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DP 23: *Phytophthora ramorum*

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ISPM 27

Diagnostic protocols for regulated pests

DP 23: *Phytophthora ramorum*

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CONTENTS

1. Pest Information	3
2. Taxonomic Information	4
3. Detection.....	4
3.1 Symptoms.....	4
3.1.1 Bleeding canker.....	4
3.1.2 Shoot dieback	5
3.1.3 Leaf blight	5
3.1.4 Needle blight	5
3.2 Sampling and sample preparation	5
3.2.1 Plant material	5
3.2.2 Water	
3.2.3 Soil or plant debris	6
3.3 Detection by serological methods	6
3.4 Isolation and culture from symptomatic or asymptomatic material	6
3.4.1 Isolation from plant samples	6
3.4.2 Isolation from water samples	7
3.4.3 Isolation from soil or plant debris samples	7
3.5 Isolation media	8
3.6 Detection by molecular methods.....	8
3.6.1 Preparation of material	9
3.6.2 DNA extraction	9
3.6.3 Conventional PCR.....	9
3.6.4 Real-time PCR	11
3.6.5 Controls for molecular tests	13
4. Identification.....	14
4.1 Morphological identification.....	15
4.1.1 Cultural characteristics and morphology.....	15
4.2 Molecular identification	16
4.2.1 ITS sequencing for species level identification using the primers of White <i>et al.</i> (1990)	16
4.2.2 Controls for molecular tests	18
5. Records	18

6. Contact Points for Further Information	18
7. Acknowledgements	18
8. References	18
9. Figures	22

1. Pest Information

Phytophthora ramorum Werres, de Cock and Man in't Veld, 2001 is an oomycete pathogen of unknown origin (Brasier *et al.*, 2004). It is considered to have been introduced into western North America and western Europe in the late twentieth century by the ornamental plant trade (Prospero *et al.*, 2007; Mascheretti *et al.*, 2008; Goss *et al.*, 2011; Grünwald *et al.*, 2012; Van Poucke *et al.*, 2012). *P. ramorum* attacks a wide range of trees and shrubs in nurseries and in the field, causing leaf blight, stem cankers, bleeding stem lesions and dieback.

In North America, the pathogen was found in the early 1990s causing mortality of *Quercus* (oak) trees and *Lithocarpus densiflorus* (tanoaks), mainly in California and Oregon (Rizzo *et al.*, 2002). Named “sudden oak death” (SOD), this disease has now reached epidemic proportions in North America. The pathogen was originally considered as affecting only woodland plants but since 2003 nursery plants in several states of the United States of America have been affected. The disease has also been found in Canada (CABI, n.d.).

In Europe, *P. ramorum* has been observed in Germany since 1993 causing twig blight of rhododendron in nurseries and on mature bushes in gardens. In the Netherlands, it was found in 1998 on diseased *Viburnum* sp. (Werres and Marwitz, 1997; Werres *et al.*, 2001). The pathogen has now been recorded in more than 20 European countries, predominantly on ornamental plants in nurseries and in a few managed gardens. In 2009, however, *P. ramorum* was unexpectedly found infecting and killing large numbers of *Larix kaempferi* (Japanese larch) trees in southwest England. Heavy dieback and mortality of plantation *L. kaempferi* trees in western Britain and Northern Ireland have resulted in the felling of 600 000 trees (Brasier and Webber, 2010; Webber *et al.*, 2010).

This unexpected finding emphasizes that although many of its hosts are known, *P. ramorum* still poses a substantial threat to tree species and other ecologically important plants such as heathland species. The pathogen is, however, most commonly observed on *Camellia*, *Magnolia*, *Pieris*, *Quercus* (in particular the red oak species *Q. acuta*, *Q. agrifolia*, *Q. cerris*, *Q. chrysolepis*, *Q. ilex* and *Q. rubra*), *Rhododendron* and *Viburnum*. Disease symptoms, recent findings and lists of the known hosts for *P. ramorum* can be found in CABI (n.d.), COMTF (n.d.) and USDA-APHIS (n.d.).

P. ramorum has a complex life cycle and is adapted to cool temperatures, with 20 °C being optimal. Although *P. ramorum* is soil-borne, deciduous, asexually produced sporangia are formed on the surface of infected leaves or twigs on some hosts and, depending on environmental conditions, are locally splash-dispersed or spread over long distances by wind and wind-driven rain (Davidson *et al.*, 2005). Rivers, streams and other waterways can also carry the sporangia and thus spread the pathogen (Defra, 2007). Sporangia that land on suitable hosts germinate to produce hyphae. In the presence of water, sporangia will release motile zoospores that encyst on the host surface, germinate and penetrate the host tissue, forming a colony from which more sporangia are produced. These sporangia repeat the cycle and with enough repetitions, under favourable environmental conditions, an epidemic can ensue. Different asexual spores, chlamydospores, are produced in abundance within infected plant tissue and allow *P. ramorum* to survive adverse conditions in infected stems and leaves on the plant, in plant debris on the soil surface, or in the soil (Grünwald *et al.*, 2012).

P. ramorum is a heterothallic species and may produce sexual oospores, but this requires both mating types. No evidence exists that natural crossing of these mating types has occurred in nature although crossing has been achieved in the laboratory (Brasier and Kirk, 2004). Currently, mating type A1 is the predominant type in Europe while A2 is the predominant type in North America (Werres and Kaminski, 2005). There are four clonal lineages known, with the first three designated as: NA1 (mating type: A2; distribution: North America; environment: forest and nurseries); NA2 (mating type: A2; distribution: North America; environment: nurseries); and EU1 (mating type: predominantly A1, rarely A2; distribution: Europe and North America; environment: nurseries and gardens) (Grünwald *et al.*, 2009). The fourth, a new lineage designated as EU2, was discovered recently in Northern

Ireland and western Scotland and is associated in particular with *L. kaempferi* (Van Poucke *et al.*, 2012).

2. Taxonomic Information

Name:	<i>Phytophthora ramorum</i> Werres, de Cock and Man in't Veld, 2001
Synonyms:	None
Taxonomic position:	Chromista, Oomycota, Peronosporae, Peronosporales, Peronosporaceae
Common names:	Sudden oak death (SOD), ramorum leaf blight, ramorum shoot dieback and sudden larch death
Reference:	MycoBank MB#474485

3. Detection

Laboratory studies have shown that the time between foliage infection and visible disease expression is typically between 3 and 14 days, depending on host and temperature. However, the period may be longer in the field and on different plant parts (Defra, 2007). Leaves selected at random can be checked for surface contamination or latent infection by baiting (section 3.4.2) or molecular methods (section 3.6). The use of fungicides in the field can make it more difficult to detect infected plant material by isolation on agar media (Hamelin *et al.*, 2000; Shishkoff, 2014). Fungicides may suppress symptom development as well as the viability of the pathogen, which may lead to false negative test results.

This diagnostic protocol describes well-established methods for the detection and identification of *P. ramorum*. It is not a comprehensive review of all methods available for the diagnosis of infection by *P. ramorum*. Detection of *P. ramorum* can be achieved by serological, biological and molecular methods. Serological methods can be used first as a screening test for the presence of *Phytophthora* spp., but may yield false negative or false positive results (Kox *et al.*, 2007). When a *Phytophthora* species has been detected by a serological method, the identity of the species must be confirmed by isolation and morphological identification or by molecular methods according to the flow chart in Figure 1. If identification of *P. ramorum* represents the first finding for a country, the laboratory may wish to have the diagnosis confirmed by another laboratory.

