National Diagnostic Protocol

Phytophthora ramorum

The cause of Sudden Oak Death

NDP 5 V2

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Purpose

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

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

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

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

Process

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

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at http://plantbiosecuritydiagnostics.net.au/sphd/sphd-reference-standards/

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 *Phytophthora ramorum* is current as at the date contained in the version control box below.

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Further information

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

Contents

1 INTRODUCTION

This diagnostic protocol provides technical information for the identification of *Phytophthora ramorum* Werres, De Cock, and Man in 't Veld, the causal agent of ramorum shoot dieback, ramorum blight and sudden oak death (Hansen *et al.*, 2002).

Phytophthora ramorum causes a leaf and shoot blight on many understory trees and shrubs and stem canker on oak trees (Figure 1).

Phytophthora ramorum was first described in 2001 as a pathogen of *Rhododendron* spp., *Viburnum* spp. and *Pieris* spp. but diseases caused by this pathogen had been observed in Europe since 1993 (Werres *et al.*, 2001) and in oak forests of coastal California since 1995 (Rizzo *et al.*, 2002). It has also been detected causing dieback on Japanese larch, *Larix kaempferi* (Webber et al 2010).

Figure 1. Symptoms of sudden oak death in Marin County, CA, USA (© Marin County Fire Department).

1.1 Host range

Phytophthora ramorum has a wide host range; it has been detected on plant species in over 70 genera representing 33 families. The host lists are being continually updated, and are not included in this protocol. An up-to-date list of hosts regulated in the USA is maintained on the APHIS website, https://www.aphis.usda.gov/aphis/ourfocus/planthealth/plant-pest-and-disease-programs/pests-and-diseases (accessed 21/03/2016). A host list can also be found on CABI: http://www.cabi.org/isc/datasheet/40991 (accessed 21/03/2016).

Some native plants in the southern hemisphere including a number of *Myrtaceae* and *Pittosporaceae* genera are susceptible to *P. ramorum,* based on field observations and pathogenicity tests. This includes *Eucalyptus gunnii* (Myrtaceae) (Brown, unpublished data), *Nothofagus obliqua* (Nothofagaceae) (Brown, unpublished data) and *Pittosporum undulatum* (Pittosporaceae, Huberli *et al.,* unpublished data).

2 TAXONOMIC INFORMATION

Taxonomic position: Chromista, Oomycota, Oomycetes, Peronosporales, Pythiaceae, *Phytophthora ramorum*

Kingdom: Chromista
Phylum: Oomycota
Class: Oomycetes
Order: Peronosporales
Family: Peronosporaceae
Genus: *Phytophthora*
Species: *Phytophthora ramorum*

Scientific Name: *Phytophthora ramorum* Werres, De Cock & Man in 't Veld
Common Names: Sudden oak death, ramorum shoot dieback, ramorum leaf blight.
Anamorph: None
Synonym: None

3 DETECTION

3.1 Symptoms

Diseases caused by *P. ramorum* can be classified by three distinct disease syndromes described by Hansen *et al.* (2002):

- Sudden oak death, characterised by lethal cankers;
- Ramorum shoot dieback, which results from foliar infection and/or direct infection of stems;
- Ramorum leaf blight, which results from foliar infection.

3.1.1 Sudden oak death

Diagnostic symptoms of the disease on large trees include cankers on the lower trunk that have brown or black discoloured outer bark and bleeding sap (Hong 2003;

Figure 2a). Sunken or flattened cankers may occur beneath bleeding areas; when the bark is removed from bleeding cankers, areas of necrotic, dead discoloured (black to dark brown) tissue with a distinct margin may be observed (

Figure 2b). Cankers develop before foliar symptoms become evident and, as the canker girdles the stem, the crown often appears to wilt and die rapidly. *Eucalyptus gunnii* and *Nothofagus obliqua* have been shown to exhibit similar symptoms (Brown, personal communication) (Figure 3).

Figure 2. Bleeding canker on tanoak infected with *Phytophthora ramorum* (left) and bark removed showing darkened areas of necrotic, dead discoloured phloem (arrows indicate distinct margin of lesion) (right) (© Dr Anna Brown, DEFRA, United Kingdom).

Figure 3. Bleeding canker on *Nothofagus obliqua* infected with *Phytophthora ramorum* in the United Kingdom and bark removed showing mottled areas of necrotic, discoloured phloem (inset) (© Dr Anna Brown, DEFRA, United Kingdom)

Similar symptoms: Bleeding cankers with dark-stained wood under the bark can occur on the trunks of several plant species caused by other species of *Phytophthora* including *P. kernoviae, P. syringae, P. citrophthora, P. cambivora, P. pseudosyringae, P. cinnamomi, P. citricola, P. cactorum* and likely others (Figure 4). Some of these species (e.g., *P. cinnamomi, P. citricola, and P. cactorum*) are known to colonize stem and root tissue, whereas *P. ramorum* has not been observed or detected in roots. However, this is not a reliable character for diagnosis. In contrast, cankers that may exude a black ooze may be caused by other species of *Phytophthora.*

There are other pests and pathogens that can cause bleeding cankers on hosts of *P. ramorum*. Species of *Armillaria* may cause bleeding cankers but can be readily identified by the presence of white mycelial fans under the bark (Figure 5a) and fruiting bodies also may be present (Figure 5b)

Other fungal pathogens that can cause bleeding cankers on eucalypts include *Cryphonectria cubensis* (Figure 6), *C. parasitica* and *Coniothyrium zuluense* (Figure 7).These pathogens cannot be readily distinguished from *P. ramorum* in the field.

Figure 4. Canker on chestnut (*Castanea sativa*) caused by *Phytophthora cinnamomi* in Victoria, Australia (©Biosciences Research Division, Department of Economic Development, Jobs, Transport and Resources)

Figure 5. Mycelial fans under bark (left) and fruiting bodies of *Armillaria* sp. (right) (©Biosciences Research Division, Department of Economic Development, Jobs, Transport and Resources)

Figure 6. Canker on *Eucalyptus grandis* caused by *Cryphonectria cubensis* (© Edward L. Barnard, Florida Department of Agriculture and Consumer Services)

Figure 7. Canker caused by *Coniothyrium zuluense* on *Eucalyptus* sp. in Kwazulu, South Africa (©FAO)

3.1.2 Ramorum shoot dieback and leaf blight

Symptoms of *P. ramorum* on shrubs and understory trees, such as *Rhododendron* spp., *Viburnum* spp., and *Umbellularia californica,* are shoot dieback and leaf blight (Figures 8, 9). Ramorum shoot dieback is characterised by blackened shoots with or without foliage attached. Symptoms of ramorum leaf blight include diffuse, brown to dark brown lesions which frequently occur at the leaf tip or elsewhere on the leaf where moisture has accumulated and encouraged infection (Figure 10). Eventually, entire leaves can turn brown to black and may drop prematurely. With the exception of *Viburnum* spp, *P. ramorum* usually does not kill shrub hosts.

