to be transported at temperatures between 15-28 °C within 3 days for isolation or 7 days for molecular testing.
- If sample transport duration is expected to be lengthy or unpredictable (e.g. from remote areas or from overseas) the stems can be preserved in 80% (v/v) ethanol for extended periods, noting this is only useful for DNA extraction. In this case, remove all leaf material so only the stem is remaining.

Both the EPPO (European Plant Protection Organization) and IPPC (International Plant Protection Consortium) protocols for the detection of *X. fastidiosa* consider molecular tests the most sensitive for the screening of symptomatic plant material (IPPC, 2018; EPPO, 2018). However, limited research is available to guide the sampling and testing of asymptomatic plants where the titre of *X. fastidiosa* is likely to be lower.

For in-field detection, during an outbreak, it is recommended that only symptomatic tissue be sampled. Sampling recommendations for a range of hosts are summarised in Table S9.1.

9.3 Sampling of Symptomatic Plants

The sample should consist of branches or cuttings representative of the suspect symptoms. For symptomatic plants, the material should be taken from areas close to symptoms but not from necrotic tissues (Valentini et al., 2017).

The approximate weight needed for laboratory samples is between 0.5 and 1 g of xylem tissue, leaf petioles or midribs from each plant (EPPO, 2018). For in-field testing, most extraction techniques use approximately 100 mg of tissue, which may only allow one to five petioles or midribs to be pooled; however, tissue from more than one leaf is recommended.

Table S9.1: Recommendations for tissue sampling of various host plants for the detection of *Xylella* spp.

Host	Best time for sampling	Types of samples	Reference
Polygala spp.	Late spring to early autumn	Stems only	IPPC, 2018; EPPO, 2018
O. europaea and *N. oleander*	Summer	Stems only	IPPC, 2018; EPPO, 2018
Prunus spp.	During summer	Stems only	Aldrich et al., 1992
Citrus fruits	Late spring to early autumn	Citrus fruit peduncles, symptomatic leaves including petioles and stems	Rossetti et al., 1990
Dormant woody plants	Year round	Mature branches and woody cuttings	IPPC, 2018; EPPO, 2018
Blueberry	Early autumn	Stem section only and roots fine roots	Holland et al., 2014
Indoor plants e.g. coffee	All year round	Stems only	IPPC, 2018; EPPO, 2018

9.4 Sampling of Asymptomatic Plants

If asymptomatic plants are tested, the sample should be representative of the entire aerial part of the plant, from the four cardinal points at different levels and include non-herbaceous twig parts, and/or mature leaves with petioles from woody twigs (Valentini et al., 2017). Recent experimental data on the detection of *X. fastidiosa* in monumental and ancient trees of *O. europaea* indicate detection was more reliable when sampling the middle-upper part of the canopy (EPPO, 2018), so consideration will be needed for gaining access to taller trees if required. For testing individual asymptomatic plants, the number of branches to be collected should be between four and ten, depending on host species and the size of the plant.

In-field testing of asymptomatic plants is not recommended due to inconsistent distribution and low *X. fastidiosa* titre in plant tissue and lower diagnostic sensitivity achieved with in-field testing methods.

There is limited knowledge about the sampling of asymptomatic host plants of lesser agricultural importance, with research focusing on key hosts of economic importance such as grapevines and olives.

9.5 Sampling from Insects

DNA may be extracted from a single insect head or a pool of five heads (Bextine et al., 2004; EPPO, 2018). Only the heads of insects are used because they contain the foregut and mouthparts where *X. fastidiosa* resides (Bextine et al., 2004). For DNA extraction from insects with big heads (e.g. *Cicadella viridis*, *Cicada orni*), only a single head should be used. For small insects (e.g. spittlebugs) whole insects can be used.

9.6 Sample Transport and Storage in the Laboratory

Once collected, samples should be kept cool (4-28 °C) and transported as soon as possible before the plant tissues deteriorate. *Xylella fastidiosa* does not survive well at cold or hot temperatures and it is better to process samples immediately for accurate detection. It is recommended that samples are processed no longer than 3 days from the collection if isolation will be attempted and up to 7 days for other testing types (i.e. molecular). For longer-term storage, processed samples may be stored as homogenate at −20 °C or −80 °C for molecular diagnostics. If plant samples originate from areas where infected vectors can occur, it is recommended to check if insects are present in the sample before opening the bags to avoid the release of infected vectors. If any insects are present, samples should be stored in the freezer (−20 °C) for approximately 12 h prior to opening.