3.1 Symptoms

Several disease syndromes caused by *P. ramorum* have been described. The symptoms within each syndrome can vary widely depending on the host. The most commonly observed host symptoms are described below and are illustrated in Figures 2 to 6. Additional disease symptoms can be found on several websites (USDA-APHIS, 2009; COMTF, n.d.; EPPO, n.d.; Fera, n.d.).

3.1.1 Bleeding canker

Despite the name sudden oak death, which is the most common name used for tree dieback caused by *P. ramorum* (McPherson *et al.*, 2001), the following symptoms can be observed on many tree species and can take several years to kill mature trees. Typically, symptoms include lethal cankers around the lower trunks of infected trees, from which dark red to black sap may ooze (called bleeding cankers or tarry spots) (Figure 2). Removing the outer bark under and around oozing areas often reveals dead and discoloured inner bark with a black zone line around the edge of the necrosis. The foliage of infected trees may die prematurely, with leaves remaining on the branches after death. Trees that show these symptoms may suddenly die. It should be noted that these symptoms are not restricted to an infection caused by *P. ramorum*; they may also be hastened by other plant pathogens (including other *Phytophthora* species) or be associated with non-pathogenic disorders or insect pests.

3.1.2 Shoot dieback

On *Rhododendron* spp., diseased twigs often have brown to black lesions that usually develop first at the tip and then spread towards the base (Figure 3). Mid-stem lesions can also be found. The cambial tissue of diseased twigs is often discoloured. Shoots and stems may have cankers near ground level, resulting in rapid wilting of shoots and causing the leaves, which remain attached, to hang down (Figure 4). Infection on *Viburnum* spp. usually occurs at the base of the stem causing plants to wilt and collapse very quickly (Figure 5). Brown necrosis can often be seen spreading into stems and twigs and leaf spots may also be observed. Infection on *Pieris* spp. tends to cause petiole blackening, leading to stem cankers and aerial dieback.

3.1.3 Leaf blight

On *Rhododendron*, *Camellia*, *Kalmia* and *Pieris* species, black–brown lesions occur on leaves, usually at the tip but often at the petiole end (Figure 6(A) and (B)). Disease develops across infected leaves, often following the midrib, and eventually leads to premature leaf fall. On *Magnolia* spp. and *Rhododendron* spp., multiple small spots can also be observed, eventually merging into larger necrotic areas.

3.1.4 Needle blight

P. ramorum causes needle blight and dieback of young shoots of the conifers *Pseudotsuga menziesii* (Douglas fir), *Sequoia sempervirens* (coastal redwood), *L. kaempferi*, *Taxus baccata* (English yew) and *Abies grandis* (grand fir) (Figure 6(C)). Typical symptoms observed on *Larix* are needle infections, shoot dieback, and branch and trunk cankers. Infected shoot tips wither and wilt and infected needles appear blackened. Early needle abscission of infected needles also occurs.

3.2 Sampling and sample preparation

Different techniques for sampling and sample preparation as described below are recommended depending on the material being tested. Samples should be kept cool and sent to the diagnostic laboratory in strong closed plastic bags or containers, or double-bagged for next day isolation, as prolonged transit times or raised temperatures can reduce the likelihood of successful isolation and detection. Placing a small amount of damp tissue with the plant material will reduce sample desiccation and may increase the chance of isolation. However, in sealed self-closing plastic bags, excessive moisture can hasten tissue degradation and saprophytic activity. Storage at 2–8 °C is highly recommended to prolong sample life but storage for longer than seven days reduces the ease of isolation.

3.2.1 Plant material

When sampling bleeding cankers from trees, the outer bark around the canker should be removed to reveal the inner bark and the margin of necrosis. Pieces of phloem and xylem can then be excised from across the leading edge (the junction between healthy and necrotic tissue) and sent for testing. Symptomatic shoots and twig samples approximately 15 cm long, spanning the leading edge of an infection, should be taken while for leaves, several, showing a range of typical symptoms, should be taken.

Non-symptomatic plants can be sampled by taking leaves at random following statistical norms. The leaves sampled are bagged together and submitted for testing.

3.2.2 Water

Water samples should be at least 1 litre in volume and be taken from the surface of the area being tested, preferably where the water is flowing and is not below 4 °C or deeper than 15 cm. The water samples should be kept cool (5–20 °C) during storage and transport and tested within 48 h of

collection. Rainwater can also be collected and tested. Water bait bags, sometimes called “bobs” (muslin bags containing leaves for baiting), are an alternative, very effective method of on-site testing of water (Defra, 2007; USDA-APHIS, 2014). They consist of cut or whole leaves of rhododendron (*Rhododendron catawbiense* ‘Grandiflorum’, *R.* ‘Cunningham’s White’ or *R. ponticum*) in muslin bags containing polystyrene to aid flotation. They have been used extensively in field situations to check water sources, including streams and irrigation ponds, for *P. ramorum* (Defra, 2007). Bait bags are best deployed where the water is flowing, however slow, rather than still. Bait bags can be used when the water to be tested is above 4 °C (Defra, 2007).

3.2.3 Soil or plant debris

About 500 g of soil or plant debris should be taken from the sampling site. This should be placed in a sealed container or bag. Alternatively, cut rhododendron leaves in bait bags (section 3.2.2) (without the polystyrene) can be buried in the soil or the plant debris for later collection, provided it will remain moist.

3.3 Detection by serological methods

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

Serological methods may be used only to pre-screen samples for the presence of *Phytophthora* spp. A low level of false negative and false positive results may occur (Kox *et al.*, 2007). Different formats are available, including lateral flow devices (Forsite Diagnostics¹) and ImmunoStrip Tests (Agdia¹), which are both suitable for field use, primarily to screen out negative samples. Larger format enzyme-linked immunosorbent assays (ELISA) are also available (from Neogen¹, Lexington¹ or Agdia¹), and are more suitable for laboratory use.

3.4 Isolation and culture from symptomatic or asymptomatic material

3.4.1 Isolation from plant samples

Symptomatic samples can be washed with water to remove loose surface contamination. At least four 1 cm² pieces should then be excised from the leading edge of infection on each sample and plated on one of the semi-selective isolation media described in section 3.5.

As much of each piece of tissue as practically possible should be slid under the media to force any *Phytophthora* present to grow through the media. A maximum of ten leaf pieces should be placed on each plate. Leaf pieces from different sampling sites (e.g. nurseries) or different hosts or locations within a site (i.e. subsamples) should be placed on different plates. Sporangia are formed more readily on unsealed plates (P. Giltrap, personal communication, 2014). The plates are incubated in daylight or in the dark (the dark favours chlamydospore production) at between 18 and 25 °C, and examined for *Phytophthora* growth after three to seven days. Samples plated onto media containing rifampicin should be incubated in the dark because rifampicin is inactivated by light. Growth should occur within ten days but morphological features can be seen after three days in some cases.

