Phytophthora cinnamomi in Australia: Protocols for isolation, culture and molecular detection

Compiled by Dr Manisha Kunadiya March 2021 version

Contents

INTRODUCTION

This diagnostic protocol provides technical information for the detection and identification of *Phytophthora cinnamomi* Rands. This species was first isolated from cinnamon trees in Sumatra in 1922 (Rands, 1922).

Phytophthora cinnamomi is a soil-borne pathogen with global a distribution (Burgess et al., 2017). It causes root and collar rot in many plant species within natural ecosystems and horticultural crops worldwide. In Australia, the inadvertent introduction of *P. cinnamomi* into natural ecosystems has caused deaths in a wide range of native plant species and has had a deleterious impact on biodiversity. Consequently, it is recognised as a 'Key threatening process to 'Australia's 'biodiversity' by the ' 'Commonwealth's Environmental and Biodiversity Conservation Act '1999'. Most significantly, it causes dieback or death within *Banksia* woodland and heathlands in the southwest of Western Australia (Hardy et al., 2001, Shearer et al., 2007), where out of the 5710 described plant species in the south-west botanical province, approximately 2300 species are susceptible and 800 highly susceptible (Weste, 2003, Shearer et al., 2004). It is also a major pathogen of several horticultural species, including macadamia, avocado and pineapple (Simmonds, 1929, Pegg et al., 1990).

Host range

Phytophthora cinnamomi has a wide host range across the globe; it causes disease in about 5000 species of plants, including some 4000 Australian native species. This includes native trees species used in horticultural and forestry in Australia include jarrah (*Eucalyptus marginata*), avocado, and macadamia. The host range is continually updated, and a list is given on CABI; https://www.cabi.org/isc/datasheet/40957 (accessed 11 December 2020).

TAXONOMIC INFORMATION

Taxonomic position:

Domain	Eukaryota
Kingdom	Chromista
Phylum	Oomycota
Class	Oomycetes
Order	Peronosporales
Family	Peronosporaceae
Genus	*Phytophthora*
Species	*cinnamomi*

Scientific Name: *Phytophthora cinnamomi* Rands
Common Name: *Phytophthora* dieback

(Hardham and Blackman, 2018)

DETECTION

Symptoms

The following symptoms can classify diseases caused by *P. cinnamomi*;

- The rot of fine feeder roots and formation of root cankers, leading to dieback and death of host plants,
- Wilt, stem cankers, yield decline, fruit and leaf size decreased, and
- Gum exudation, collar and heart rot.

The primary symptom of *P. cinnamomi* infection is the rot of the fine and small woody roots, where the disease causes necrotic lesions. The pathogen penetrates the epidermis and cortex, grows into the stele, kills the phloem and cambium, and extends along the roots. In many woody hosts, it can also girdle the collar (Fig 1).

Fig 1: Necrotic girdling collar lesion in *Eucalyptus marginata* caused by *Phytophthora cinnamomi.*

The secondary symptoms can be chronic or acute and include leaf chlorosis, abscission, and primary branch death (Fig 2). Woody shrubs become chlorotic, die back, and collapse, and

their root systems can be destroyed. In more tolerant hosts, epicormic shoots can sprout, but this wilt, turn brown and die (Fig 2b). Trees may die suddenly with brown leaves attached. While trees often take three or more years to die, some susceptible species have regenerated and survived 30 years after the initial infection (Dawson and Weste, 1984, Weste and Ashton, 1994). In these instances, the root systems are not extensively rotted, and the base of the trunk is not girdled (Weste and Marks, 1987).

The disease is promoted under conditions favourable for zoospore production and dispersal which require sufficient water and a suitable temperature (Shearer, 1989). When overstorey species have been killed many understory species which may or may not be susceptible to *Phytophthora* are lost as they do not tolerate exposure (Shearer, 1989).