9.7 In-Field Tests

Two in-field tests, a loop mediated isothermal amplification (LAMP) assay (Harper et al., 2010, erratum 2013) and a commercial recombinase polymerase amplification (RPA) assay from Agdia have been recommended for inclusion in the NDP after extensive validation under Australian conditions. Assessment of Harper et al., (2010) LAMP assays and the commercial Agdia RPA assay "AmplifyRP® XRT+ for Xf" identified that *X. taiwanensis* was also detected with both tests reported to be specific for *X. fastidiosa* and both can be used as generic in-field triage tools. However, it is only recommended for use with symptomatic hosts based on the European experience.

The LAMP assay (Appendix 8.4) is performed in real time using a portable fluorescence reading device (e.g. Genie II or III instruments from Optigene). The primer mix for the LAMP assay is made in the lab in 1:8:4 ratio of B3-F3/ BIP-FIP/ LF-LB, respectively according to Tables 16a-16b in section 4.4.4.

"Alternative molecular methods for the laboratory detection of *Xylella fastidiosa*" or the commercial Optigene Xf LAMP kit (contains both Isothermal master mix and ready to use primes mix) (Appendix 8.5) can be ordered. Two Isothermal reagents from Optigene are available; DR004 which completes the assays in 20 mins and DR001 which takes 30 mins to finish. Note the time to positive is important when using these two master mixes. The LAMP is 10 times less sensitive than the Harper qPCR.

9.7.1 DNA extraction for LAMP assay

9.7.1.1 Extraction from plant hosts using Plant Lysis kit (Optigene)

The Plant Lysis Kit is recommended for plant DNA extraction for use in LAMP, based on the comparison of extraction methods.

(1) Materials, equipment, kits and reagents required.

The Plant Lysis kit (Optigene) includes the following components:

- Bijoux with ball baring
- Lysis buffer
- Transfer loops (10 µl)
- Dilution buffer

- Pipette (100-1000 µL)
- Filter tips (1000 µL)
- Scalpel blades
- Cleaning – 80% (v/v) ethanol

Figure S9.1: Plant Lysis Kit components

(2) Procedure (Figure S9.2)

1. Transfer 1 mL of lysis buffer to the red capped bijoux. *Note: this can be done before heading into the field.*
2. Add plant tissue to the lysis tube.
3. Close the lid firmly and shake the tube by hand for 1 min.
4. Use the 10 µL loop to transfer homogenised tissue extract from the bijoux to the tube labelled 'dilution buffer' and mix the loop into the buffer.
5. The sample in dilution buffer serves as the template for the LAMP reaction.