¹ In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

Where no semi-selective medium is used, surface sterilization is recommended. For example, the 1 cm² pieces can be dipped in an aqueous solution of bleach (1% active sodium hypochlorite) for 2–5 min depending on the thickness of the material (e.g. thin leaves may need less time than thicker stems) or 70% ethanol for 30 s, then rinsed in sterile distilled water and dried. The stem sections are split lengthwise before plating to aid culture growth.

Isolation of *P. ramorum* from woody tissue is difficult and can lead to false negative results. For woody tissue, therefore, the use of more than one method of detection for a sample is advisable. Isolation is as for soil or plant debris (section 3.4.3), covering the woody material in Petri's mineral solution and using whole or cut rhododendron leaves as bait, which are then plated or tested by molecular methods.

Non-symptomatic plants may be tested by baiting (section 3.4.2).

3.4.2 Isolation from water samples

In the laboratory, water samples are placed in a sterilized container of appropriate volume with a large surface area (e.g. a Ziploc¹ 946 ml square disposable plastic box wiped with 50% ethanol and dried before use). To promote infection from zoospores, a sterilized metal screen or cheese cloth may be used in the box to keep floating debris from touching the leaf baits. At least four 1 cm² pieces of healthy rhododendron leaf that has not been treated with fungicide are placed on the water surface. Alternatively, fully developed rhododendron leaves that have not been treated with fungicide and have been cut several times on the leaf margin with a sterile scalpel can be used. *R. 'Cunningham's White'*, *R. catawbiense 'Grandiflorum'* and *R. ponticum* are recommended because they are highly susceptible to *P. ramorum*; however, many other rhododendron species are as susceptible (De Dobbelaere *et al.*, 2010).

The box is sealed and incubated at room temperature (18–25 °C). Within three to seven days, symptoms of *P. ramorum* infection usually develop if the pathogen is present; however, the lack of symptoms is not conclusive evidence for the absence of *P. ramorum*. The bait leaves should be plated as described in section 3.4.3 or used directly for DNA extraction. Alternatively, whole or partial leaf baits can be slipped under the selective media with the aid of a sterile spatula to help discourage bacterial contamination and allow the suspect *Phytophthora* to grow through the media. It can then be excised from the surface and transferred to a non-selective medium.

Where bait bags have been used, the rhododendron leaves are retrieved after three to seven days, and washed and plated (section 3.4.1) or used directly for DNA extraction.

Baiting with rhododendron has been demonstrated as detecting *P. ramorum* at sporangial concentrations of 1 to 40 000 per litre of water (Defra, 2007). Other baiting substrates have been described, such as *Pyrus communis* (pear fruit) (Themann *et al.*, 2002), but rhododendron leaves have been used most commonly, work very well and are easy to handle.

Baiting is not specific to *P. ramorum* and may pick up other *Phytophthora* species, as well as *Pythium* species. Using selective media when plating out helps reduce the growth of other organisms, making morphological identification of *P. ramorum* easier.

3.4.3 Isolation from soil or plant debris samples

Approximately 250 g soil to be tested is placed in a large sterilized plastic box, covered with about 500 ml Petri's mineral solution (1 litre distilled water with CaNO₃ 0.4 g; MgSO₄·7H₂O 0.15 g; KH₂PO₄ 0.15 g; and KCl 0.06 g) or sterile demineralized water, and whole or cut rhododendron leaves are placed as bait on the surface of the solution, as described in section 3.4.2. Plant debris can be treated in the same manner. The box is incubated for three to seven days, then the sample is checked for the presence of *P. ramorum* by plating (section 3.4.1) or molecular methods (section 3.6). Where bait bags have been used, these are treated as for water samples (section 3.4.2).

3.5 Isolation media

For isolation, P₅ARP(H) (pimaricin, ampicillin, rifampicin, pentachloronitrobenzene, hymexazol) culture medium (Jeffers and Martin, 1986) is recommended, as this is semi-selective for *Phytophthora* spp. and on it, characteristic features of *P. ramorum* are readily observed. Hymexazol is included in this medium to suppress *Pythium* spp. and can be particularly useful when working with soil and water. Hymexazol has been shown to slow the growth of certain *Phytophthora* spp., including *P. ramorum*; however, adding up to 25 mg/litre hymexazol has been shown to have minimal effects on *P. ramorum* (Murphy *et al.*, 2007).

P₅ARP(H) medium is made by adding 17 g cornmeal agar to 1 litre distilled water, stirring thoroughly, then autoclaving at 121 °C for 15 min before cooling to 50 °C in a water bath (EPPO, 2012). Additions, where necessary, are prepared by suspending them in 10 ml sterile distilled water or dissolving them in ethanol before adding to the medium. For 1 litre P₅ARP(H) medium, 5 mg pimaricin, 250 mg ampicillin (sodium salt), 10 mg rifampicin (dissolved in 1 ml of 95% ethanol), 100 mg pentachloronitrobenzene and 75 mg (final concentration: 22.5 parts per million (ppm)) hymexazol (30% active substance) are added to the cooled (50 °C) medium, which is then stirred thoroughly and poured onto plates. The plates should be stored at 2–8 °C in the dark and used before five to seven days have elapsed since they were made (Jeffers and Martin, 1986).

The final concentration of hymexazol should be considered when making any amended medium. When isolating the pathogen from leaves or woody tissue, hymexazol can be considered optional. Another semi-selective medium including hymexazol and similar bactericides is PARP-V8 (Fergusson and Jeffers, 1999).

Another medium that can be used for isolation is cherry decoction agar. Cherry juice is made by boiling 1 kg cherries, free of stones and petioles, in 1 litre tap water for approximately 2 h. The juice is filtered through muslin or cheesecloth, poured into bottles, sterilized at 110 °C for 30 min, adjusted to pH 4.5 with 1 N KOH or 1 N HCl, and stored until use. In a bottle containing 0.8 litre distilled water, 20 g Technical Agar No. 3 is added and the mixture is sterilized at 121 °C for 15 min. Immediately after sterilization, 0.2 litre sterilized cherry extract is added, mixed well and sterilized at 102 °C for 5 min (Gams *et al.*, 1998).

For extended culturing, isolates should be transferred to carrot piece agar, made by first finely grating 50 g carrots. Twenty-two grams of Technical Agar No. 3 is dissolved in 1 litre water in a 2 litre beaker, and stirred thoroughly before adding the grated carrots and stirring again. When the contents are thoroughly mixed, the beaker is covered with foil and placed into a steamer for 1 h. Before removing it from the steamer, thorough stirring of the medium is recommended. The medium is then transferred to bottles, ensuring that the carrot pieces are divided equally between them. The bottles are autoclaved at 121 °C for 15 min before the medium is poured onto plates, which are stored at room temperature (Gams *et al.*, 1998).

3.6 Detection by molecular methods

Molecular tests have been developed to detect *P. ramorum* from culture or *in planta* using conventional or real-time polymerase chain reaction (PCR). Many of these methods were compared by Kox *et al.* (2007) and Martin *et al.* (2009). For this protocol, four methods have been selected based on the experience obtained by laboratories with them and the availability of validation data, and these methods are described below. However, other PCR methods can be used. PCR methods will detect non-viable *P. ramorum* in infected plant material, which would not be detected by isolation and culture (Bilodeau *et al.*, 2007). Real-time PCR may be preferred for high throughput, routine testing as the closed-tube format reduces the risk of carrying over contamination due to processing of amplification products (e.g. for nested PCR or gel electrophoresis).