Similar symptoms: As with sudden oak death, these symptoms are not unique to ramorum shoot dieback and leaf blight. Several other species of *Phytophthora*, including *P. nicotianae*, *P. citrophthora, P. heveae* and *P. kernoviae,* also may cause foliar symptoms similar to those of ramorum dieback. Additionally, species of *Colletotrichum, Botryosphaeria* and *Botrytis* and abiotic factors such as sunburn also may express similar symptoms (

Figure 11, 12).

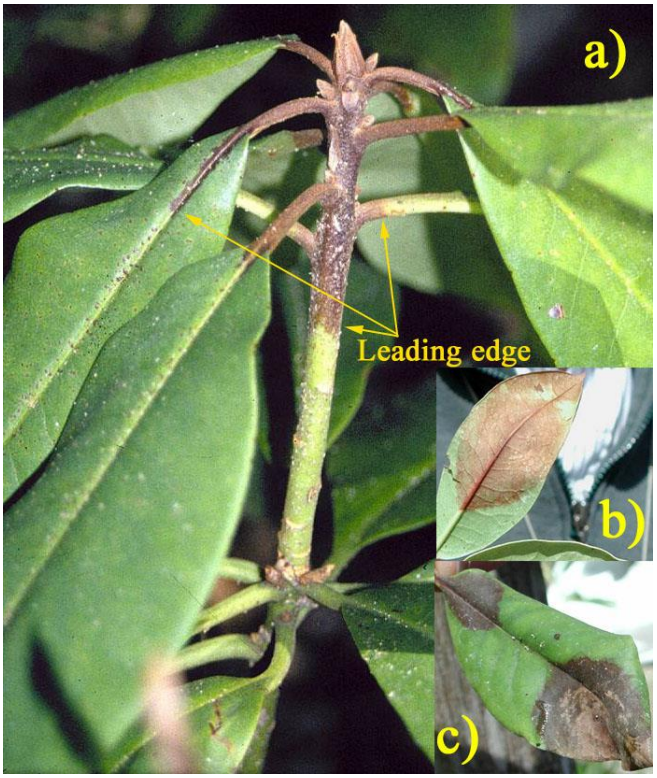

Figure 8. Ramorum shoot dieback and leaf blight: shoot of rhododendron with arrows depicting leading edge of infection (a) (©Everett Hansen, Oregon State University); abaxial side of rhododendron leaf (insert b) (© Bruce Moltzen, Missouri Dept. of Conservation); and adaxial side of tanoak leaf (insert c) (© Bruce Moltzen, Missouri Dept. of Conservation).

Figure 9. Shoot dieback of *Viburnum* sp.: shoot dieback (a) (Oregon Department of Agriculture); seedlings in pots killed by *P. ramorum* (b) (Oregon Department of Agriculture); and leaf symptoms (c) (Jennifer Parke, Oregon State University).

Figure 10. Leaves of California bay laurel (*Umbellularia californica*) infected by *Phytophthora ramorum* (© Joseph O'Brien, USDA-Forest Service)

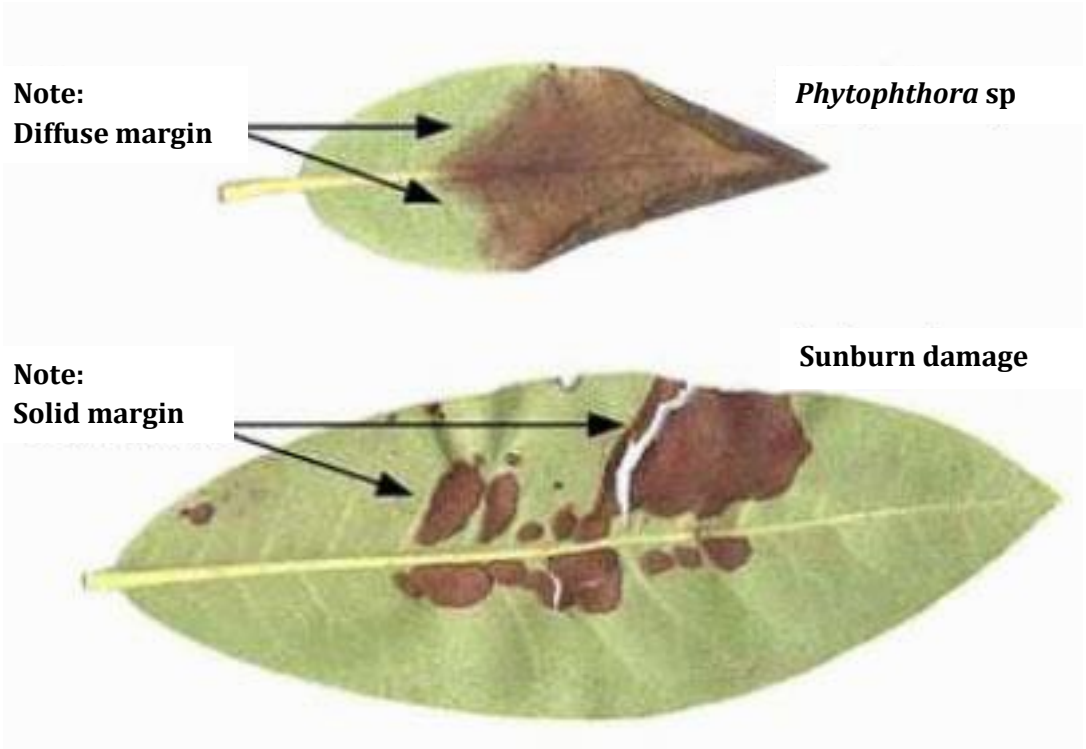

Figure 11. Comparison of leaf lesions on *Rhododendron* sp. caused by *Phytophthora* sp. compared to sunburn damage (© Tim Tidwell, CA Dept of Food and Agriculture)

Figure 12. Kino bleeding from the trunk of *Corymbia ficifolia* associated with *Botryosphaeria* infection in Victoria, Australia. (©Biosciences Research Division, Department of Economic Development, Jobs, Transport and Resources)

3.2 Detection in plant material

3.2.1 Sampling

Bleeding cankers

Remove the outer bark in the area directly around the oozing sap until the margin of the lesion is evident. Remove pieces of cambium (approximately 7-10 cm length and width and 2-4 cm thick) which capture the margin between healthy tissue and diseased tissue; sample from multiple areas around the canker and place in a sealed container for isolations in the laboratory. Ideally, wrap samples in damp paper towel to avoid desiccation. Additionally, small pieces (approximately 1 to 2 cm^3) from the same areas as described above also may be removed aseptically and embedded directly in an agar medium (preferably a semi-selective medium) (Rizzo *et al*., 2002).