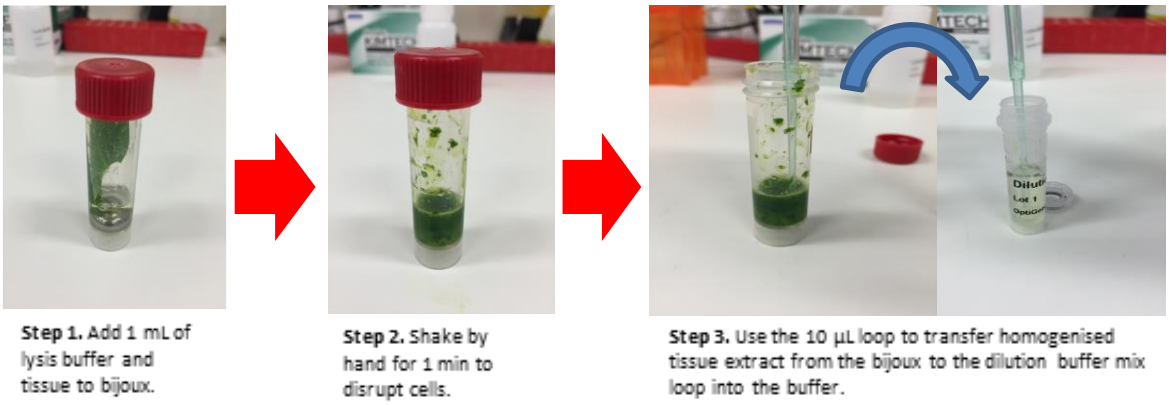


Figure S9.2: Steps of DNA extraction using the Plant Lysis Kit

9.7.1.2 Extraction from insect hosts using QuickExtract DNA

QuickExtract DNA solution (Lucigen) is recommended for insect DNA extraction for use with LAMP based on comparative extraction method results and validation across a range of insect genera generated by AVR Projects and publications. These include:

- AJIF Horticulture Development Plan (HDP) CMI 106199
- Phylloxera LAMP (AVR project CMI 106128)
- Brown Marmorated Stink Bug LAMP (AVR project CMI 106136)
- Khapra Beetle LAMP (AVR project CMI 106136)
- Varroa mites LAMP (AVR project CMI 106136)
- Blacket et al., 2020

(1) Materials, equipment, kits and reagents required

- Pipette (20-200 µL)
- Filter tips (200 µL)
- 0.2 mL LAMP strip tubes
- QuickExtract DNA solution
- Scalpel blades
- Cleaning – 80% (v/v) ethanol
- Genie III machine or heat block

(2) Procedure

1. Transfer 100 µL of QuickExtract solution into 0.2 mL LAMP strip tubes *Note: this can be done before going out into the field.*
2. Sever the heads of insects using a scalpel blade and add 1-5 heads to the QuickExtract DNA solution using forceps and close the lid. *Note: the number is dependent on the size of the insect. For small insects (<7mm) the whole specimen can be added to the mix.*
3. Transfer the strip tube to the Genie III or heat block and run it at 65 °C for 6 min followed by 98 °C for 2 min (Total run time = 8 min).
4. The resulting solution can now be used directly as DNA template in LAMP tests.

9.7.2 LAMP testing for X. fastidiosa

(1) Materials, equipment, kits and reagents required

- Filter tips (10 µL, 20 µL, 200 µL and 1000 µL)
- Pipettes (0.5-10 µL, 2-20 µL, 20-200 µL, 100-1000 µL and 8-channel 10 µL multichannel)
- Genie III machine
- 0.2 mL LAMP strip tubes
- Isothermal Mix (In OptiGene kit)
- Sterile molecular grade water
- Gloves
- 1.5 mL Eppendorf tubes
- Positive control
- Sample DNA (from extractions)
- PCR grade water
- Xf LAMP primer mix (preparation instructions below)
- COX LAMP primer mix (preparation instructions below)

9.7.3 Specific LAMP detection of Xylella fastidiosa (Table S9.2)

A positive and negative amplification control should be included in the testing.

A housekeeping LAMP (procedure described in section 4.4.4) must be run in parallel on plant host extracts to ensure extracted DNA can be amplified.

Table S9.2: Reaction set up for the LAMP assays used to detect *Xylella fastidiosa*

Component	Per reaction (25 µL)	X number of reactions
Sterile water	3 µL	
Isothermal Mix	15 µL	
LAMP Primer Mix (prepared as instructed in Table 16b).	5 µL	
Template (extraction)	2 µL	
Reaction total	25 µL	

Note: when calculating for the master mix add a minimum of three reactions to the number of samples (positive and negative controls, spare)

1. Make master mix and mix thoroughly by slowly pipetting 10 times.
2. Aliquot 15 µL of master mix into each tube of the LAMP strip tubes.
3. Add 3 µL of sterile water to the negative control tube and close the lid.
4. Add 2 µL of the template (DNA extraction) into the reaction tubes and close the lids.
5. Add 2 µL of the positive control DNA into the positive control tube and close the lid.
6. Load the LAMP strip tube into the Genie III and run the below protocol, this protocol can also be saved as a template in the machine.

9.7.4 LAMP Protocol

Run at 65 °C for 30 mins, followed by an annealing step of 98 °C to 80 °C (ramp rate of 0.05 °C/sec)

(1) Result interpretation

If target DNA is present, dsDNA will be amplified and an increase in fluorescence from the dsDNA intercalating dye will be visualised on the Genie III. A positive amplification plot will show a sigmoid curve reflecting the increase in fluorescence detected (Figure S9.3). A negative result will remain relatively flat.

