Diagnostics[1]
Diagnostic

Phytophthora ramorum

Specific scope

This standard describes a provisional diagnostic protocol for *Phytophthora ramorum*. Because the methods have been developed very recently in different European laboratories, there has not been sufficient time for them to be compared and evaluated. The text accordingly proposes a selection of parallel methods. EPPO proposes to prepare a harmonized revision of this Standard as soon as possible.

Specific approval and amendment

Approved in 2005-09.

Introduction

Phytophthora ramorum is a relatively recently described species of *Phytophthora* (Werres *et al*., 2001) causing high mortality of oak trees in California (US), where the disease is known as 'sudden oak death' (Rizzo *et al*., 2002). The pathogen has also been found causing 'ramorum dieback' and 'ramorum leaf blight' on a range of native plants and species of conifer in California and on a range of ornamental plants in parts of Europe.

Damage caused by *P. ramorum* in Europe was first reported in 1997 (Werres & Marwitz, 1997). The authors described a twig blight disease of *Rhododendron catawbiense* 'Grandiflorum' and *Rhododendron* 'Roseum elegans' occurring in nurseries and occasionally on large bushes in Germany during the preceding three years. The symptoms were similar to those caused by *Phytophthora. syringae,* but the pathogen did not fit with any known species described and the authors indicated it might be a new species. The same damage to rhododendrons was also seen in the Netherlands (de Gruyter *et al*., 2002). Subsequently, the pathogen was found sporadically causing stem base necrosis on *Viburnum* spp. (Werres *et al*., 2001).

In Europe, following increased awareness and emergency EU legislation, ongoing surveys were intensified or initiated to look for the presence of *P. ramorum*. This led to findings on rhododendron and viburnum plants in nurseries and to a lesser extent on shrubs in public gardens in other parts of Europe (Lane *et al*., 2003a; Man in't Veld *et al*., 2002a; Werres, 2002). Since then, there have been additional reports of damage to several new host plants. In autumn 2003, first findings of *P. ramorum* were reported in Europe on two American red oak species (*Quercus falcata* (Web Fig. 3), *Q. rubra* (Web Fig. 2)) with bleeding cankers and other symptoms characteristic of sudden oak death (Brasier *et al*., 2004). In the UK, this was later followed by findings of *P. ramorum*-infected European beech (*Fagus sylvatica*), holm oak (*Quercus ilex*), turkey oak (*Quercus cerris*), horse chestnut (*Aesculus hippocastanum*), and sweet chestnut (*Castanea sativa*). In the Netherlands, *P. ramorum* was positively identified from bark cankers found on *Q. rubra*-trees. Updated lists with natural hosts for *P. ramorum* can be found at http://www.suddenoakdeath.org at http://www.defra.gov.uk/planth/pramorum.htm.

Identity

Name: *Phytophthora ramorum* Werres, De Cock & Man in't Veld.
Taxonomic position: *Chromista*: *Oomycetes*: *Peronosporales*: *Pythiaceae*.
EPPO code: PHYTRA.
Phytosanitary categorization: EPPO Alert list, provisionally regulated by the EU (2002, 2004).

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Detection

Symptoms

Symptoms caused by *P. ramorum* can be diverse. Hansen *et al*. (2002) distinguished three distinct disease syndromes: (1) 'sudden oak death', characterized by lethal cankers; (2) 'ramorum shoot dieback', which results from foliar infection and/or direct infection of stems; (3) 'ramorum leaf blight', which results from foliar infection. The three distinct disease-types are described below (Web Figs 1–15).

Sudden oak death

Trunk: presence of *P. ramorum* is characterized by dark red to black sap oozing from the trunk (bleeding cankers or tarry spots) (Web Fig. 1). This usually occurs on the lower portion of the trunk, but may also occur up to 20 m up the stem. Sunken or flattened cankers may occur beneath bleeding areas. When the outer bark is removed from bleeding cankers, mottled areas of necrotic discoloured inner-bark tissues may be seen (this can be confused with the normal oxidative reddening of the phloem tissues). Black 'zone lines' are often present within and around edges of the necrotic areas. On young or thinner trees, a distinct edge between necrotic and healthy tissues may be visible.

Foliage: girdling necroses often result in sudden death of the tree (Web Fig. 4). As a result, foliage may change colour rapidly and uniformly throughout the crown, with leaves still remaining on the branches after death.

Roots: *P. ramorum* does not primarily inhabit roots.

Similar symptoms: European oaks are already subject to a local mortality and dieback of complex cause known as 'oak decline'. In some cases, this is associated with infection by root-infecting *Phytophthora* species. It also involves recurrent droughts, root-disease fungi such as *Collybia* and *Hypoxylon*, recurrent defoliation by leaf-feeding insects and infestation by scale insects. However, bleeding cankers on the main stem are not usually observed on European oaks. Occasionally, declining trees exhibit dark, watery fluxes from bark cracks. These tend to run freely down the tree. Decline mainly affects mature oaks; and the severity of foliar symptoms often increases gradually, affected trees becoming 'stag-headed'. Oak decline tends to be in pockets, with the longer dead and dying trees in the centre. However, scattered individual trees exhibiting decline symptoms are common, especially in open spaces.

Ramorum shoot dieback

Symptoms on the most commonly affected host plants are given below.