Shoots and twigs

Remove a piece of shoot or twig which captures the leading edge of the lesion (Figure 8) and place in a sealed container. Allow up to 5-7 cm on either side of the leading edge or, if possible, remove the entire shoot to allow for isolations in the laboratory. Multiple samples from one plant are preferable. Place a damp tissue with each sample to prevent desiccation.

Leaves

Remove 4-6 leaves, if possible, with symptoms as described above. Note that not all hosts display the same symptoms; therefore, if unsure, collect a sample which adequately represents the symptoms observed. Place samples in a sealed container with a damp tissue.

3.2.2 Culturing

Surface-sterilization may not be necessary if aseptic techniques are used (i.e., cutting material directly from wood) or if a semi-selective medium is used. If a non-amended medium is used, then surface-sterilization is required. Submerge cut pieces (approximately 1 cm^2) in 0.5 % sodium hypochlorite for 2-5 min followed by a rinse in sterile water before embedding in agar medium; delicate tissues such as thin leaves should be submerged for less time than woody or stem tissues.

3.2.3 Serological testing

Serological testing can be used as a pre-screening technique for *Phytophthora* spp. There are several serological test kits that are commercially available from Agdia Inc and Forsite Diagnostics Ltd., Surrey, England. They all detect to the genus *Phytophthora* but not specific to *P. ramorum* and identification must be confirmed by another method (i.e., isolation or molecular assay). Instructions for use of each the test kits are provided by the manufacturer.

3.3 Detection in soil and water

P. ramorum can be detected in soil and water using traditional baiting techniques. As this is more commonly used for surveillance the sampling and detection methods are included in Appendix.

4 IDENTIFICATION

The EPPO diagnostic protocol for *P. ramorum* (EPPO Bulletin, 2006) recommends that for a positive identification, the pathogen should be identified unambiguously by any one of these three methods: morphological examination, real-time PCR or conventional PCR.

The USDA recommends ELISA pre-screen followed by PCR (http://www.aphis.usda.gov/plant_health/plant_pest_info/pram/).

4.1 Morphological methods

4.1.1 Growth characteristics and morphology

Phytophthora ramorum produces a unique collection of morphological characters which allows for ease of identification. Culture characteristics and morphological features are summarized below (Table 1) as described in Werres *et al.* (2001). Note that some characters may vary according to media type.

Table 1 Growth characteristics on a selective (P_5ARP(H))and non-selective (Carrot piece agar) medium.

Character	**P_5ARP(H) *- selective media**	**Carrot piece agar * - non-selective media**
Colony Figures 14a & b	relatively slow growing, approximately 2 mm per day	weak rosette-like pattern, pronounced concentric rings, growth rate approximately 3 mm per day
Mycelium Figure 14 c	weakly coralloid, growing within the agar with little superficial growth, no hyphal swellings	aerial mycelium sparse, no hyphal swellings
Sporangia Figures 13 e & f & 14a	produced abundantly on the agar surface, semi-papillate, caducous with short or no stalk. Size: 20-32 x 40-80 µm, average 24 x 52 µm; average length/width ratio 2.16	
	ellipsoid, frequently in small clusters and relatively narrow, initial sporangium commonly producing secondary, smaller sporangia	ellipsoid, spindle-shaped or elongated-ovoid, single or in clusters
Chlamydospores Figures 14 c & d	more common in older colonies (7-10 days), very large (up to 80 µm diameter), hyaline to pale brown to brown	after 3 days incubation in the dark, in the older parts but very often also in the young parts of the colony, thin-walled, hyaline to pale brown up to 88 µm

* Characteristics are based on observation at 20°C with 12 hour light and dark cycles on P_5ARP(H) after 4-6 days and on carrot-piece afar after 3-5 days.

Colony pattern on V8 agar is a rosette-like pattern with concentric rings (Figure 14b); rings may be more or less pronounced on other media. Aerial mycelium is sparse to absent on most media.

P. ramorum produces large (22 to 72 µm) chlamydospores that are mainly terminal (Figure 14c, 14d). Chlamydospores change from hyaline to cinnamon brown as they mature and are abundant when host material is present in the culture.

Sporangia are semi-papillate, caducous (Figure 14e, 14f, 13), and often form in clusters on the surface of the agar. If sporangia are not present in culture plates, plugs of colonized agar may be removed, placed on a sterile petri dish, and flooded with soil water (recipe in appendix) to induce production of sporangia. Plugs should be maintained at room temperature with adequate light and observed under the stereo microscope at 1 to 2 days after flooding for the presence of semi-papillate sporangia; examine the surface of the water for sporangia that have released from the sporangiophore.