Jarrah Dieback

Diagnostic symptoms of the disease on large trees included severe necrosis of the root system and cankering in the basal stem region, resulting in either plant death or decline symptoms in the crown. Jarrah may show crown decline symptoms, including leaf yellowing and death of primary leaf-bearing branches. Leaves are pale green, wilted, and fall readily (Fig 2a). Shoots die back from the tips, and the tree canopy is eventually reduced to a bare framework of dying branches (Fig 2b). The trees exhibit drought-like symptoms due to the lack of nutrients and water. Infected roots become dark brown. Waterlogging plays a significant role in the death of jarrah together with the presence of *P. cinnamomi* (Davison, 2015).

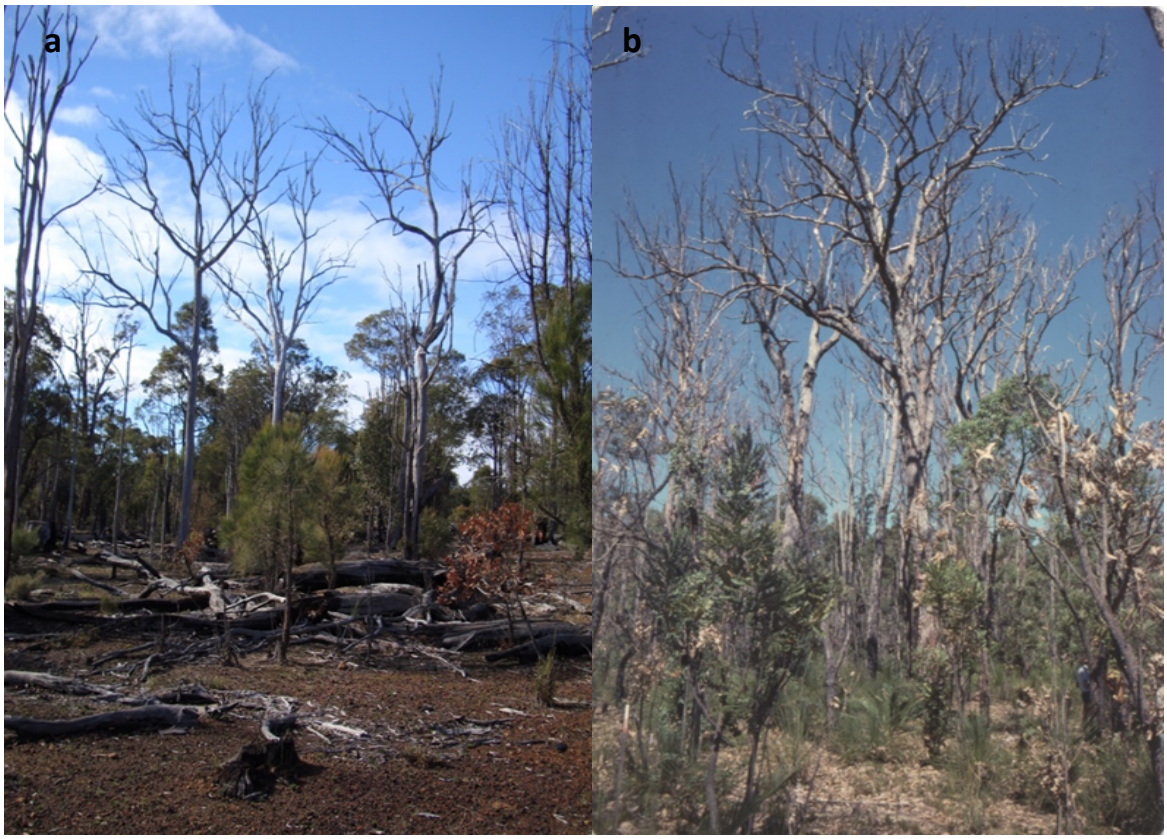

Fig 2: (a) Severely impacted jarrah *(Eucalyptus marginata*) forest showing dead jarrah trees and few understorey species remaining, and (b) dead jarrah trees in background, and in the foreground living and dead *Banksia grandis* and some which have also succumbed.