Rhododendron: the pathogen primarily causes an aerial dieback on both nursery plants and mature bushes. Other *Phytophthora* species, other fungi (e.g. *Glomerella*, *Botryosphaeria*, *Botryotinia*) and disorders can mask or cause similar symptoms so care should be taken with the diagnosis. Diseased twigs have brown to black lesions that usually begin at the tip and move towards the base. However, cankers may form on any part of the shoot (Web Fig. 5) or stem. The cambial tissue of the diseased twig is killed causing a discoloration that is visible when the bark is removed. Cankers near ground level can result in rapid wilting of shoots, causing the leaves, which remain attached, to hang down (Web Fig. 6). The most characteristic leaf symptoms are the blackening of the leaf petiole extending into the leaf base (Web Fig. 7). This may spread further along the midrib causing blackening of the leaf. Leaves can become diseased with and without shoot infection.

Although *P. ramorum* has been isolated from roots and stem bases, the pathogen is not considered to cause significant rotting in these areas, unlike other *Phytophthora* species.

Viburnum: infection usually occurs at the stem base (Web Fig. 8) causing plants to wilt and collapse very quickly. Brown necrosis at the collar level can spread up into the stem. The cambial tissue in the stem initially turns brown, but eventually becomes dark brown. Twig or shoot blight, and leaf spots, may also occur (Web Fig. 9). Minor root rotting has been observed.

Pieris: leaf symptoms are similar to those on Rhododendron. The pathogen causes leaf petiole blackening, which may extend down the midrib as well as causing a general leaf blight (Web Fig. 10). The infection can spread out of the leaf into shoots and vice versa. Individual stem cankers lead to aerial dieback.

Syringa: symptoms are initially observed on buds (Web Fig. 11) leading to a brown to black discoloration of shoots. On leaves, there is a typical tip and marginal necrosis.

Pseudotsuga menziesii and *Sequoia sempervirens*: limited dieback of needles and shoots. On *P. menziesii*, *P. ramorum* produces symptoms that are very similar to those of attacks by *Botryotinia fuckeliana* or frost injury.

Ramorum leaf blight

Symptoms on the most commonly affected host plants are given below.

Rhododendron: diffuse brown (dark-brown) spots or blotches frequently occur at the leaf tip (where moisture can accumulate and remain for extended periods encouraging infection), but spots can form elsewhere (drops with zoospores falling down on the leaf surface cause round, dark-brown patches). Eventually, entire leaves can turn brown to black and may fall prematurely. Sometimes early leaf spots caused by rust (*Chrysomyxa rhododendri*) look very similar to *P. ramorum* leaf spots.

Kalmia: infection usually occurs at the leaf tip. The pathogen subsequently grows through leaf tissues towards the leaf base causing a brown to black discoloration (Web Fig. 12). Other *Phytophthora* spp. may cause similar symptoms.

Camellia: brown to black leaf-tip spots (Web Fig. 13) form that may result in entire leaf death. Stems have been found to be susceptible in experiments so it is possible that dieback could occur.

Leucothoe: symptoms to date include leaf base and petiole necrosis (Web Fig. 14).

Taxus baccata: the pathogen causes a dieback of needles on young shoots (Web Fig. 15). The spots initially appear quite water-soaked leading to a general necrosis.

Sampling procedures

Depending on the type of material to be sampled, different methods are used (Rizzo *et al*., 2002), as described below.

Plant material
Trees: for bleeding cankers, the inner bark in the area directly around the oozing sap is cut until a canker margin is evident. Pieces of phloem and xylem are removed and placed in a sealed container, or small pieces may also be plated out on different media directly (Rizzo *et al*., 2002).

Shoots or twigs: the leading edge (junction between diseased and healthy tissue) is located and a piece of stem approximately 15 cm long, with 7.5 cm either side of the leading edge is removed and placed in a sealed plastic bag, with a small piece of damp tissue to prevent desiccation.

Leaves: 4–6 leaves showing a suitable range of symptoms are removed and placed in a sealed plastic bag with a piece of damp tissue.

All samples of plant material should be sent to the laboratory to arrive the next day. Overheating or desiccation of samples prior to despatch should be prevented. Samples may be stored in a refrigerator (4–10°C) for several weeks if necessary.

Water
At least 1 L of water, including sediment and any floating debris from the surface of the water, is skimmed off for testing. It is placed in a strong plastic bottle kept cool (4–10°C) and sent to the laboratory to arrive the next day.

Soil
At least 500 g or 200 mL of soil and debris from affected areas is collected and placed in a strong, sealed plastic bag. It should be kept cool (4–10°C), and sent to the laboratory to arrive the next day.

Preliminary screening by serological methods

Serological methods can be used to pre-screen for the presence of *Phytophthora* spp. These methods are not specific to *P. ramorum* and false negatives and positives may occur. Kits are commercially available, e.g. from ADGEN Agri-foods Diagnostic Systems (Ayr, GB; www.adgen.co.uk) and Central Science Laboratory (York, GB).

Isolation from plant material

Various alternative methods may be used for surface disinfestation or decontamination of plant material. To eliminate surface-colonizing microorganisms in cases where *P. ramorum* is supposed to be present inside tissues, alcohol treatments or a solution with 0.5% active sodium hypochlorite for 2–5 min may be used. The plant material should then be rinsed with sterile water and dried. Other alternatives (Appendix 1) depend on:

- the type of substrate (thin root or leaf tissue should not be treated with alcohol)
- the type of test to be performed (culturing is prone to contamination by other microorganisms but molecular methods are not)
- the risk of false negatives (water rinses may partially remove contamination with irrelevant organisms but allow the remaining ones to suppress *P. ramorum*, whereas alcohol treatments may remove the contaminants as well as *P. ramorum*).