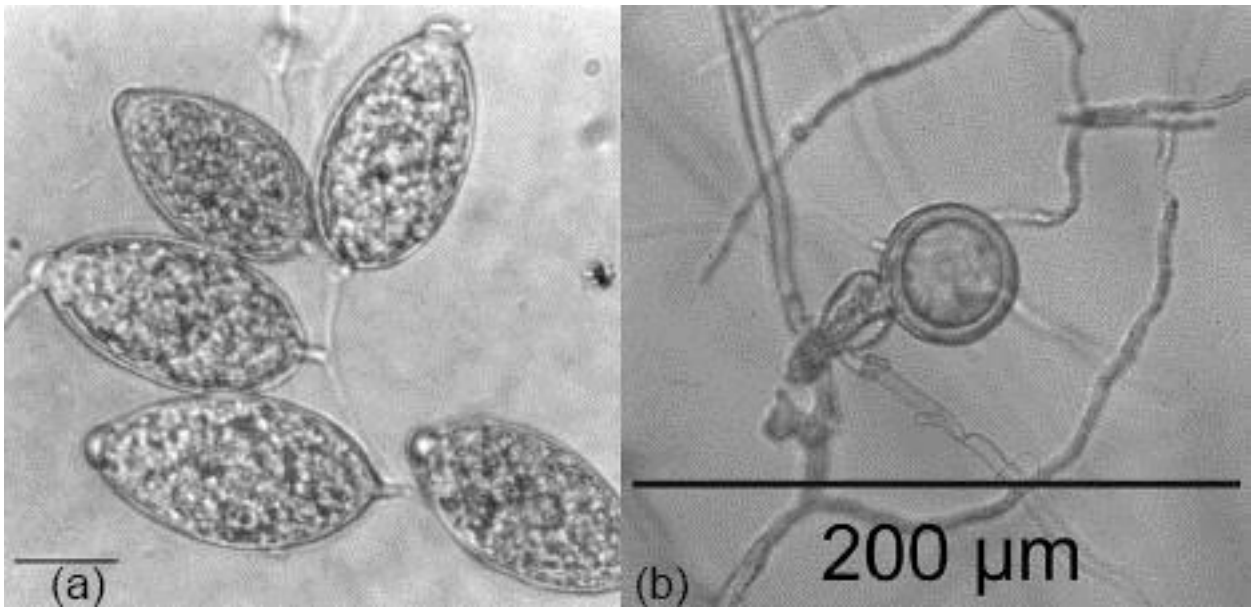

Figure 13. Semi-papillate sporangia (a)(Werres & Zielke 2003) and oogonium with oospore of *Phytophthora ramorum* (b) (Davidson *et al.*, 2003).

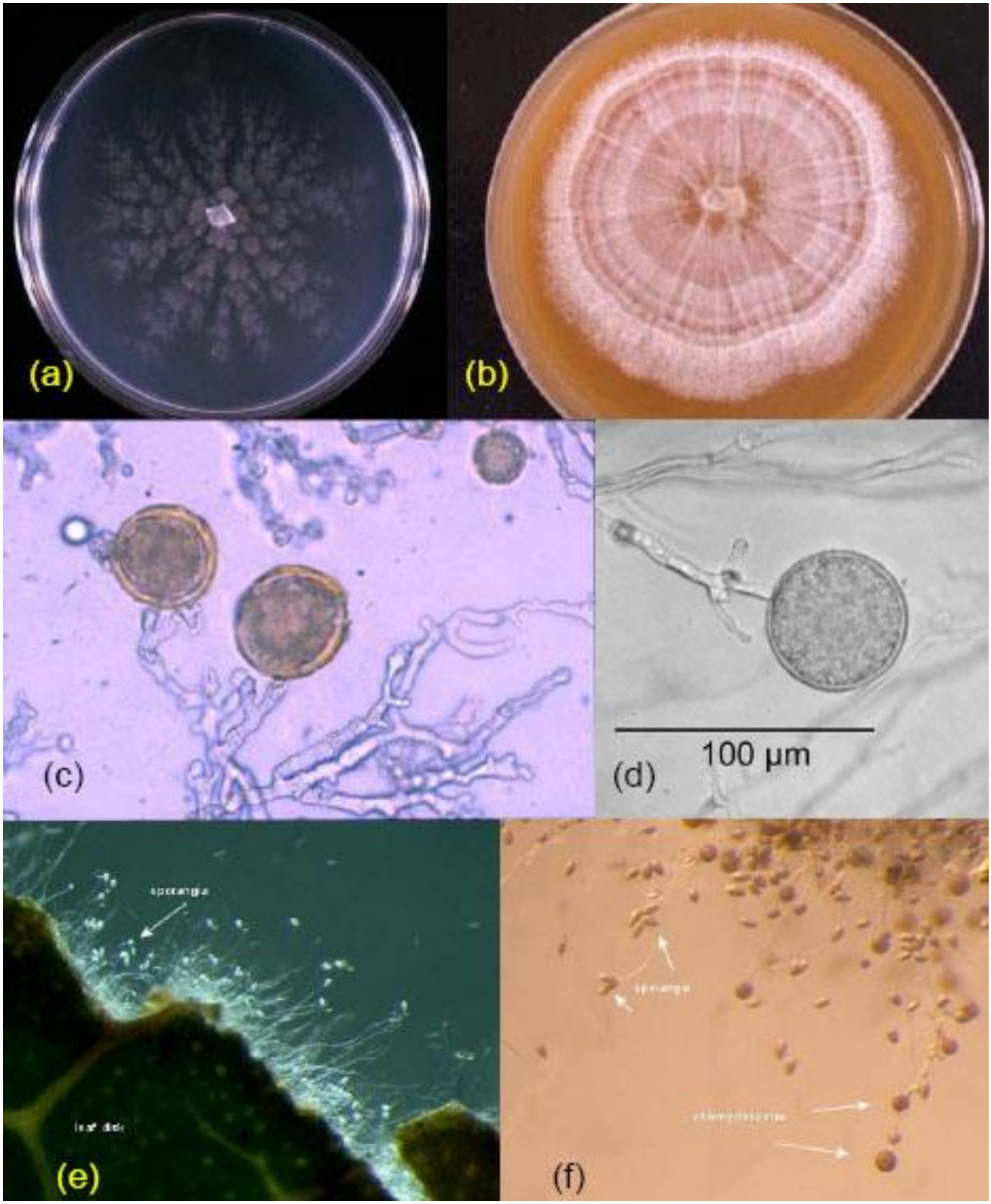

Figure 14. Characteristics of *Phytophthora ramorum*: colonies on water agar (a) and V8 juice agar after 15 days (b) (© USDA 2005); mycelium and chlamydospores (c, d)(© UC Davis and UC Berkeley); and sporangia (e, f) and chlamydospores (f) on the edge of a bay leaf disk at 20 hours (e) and 7 days (f) after inoculation (© J. L. Parke, Oregon State University).

4.1.2 Morphology of other Phytophthora species

Although *P. ramorum* has morphologically-distinguishable characteristics, other species of *Phytophthora* may be commonly isolated or may be confused with *P. ramorum*. Below are some brief distinguishing characteristics of these species (Brasier *et al.*, 2005; Erwin and Ribeiro, 1996; Gallegly and Hong 2008).

Phytophthora cinnamomi forms coralloid hyphae with characteristic grape-like clusters of chlamydospores produced laterally on the hyphae. Sporangia of *P. cinnamomi* are non-papillate, persistent, and sparse in soil extract solution.

Phytophthora lateralis is the closest-known relative of *P. ramorum* but is easily distinguished from *P. ramorum* because the geographic and host ranges of *P. lateralis* are limited to *Chamaecyparis lawsoniana* and *Taxus* spp., in the Pacific Northwest of the USA and parts of the UK. *Phytophthora lateralis* is homothallic (produces oospores in culture), forms abundant chlamydospores laterally on hyphae, and forms non-papillate sporangia.

Phytophthora nemorosa is morphologically similar to *P. ilicis* but both can be distinguished from *P. ramorum* by the presence of oospores in culture with amphigynous antheridia and deciduous, semi-papillate sporangia. *Phytophthora nemorosa* causes symptoms similar to *P. ramorum* and has a similar host range (Hansen *et al.*, 2003).