The mass death of Banksia woodland species

Diseased *Banksia* plants show discolouration of the foliage, most commonly reds and yellows, depending on the species before the foliage dries out. Symptoms of infection vary with the time of the year but usually reflect water stress in the plant shoot (Fig 3). Plants are rated dead when leaves and branches are brittle and typically white to pale green (Fig 4 a, b, e, and f) compare to healthy banksia woodland (Fig 4c, and d) (McCredie et al., 1985). It was also infected other susceptible plant species like *E. marginata* and *C. calophylla* through the lower stem periderm tissue and colonize stem tissue under condition of ponding after rainfall in rehabilitated bauxite mines areas (Fig 5 a, and b) (Colquhoun and Hardy, 2000)

Susceptibility of species of the heathlands in Western Australia

The heathland vegetation of the northern and southern sandplains of Western Australia is particularly biodiversity and highly susceptible to *Phytophthora* (Steady State Consulting 2009) (Fig 4a, b).

Fig 3: Banksia woodland with dead and dying banksias. Many understorey species have also been killed leaving resistant species of sedges (Cyperaceae) and rushes (Restionaceae).

Fig 4: (a) The Lesueur National Park infected with *Phytophthora cinnamomi* in a Coomallo area (Photo: K Howard), (b) loss of the complex Proteaceous community in the foreground with healthy and dying plants in the background (Fitzgerald River National Park), (c and d) healthy Banksia woodland, and (e and f) after *P. cinnamomi* has been introduced (Banksia woodland Swan Coastal Plain, Western Australia).

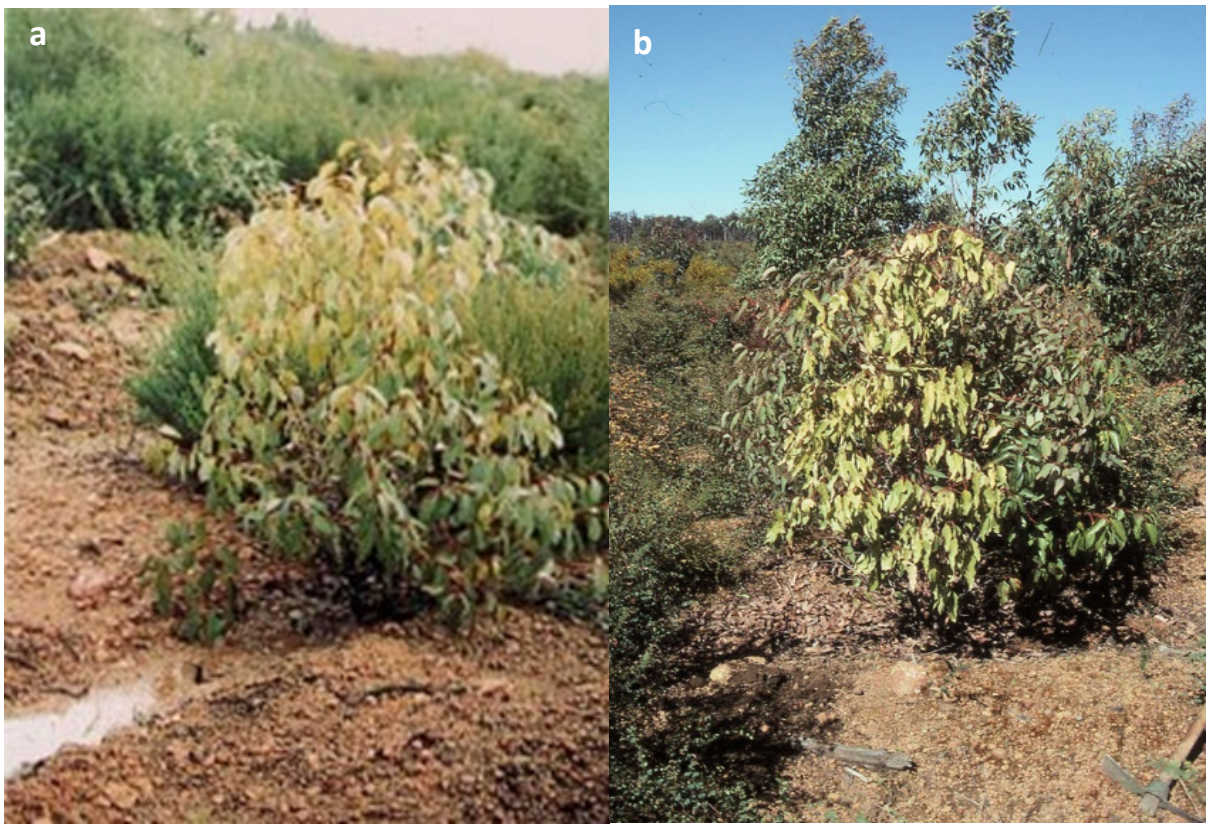

Fig 5: Chlorotic (a) *Eucalyptus marginata* and (b) *Corymbia calophylla* showing early symptoms of *Phytophthora* root and collar rot.