3.6.1 Preparation of material

When testing symptomatic plant material, it may be beneficial to sample from the leading edge of the lesion. Depending on the sample matrix (leaves or stems, or soil), different methods may be used for homogenization or disruption of the tissue. Plant tissue (from leaves) or mycelium (from cultures) may be disrupted using a tissue pulverizer or bead beater. Pre-freezing in liquid nitrogen can be beneficial for disruption. Various grinding methods can be used, providing they produce a homogenously ground sample; for example, mortar and pestle with liquid nitrogen (for leaves and cut stems), bead mills, TissueLyser (Qiagen¹) or the Homex grinder (Bioreba¹) (for cultures and tough woody tissue).

3.6.2 DNA extraction

DNA extraction from plant material or from cultures can be performed using commercial kits (e.g. the NucleoSpin Plant II Extraction Kit (Macherey-Nagel¹) or the DNeasy Plant Mini Kit (Qiagen¹), following the manufacturers' instructions. For DNA extraction from cultured isolates, the same kits can be used. DNA should be stored at -20 °C until use. Refer to the source papers in the following sections for the extraction methods originally used; however, laboratories may find that alternative extraction techniques work equally well.

3.6.3 Conventional PCR

There are several *P. ramorum*-specific conventional PCR methods described in the literature. Two of these are described below.

3.6.3.1 Conventional PCR of Kox et al. (2002) targeting *P. ramorum*

The primers Phyto 1 (forward) and Phyto 4 (reverse) from the internal transcribed spacer (ITS) ribosomal (r)DNA were developed by M. Garbelotto (Hayden *et al.*, 2004) and used for the detection of *P. ramorum* by conventional PCR (Kox *et al.*, 2007). The primers are listed below, and the details for the PCR are in Table 1.

Phyto 1: 5'-CAT GGC GAG CGC TTG A-3'

Phyto 4: 5'-GAA GCC GCC AAC ACA AG-3'

Table 1. Master mix composition, cycling parameters and amplicons for conventional PCR with primers Phyto 1/Phyto 4

Reagent	Final concentration
PCR-grade water	—†
10x PCR buffer	1x
MgCl ₂	1.5 mM
dNTPs	200 µM
Primer Phyto 1	0.2 µM
Primer Phyto 4	0.2 µM
DNA polymerase	0.5 U
DNA (volume)	5 µl
Cycling parameters	
Initial denaturation	95 °C for 15 min
Number of cycles	35
- Denaturation	94 °C for 15 s
- Annealing	62 °C for 1 min
- Elongation	72 °C for 45 s
Final elongation	72 °C for 10 min
Expected amplicons	
Size	687 bp

† For a final reaction volume of 25 µl.

bp, base pairs; PCR, polymerase chain reaction.

3.6.3.2 Conventional PCR of Ioos et al. (2006) targeting *P. ramorum*

This PCR is based on the amplification of DNA from intronic regions using two pairs of specific primers: TRP-PRAM-F (forward) and TRP-PRAM-R (reverse) from intron TRP1, and GPA-PRAM-F (forward) and GPA-PRAM-R (reverse) from intron GPA1. The primers TRP-PRAM-F/TRP-PRAM-R can be used for detection and GPA-PRAM-F/GPA-PRAM-R for confirmation, and both pairs of primers have been fully validated and characterized (Ioos *et al.*, 2006). The primers are listed below, and the details for the PCR are in Table 2.

TRP-PRAM-F: 5'-GAG TAG AAA CTT CGG GAA TG-3'

TRP-PRAM-R: 5'-GTT CGG CAC ATT AAC GCA G-3'

GPA-PRAM-F: 5'-TAA GGA ACA AGG TAC CAA AG-3'

GPA-PRAM-R: 5'-CTC AGG AAT TCA CTC TCA CG-3'

Table 2. Master mix composition, cycling parameters and amplicons for conventional PCR with primers TRP-PRAM-F/TRP-PRAM-R and GPA-PRAM-F/GPA-PRAM-R

Reagent	Final concentration
PCR-grade water	—†
10x PCR buffer	1x
MgCl ₂	2 mM
dNTPs	200 µM
Bovine serum albumin	0.60 µg/µl
Primer TRP-PRAM-F or GPA-PRAM-F	0.45 µM
Primer TRP-PRAM-R or GPA-PRAM-R	0.45 µM
DNA polymerase	0.5 U
DNA (quantity/volume)	2 µl (30–80 ng)
Cycling parameters‡	
Initial denaturation	95 °C for 3 min
Number of cycles	35
- Denaturation	94 °C for 30 s
- Annealing	58 °C for 30 s
- Elongation	72 °C for 1 min
Final elongation	72 °C for 7 min
Expected amplicons	
TRP-PRAM-F/TRP-PRAM-R	527 bp
GPA-PRAM-F/GPA-PRAM-R	248 bp

† For a final reaction volume of 20 µl.

‡ The maximum temperature ramping rate should be used between steps.

bp, base pairs; PCR, polymerase chain reaction.

3.6.4 Real-time PCR

There are several *P. ramorum*-specific real-time PCR methods described in the literature. Two of these are described below.

3.6.4.1 Real-time PCR of Hughes *et al.* (2006) targeting *P. ramorum*

The primers and probe described by Hughes *et al.* (2006) target the ITS-1 region of the nuclear ribosomal (nr)RNA gene. Primer and probe sets have been developed that target other genes such as genes for cytochrome oxidase subunit I (*COXI*) (Tooley *et al.*, 2006), beta-tubulin and elicitin (Bilodeau *et al.*, 2007) and the ras-related Ypt1 protein (Schena *et al.*, 2006).

Hughes *et al.* (2006) reported a limit of detection of 10 pg genomic DNA, and no cross-reactivity with 29 species of non-target *Phytophthora*, with the exception of *Phytophthora lateralis*, which was detected at or above concentrations of approximately 10 ng per 25 µl reaction. For a full list of species used for the assessment of specificity, see Hughes *et al.* (2006).

The primers and probe are listed below, and the details for the PCR are in Table 3.

Pram 114-Fc: 5'-TCA TGG CGA GCG CTG GA-3'

Pram 190R: 5'-AGT ATA TTC AGT ATT TAG GAA TGG GTT TAA AAA GT-3'

Pram 134-T probe: 6-FAM 5'-TTC GGG TCT GAG CTA GTA G-3' TAMRA

Table 3. Master mix composition and cycling parameters for real-time PCR with primers Pram 114-Fc/Pram 190R and probe Pram 134-T

Reagent	Final concentration
PCR-grade water	— [†]
10x PCR buffer	1x
MgCl ₂	6.0 mM
dNTPs	240 µM
Primer Pram 114-Fc	300 nM
Primer Pram 190R	300 nM
Probe Pram 134-T	100 nM
DNA polymerase	1 U
DNA (quantity/volume)	1 µl (20–100 ng)
Cycling parameters	
Initial denaturation	95 °C for 10 min
Number of cycles	40
- Denaturation	95 °C for 15 s
- Annealing	—
- Elongation	60 °C for 1 min
Expected amplicons	
Size	n/a

[†] For a final reaction volume of 25 µl.

n/a, not applicable; PCR, polymerase chain reaction.