Phytophthroa kernoviae causes symptoms similar to *P. ramorum*, but forms caducous, papillate sporangia that are sometimes asymmetric, and produces oospores with amphigynous antheridia in culture.

Note: Of the species listed, only *P. cinnamomi* has been detected in Australia; the remainder (*P. lateralis*, *P. ilicis*, *P. kernoviae* and *P. nemerosa*) are not known to be present in Australia at this time and therefore are less likely to be encountered. However, there are numerous other species of *Phytophthora* present that may be encountered. A list of these, their known distribution and host species are provided in Burgess et al (2009), Brasier et al (2005), Erwin & Ribeiro (1996), Gallegly & Hong (2008) and Irwin et al (1995).

4.2 Molecular methods.

The real-time PCR method developed by Hughes *et al.* (2006) and included in the EPPO Diagnostic Protocol (EPPO 2006) is the recommended method as it is reliable, sensitive, and efficient. The test includes universal internal control primers to ensure the DNA extraction was successful.

The conventional PCR method included here (Lane *et al* 2003b) and verified in Australia is also included in the EPPO diagnostic protocol (EPPO 2006). Other conventional PCR methods are included in the EPPO protocol (Kox *et al*., 2002; Wagner & Werres, 2003) and a method for *P. lateralis* has been published (Winton and Hansen, 2001) which is able to detect *P. ramorum*.

The conventional PCR primers amplify DNA from *P. lateralis* and *P. hibernalis;* however, the former has not been reported in Australia and both organisms are unlikely or seldom to be encountered on hosts of *P. ramorum*. The real-time PCR primers also amplify DNA from *P. lateralis* but only at high concentrations that are unlikely to be encountered in plant material. Positive results from either method may be confirmed by sequencing the ITS region (Section 4.2.3).

4.2.1 DNA extraction

A 0.5 cm x 1 cm sample from a test culture is cut aseptically, or several small pieces of tissue from the leading infection edge of suspect plant material are removed and placed in a thick-walled plastic bag.

The bagged sample is placed in liquid nitrogen. Once the sample is frozen, the bagged sample is put on the laboratory bench and ground by rolling the bag with a wallpaper seam roller or similar device. Alternatively, samples may be ground up by cutting them into small pieces and placing these in a 2 mL centrifuge tube containing approximately 150 mg siliconised 0.5 mm glass beads (Biospec products, Bartlesville, USA). The tube is closed with a screw-fitting lid containing an o-ring and the tube is oscillated in a Mini-Beadbeater (Biospec products) at full power for at least 20s.

DNA is extracted from ground-up samples using a commercial kit such as DNeasy® Plant mini Kit (Qiagen), a kit such as the NucleoSpin plant extraction kit (Macherey-Nagel, Düren, DE, Cat. ref. 740 570.250), or a more traditional method such as described in Hughes *et al*. (2000). Extracted (neat) DNA is stored at 4°C for immediate use or at -20°C if testing is not to be performed on the same day.

4.2.2 Identification at species level by real-time PCR (Hughes et al., 2006)

The following method may be used for TaqMan®-PCR identification of *P. ramorum* from cultures and plant material. Internal control primers should be used when plant material is tested directly; this is not necessary when using pure cultures. The internal primers and probe are based on sequences by Weller *et al*. (2000) and amplify plant DNA present in the test extracts. Their use confirms that amplifiable DNA is present in test extracts from plants which are PCR- negative for *P. ramorum*.

Primers/ TaqMan®- probe: the primer sequences are:

Pram 114-FC: 5' TCA TGG CGA GCG CTG GA 3',
Pram 1527-190-R: 5' AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT 3',

and the TaqMan®- probe is :

Pram 1527-134-T: 5' TTC GGG TCT GAG CTA GTA G 3'.

The TaqMan®-probe is labelled at the 5' end with the fluorescent reporter dye 6- carboxyfluorescein (FAM) and at the 3' end with the quencher dye, 6-carboxytetramethyl- rhodamine (TAMRA).

DNA from samples is prepared at approximately 20-100 ng/µl.

Amplification and analysis

In optical quality reaction tubes/plates (Applied Biosystems) at least two replicate reactions for each test sample and control samples of known *P. ramorum* DNA (positive control) and water (negative control) are prepared.

The reaction mixture (25 µL) should contain: 12.5 µL of 2 X Taqman Universal master mix (Applied Biosystems); 1.5 µL 5 µM primer Pram 114-FC; 1.5 µL 5 µM primer Pram 1527-190-R; 0.5 µL 5 µM probe Pram 1527-134-T; 1.0 µL c. 20-100 ng DNA test suspension; 8.0 µL sterile molecular grade water.

Test reactions are cycled in a suitable instrument for detection of reporter fluorescence, for example an ABI Prism 7700 or 7900 Sequence Detection System (Applied Biosystems) using the following conditions: 10 min at 94°C; then 40 cycles of 15 s at 94°C and 60 s at 60°C.

Assessment of PCR

Data from the TaqMan® run are analysed as per manufacturer's instructions. Samples with cycle threshold (Ct) values less than 36 are considered as positive for *P. ramorum*, typically Ct values are between 25 and 35. A Ct value of 36 indicates a negative result.

Using internal control primers

Internal control primers should be used when plant material is tested directly; this is not necessary when using pure cultures. The internal primers and probe are based on sequences by Weller *et al*. (2000) and amplify plant DNA present in the test extracts. Their use confirms that amplifiable DNA is present in test extracts from plants which are PCR- negative for *P. ramorum*.

Internal control primers: the primer sequences are:

COX-F 5' CGT CGC ATT CCA GAT TAT CCA 3', and

COX-RW 5' CAA CTA CGG ATA TAT AAG RRC CRR AAC TG 3'

N.B. Primer COX-RW contains degenerative nucleotides indicated by the IUPAC code R, indicating that both adenine and guanine are inserted at these positions in equal amounts.

Internal control TaqMan®- probe:

COX-P 5' AGG GCA TTC CAT CCA GCG TAA GCA 3'

The TaqMan®-probe is labelled at the 5' end with the fluorescent reporter dye VIC (Applied Biosystems) and at the 3' end with the quencher dye TAMRA.

Amplification and analysis

Test reactions and positive/ negative controls are prepared using the master mix as described below, and each sample is cycled as described above for testing cultures by TaqMan® PCR.