Root rot of a horticultural species: avocado

Young feeder roots turn brown to black, become brittle, and die. As the disease progresses, only remnants of the root systems remain. *P. cinnamomi* can also cause cankers in the collars of trees as it kills the cambium and phloem. The cankers appear as water-soaked to dark brown areas at or below ground level and can girdle the tree (Fig 6b). The cankers can often exude red resin, which becomes brownish to white and powdery as it dries (Fig 6a). Foliar symptoms of affected trees show a loss of vigour, chlorosis, loss of leaves, wilting, reduced size of fruits, and a decline of the canopy through branches' death (Silva et al., 2016).

Fig 6: (a) Avocado trunk canker and collar rot symptoms. Red resin can be seen leaking from the lesion. It becomes brownish to white and powdery as its dries. (b) Avocado trunk canker symptom under the bark. This symptom occurs only on very susceptible rootstocks. The bark has been peeled off to show the brown lesion in the wood. (Images of avocado trunk canker disease symptoms acquired from California Avocado Commission) (Eskalen, 2013).

Detection in plant material

Sampling directly from symptomatic plants

Stem or basal cankers

Phytophthora cinnamomi can be readily isolated from the active lesion front of infected woody plants and trees. On symptomatic trees, lesions can often be seen in the stem collar or roots. However, the lesion front may not always reach the collar before the plant dies. Lesions may be identified by removing the bark and slicing to expose the cambium layer at the plant's collar (Fig 1) or exposing roots using a machete, mattock, or similar tool. Samples should be taken from several places along the lesion front (to include healthy and diseased tissue) to isolate the pathogen. Samples should be stored in a clean container with damp paper to avoid desiccation. Isolations from lesions similar to that shown in Figure 1 can be made directly on *Phytophthora* selective agar plates in the field. Plates should be sealed and placed in a cooler box.

Surface sterilisation of tissue

Only small pieces (≤5 mm^2) of tissue or samples of fine roots can be used for direct plating without surface sterilisation. They should be washed in sterile water and blotted dry with

sterile filter paper. Surface sterilisation is only required for larger samples of roots or stems may be surface sterilised by immersing in 70% ethanol for ~30 sec. Thick stems (0.5-1 cm diameter) can be dipped into 70% ethanol for 10-30 sec, and then quickly flamed to burn off excess alcohol. The flamed material's outer sections can be discarded before placing small pieces of material onto the selective medium. Samples are placed on a *Phytophthora* selective medium (e.g., NARH) and cultured at 20-22°C for 3-10 days in the dark. Plates should be examined regularly for the slow emergence of non-septate hyphae. These should then be sub-cultured onto fresh plates for further examination and if not contaminant free, hyphal tips subculture until aseptic cultures are obtained. Isolates should be maintained and stored into long-term storage McCartney bottles.

Sampling by soil and rhizosphere baiting

Soil or root samples should be taken from the top 10-15 cm of the rhizosphere soil, emphasising including as much root material as possible, preferably from plants showing symptoms typical of *P. cinnamomi*. At least four samples per site should be collected to give ~500g of material. The soil sample should be stored in a sterile Ziplock bag in an insulated container and processed as soon as possible.