For the real-time PCR carried out by Hughes *et al.* (2006) the cycle threshold (Ct) value was assessed using a default threshold setting of 0.2 ΔR_n (fluorescence units).

Under the Hughes *et al.* (2006) conditions, samples with Ct values less than 36 may be considered positive for *P. ramorum*. Ct values between 36 and 40 may be a result of aerosol contamination or cross-reaction with non-target DNA at high concentrations (e.g. *Phytophthora foliorum* or *P. lateralis*). Samples giving these results should be resampled or retested and if the result is still in doubt, the presence of *P. ramorum* confirmed by another method described in the protocol. Samples with Ct values of 40 are considered negative. However, the cut off Ct value should be verified in each laboratory when implementing the test for the first time.

3.6.4.2 Real-time PCR of Schena *et al.* (2006) targeting *P. ramorum*

Schena *et al.* (2006) developed a multiplex real-time PCR based on the *Ypt1* gene to detect *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in infected plant material. For *P. ramorum*, in a singleplex PCR, the authors report a limit of detection of 100 fg per 25 µl reaction, and there is no cross-reaction with *P. lateralis*. The primers and probe for detecting *P. ramorum* in the singleplex PCR are listed below, and the details for the PCR are in Table 4.

Yram4F: 5'-TTT GTC AGT GAC CTC TCT CTC TCT C-3'

Yram3R: 5'-GCA TAA GTA TAA GTC AGC AAG CCT GT-3'

YramP probe: 6-FAM 5'-AGA ACA CGA TCC CCT CGT CAG CAG TC-3' BHQ

Table 4. Master mix composition and cycling parameters for real-time PCR with primers Yram4F/Yram3R and probe YramP

Reagent	Final concentration
PCR-grade water	—†
10x PCR buffer	1x
MgCl ₂	5.0 mM
dNTPs	200 µM
Primer Yram4F	330 nM
Primer Yram3R	330 nM
Probe YramP	130 nM
DNA polymerase	0.5 U
DNA (quantity/volume)	1 µl (10–100 ng)
Cycling parameters	
Initial denaturation	50 °C for 2 min
	95 °C for 10 min
Number of cycles	40
- Denaturation	95 °C for 20 s
- Annealing	—
- Elongation	62.5 °C for 20 s
Expected amplicons	
Size	n/a

† For a final reaction volume of 25 µl.

n/a, not applicable; PCR, polymerase chain reaction.

The real-time PCR of Schena *et al.* (2006) uses a qPCR Core Kit. Amplifications are performed using a Chromo 4¹ Detector, and data acquisition and analysis are realized using the Opticon Monitor software version 2.03 (MJ Research¹) supplied with the thermocycler.

A cut off Ct value of 36 (corresponding to the detection of 100 fg of target DNA) was obtained with the PCR described by Schena *et al.* (2006). The cut off Ct value should be verified in each laboratory when implementing the test for the first time.

3.6.5 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR, a positive nucleic acid control and a negative amplification control (no template control) are the minimum controls that should be used. The use of an internal control assay for the detection of host plant DNA, to be used in multiplex with the pathogen-specific assay, in parallel singleplex reactions, or in parallel tests for conventional and real-time PCR, can assist in the interpretation of *P. ramorum*-negative results. The use of a plant internal control is highly recommended to confirm the quality of the extracted DNA, especially where molecular methods are being used as a primary screen.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction). Pre-prepared (stored) genomic DNA, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product) may be used. A good positive control for *P. ramorum* is DNA extracted from a host plant (e.g. *Rhododendron*) infected with *P. ramorum* with a Ct value near the limit of detection (LOD).

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Internal control. To eliminate the possibility of PCR false negatives due to DNA extraction failure, nucleic acid degradation or the presence of PCR inhibitors, primers and probe targeting plant internal control DNA (e.g. *COX* as used by Hughes *et al.* (2006)) can be incorporated into the protocol.

The internal control primers can be used in a multiplex reaction with the pathogen-specific primers or they can be used in parallel singleplex reactions. Performing the reactions in singleplex may help to avoid a reduction in the sensitivity of detection of *P. ramorum*. Laboratories may choose to establish a cut-off Ct value to be used to identify samples for which extraction or amplification has not failed but was suboptimal (which could lead to false negative results). The appropriate cut-off Ct values may need to be determined for each sample type (host, tissue, etc.). Samples with failed internal controls should be plated onto selective media to try to derive a culture for DNA extraction and subsequent PCR. A dilution (e.g. 1:10) of the DNA extract can also help to overcome a problem due to the presence of inhibitors.

Alternative internal controls may be used. For example, Hayden *et al.* (2006) describe a universal primer and probe set targeting a conserved region of the small subunit of the rDNA gene, which was developed to detect any eukaryote.

3.6.5.1 Additional controls (optional)

Positive extraction control. This control is used to ensure that target nucleic acid extracted is of sufficient quantity and quality for PCR and that the target is detected. Nucleic acid is extracted from infected host tissue or, if suitable infected material is not available, healthy plant tissue that has been spiked with the target.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. It is recommended that multiple controls be included when large numbers of positives are expected.

Alternatively, extraction blanks (sterile water) can be processed with the samples to be tested if sufficient uninfected host tissue is not available. This will allow contamination of extraction reagents and cross-contamination between samples to be identified.

4. Identification

P. ramorum may be identified either by its growth characteristics and morphology in culture or by sequence analysis.

Possible confusion in morphology and cultural characteristics is most likely to occur with *Phytophthora palmivora* while *Phytophthora hibernalis*, *P. lateralis* and *P. foliorum* may give a cross-reaction in the conventional PCR test (section 4.2).

A flow chart for the diagnosis of *P. ramorum* on symptomatic plant material is given in Figure 1. A positive diagnosis can be based on morphology; however, experience with the identification of *Phytophthora* species is required. Further PCR or sequencing is recommended.

A very low percentage of cross-reactivity has been observed with Hughes *et al.* real-time PCR primers, when *P. foliorum* or *P. lateralis* are present in very high concentration. The Ct values are usually more than 36, and for those cases, morphological (section 4.1) or sequencing (section 4.2) studies of pure cultures are needed for a conclusive identification.

4.1 Morphological identification

4.1.1 Cultural characteristics and morphology

The growth characteristics and morphological features of *P. ramorum* on agar, described in Werres *et al.* (2001), can be affected by the type of agar, substrate or host plant (P. Giltrap, personal communication, 2014). Colonies on carrot piece agar, PARP-V8 agar and cornmeal agar are submerged, showing pronounced (PARP-V8 agar) or weak (carrot piece and cornmeal agar) concentric rings. On cherry decoction agar, colonies have an appressed aerial mycelium with weak rosette-like patterns. Sporangia are ellipsoid, elongate-ovoid, caducous, often with a short pedicel, semipapillate, hyaline, $45.6\text{--}65 \times 21\text{--}28.3\text{ }\mu\text{m}$, single but in clusters; chlamydospores are numerous, thin-walled, globose, hyaline to brown, mostly $46\text{--}60\text{ }\mu\text{m}$, and terminal or intercalary. Generally, characteristic chlamydospores allow accurate identification of *P. ramorum* in culture. Possible confusion in morphology and cultural characteristics is most likely to occur with *P. palmivora*. The key characteristics are illustrated in Figures 7, 8, 9 and 10. The features that are essential for accurate identification, as formed on examples of selective and non-selective media, are given in Table 5.