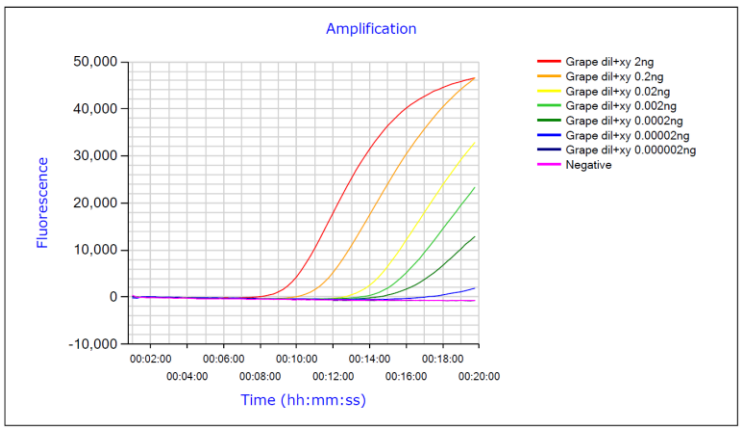
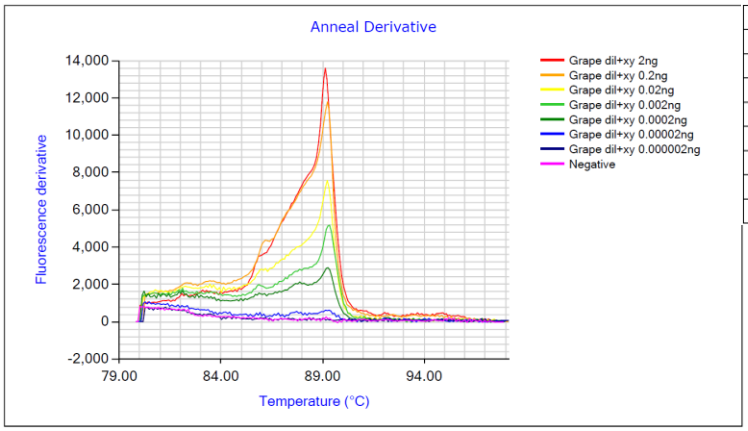


Figure S9.3: LAMP amplification rate data for six positive (sigmoid) amplification curves (red, orange, yellow, green, dark green, and blue) and two negative amplifications (flat) (navy and pink). The assay was done using isothermal mix DR004 therefore the amplification was recorded until 20 mins.

As confirmation of amplification of the correct target, an anneal step is performed after the isothermal step. Each target will have its own signature anneal temperature, which is related to the GC content of the generated amplicon (Figure S9.4). Positive amplification followed by an anneal temperature at the expected range is confirmation of a true positive in a sample.



Well Number	Well Name(°C)	Peak Value(°C)
1	Grape dil+xy 2ng	89.1
2	Grape dil+xy 0.2ng	89.2
3	Grape dil+xy 0.02ng	89.2
4	Grape dil+xy 0.002ng	89.3
5	Grape dil+xy 0.0002ng	
6	Grape dil+xy 0.00002ng	
7	Grape dil+xy 0.000002ng	
8	Negative	

Figure S9.4: Anneal derivative data of the amplification products generated in the LAMP reaction shown in Figure S9.3. There is a consistent annealing temperature peak value indicating that the nucleic acid composition of the products is the same, confirming a positive result. A temperature peak value different from that of a positive control would indicate off-target products.

The LAMP test will be considered valid only if all following criteria are met:

- The positive control produces a sigmoidal amplification curve with the *X. fastidiosa*-specific primers.
- Negative amplification control does not produce an amplification curve with the *X. fastidiosa*-specific primers.
- The extracted DNA template produces a sigmoidal amplification curve with the Cox housekeeping LAMP primers for plant extracts.
- Positive samples have the same annealing temperature as the positive control.