Our standard CPSM baiting protocol uses ~ 200 g soil, including roots if present, placed into a 1 L plastic container (11.5X16.5X7.5 mm deep, Genfac Plastics P/L), covered with 100 ml distilled water and left overnight. Additional 500 ml distilled water was added to samples. Bait leaves of either *Q. ilex*, *Chamelaucium uncinatum, Hibbertia scandens, Scholtzia decussata, Pimelia ferruginea*, or *Hedera canariensis* were floated on the surface. The containers are then placed on laboratory benches at 20-22°C for 7-10 days. As soon as lesions are observed, baits should be removed and blotted dry on paper towel, cut into small pieces (1-3 mm), and plated on NARH *Phytophthora* selective medium (Simamora et al., 2017). The plates are then incubated at 22°C in the dark for 3-5 days and checked daily for the growth of any *Phytophthora* spp. Roots showing active lesion fronts can be washed of soil, dried, and plated directly onto *Phytophthora* selective agar. In larger roots, outer layers of bark with soil can be removed with a sharp knife and necrotic tissue plated as described above (Burgess et al., 2020).

Serological testing

Serological testing can be used to screen for *Phytophthora* spp. There are two commercially available serological assay kits for detecting *Phytophthora* at the genus level: the multiwall E kit and the rapid assay F kit from Agdia Inc. (https://www.agdia.com/) and Forest Diagnostics Ltd., York, UK (https://orders.agdia.com/agdia-immunostrip-for-phyt-isk-92601). An immunological dipstick assay was developed for *P. cinnamomi* zoospores based on the antigen-antibody reaction (https://www.pocketdiagnostic.com/onlineshop/pocketdiagnostic/phytophthora/). These kits are simple to use and provide a result within 3-5 min. Although this technique has been useful to detect several plant pathogens, they have limited sensitivity and specificity for *Phytophthora* (Cahill and Hardham, 1994, Lane et al., 2007, Tomlinson et al., 2010). Instructions for the use of each kit are provided by the manufacturer (Benson, 1991).

Media Recipes

All solid media should use Grade A agar or Bacto Agar. Media are autoclaved at 121°C for 20 min. Solidified media in Petri plates are stored upright and are kept in the dark if they contain antibiotics.

NARH *Phytophthora* selective medium

The original formulation of the selective medium NARPH (Hüberli et al., 2000) has been modified by the removal of the carcinogen PCNB and antibiotics at the concentrations given below have been shown to not significantly inhibit the growth of most *Phytophthora* species occurring in Australia (Sakar et al., 2020).

To 1 L of deionized water, add 17 g of Oxoid cornmeal agar (Thermo Fisher Scientific) dissolve then autoclave at 121 °C for 15-20 min. Dissolve the following antibiotics in 10-15 sterile water and add to the agar when it has cooled to about 50°C: 22.72 Nystatin (Omegapharma Pharmaceuticals Pty Ltd)), 100 ppm ampicillin sodium (Ampicyn; Alphapharma Pty Ltd), 10 ppm rifampicin (Rifadin; Sanofi-aventis Australia Pty Ltd), and 50 ppm hymexazol (Tachigaren; Sankyo Company, Toyota, Japan).

Half-strength potato dextrose agar (PDA)

To 1 L of deionized water, add 19.5 g PDA (half Potato Dextrose Agar) (Difco, Becton Dickson, NJ, USA), and 7.5 g agar.

Carrot Agar (CA)

To 0.9 L of deionized water, add 0.1 L filtered carrot juice, 17 g agar (all from Difco, Becton Dickson, NJ, USA).

V8 Agar

Clarify Vegetable-8 (V8) juice by centrifugation at 1800 g for 10 min, then vacuum filter the decanted juice through a Whatman No. 1 filter paper. Dissolve 0.05 g $CaCO_3$ in 50 ml cleared V8 juice. Add 450 ml distilled water and adjust the pH to 7 using 1 M NaOH, then add 8.5 g Bacteriological agar (Miller, 1955).

Cornmeal Agar (CMA)

To 1 L of deionised water, add 17 g of Cornmeal agar (CMA).

IDENTIFICATION

Morphological methods

Growth characteristics and morphology

Phytophthora cinnamomi is easy to identify due to its morphological features, particularly the presence of coralloid hyphae. Other key features include the presence of chlamydospores and hyphal swellings, antheridium attachment, and heterothallic or homothallic sexual reproduction (Table 1).

Table 1: Growth characteristics and morphology of *Phytophthora cinnamomi* after seven days growth on NARH medium and V8 agar.