Table 5. Growth characteristics of *Phytophthora ramorum* on selective and non-selective media

Characteristic	P5ARP(H) [†] (selective)	Carrot piece agar ^{†,‡} (non-selective)
Colonies	Relatively slow growing, approximately 2 mm per day	Weak rosette-like pattern, pronounced concentric rings, growth rate approximately 3 mm per day
Mycelia	Weakly coralloid, growing within the agar with little superficial growth, no hyphal swellings. Superficial, fluffy growth may be observed when growing out of plant material and coralloid appearance can differ according to the host out of which the mycelium is growing [§] .	Aerial mycelium sparse, no hyphal swellings
Sporangia	Produced abundantly on the agar surface, semipapillate, caducous with short (5 μm) or no stalk. Size: $40\text{--}80 \times 20\text{--}32\text{ }\mu\text{m}$, average $24 \times 52\text{ }\mu\text{m}$; average length/width ratio: 2.16.	Ellipsoid, spindle-shaped or elongated ovoid, single or in clusters
	Ellipsoid, frequently in small clusters and relatively narrow, initial sporangium commonly producing secondary, smaller sporangia. When growing out of plant material, can appear papillate when about to germinate. Sporangia with constrictions (central or at pedicel end) have been observed [§] , particularly when growing out of plant material.	
Chlamydospores	More common in older colonies (seven to ten days) unless growing out of plant material. Very large (up to 80 μm diameter), hyaline to pale brown to brown. Hyphal swellings present.	After three days' incubation in the dark, in the older parts but very often also in the young parts of the colony. Up to 88 μm diameter, thin-walled, hyaline to pale brown.

Source: Werres *et al.* (2001).

[†] On P5ARP(H), characteristics can be observed after four to six days' incubation at 20 °C, 12 h light/12 h dark. On carrot piece agar, characteristics can be observed after three to five days' incubation at 20 °C in darkness.

[‡] Sexual structures can be observed on carrot piece agar after pairing with an opposite mating type; for example, *Phytophthora cryptogea* (Werres and Kaminski, 2005). A *P. ramorum* \times *P. ramorum* pairing is also possible *in vitro* (not with all isolates) (Brasier and Kirk, 2004) and in rhododendron twigs (Werres and Zielke, 2003).

[§] P. Giltrap, personal communication, 2014.

If no sporangia are produced on agar, sporulation can be encouraged by cutting 6 mm plugs from four-day-old colonies and placing these in a sterile Petri dish, mycelium side up, along with enough sterile tap water or Petri's mineral solution to be level with the top of the plugs but not covering the mycelium. Non-sterile pond water or soil extract water can be used, provided contamination with *P. ramorum* has been ruled out. The dishes are placed in the dark at 18 °C or cooler for 24–48 h. This should encourage sporangia to form on the edge of the plugs. Clusters of *P. ramorum* sporangia may be seen also in the water, having broken away from the agar plug.

A positive morphological identification would be recorded if caducous, semipapillate sporangia in the correct size range and shape with short pedicels (5 µm) were observed along with the characteristic chlamydospores.

4.2 Molecular identification

The following tests are recommended for identification of *Phytophthora* species, including *P. ramorum*, from clean cultures. The conventional PCR and real-time PCR methods described in section 3.6 for *in planta* detection of *P. ramorum* are species-specific and are used for detection of the pathogen in infected material or in cultures. Molecular diagnostic tests detect DNA, not the viable organism, and cross-reaction with closely related species, including *P. lateralis*, *P. hibernalis* and *P. foliorum*, is possible at high DNA concentrations with some methods. In addition, environmental samples (infected samples) that have very low titre can yield negative results, so care should be taken in the interpretation of results when testing DNA extracts from cultures, which may be at a higher concentration than extracts from plant material. ITS sequencing is described in section 4.2.1 as an example of a method that may be used for species level identification of *Phytophthora* isolates. Sequencing can also be performed for other genes such as *COXI* and *II* (Martin & Tooley, 2003; Martin *et al.*, 2004) and *Ypt1* (Schena *et al.*, 2006).

4.2.1 ITS sequencing for species level identification using the primers of White *et al.* (1990)

The identity of *P. ramorum* isolated in culture can be confirmed by sequencing the amplified ITS-1, 5.8S and ITS-2 region of the nrRNA gene with the primers listed below and the PCR described in Table 6. These primers can be used to generate amplification products for sequencing from all species of *Phytophthora*.

ITS5: 5'-GGA AGT AAA AGT CGT AAC AAG G-3'

ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'

Table 6. Master mix composition, cycling parameters and amplicons for conventional PCR with primers ITS5/ITS4

Reagent	Final concentration
PCR-grade water	—†
10x PCR buffer	1x
MgCl ₂	1.5 mM
dNTPs	200 µM
Primer ITS5	0.2 µM
Primer ITS4	0.2 µM
DNA polymerase	0.5 U
DNA (quantity/volume)	1 µl (50–500 pg)
Cycling parameters	
Initial denaturation	95 °C for 1 min 25 s
Number of cycles	34
- Denaturation	92 °C for 35 s
- Annealing	62 °C for 55 s
- Elongation	72 °C for 50 s
Final elongation	72 °C for 10 min
Expected amplicons	
Size	800–900 bp

† For a final reaction volume of 25 µl.

bp, base pairs; PCR, polymerase chain reaction.

Amplification products may be visualized by agarose gel electrophoresis: a single amplicon of 800–900 base pairs is produced by DNA from *Phytophthora* spp. The remaining amplification product can be purified using a suitable PCR purification kit following the manufacturer's instructions and the purified amplicon can be two-way sequenced with ITS5 (forward) and ITS4 (reverse) primers. The quality of the resulting sequence should be checked by visual assessment of the electropherograms. Consensus sequences may be built from the forward and reverse reads and compared with published sequences using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, United States; <http://www.ncbi.nlm.nih.gov/>). In order to make a correct identification of the generated sequences to *Phytophthora* species level, use of the GenBank accession number that corresponds to the ex-type of *P. ramorum* P10103 (WPC) is recommended, which is FJ801269.

The following steps are suggested for processing sequences by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq):

- (1) select “Align two or more sequences using BLAST” (under Specialized BLAST)
- (2) paste the obtained sequence in a FASTA format in the first box
- (3) paste the GenBank accession number (FJ801269) in the second box
- (4) select “Highly similar sequences (megablast)”
- (5) click on BLAST.

In the absence of a >99% match to *P. ramorum*, phylogenetic trees may be compiled to assess intraspecific and interspecific variation in order to make the identification.

4.2.2 Controls for molecular tests

The required controls are a negative amplification control and a positive nucleic acid control for the PCR. See section 3.6.5 for more details on controls for molecular tests.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

Cultures of *P. ramorum* can be stored on carrot piece or oatmeal agar slopes at room temperature or in sterile distilled water at 5 °C. DNA can be stored at –80 °C or –20 °C.