Incubation
Sometimes, presence of *P. ramorum* can be seen directly on the collected material (i.e. presence of typical sporangia). If not, material can be incubated for 3–5 days in a closed box with a small piece of damp tissue on the bottom to promote sporulation.

Plating on medium
P. ramorum is then isolated by plating on a suitable medium (Appendix 2). The most commonly used medium is P_5ARP [H] as this is semi-selective for *Phytophthora* spp. and characteristic features of *P. ramorum* are readily observed. From surface-disinfested material, at least four small pieces (about 2 mm^2) of tissue are excised with a sterile scalpel and transferred aseptically onto one or more appropriate agar media. Plates are incubated on the laboratory bench (20–25°C), or in an incubator at 20–22°C.

Isolation from water or soil

Water samples
For all water samples, the 'rhododendron leaf test' should be used (Themann & Werres, 1998; Themann *et al*., 2002; www.bba.de/phytoph/diagn_r.htm). Leaves should be taken from plants which have not previously been sprayed with fungicides. For small samples, known healthy leaf pieces of *Rhododendron*, preferably *R. catawbiense* 'Cunningham's White', are floated on the surface of water samples. Within 3–7 days of incubation, suspect leaf areas should be cut out for isolation onto nonselective (e.g. CPA) or selective media (e.g. P_5ARP[H]), or for testing by PCR. For media, see Appendix 2. For larger samples (e.g. irrigation ponds, streams, etc.), 5–10 leaves (as above) are placed into a small piece of muslin, and pieces of polystyrene or similar material are added to aid flotation. The material is placed in a bag, tied with string, and floated on the water for 3–7 days. The presence of *P. ramorum* is checked as described for small samples.

Soil
The Rhododendron leaf test is used (as above). The soil is placed in a large plastic box, and sufficient sterile, demineralized water or Petri's mineral solution is added to allow leaf pieces (as above) to be floated on the surface. After 3–7 days incubation, the presence of *P. ramorum* is checked as described for the water samples.

Table 1 Growth characteristics on a selective and non-selective medium

Character	P_5ARP(H)*	Carrot piece agar*
Colony	relatively slow growing, approximately 2 mm per day	weak rosette-like pattern, pronounced concentric rings, growth rate approximately 3 mm per day
Mycelium	weakly coralloid, growing within the agar with little superficial growth, no hyphal swellings	aerial mycelium sparse, no hyphal swellings
Sporangia	produced abundantly on the agar surface, semi-papillate, caducous with short or no stalk. Size: 20–32 × 40–80 µm, average 24 × 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	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
Sexual structures	can be observed on carrot piece agar after pairing with the opposite mating type, e.g. that of *P. cryptogea* (Werres & Zielke, 2003). *P. ramorum* × *P. ramorum* pairing is also possible *in vitro* (not with all isolates) (Brasier, pers. comm.) and in Rhododendron twigs (Werres & Zielke, 2003)	

*On P_5ARP(H) characters can be observed after 4–6 days incubation on the bench at 20°C, 12 h light/12 h dark. On carrot piece agar this is after 3–5 days incubation at 20°C in darkness.

Identification

P. ramorum may be identified at species level either by its growth characteristics and morphology in culture (with confirmatory biochemical or molecular tests if necessary), or by appropriate molecular methods. A flow diagram indicating combinations of methods is given in Fig. 1.

Growth characteristics in culture and morphology

The growth characteristics and morphological features on agar are described in Werres *et al*. (2001). The most essential features on a selective and a non-selective medium are given in Table 1. Generally, the unique morphological features described make *P. ramorum* a relatively easy organism to identify in culture. The greatest possibility of confusion in morphology and cultural characteristics is with *Phytophthora palmivora*. The key characteristics are illustrated in Web Figs 16–22.

Biochemical methods

Identification of *P. ramorum* by isozymes is described in Werres *et al*. (2001) with reference to Man in't Veld *et al*. (1998). Both enzymes used, malate dehydrogenase (MDH) and malic enzyme (MDHP), are monomorphic within the *P. ramorum* population (40 strains tested; Man in't Veld *et al*., 2002b). The closely related *P. lateralis* and *P. hibernalis* differ from *P. ramorum* on the isozyme loci concerned. These data were validated using the PhastSystem described below. Details of the methods are given in Appendix 3.

Molecular biological methods

Several molecular methods have been developed to identify *P. ramorum* from culture plates as well as directly *in planta*, using either conventional PCR or Real-time PCR.

Methods for conventional PCR, with primers designed by M. Garbelotto (US) (Method A: Kox *et al*., 2002; Method B: Wagner & Werres, 2003) or by K. Hughes (GB) (Method C: Lane *et al*., 2003b), are presented in Appendix 4. Methods for real-time PCR (Method A, Hughes *et al*., 2005; Method B, Hayden *et al*., 2004) are presented in Appendix 5.

In general, the conventional PCR primers are quite specific for *P. ramorum* with a small number of cross reactions. Conventional PCR using Garbelotto's primers may show cross reaction with *Phytophthora lateralis* (L.F.F. Kox, pers.comm.), a fungus that is not native to Europe and does not occur on hosts of *P. ramorum*, and with *P. cambivora* at high DNA concentrations (Davidson *et al*., 2003). The British Real-time PCR primers are specific to *P. ramorum* (Hughes, *pers. comm*.). Further identification methods include sequencing of the ITS region to permit identification to species (Appendix 6).