Character	NARH selective medium	V8 agar - non-selective medium
Colony Fig 7 a) and b)	Coralloid-type colony, rosaceous, petaloid, or has no patterns, rarely symmetrical.	Medium-dense, woolly colony, uniform, filling the space between lid and agar surface (Coffey 1992; Hardham 2005).
Mycelium Fig 8 a) and b)	Prolific hyphal swellings, swollen vesicles, and terminal or lateral clustered protuberances.hyphal diameter 5.8-6.0 µm	Mycelium coralloid, with abundant hyphal swellings and spherical and in clusters, profuse tough hyphae diameter (8 µm wide).

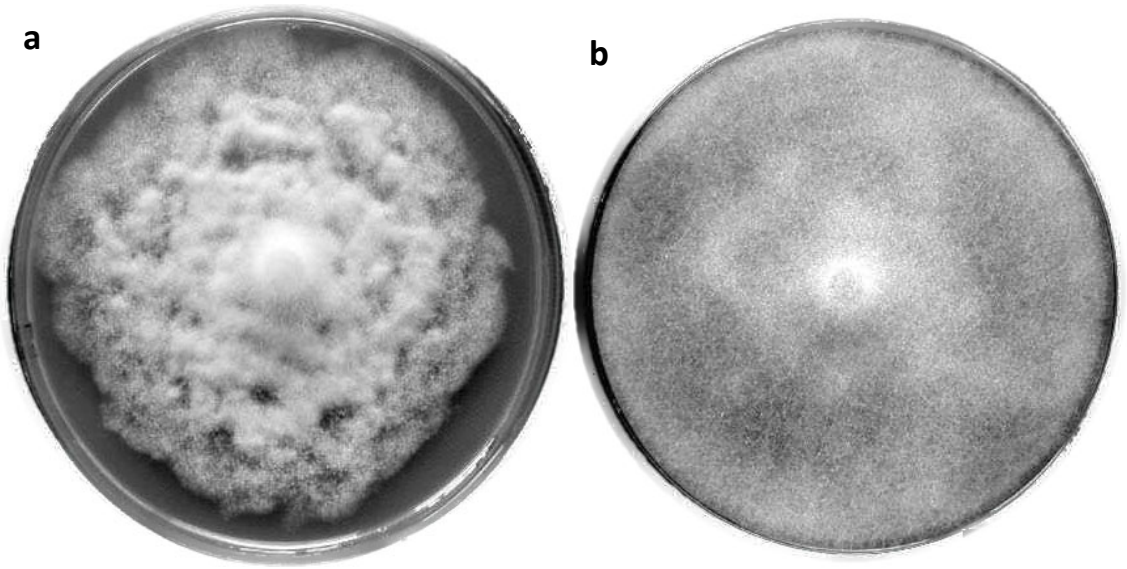

Fig 7: a) *Phytophthora cinnamomi* colony morphology after 6-7 days growth at 20°C on; a) NARH, b) V8 agar medium. Characteristic based on observation at 20°C after 6-7 days for NARH medium and after 7 days on V8 agar medium