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Fera Science Ltd. (Fera), Sand Hutton, York YO41 1LZ, United Kingdom (Ann Barnes; e-mail: ann.barnes@fera.co.uk; tel.: +44 (0) 1904 462494 or Jennifer Tomlinson; e-mail: jenny.tomlinson@fera.co.uk; tel.: +44 (0) 1904 462000 extension 3207).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

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The protocol was updated by A. Barnes, P. Giltrap and J. Tomlinson (Fera, United Kingdom), S.C. Brière (Canadian Food Inspection Agency, Canada) and Z.G. Abad (USDA-APHIS-PPQ-Center of Plant Health Science and Technology, United States of America).

L. Laurenson (Fera, United Kingdom) assisted with reviewing comments relating to molecular detection and identification received from the IPPC first consultation period.

8. References

The present annex may refer to ISPMs. ISPMs are available on the International Phytosanitary Portal (IPP) at <https://www.ippc.int/core-activities/standards-setting/ispm>.

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9. Figures

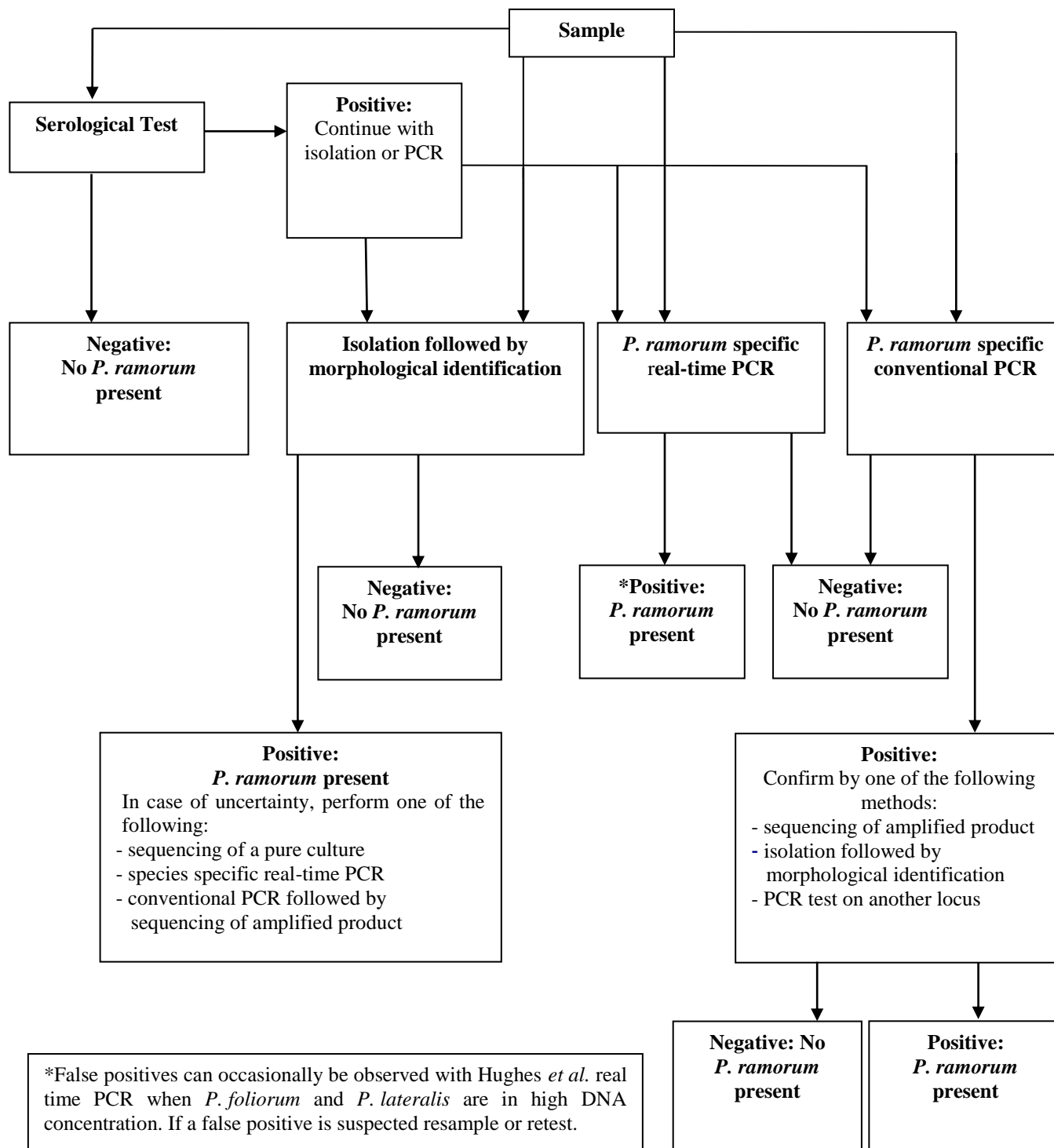
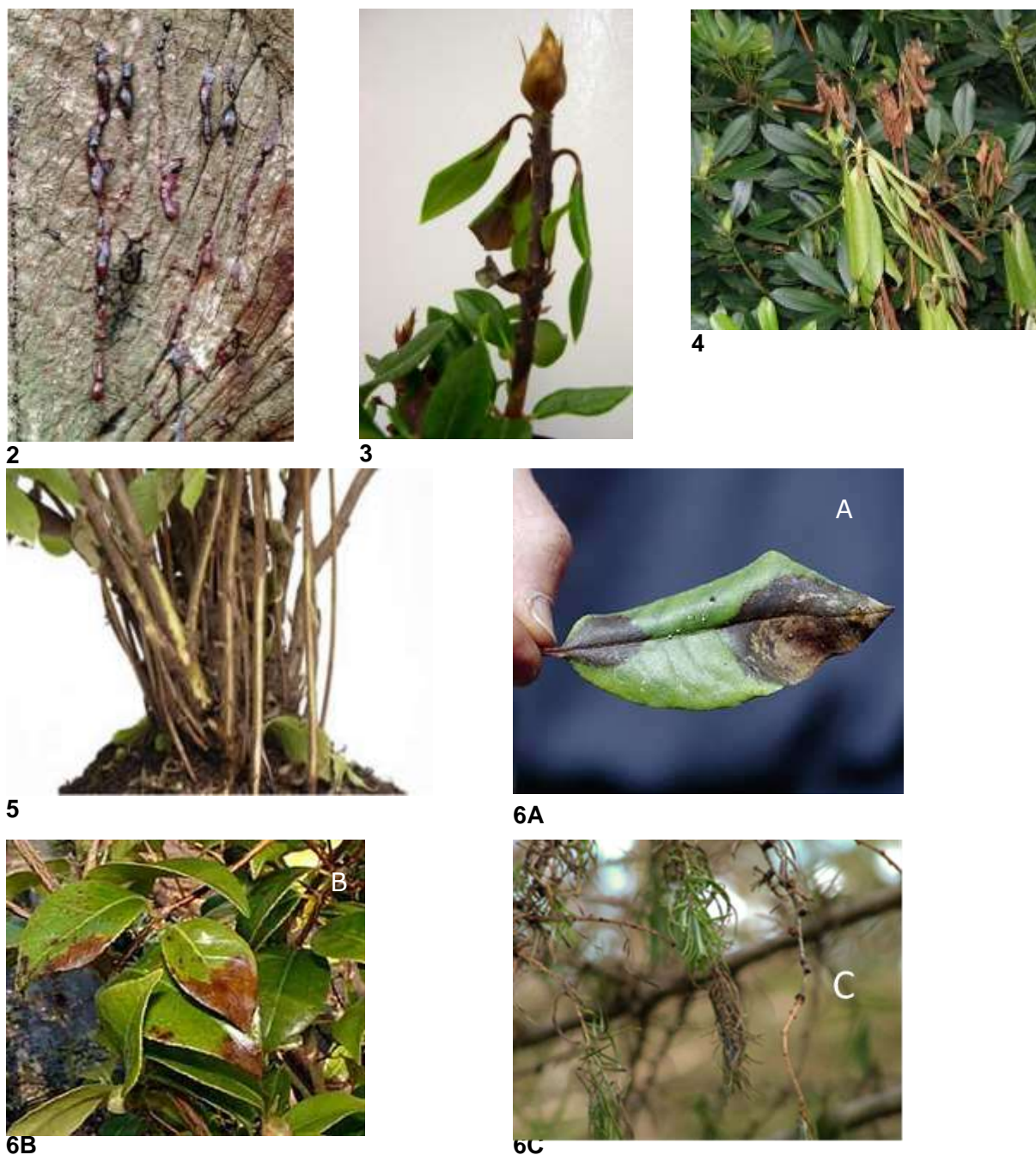
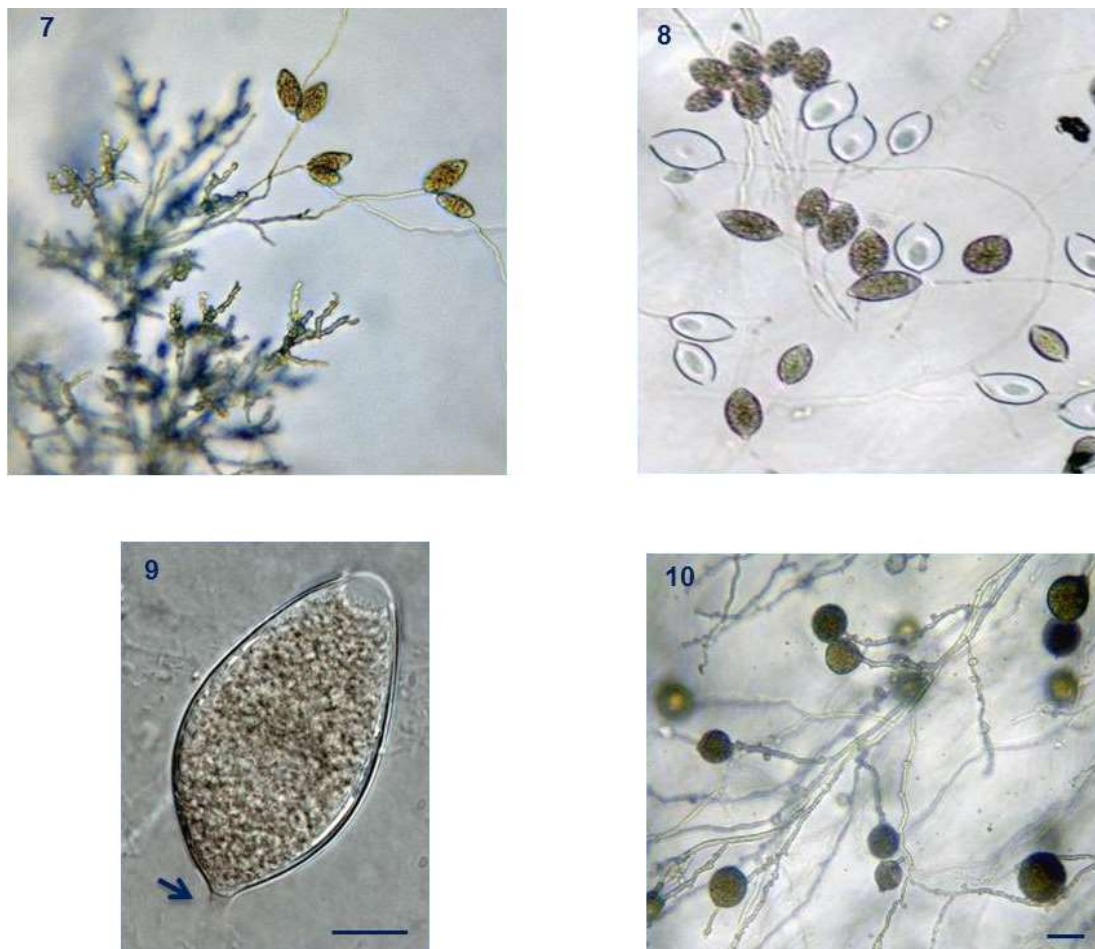