The reaction mixture (25 µL) should contain: 12.5 µL 2 X Taqman Universal master mix (Applied Biosystems); 1.5 µL 5 µM primer Pram 114-FC; 1.5 µL 5 µM primer Pram 1527-190-R; 0.5 µL 5 µM probe Pram 1527-134-T; 1.0 µL 5 µM primer COX-F; 1.0 µL 5 µM primer COX-RW; 0.5 µL 5 µM probe COX-P; 1.0 µL c. 20-100 ng DNA test suspension; 5.5 µL sterile molecular grade water.

Assessment of PCR

Samples containing amplifiable *P. ramorum* DNA produce FAM fluorescence as recorded by Ct FAM values of < 40. These samples may also produce VIC fluorescence as recorded by Ct values of < 40 as should all other samples NOT containing *P. ramorum* DNA. VIC fluorescence indicates that the COX primer/probe set has amplified viable DNA present in the test sample. If neither FAM nor VIC fluorescence is recorded this indicates that the sample contains no amplifiable DNA and that sample should be re-extracted and tested again.

4.2.3 Identification at species level by conventional PCR (Lane et al 2003b)

The following protocol is for the conventional PCR identification of *P. ramorum* from cultures and plant material.

Primers

A primer pair (Pram F1 and Pram R1) has been developed by Hughes (Lane *et al.*, 2003b) for conventional PCR. The primer sequences are:

Pram F1 : 5' CTA TCA TGG CGA GCG CTT GA 3' and
Pram R1 : 5' GAA GCC GCC AAC ACA AG 3'.

Amplification and analysis

Extracted DNA is defrosted if necessary and a ten-fold dilution of each extract is prepared in sterile molecular grade water. Then in an area dedicated for PCR work and using dedicated pipettes with filtered tips, enough reaction mix for testing at least two replicates of the neat and ten-fold dilution for each extract is prepared. For each PCR run positive control reactions of master mix plus *P. ramorum* DNA and negative control reactions of reaction mix loaded with water rather than DNA are included.

The reaction mixture (25 µL) should contain: 1.0 µL DNA suspension; 2.5 µL 10 X reaction buffer containing 15 mM $MgCl_2$ (Applied Biosystems); 2.0 µL 10 mM dNTPs; 2.5 µL 5 µM of each primer Pram F1 and Pram R1; 0.125 µL AmpliTaq (Applied Biosystems) (5 U/µl), and 14.375 µL sterile molecular grade water to give a final volume of 25 µL.

Amplification is performed in thin-walled PCR tubes in a PCR thermocycler programmed as follows: 2 min at 94°C; then 30 cycles of 30 s at 94°C, 30 s at 57°C, 30 s at 72°C. One cycle for 10 min at 72°C should be conducted after the 30 cycles. After amplification, 10 µL from the cycled reactions is mixed with 2 µL of loading dye (25 µg bromophenol blue and 25 µg xylene cyanol FF in 10 mL 50 % glycerol) and amplification products are resolved by electrophoresis on a 1.5 % agarose gel made with 1X TBE buffer at pH 8.0 (9.0 mM Tris, 8.9 mM boric acid and 2.5 mM EDTA). At least one replicate of a 100 base pair (bp) marker is added to each gel for amplicon size determination. Following electrophoresis,

stain the gel for 30 min with ethidium bromide [0.5 µg/mL] then wash off excess stain and view the gel on a UV transilluminator.

Assessment of PCR

Reactions containing amplifiable DNA from *P. ramorum* produce a single c. 700 bp amplicon while no bands should be produced for the negative controls. Following extensive testing, some isolates of other *Phytophthora* species simultaneously amplify two bands, one between 100 and 500 bp and the second at c. 700 bp. Samples should only be considered positive for *P. ramorum* if a single 700 bp band is amplified. As DNA concentration can affect PCR amplification it may be that only one concentration of positive test samples is amplified, this is normal and the reason why two concentrations of test DNA are tested. If neither concentration is amplified, the DNA should be tested with the universal ITS primers ITS1 and ITS4 (White *et al*., 1990), and their cycling conditions described below ('Identification by sequencing part of the ITS-region'). Amplification with these primers shows that the test DNA is of an amplifiable quality and that a true negative for *P. ramorum* has occurred. However, if amplification is still not produced, fresh DNA should be extracted and retested.

4.2.4 Identification at species level by sequencing part of the ITS region

Morphological identification of *P. ramorum* in culture can be confirmed by sequencing the ITS region. Only DNA from pure isolates can be tested using this method, otherwise sequences from multiple organisms may be amplified in the same reaction.

The verified method outlined here uses ITS1/ITS4 regions. The combination of ITS6/ITS4 also amplifies DNA from *Phytophthora* (Cooke and Duncan, 1997, Cooke *et al,* 2000)

The primer sequences are

ITS 1 : 5' TCC GTA GGT GAA CCT GCG G 3' and

ITS 4 : 5' TCC TCC GCT TAT TGA TAT GC 3' (White *et al*., 1990).

Amplification and analysis

The reaction mixture should contain: 1.0 µL DNA suspension; 10.0 µL 10 X reaction buffer containing 15 mM $MgCl_2$ (Applied Biosystems); 8.0 µL 10 mM dNTPs; 10 µL5 µM of each primer ITS 1 and ITS 4; 0.5 µL Taq polymerase (Applied Biosystems) (5 U/µL), and 60.5 µL sterile molecular grade water to give a final volume of 100 µL.

Amplification is performed in thin-walled PCR tubes in a PCR thermocycler programmed as follows: 2 min at 94°C; then 30 cycles of 1 min at 94°C, 1 min at 53°C, 1.5 min at 72°C. One cycle for 10 min at 72°C must be conducted after the 30 cycles. Samples are resolved on a 1.5 % agarose gel as previously described. Using this method samples containing *Phytophthora* DNA produce single amplicons of c. 900 bp in size.

Sequencing of amplicons

The remaining product from positive test reactions is purified using a suitable PCR purification kit such as QIAquick PCR purification kit (Qiagen, Crawley, GB, Cat. ref. 28106) following the manufacturer's instructions. Send samples for two-way sequencing with forward primer ITS1 and reverse primer ITS4. Finally, consensus sequences are compared for test samples with those on

GenBank (www.ncbi.nlm.nih.gov). The sequence should differ by no more than 2 bases from authentic sequences those on GenBank.

The type strain is 'CBS 101553' (see 6. Reference Material) and the GenBank number for the ITS region for this strain is 'HQ643339'.