9.8 Field detection of *Xylella* species using the Agdia Amplify RP® XRT kit

9.8.1 Introduction

The Agdia Amplify RP® XRT kit for *X. fastidiosa* is an isothermal DNA amplification and detection system based on recombinase polymerase amplification (RPA). Detection of amplified DNA can be measured in two different ways depending on the resources available; real-time detection using a portable fluorometer, or at the end of the test (end-point detection) using lateral flow devices. The reaction kit, fluorometer, and amplicon detection chambers (which includes the lateral flow strips) are commercially available from www.agdia.com:

1. Part Number XCS 34501/0048 (contains reaction pellets in tubes).
2. ADC 98800/0001 AmplifyRP amplifier detector chambers for endpoint detection.

For real-time detection, any portable fluorometer can be used; however, Agdia offers a device called an AmpliFire® Isothermal Fluorometer (AFR 60400) that is compatible with the tubes from the reaction kit.

Australian distributor

Department of Natural Resources & Environment Tasmania
New Town Laboratories 13 St John's Avenue New Town, Tasmania, 7008
Ph. (03) 61653243
Web https://nre.tas.gov.au/biosecurity-tasmania/plant-biosecurity/plant-diagnostic-services

The Agdia Amplify RP® XRT kit has been validated for use in Australia with a method comparison that found the test to be accurate, and 100 times less sensitive than qPCR and 10 times less sensitive than a LAMP or cPCR. This finding is consistent with previous studies (Waliullah et al., 2019). The sensitivity of the detection of isolates from the Xf subspecies was 10 times lower than the Harper LAMP, but essentially equivalent to the Optigene Xf LAMP kit and the protocol of Yaseen et al. (2015).

9.8.2 Materials, equipment, consumables and reagents

Details of all equipment, instrumentation, consumables and reagents required for the SOP.

- biosecurity waste bags/biohazard bags (waste disposal)
- Gloves
- 0.2 mL LAMP strip tubes
- Ethanol (70%; v/v)
- Scalpel blades
- Forceps
- Weighing machine (optional)
- 1000 µL filter tips
- 200 µL filter tips
- 20 µL filter tips
- Pipette Adjustable single channel 2 - 20 µL
- Pipette Adjustable single channel 20 - 200 µL
- Pipette Adjustable single channel 200 - 1000 µL
- 1.7 mL Eppendorf tubes
- Sterilised molecular grade water
- XCS 34501/0048 kit
- AFR 60400 -AmpliFire® OR Genie III LAMP
- ADC 98800/0001 AmplifyRP Amplicon Detection Chambers

The XCS 34501/0048 kit contains the following items:

XRT+ reaction pellets for Xf (48 individual tubes)
PD1 Pellet diluent (55 mL)
Amp1 extraction buffer (3 x 55 mL)

Microcentrifuge tubes (50)
Mesh extraction bags (50)

All kit components must be stored refrigerated (2 - 8 °C).
Before use, allow all kit components to warm to room temperature (18 - 30°C) for 20 to 30 min.

DNA extraction

Select 0.3 g or 4-5 petioles, 5-7 cm long, of suspect tissue and insert the sample between the mesh linings of the sample extraction bag (Figure S9.5). Add 3.0 mL of AMP1 extraction buffer to the tissue inside the bag. Extract the tissue by thoroughly macerating it with a blunt object such as a pen. Take 1 mL of PD1 diluent in a 1.5 mL tube and add 10 µl of the sample extract for testing. For maximum sensitivity, incubate for 30 min at room temperature before testing. *Note: This test was optimised using a 1:10 tissue-to-buffer ratio for sample extraction.