Figure 1. Flow chart for the diagnosis of *Phytophthora ramorum* on symptomatic plant material. PCR, polymerase chain reaction.



Figures 2–6. *Phytophthora ramorum* symptoms on different hosts: **2** *Quercus*, bleeding canker; **3** *Rhododendron*, shoot dieback; **4** *Rhododendron*, shoot tip wilt; **5** *Viburnum*, stem base discoloration; **6(A)** *Rhododendron*, leaf blight; **6(B)** *Camellia*, leaf blight; and **6(C)** *Larix*, needle blight.

Photos courtesy Fig. 2 M. Garbelotto, UC Berkeley, United States of America; Fig. 3 J.C. Bienapfl, USDA-APHIS-CPHST Beltsville Laboratory, MD, United States of America; Figs 4 and 5 P. Beales and D. Crossley, Fera, United Kingdom; Fig. 6(A) Joseph O'Brien, USDA Forest Service, <https://www.forestryimages.org>; Fig. 6(B) S. Ashby, Department of Environment, Food and Rural Affairs, United Kingdom; Fig. 6(C) © Crown copyright.



Figures 7–10. Typical morphological features of the asexual phase of *Phytophthora ramorum* on P5ARP(H) isolation medium (section 3.5): **7** coraloid mycelium and sporangia; **8** sporangia attached to sporangiophores; **9** sporangium semipapillated, caducous, with short pedicel (scale bar: 10 µm); and **10** characteristic chlamydospores (scale bar: 30 µm).

Photos courtesy Z.G. Abad, USDA-APHIS-CPHST Beltsville Laboratory, MD, United States of America.

Publication history

This is not an official part of the standard

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2006-04 Commission on Phytosanitary Measures (CPM) added work programme topic: *Fungi and fungus-like organisms* (2006-006).

2015-03 Expert consultation on draft diagnostic protocols (DPs).

2015-06 Technical Panel on Diagnostic Protocols (TPDP) revised draft.

2016-01 TPDP recommended draft to SC for approval for first consultation (2016_eTPDP_Jan_01).

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2016-07 First consultation.

2017-02 TPDP recommended draft to SC for adoption (2017_eTPDP_Feb_01).

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- ◆ There are over 180 contracting parties to the IPPC.
- ◆ Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- ◆ Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- ◆ IPPC liaises with relevant international organizations to help build regional and national capacities.
- ◆ The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

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