Positive identification

For a positive identification the fungus should have been identified unambiguously by any one of the following:

- morphological examination: determination of the presence of semi-papillate, caducous sporangia with short or no stalk, and large chlamydospores (up to 80 µm), hyaline to pale brown
- Real-time PCR, satisfying the criteria in Appendix 5
- Conventional PCR, satisfying the criteria in Appendix 4.

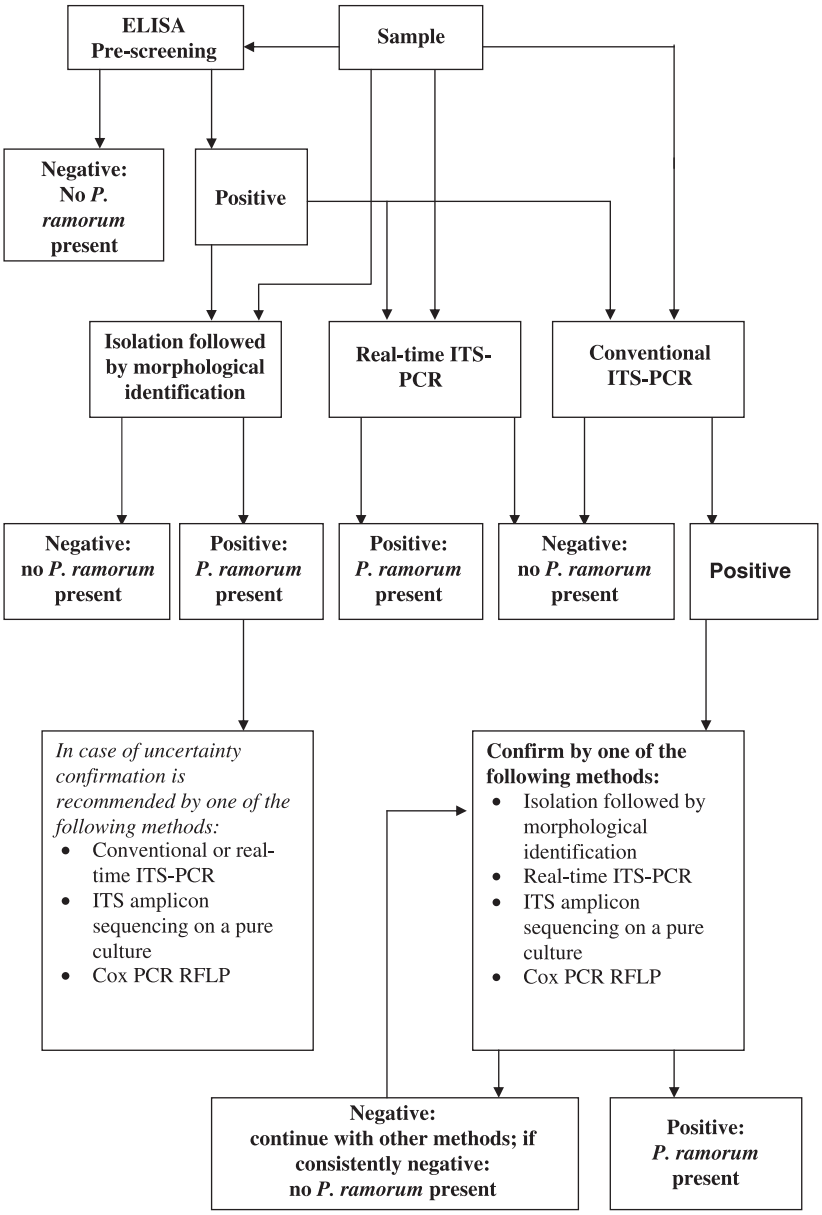

Fig. 1 Flow diagram for diagnosis of *Phytophthora ramorum* on plants and plant products.

Mating type may be determined using A1 and A2 tester strains (Werres *et al*., 2001; Werres & Zielke, 2003; Brasier & Kirk, 2004). Molecular biotypes may be identified by PCR-RFLP analysis or sequencing of the mitochondrial cytochrome oxidase (Cox)-gene (Kroon *et al*., 2004). A flow diagram describing the appropriate tests necessary for a positive diagnosis is given in Fig. 1.

Reference cultures

Type strain of *P. ramorum*: BBA 9/95 (A1) = CBS 101553. Available from BBA-Braunschweig, 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 (NL).

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

Further information

Further information may be obtained from:
Mycology Section, Plant Protection Service, PO Box 9102, 6700 HC Wageningen (Netherlands) (Fax 31.317.421701, Tel. 31.317.496111, E-mail: g.c.m.van.leeuwen@minlnv.nl)
Central Science Laboratory, YO41 1LZ York (UK) (Fax 44 1904462111, Tel. 44 1904462000, 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 (Germany) (Fax 49 531299 3009, Tel. 49531 2994407, E-mail: S.Werres@bba.de).

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Appendix I Disinfection techniques

Aerial plant parts

Sodium hypochlorite dipping and rinsing

Disinfest plant material superficially with a tissue soaked in 1% sodium hypochlorite solution. Excise small pieces of material (0.5–1 cm^2 each) from the leading edge then wash in tap water (10 s), ethanol 50% (10 s) and then finally tap water (10 s).