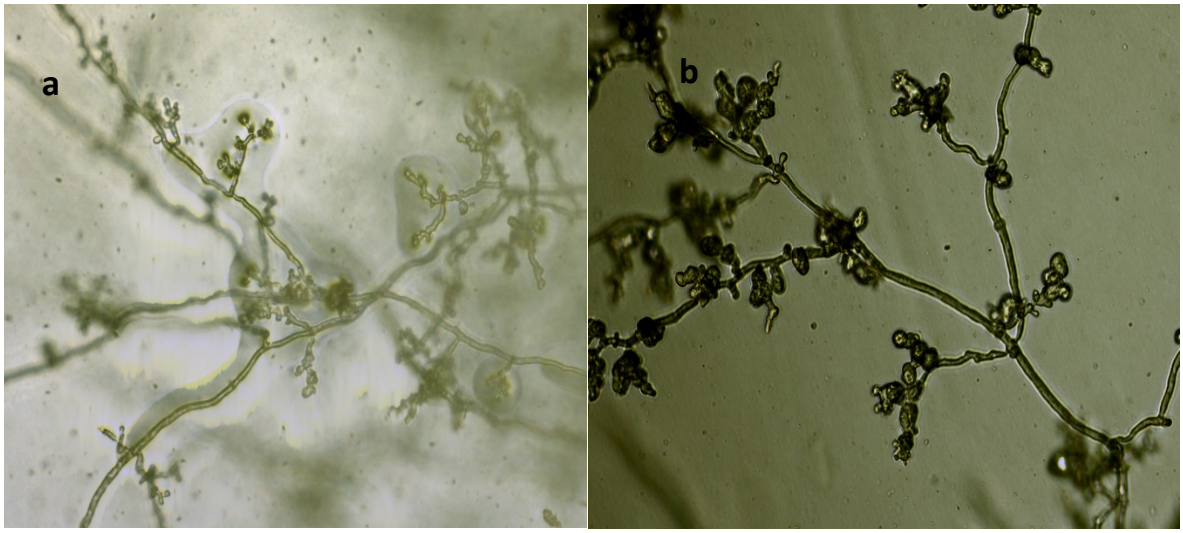

Fig 8: Typical characteristic coralloid hyphae of *Phytophthora cinnamomi* on a) NARH medium, b) V8 medium

Sporangia are ovoid, obpyriform, or ellipsoid to elongate-ellipsoid shaped. New sporangia are produced by internal or external proliferation or sympodial development of the sporangiophore immediately below empty sporangia (Erwin and Ribeiro 1996). Sporangia are not produced readily in axenic culture. However, incubation of mycelium disks in non-sterilized soil extract (10 g of soil /L water) or frequent washing of mycelium-agar disks in a salt solution usually induces sporangium formation (Chen and Zentmyer, 1970). Sporangiospores are simple, or sympodially branched either in a lax or close arrangement and exhibit internal proliferation (Erwin and Ribeiro, 1996).

Molecular methods

Many molecular techniques have been developed to detect *P. cinnamomi* from infected soil, whole plants, or plant tissue, with varied success (Duncan and Cooke, 2002, Cooke et al., 2007, Williams et al., 2009). Molecular genetic tools enable rapid identification of plant pathogens in various environmental samples, including infected plant tissue. Many PCR-based assays have been designed for *P. cinnamomi* (Kunadiya et al., 2017; O'Brien et al., 2009). However, most published primer sets have not been tested for specificity against closely related species, and most are not specific to *P. cinnamomi* (Kunadiya et al., 2017).

Described below is a *P. cinnamomi* specific probe-based qPCR assay based on the mitochondrial locus encoding subunit 2 of cytochrome c oxidase (*cox2*).

DNA extraction

Mycelium

Place 50-100 mg (wet weight) of mycelium (grown on ½ PDA) in a 2 ml Eppendorf tube and extract genomic DNA using a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions.

Plant materials

Infected plant tissue from the leading edge of lesions (roots, collar, stem, bark, and leaves) can be stored at -80°C until DNA extraction. Samples are cut into small pieces (~ 50 mg) and transferred into bead beating tubes (MO BIO, Carlsbad, USA). DNA extraction is performed using the MOBIO Power Plant DNA isolation kit (MO BIO, Carlsbad, USA).

Leaf baits

After 5-7 days, floating bait leaves (as described above), both lesioned and asymptomatic leaves are removed, washed in water, blotted dry, cut into 2 mm^2 pieces, and thoroughly mixed. A sub-sample is then taken and chopped into 0.5 mm^2 pieces to give 50 mg for DNA extraction using a MOBIO Power Plant DNA isolation kit (MO BIO, Carlsbad, USA).

Soil

DNA can be extracted from 250 mg of soil using the PowerLyzer® PowerSoil® DNA Isolation Kit (MOBIO Laboratories, CA, USA).

The use of all kits should follow the manufacturer's instructions. Extracted DNA can be stored in a DNA elution buffer at -20 °C.

Identification of *P. cinnamomi* from environmental samples

The following method can be used for qPCR identification and quantification of *P. cinnamomi* from culture, plant material, and soil samples. The nested qPCR approach can be used to increase the sensitivity of detection.