5 CONTACTS FOR FURTHER INFORMATION

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Central Science Laboratory, YO41 1LZ York, England, GB (fax: 44 1904 462111, tel: 44 1904 462000, e-mail: c.lane@csl.gov.uk, or k.hughes@csl.gov.uk)

BBA, Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Horticulture, Messeweg 11/12, D- 38104, Braunschweig, DE. (fax: 49 531 299 3009, tel: 49531 299 4407, e-mail: S.Werres@bba.de)

5.1 Reference material

Reference cultures (Not available in Australia)

Type strain of *P. ramorum*: BBA 9/95 (A1) = CBS 101553. Available from BBABraunschweig, DE (Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Protection in Horticulture, Messeweg 11/12, D- 38104, Braunschweig, DE)), or from CBS, Utrecht, the Netherlands.

For DNA repository see: http://www.biosecuritybank.com/

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7 REFERENCES

Brasier CM, Beales, PA, Kirk, SA, Denman S, and Rose J. (2005) *Phytophthora kernoviae* sp. nov., an invasive pathogen causing bleeding stem lesions on forest trees and foliar necrosis of ornamentals in the UK. Mycological Research 109, 853–859.

Brasier C and Kirk S. (2004) Production of gametangia by *Phytophthora ramorum* in vitro. Mycological Research 108, 823–827.

Burgess TI, Webster JL, Ciampini JA, White D, Hardy GES, and Stukely MJC. (2009) Re-evaluation of *Phytophthora* species isolated during 30 years of vegetation health surveys in Western Australia using molecular techniques. Plant Disease 93(3), 215–223.

Colburn G, and Jeffers S. (2011) Use of real-time and nested PCR to detect *Phytophthora ramorum* in infested nursery container mixes and soils. Phytopathology 101:S38.

Cooke DEL, and Duncan JM. (1997) Phylogenic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. Mycological research 101, 667–677.

Cooke DEL, Drenth A, Duncan JM, Wagels G and Brasier CM. (2000) A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genetics and Biology 30,17–32.

Davidson J, Werres S, Garbelotto M, Hansen EM, and Rizzo DM. (2003) Sudden Oak Death and Associated Diseases caused by *Phytophthora ramorum*. Online journal 'Plant Management Network'. www.plantmanagementnetwork.org/pub/php/diagnosticguide/2003/SOD

EPPO (2006) *Phytophthora ramorum*. EPPO Bulletin, 36, 145–155. doi: 10.1111/j.1365-2338.2006.00927.x

Erwin DC, and Ribeiro OK. (1996) *Phytophthora* diseases worldwide. APS Press, St Paul, USA.

Gallegly ME, and CX Hong. (2008) *Phytophthora*: Identifying Species with Morphology and DNA Fingerprints. APS Press. 158 pp. ST. Paul, MN USA.

Hansen E, Sutton W, Parke J, and Linderman R. (2002) *Phytophthora ramorum* and Oregon forest trees – One pathogen, three diseases. Abstract in proceedings of "Sudden Oak Death, a Science Symposium", December 2002, Monterey, CA (US) pp. 78.

Hansen EM, Reeser P, Davidson JM, Garbelotto M, Ivors K, Douhan L, and Rizzo DM. (2003) *Phytophthora nemorosa*, a new species causing cankers and leaf blight of forest trees in California and Oregon, USA. Mycotaxon 88, 129–138.

Hayden KJ, Rizzo D, Tse J, and Garbelotto M. (2004) Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay. Phytopathology 94, 1075–1083.

Hong C. (2003) Sudden Oak Death. Virginia Cooperative Extension Publication Number 450–801. http://www.ext.vt.edu/pubs/plantdiseasefs/450-801/450-801.html

Hughes KJD, Tomlinson JA, Griffin RL, Boonham N, Inman AJ, and Lane CR. (2006) Development of a One-Step Real-Time Polymerase Chain Reaction Assay for Diagnosis of *Phytophthora ramorum*. Phytopathology 96, 975–981.

Irwin JAG, Cahill DM, and Drenth A. (1995) *Phytophthora* in Australia. Australian Journal of Agricultural Research 46, 1311–1337.

Kox L, de Gruyter J, Garbelotto M, van Brouwershaven I, Admiraal J, and Baayen RP. (2002) Validation of a PCR method for detection and identification of *Phytophthora ramorum*. Abstract in proceedings of "Sudden Oak Death, a Science Symposium", December 2002, Monterey, CA (US) pp. 57–58.

Lane CR, Barnes AV, Beales PA, Griffin RL, Hughes KJD, Inman AJ, Townend, VC, Brasier CM, and Webber JF. (2003b) First report of *Phytophthora ramorum* in the UK. ICPP Paper 19.35, 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2-7th February 2003. ISBN 086476 151-1.

Rizzo DM, Garbelotto M, Davidson JM, Slaughter GW, and Koike ST. (2002) *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Disease 86, 205–214.

Wagner S, and Werres S. (2003) Diagnosmöglichkeiten für *Phytophthora ramorum*. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 55, 245–257.

Webber JF, Mullett M, and Brasier CM. (2010) Dieback and mortality of plantation Japanese larch (*Larix kaempferi*) associated with infection by *Phytophthora ramorum*. New Disease Reports 22, 19. [doi:10.5197/j.2044-0588.2010.022.019]

Weller SA, Elphinstone JG, Smith NC, Boonham N, and Stead DE. (2000) Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time flurogenic PCR (TaqMan) assay. Applied and Environmental Microbiology 66, 2853–2858.

Werres S, and Zielke B. (2003) First studies on the pairing of *Phytophthora ramorum*. Journal of Plant Diseases and Protection 110, 129–130.

Werres S, Marwitz R, Man in 't Veld WA, de Cock AWAM, Bonants PJM, De Weerdt M, Themann K, Ilieva E, and Baayen RP. (2001) *Phytophthora ramorum* sp. nov., a new pathogen on Rhododendron and Viburnum. Mycological Research 105, 1155–1165.

White TJ, Bruns T, Lee S, and Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols, a guide to methods and applications. Eds. Innis MA, Gelfand DH, Sninsky JJ and White TJ. Academic Press, London (GB). pp 315–322.

Winton LM, and Hansen EM. (2001) Molecular diagnosis of *Phytophthora lateralis* in trees, water, and foliage baits using multiplex polymerase chain reaction. Forest Pathology 31, 275–283.

8 APPENDICES (OPTIONAL)

8.1 Recipes cited in protocol

***Vegetable juice agar* (V8)**: vegetable juice 250 mL; $CaCO_3$ 5 g; agar 15 g; distilled water 1000 mL. Add $CaCO_3$ to the vegetable juice and stir firmly during 15 min. Centrifuge the mixture for 20 min at 5000 rpm, and pour off the supernatant. Make up the resultant to 1 L with distilled water, and autoclave at 120°C for 20 min.