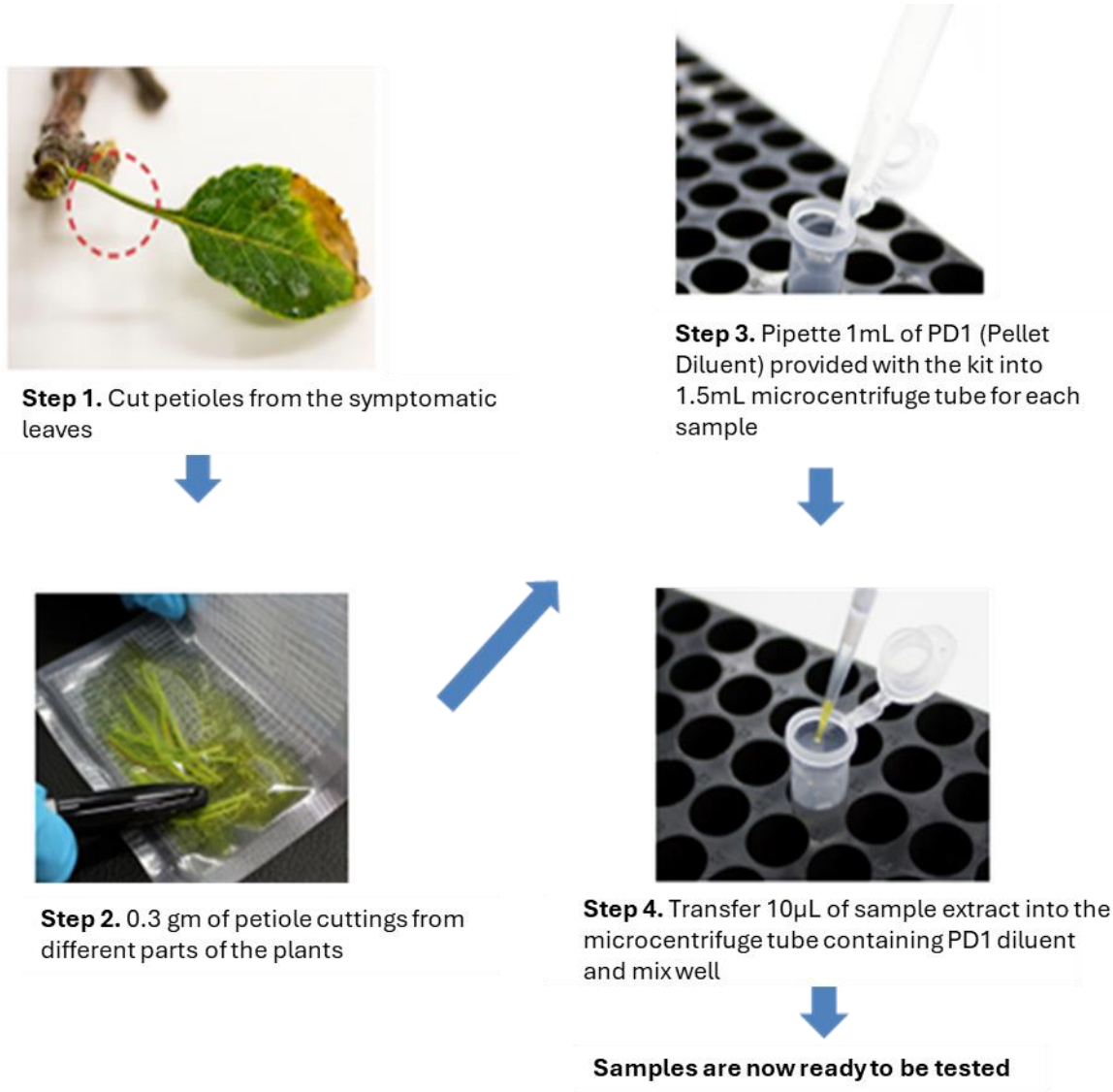


Figure S9.5: Steps involved in the extraction of DNA from plant hosts for *Xylella* detection using the Agdia Amplify RP® XRT kit

9.8.3 Detection

Detection of *Xylella* can be carried out in two ways depending on the available equipment.

1. Real-time detection using
   AmpliFire® provided from Agdia; or
   Genie III LAMP machine
2. End-point detection using lateral flow chambers provided by Agdia

1a. Real-time detection using AmpliFire

1. Press the 'Execute Reaction' button on the AmpliFire®. Then press 'Scan Product Code'.
2. (Optional) Scan the barcode found by following the hyperlink on page 1. The barcode scanner is located on the left side of the AmpliFire. Once the AmpliFire has accepted the scan and displayed the run method, click 'Next'.
3. Follow the prompts on the screen to name your reaction and individual sample IDs.
4. Remove a canister of reaction pellets from the white foil pouch labelled with the barcode. Then remove a strip of reaction pellets from the desiccated container. When securing the pellet strip on a 200 µL PCR tube rack, cut the number of reaction pellets from the strip that are intended for use.