Primers

For the nested PCR, the *Phytophthora* specific primer pair Cox2F (Hudspeth et al., 2000) and Cox2RC4 (Choi et al. 2015) are used in a conventional PCR in the first round. The primer sequences are:

Cox2F: 5'GGC AAA TGG GTT TTC AAG ATC C3'

Cox2RC4: '5'TGA TTW AYN CCA CAA ATT TCR CTA CAT 'TG3'

In the nested qPCR assay, the second round of the PCR reaction uses 2 µl of amplified product from the first PCR as the template. This assay can also be used alone without the first round of PCR. The primer sequences are

PCIN 246R2: 5’-ATA ATA AAG CAA ATG ATG GTA TA-3’

PCIN 147F2: 5’-CCA GCA ACT GTT GTG CAT GGA-'3'

The TaqMan probe is labelled at the '5' end with the fluorescent reporter dye 6-carboxyfluorescein (FAM), ZEN, internal ZEN fluorescence quencher, and at the '3' end with the quencher dye Iowa Black fluorescence (IBFQ).

The probe sequence is

PCIN PROBE: 5'-/56-FAM/TGA AAT TAT/ZEN/TTGGACTTCTATACCTGC/3IABkFQ/-'3'

Amplification and analysis

In the first round, PCR the reaction mixture (25 µl) contains 2 µl genomic DNA (1.5 ng), 23 µl of master mix with 5 µl of 5X colourless Go Taq® Flexi reaction buffer (Promega), water 11.875 µl (amplification grade, Promega, USA) 2.5 µl of 25mM $MgCl_2$ (Promega, USA), 1.5 µl of 100 nM dNTP, 1 µl of 10 ng Bovine Serum Albumin (Taylor et al. 2000), and Taq polymerase 5 units/µl 0.125 µl, and 0.5 µl (1 µM) each primer. For each PCR run, positive control reactions of master mix plus *P. cinnamomi* DNA and negative control reactions of reaction mix are loaded with water rather than DNA. Amplification is performed in thin-walled PCR tubes in a Bio-Rad thermal cycler (Bio-Rad, CA, USA) as follows: An initial denaturation temperature is set at 95°C for 4 min, followed by 36 cycles of 95°C for 40 sec, the annealing at 52°C for 40 sec, extension at 72°C for 60 sec; with a final extension at 72°C for 5 min and held at 4°C (Kunadiya et al., 2019).

For the qPCR, the reaction mixture (20 µl) contains 2 µl of template DNA of the first round PCR product. If the nested approach is used, 10 µl of iTaq™ Universal Probes Super mix (Bio-Rad, USA), 7.5 µl water (amplification grade. Promega, USA), and 0.5 µl of Prime Time® qPCR assay (Integrated DNA Technology, Iowa, USA). The negative controls contained nuclease-free water instead of DNA, and a positive control of *P. cinnamomi* DNA is included in each run. *Phytophthora cinnamomi* DNA concentration in the unknown samples can be accurately determined by including a serial dilution of a known concentration of *P. cinnamomi* DNA.

Reactions are cycled in a suitable instrument for detection of the reporter fluorescence, for example, in a Rotor-Gene 6000 (Qiagen, Germany) using the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec, 30 sec at the annealing temperature (AT) 59°C and 10 sec extension at 72°C (Kunadiya et al., 2019) . Data from the qPCR assay are analysed as per the manufacturer's instructions. Typically cycle threshold (Ct) values are between 20 and 35, samples <35 are considered positive, and >35 indicate a negative result for *P. cinnamomi.*

Hybrid method to detect P. cinnamomi in asymptomatic bait leaves

The soil collection and CPSM baiting protocols are as described above.

After 10 days, all asymptomatic baits are removed, washed in water, blotted dry, cut into 2 mm^2 pieces, and thoroughly mixed. A sub-sample is chopped into 0.5 mm^2 for DNA extraction using MOBIO Power Plant DNA isolation kit (MO BIO, Carlsbad, USA). The detection of *P. cinnamomi* is then undertaken using the nested qPCR protocol as described above.

This protocol is recommended to detect *P. cinnamomi* from agricultural, forest, and other natural ecosystems, especially when the pathogen is suspected to be present but cannot be detected by traditional methods.

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