P_5ARPH (Jeffers & Martin, 1986): cornmeal agar 17 g; distilled water 1000 mL. Autoclave, then cool to 50°C in a water bath. Then prepare pimaricin 5 mg; ampicillin (Na salt) 250 mg; rifampicin (dissolved in 1 mL 95% Ethanol) 10 mg; PCNB 100 mg; hymexazol 22.5 mg and dissolve all in 10 mL sterile distilled water. Add to cooled media, pour, store at 4°C in the dark, use within 5 days.

Note: Most species of *Pythium* are inhibited by hymexazol which typically grow faster than *Phytophthora* spp. and, therefore can make isolation of *P. ramorum* challenging. However, as hymexazol can be difficult to obtain, it may be excluded from the recipe.

Carrot Piece Agar (Werres *et al*., 2001): agar 22 g, carrot pieces 50 g, distilled water 1000 mL.

Soil water: Soil water is prepared by agitating 400g of sandy soil in 1L of water overnight; the extract should be filtered using cheesecloth and autoclaved.

8.1.1 References

Jeffers SN, and Martin SB. (1986) Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease 70, 1038–1043

Werres S, Marwitz R, Man in 't Veld WA, de Cock AWAM, Bonants PJM, De Weerdt M, Themann K, Ilieva E, and Baayen RP. (2001) *Phytophthora ramorum* sp. nov., a new pathogen on Rhododendron and Viburnum. Mycological Research 105, 1155–1165.

8.2 Sampling Methods

8.2.1 Soil

Collect a composite sample (i.e., collect several scoops of soil from around a tree or block of plants into one bag) of approximately 1 L of soil (including debris) from affected areas; samples should be collected in a sealable plastic bag and placed inside a second bag to contain any leakage.

Mix each soil sample before sub-sampling for baiting to ensure a uniform sample. Place approximately 100 ml of soil in a plastic container (approximately 500-750 ml) and add approximately 200 ml sterile, deionized water. Place 4-6 pieces of rhododendron leaf (approximately 1 cm^2) on the water. Alternative baits may be used as mentioned above (Section 3.3.1.2.1). After three days at room temperature (20-22°C), remove baits, blot dry on a paper tissue, and embed in an appropriate medium.

8.2.2 Water

Water samples can be collected from any type of water body where *P. ramorum* is suspected including river or stream water, run-off water (e.g., from plant nurseries), ditches, and puddles. Collect a minimum of 1 L of water from each sampling area; allow any sediment or debris to remain in the bottle. Samples should be kept in a cool ice chest (4-10°C) and should be processed within 48 hours.

Alternatively, bodies of water can be baited *in situ* for an extended period of time (i.e., several days to two weeks depending on lesion development). This method is preferred as, in theory, the baits are exposed to more water. However, this method requires a longer sampling time and two visits to the baiting site (deployment and retrieval) rather than one visit for *in vitro* baiting.

Baiting

Water samples can be baited either *in vitro* (in the plastic bottle) or *in situ* (in place). For both methods, rhododendron leaves, preferably *Rhododendron* hybrid cv. 'Cunningham's White'**,** should be used; however, if these are not available whole pears or leaves of *Viburnum* spp. can be used (NPDN 2006; Themann & Werres, 1998; Themann, *et al.*, 2002). Leaves should be taken from plants which have not been treated with fungicides and are known to be healthy. Entire, one-year-old leaves are preferable as succulent new growth is more susceptible to colonization by species of *Pythium* and older leaves are more likely to be colonized by other organisms.

***In vitro* baiting:** Place 1-2 entire leaves of rhododendron in each water sample. Leaves may be wounded by trimming 1 cm long cuts perpendicular to the margin of the leaf so as to hasten infection. Incubate the sample at room temperature (20-22°C) for 3 days. Symptoms of infection by Phytophthora species include water-soaked areas and brown lesions; suspect areas should be cut out for isolation onto non-amended or semi-selective media as described above. Bait tissues also may be used for real-time PCR (Colburn and Jeffers, 2011).

If you are using Themann's protocol, then specify entire leaves. In some protocols, one side of the leaf is wounded with ~1-cm cuts perpendicular to the margin of the leaf along one side of the leaf. They are easily sliced off and embedded in agar (Hwang et al., 2008).

***In situ* baiting:** Place 5-10 healthy leaves of rhododendron into a small piece of muslin (or similar porous material like flywire) and pieces of polystyrene or similar material to aid flotation. Draw the material up into a bag, tie with string and float on the water for 1 to 2 weeks (Figure 1). Bait bags can be produced using various methods (Hwang *et al.*, 2008) When bags are retrieved, leaves may be wiped down with a dry tissue to remove sediment that obscures observation of the symptoms. Remove 4-6 pieces of symptomatic tissue (approximately 1-2 cm^2) from each leaf and embed in an appropriate medium.

Filtration

As an alternative to baiting, water may be filtered to detect *Phytophthora*. Vacuum-filter aliquots of 100 mL of sample water through nitrocellulose or polycarbonate membrane filters with 5-µm pore size (Sterlitech Corp., Kent, WA, USA). For each aliquot, remove the filter paper and invert onto a semi-selective medium (e.g. P_5ARP[H]). Repeat until the entire sample has been assayed.

Figure 15. Rhododendron leaf baits and bags used in stream monitoring for *Phytophthora ramorum* in the USA. (Murphy *et al.* 2005).

8.2.3 References:

Colburn G, and Jeffers S. (2011) Use of real-time and nested PCR to detect *Phytophthora ramorum* in infested nursery container mixes and soils. Phytopathology 101:S38.

Hwang J, Oak SW, and Jeffers SN. (2008) Detecting *Phytophthora ramorum* and other species of *Phytophthora* in streams in natural ecosystems using baiting and filtration methods. *In* "Proceedings of Sudden Oak Death Third Science Symposium", March 2007, Santa Rosa, CA, USA. pp. 55.

NPDN (2006) National Plant Diagnostic Network. Standard Operating Procedure for Plant Diagnostic Laboratories *Phytophthora ramorum. Draft 5.5.* http://spdn.ifas.ufl.edu/Phytophthora%20ramorum-Draft%205.5.doc.

Themann K, and Werres S. (1998) Verwendung von Rhododendronblättern zum Nachweis von Phytophthora-Arten in Wurzel- und Bodenproben. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 50, 37–45.

Themann K, Werres S, Diener H-A, and Lüttmann R. (2002) Comparison of different methods to detect *Phytophthora* spp. in recycling water from nurseries. Journal for Plant Pathology, 84, 41–50.