Alcohol swabbing
Quickly wipe the surface of selected plant tissue with cotton wool impregnated with 70% ethanol, then excise small pieces of material and transfer aseptically to selective medium. For stems, remove the bark prior to plating out.

Immersing in water or disinfestant solution
Either (1): select appropriate plant parts, place in a large plastic bag, add about 20–30 mL of distilled water and allow to soak for several minutes, agitate the sample within the bag for 10 s, drain off the water, repeat washing, then remove the plant material for isolation; or (2) place in approximately 0.5% active sodium hypochlorite solution for 2–5 min in a laminar flow cabinet, wash in sterile distilled water twice, dry carefully (on filter paper), then transfer aseptically to one of the media listed below in 'Media and incubation'.

Appropriate plant parts are, for twigs, at least three pieces per twig, one from the dark brown area, one from the edge of the lesion and one from the healthy looking tissue just under the lesion; for leaves: little pieces (about 0.5 × 0.5 cm) from the edge of a necrosis area or spot.

Stem base, roots or heavily contaminated samples

Flushing with water
Excise suitable pieces of tissue, place in a 250 mL side-arm conical flask and over the neck place a porous cover (e.g. muslin, fine wire mesh, Parafilm with small holes pierced in it). Connect the flask to the tap and flush with water for at least 2 h.

Alcohol treatment
Cut out large pieces (minimum 10 × 10 cm). In the laboratory, dip these pieces into 98% alcohol, let them dry and cut out little pieces to insert into carrot piece agar or selective medium. It is also possible to insert small pieces directly into medium in the field, without alcohol treatment.

Appendix 2 Media for Phytophthora ramorum

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 r.p.m., and pour off the supernatant. Make up the resultant to 1 L with distilled water, and autoclave at 120°C for 20 min.
P_5ARP[H] (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, poor, store at 4°C in the dark, use within 5 days.
P_5ARP If hymexazol is unavailable, then PARP is still very useful.
PARB [H] (Robin *et al.,* 1998): cornmeal agar 17 g; distilled water 1000 mL. Autoclave, then cool to 50°C in a water bath. Then prepare pimaricin 10 mg; ampicillin 250 mg; rifampicin 10 mg; benomyl 15 mg; hymexazol 50 mg.
Carrot Piece Agar (Werres *et al*., 2001): agar 22 g, carrot pieces 50 g, distilled water 1000 mL.
Carrot Juice Agar 5% (Kröber, 1985): agar 5–22 g; carrot juice (without honey) 50 mL; distilled water 950 mL.
CSL Dark Carrot Agar (DCA): carrots 200 g; agar Oxoid No. 3 15 g; distilled water 1000 mL.
Slice the fresh carrots and comminute in a blender with 500 mL of distilled water for 1 min at high speed. Filter through four layers of cheesecloth and squeeze out the juice from the residue. Make up the resultant filtrate to 1 L and add the agar. Heat to dissolve the agar, pour into bottles and autoclave at 121°C for 15 min.
Cherry decoction Agar (CHA): agar 60 g; distilled water 3600 mL; cherry juice 400 mL. Filter the cherry juice, and adjust the pH to 4.4 with KOH. Dissolve the agar thoroughly first, then add cherry juice. Autoclave at 102°C for 5 min.
Snyder and Nash Agar (SNA): KH_2PO_4 1 g; KNO_3 1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; KCl 0.5 g; glucose 0.2 g; saccharose 0.2 g; agar 15 g; distilled water 1000 mL. Autoclave for 15 min at 120°C.

Appendix 3 Identification on basis of isozymes

Culturing*:* isolates are grown in 50 mL of Tryptone Soy Broth (TSB, Oxoid) medium in 250 mL Erlenmeyer flasks on a rotary shaker at 40 r.p.m. in the dark at 23°C. Flasks are inoculated with three V8-agar plugs (diameter 5 mm) bearing mycelium, from the margin of actively growing colonies (three days old). After 7 days, the mycelium is collected by pouring the content of the flask through a sieve (pore size approximately 1 mm). The fungal tissue is dried by pressing it between filter paper. The mycelium is stored overnight at –70°C before extracting enzymes.

Extraction: frozen mycelium is thawed at 4°C for 3 h before enzyme extraction. Then approximately 0.5 g of mycelium is ground for three minutes in a chilled mortar with sand and 70 µL of extraction medium (0.1 M Tris-HCl (pH 7.0), 1 mM dithiothreitol, 50 mM Ethylene Diamine Tetra-acetic Acid (EDTA), 10% Poly Vinyl Pyrrolidone (PVP; M.W. 25000) w/v, 50 µg/mL soybean trypsin inhibitor, 0.1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF) and 5% glycerol (v/v)). The homogenate is transferred to microcentrifuge tubes and centrifuged for 10 minutes at 14 000 r.p.m. (4°C). The supernatant (40–80 µL) is collected, and stored at –70°C before use.

Electrophoresis and enzyme staining: electrophoresis is performed at 4°C, for example by using an automated Phast-System®. Crude extracts, obtained as above are analyzed for any one of the following enzymes:

1 malic enzyme (MDHP, EC 1.1.1.40) on native, 12.5% homogeneous polyacrylamide gels, or
2 malate dehydrogenase (MDH, EC 1.1.1.37) on native, 8–25% gradient polyacrylamide gels.