Note: Reaction pellets are light-sensitive. The remaining reaction pellets are immediately placed back into the desiccated tube, and then the desiccant tube is inserted into the foil pouch to protect them from light.

5. Transfer 25 µL from the microcentrifuge tube containing the diluted sample extract to the reaction pellet (clear tube).
6. Recap the reaction tube tightly. Mix well and centrifuge. If you cannot vortex the reaction, mix by gently flicking the side of the tube. If you do not have a centrifuge available, you can manually shake the liquid to the bottom of the reaction tube.

Important: do not transfer more than the prescribed 25 µL during this step. Immediately proceed to the next step once the reaction has been rehydrated.

7. Press 'Start' on the AmpliFire. Immediately follow the instructions to add your reactions, press 'OK' and remove the lid.
8. After 4 min of incubation, remove the reaction(s) from the AmpliFire instrument. Quickly mix, spin, and reinsert the reaction(s) into the AmpliFire instrument to continue monitoring results. Take care to ensure that the tubes are in their original positions and orientations.
9. After 20 min of total run-time, the instrument will beep, indicating the test is complete. The test results will be visible next to the well designation on the screen (Figure S9.6) and should be interpreted as follows:
   (+) = Positive for Xf (-) = Xf not detected (!) = Invalid

Figure S9.6. Example test results displayed next to the well designation on the screen of an AmpliFire instrument. Results are interpreted as (+) = Positive for Xf (-) = Xf not detected (!) = Invalid

1b. Detection in a Genie III LAMP machine

1. Take out reaction pellets as described above in Step 4 based on the samples to be tested.
2. Follow steps 5, 6 and 8. Make sure you have the 'press to continue setting on (tick to wait box in the profile setup). After mixing and flicking down the sample, place back in the Genie and press continue (a red box should open)
3. To run samples on the Genie III LAMP machine, load a program for a constant temperature of 42 °C for 30 min.
4. Samples mixed with pellets are transferred to 8-well strips for Genie III compatibility.
5. Run the program.
6. The positive sample will produce an S-shaped amplification curve, while the negative result showed a flat line. The genie instrument also shows the results in real-time.

2. Endpoint detection using amplicon detection chambers

Amplification

1. Allow the heat block to warm to 42 °C before preparing reactions. If using an Agdia-supplied heat block, allow 2 to 3 min for this step.
2. Remove the reaction pellet strip from the desiccated container included in the kit. When securing the pellet strip on a 200 µL PCR tube rack, cut the number of reaction pellets from the strip that are intended for use. Immediately place the remaining reaction pellets back into the desiccated tube for later use.
3. Transfer 25 µL from the microcentrifuge tube containing the sample extract to the reaction pellet (clear tube).
4. Tightly recap the reaction tube. Mix well and centrifuge. If you cannot vortex the reaction, mix by gently flicking the side of the tube. If a centrifuge is not available, the liquid can be shaken manually to the bottom of the reaction tube.

Important: do not transfer more than the prescribed 25 µL during this step! The reaction immediately proceeded to the next step once the reaction was rehydrated.

5. Add the reaction to the portable heat block for 4 min. After 4 min of incubation, remove the reaction from the heat block. Quickly mix, spin, and reinsert the reaction into the heat block for an additional 16 min.
6. Immediately remove the reaction from the heat block and proceed to the detection steps.

Detection

Important: To avoid possible contamination of future tests, do not open the reaction pellet.

Step 1. Open the foil pouch containing the amplicon detection chamber (ADC). There are two pieces to the chamber, as indicated in Figure S9.7 to the right.

Step 2. Add the unopened reaction tube to the reaction apparatus as per the manufacturer's directions. Once the tube has been added, snap the apparatus shut, which will immobilize the reaction tube.