Gels are prepared with 0.11 M Tris-acetate buffer (pH 6.4). The running buffer, contained in 2% agarose gel, consists of a 0.25 M Tris and 0.88 M L-alanine buffer (pH 8.8). Glycine is added to the samples to produce a final concentration of 0.22 M.

MDH activity is assayed by diluting 5–10 times to avoid smearing. Following electrophoresis, gels are immersed in freshly prepared staining solutions in the dark at 37°C for 5 and 20 min for MDH and MDHP, respectively. The reaction ingredients of the enzymatic stains are as follows:

MDH: 25 mL 0.2 M Tris-HCl pH 8.0, 440 mg L-malic acid (di-sodium salt), 12.5 mg Nicotineamide-Adenine-Dinucleotide (NAD), 7.5 mg Nitro Blue Tetrazolium (NBT) (Sigma N 6876), 1 mg Phenazine Metho Sulfate (PMS) (Sigma P 9625).

MDHP: 25 mL 0.2 M Tris-HCl pH 8.0, 440 mg L-malic acid (di-sodium salt), 12.5 mg Nicotinamide Adenine Dinucleotide Phosphate (NADP), 7.5 mg NBT, 1 mg PMS.

Analysis: the bands appearing on the gel are identified by using a reference strain of *P. ramorum*.

Appendix 4 Identification at species level by conventional PCR

Conventional PCR: Method A (Kox *et al.*, 2002)

Primers: a primer pair (Phyto 1 and Phyto 4) has been developed by M. Garbelotto (Hayden *et al*., 2004) for the detection of *P. ramorum* by conventional PCR. The primer sequences are:

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

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

Sample handling: plant material (leaves, twigs, stems, stem base) is disinfected superficially with a tissue using 1% sodium hypochlorite. Small pieces of material (0.5–1 cm^2 each) are cut from the edge of symptomatic tissue with a sharp knife. In general 6–10 pieces are collected and washed in a laminar flow cabinet according to the following procedure: tap water (10 s), alcohol 50% (10 s), tap water (10 s), 1% sodium hypochlorite (10 s), and finally tap water (10 s). The material is then dried on sterile filter paper for 20–30 s. The pieces are cut into very small parts (each approximately 1 mm^2) with a sterile scalpel, and placed in a clean 1.5 mL screw cap tube. When not immediately processed, the tubes are stored with material in a freezer (–20°C). Cultures can also be used as starting material for extraction. A piece of approximately 1 cm^2 from the agar (taking as little agar as possible) is cut, and put it into a 1.5 mL screw cap tube.

DNA extraction: 500 µL extraction buffer (0.02 M PBS, 0.05% Tween T25, 2% polyvinylpyrolidone, 0.2% bovine serum albumin) and beads are added to the tube which is beated for 80 s at speed 5 (Hybaid Ribolyzer). The tube is centrifuged for 5 s in a microcentrifuge at maximum speed (16 100 g) and 75 µL of the supernatant is transferred to a new 1.5 mL tube.

DNA isolation: DNA for PCR is isolated using the Dneasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions (Qiagen, 2000), and is eluted with 100 µL AE buffer. It is further purified using polyvinylpolypyrrolidone (PVPP) columns. These columns are prepared by filling a Micro Bio-Spin chromatography column (Biorad, cat no: 732–6204) with 0.5 cm PVPP, and placed in an empty tube. Then 250 µL RNAse- and DNAse-free water are applied to the PVPP column which is centrifuged for 5 min at 4000 r.p.m. in a microcentrifuge. This last step is repeated. The columns are put on a clean tube, and DNA is applied to the column which is centrifuged for 5 min at 4000 r.p.m.

Amplification and analysis: the reaction mixture (25 µL) should contain: 5 µL DNA suspension; 2.5 µL of 10 × concentrated reaction buffer containing 15 mM $MgCl_2$ (Qiagen); 0.5 µL 10 mM dNTPs; 0.1 µL 100 µM of each primer Phyto 1 and Phyto 4; 0.1 µL HotStarTaqTaq DNA polymerase (5 U/µL; Qiagen), and 16.7 µL DNAse- and RNAse-free water to give a final volume 25 µL.

The amplification is performed in thin-walled 200 µL PCR tubes in a Peltier type thermal cycler with a heated lid using the following conditions: 15 min at 95°C; then 35 cycles of 15 s at 94°C, 60 s at 62°C, 45 s at 72°C. One cycle for 10 min at 72°C should be conducted after the 35 cycles, and finally 1 s at 20°C. After amplification, 10 µL of the reaction mixture is loaded onto a 1.0% agarose gel in 0.5 × TBE buffer, separated by electrophoresis, stained with ethidium bromide, and viewed and photographed under UV light. A negative control (DNAse- and RNAse-free water) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen) to monitor the performance of the PCR. The positive control should yield an amplicon of 687 bp, but not the negative control. Strains yielding an amplicon of this size are positive for *P. ramorum*. Samples not yielding such an amplicon can be considered negative for *P. ramorum*. If either the negative or positive control fails the electrophoresis and/or the PCR should be repeated. To monitor for false negatives resulting from inhibition of the PCR reaction, duplicate reactions are spiked with *P. ramorum* DNA. If the spiked reaction gives a negative result, the PCR should be repeated with diluted DNA extract.

Conventional PCR: Method B (Wagner & Werres, 2003)

For Garbelotto's primers see above.

Sampling and sample preparation: if possible at least five twigs or leaves with disease symptoms are taken per plant. About five tissue pieces per twig or leaf (each 0.5 cm^2, per sample about 100–200 mg total weight) are cut out from the discoloured tissue using a sterile knife.

DNA extraction: 500 µL CTAB-buffer (2%) and 50 mg of sterile quartz sand are added to the sample, which is then homogenised with a pestle and mortar for 2 min. This preparation is transferred into a 2 mL tube and vortex for 20 s. It is then freezed at –20°C defrosted at 75°C. The operation is repeated two times for two min, and a third time for 30 min 350 µL of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) is added and the preparation is vortexed for 1 min at maximum speed. It is then centrifuged for 15 min in a microcentrifuge at maximum speed (16 100 g), and the supernatant is removed and pipetted into a new sterile 1.5 mL tube.

DNA isolation: the DNA is purified with GeneClean Turbo Kit (Nucleic Acid Purification Kit: OBIOGENE).

Amplification and analysis: the reaction mixture (25 µL) should contain: 6.25 µL DNA suspension; 2.5 µL of 10 ×

concentrated reaction buffer containing 7.5 mM $MgCl_2$ (INVITROGEN); 2.5 µL 2 mM dNTPs; 0.25 µL 50 µM of each primer Phyto 1 and Phyto 4; 0.25 µL Taq DNA polymerase (INVITROGEN, recombinant cloned), and 12.25 µL DNAse- and RNAse-free water.

Thermocycler conditions should be as follows: 1.25 min at 94°C; then 34 cycles of 35 s at 93°C, 55 s at 62°C, 50 s at 72°C adding 5 s at each cycle. One cycle for 10 min at 72°C should be conducted after the 34 cycles. Separate the PCR product after amplification. Load 10 µL of the reaction mixture onto a 1.0% agarose gel in 1 × TAE buffer, separate by electrophoresis, stain with ethidium bromide, and view and photograph under UV light.

A negative and a positive control should be included in every PCR. For the negative control 6.25 µL DNAse- and RNAse-free water and 18.75 µL PCR mix are taken. For the positive control DNA from the type strain is used. The DNA of the type strain should yield an amplicon of 687 bp. Samples yielding an amplicon of this size should be identified as *P. ramorum*, assuming that the controls have reacted properly.

Conventional PCR: Method C (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′.

Sample handling: a 0.5 cm × 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.

DNA extraction: 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 20 s.

DNA isolation: DNA is extracted from ground-up samples using a suitable 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.

Amplification and analysis: extracted DNA is defrosted if necessary and a 10-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 10-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 are 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 then be extracted and retested.

Appendix 5 Identification at species level by Real-time PCR

The following two equivalent and validated methods may be used for TaqMan®-PCR identification of *P. ramorum* from cultures and plant material.

RT-PCR: Method A (Hughes *et al.*, 2005)

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-carboxy-tetramethyl-rhodamine (TAMRA).

Sample handling/DNA extraction/DNA isolation: DNA from samples is prepared at approximately 20–100 ng/µL as described above for the conventional PCR-method C.

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 analyzed 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.

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 or COX fluorescence is recorded this indicates that the sample contains no amplifiable DNA and that sample should be re-extracted and tested again.

RT-PCR: Method B (Hayden *et al*., 2004)

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

Pram-5: 5′-TTA GCT TCG GCT GAA CAA TG-3′,

Pram-6: 5′-CAG CTA CGG TTC ACC AGT CA-3′,

and the TaqMan®-probe is:

Pram-7 : 5′-ATG CTT TTT CTG CTG TGG CGG TAA-3′.

The hybridization oligonucleotide is labeled with 6-FAM at the 5′ end (reporter) and TAMRA at the 3′ end (quencher).

Sample handling/DNA extraction/DNA isolation: DNA from samples are prepared as described in section 'Identification at species level by conventional PCR', Method (A).

Amplification and analysis: the reaction mixture (15 µL) should contain: 1 X Taqman Universal PCR Master Mix (Applied Biosystems part no. 4324018); 0.2 µM of each primer; 0.2 µM of probe; 5.0 µL template DNA.

Cycle test reactions should be performed in a suitable instrument, for instance ABI Prism 7700 or 7900 Sequence Detection System (Applied Biosystems) under the following conditions: 1 cycle at 95°C for 3 min; then 35 cycles at 95°C for 15 s, 60.5°C for 1 min. Store end products between 4 and –20°C.

A negative control (DNAse- and RNAse-free water) should be included in every experiment to test for contamination as well as a positive control (DNA from a reference strain of the pathogen) to monitor the performance of the PCR. If either the negative or positive control does not give the proper result, the PCR should be repeated. To monitor for false negatives resulting from inhibition of the PCR, duplicate reactions are spiked with *P. ramorum* DNA. If the spiked reaction gives a negative result, the PCR should be repeated with diluted DNA extract.

Assessment of PCR: samples containing amplifiable *P. ramorum* DNA produce FAM fluorescence as recorded by Ct FAM values of < 35.

Appendix 6 Identification at species level by sequencing part of the ITS region

The identity of *P. ramorum* isolates from new hosts or isolates that do not morphologically match published descriptions can be confirmed by sequencing. Only DNA from pure isolates can be tested using this method, otherwise sequences from multiple organisms may be amplified in the same reaction.