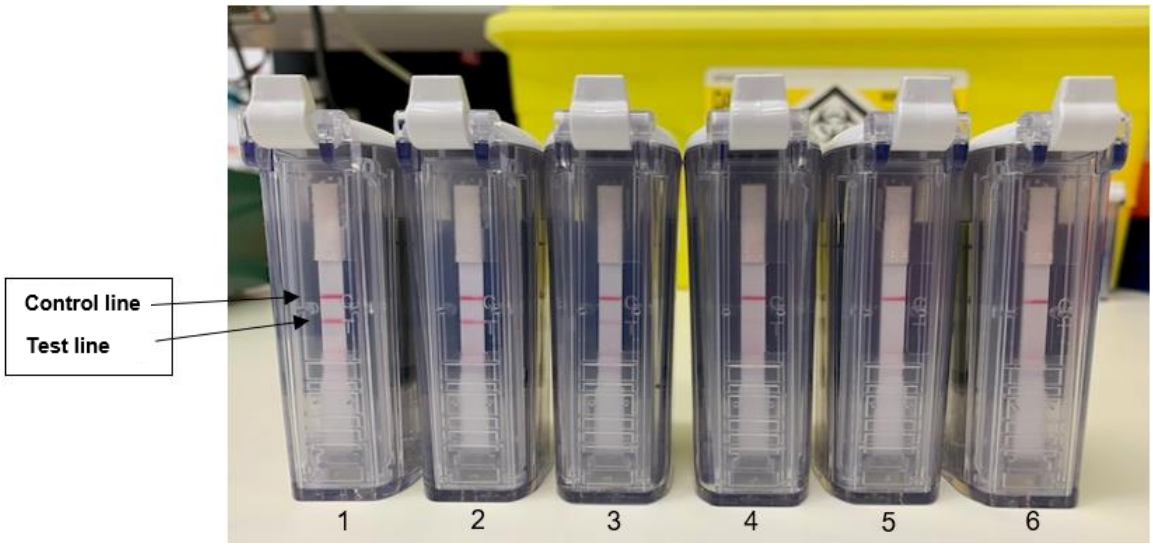
Step 3. Add the reaction apparatus to the housing of the detection chamber as indicated in the manufacturer's directions. IMPORTANT: The reaction tube should be facing toward the lateral flow strip, contained in the housing, during this step.

Step 4. Push down on the handle of the detection chamber housing until it shuts off. Wait 20 minutes before interpreting the results. Positive results may be visible in as little as 5 to 10 minutes. Samples containing lower copy numbers may take up to 20 minutes to produce a positive test line.

Results interpretation

The chamber has lateral strips; the buffer starts to rise upwards as soon as the chamber is closed. A positive sample displays two lines, the upper line is a control line, whereas the lower line is the test line (Figure S9.7).

Figure S9.7. The lateral flow chambers 1-4, (4 with a faint test line) showed two positive lines whereas samples 5 and 6 had a single control line as negative.



Precautions/ Limitations

Reaction Volume: Care must be taken to ensure that the volume used to rehydrate the reaction is within +/- 10 % of the prescribed 25 µL mentioned in Steps 5 and 3 of the test protocols. Deviating outside this tolerance may result in a test failure.

Inhibition: Some plant species may cause inhibition with this assay if the sample extract is too concentrated. Follow the extraction and sample dilution steps carefully. Addition of sample extract to reaction pellet: It is important to add only the prescribed amount of sample extract to the pellet diluent tubes. Adding too much extract may cause a test failure.

Storage: The test results may be weak, or the test may fail if the storage instructions are not followed properly. The lyophilised test components must remain protected from light to prevent bleaching and sealed with desiccant when not in use to prevent moisture degradation, which may affect test results. Do not store pellets at temperatures greater than 42 °C, even for short periods, as this may cause a test failure. An important point to note is that a field test negative is not necessarily definitive and that any suspicious symptoms and any suspect positive detection should always be reference to the nearest diagnostic laboratory for diagnostic confirmation ASAP.

9.9 Laboratory Tests

Relevant laboratory tests can be found in Section 4 of the NDP for *X. fastidiosa.* These include PCR and direct isolation.

9.10 Acknowledgements

For the development of this report, considerable use was made of the information contained in the EPPO diagnostic protocol (EPPO, 2019) and the IPPC diagnostic protocol (IPPC, 2018).

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