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 µL 5 µM of each primer ITS 1 and ITS 4; 0.5 µL AmpliTaq (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 90 µL 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).

Web Fig. 1 Oak bleeding canker.
Web Fig. 2 Canker on Quercus rubra.
Web Fig. 3 Canker on Quercus falcate.
Web Fig. 4 Oak – dead tree.
Web Fig. 5 Rhododendron shoot canker.
Web Fig. 6 Rhododendron – wilted leaves.
Web Fig. 7 Rhododendron – leaf blackening.
Web Fig. 8 Viburnum – stem base necrosis.
Web Fig. 9 Viburnum – leaf blight.
Web Fig. 10 Pieris – leaf blight.
Web Fig. 11 Syringa – bud/leaf blight.
Web Fig. 12 Kalmia – leaf blight.
Web Fig. 13 Camellia – leaf blight.
Web Fig. 14 Leucothoe – leaf blight.
Web Fig. 15 Taxus – needle dieback.
Web Fig. 16 Typical slow growing colony on a selective medium (P_5ARP(H)).
Web Fig. 17 Typical coralloid mycelium on a selective medium (P_5ARP(H)).
Web Fig. 18 Sporangia on a selective medium (P_5ARP(H)).
Web Fig. 19 Chlamydospore on a selective medium (P_5ARP(H)).
Web Fig. 20 Typical outgrowth of colony on a nonselective medium (CPA).
Web Fig. 21 Sporangia (bar = 20 µm) on a nonselective medium (CPA).
Web Fig. 22 Chlamydospore (bar = 20 µm) on a nonselective medium (CPA).
Web Fig. 23 Flow diagram for diagnosis of *Phytophthora ramorum* on plants and plant products.

Webfig. 1. Bleeding canker on oak

Webfig. 2. Canker on *Quercus rubra*.

Webfig. 3. Canker on *Quercus falcata*.

Webfig. 4. Oak – dead tree.

Webfig 5. *Rhododendron* shoot canker.

Webfig. 6. *Rhododendron* shoot tip wilt.

Webfig.7. *Rhododendron* leaf necrosis of midrib from petiole.

Webfig. 8. *Viburnum* stem base discoloration.

Webfig 9. *Viburnum* - leaf blight.

Webfig. 10. *Pieris* - leaf blight.

Webfig. 11. *Syringa* – leaf blight.

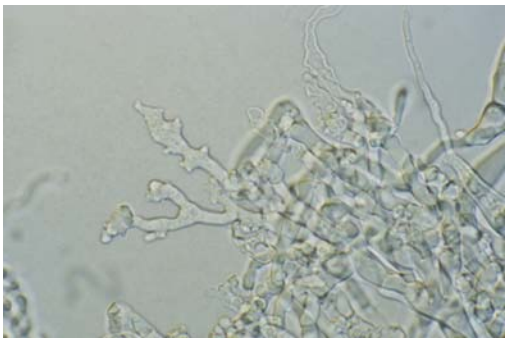
Webfig. 12. *Kalmia latifolia* – leaf blight.

Webfig. 13. *Camellia* – leaf blight.

Webfig. 14. *Leucothoe* – leaf blight.

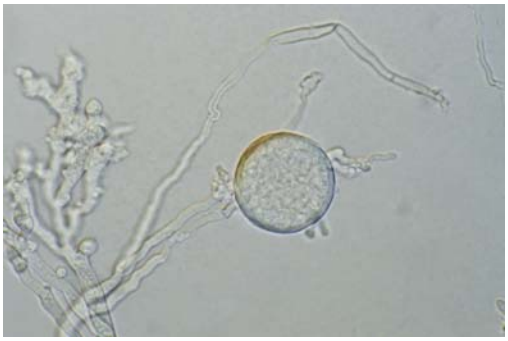
Webfig. 15. *Taxus* – needle dieback.

Webfig. 16. Typical slow growing colony on a selective medium P_5ARP(H)



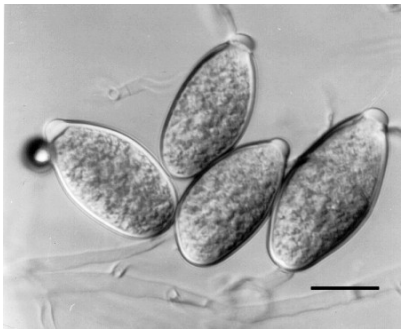
Webfig. 17. Typical coralloid mycelium on a selective medium P_5ARP(H)

Webfig. 18. Sporangia on a selective medium P_5ARP(H)

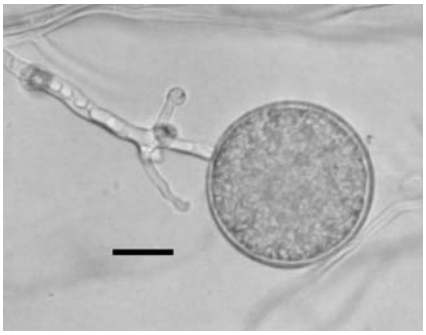


Webfig. 19. Chlamydospore on a selective medium P_5ARP(H)

Webfig. 20. Typical outgrowth of colony on a non-selective medium (CPA)



Webfig 21. Sporangia (bar = 20 µm) on a non-selective medium (CPA)



Webfig. 22. Chlamydospore (bar = 20 µm) on a non-selective medium (CPA)