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False-negative isolations or absence of lesions may cause mis-diagnosis of diseased plants infected with *Phytophthora cinnamomi*

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Abstract
In a series of growth cabinet, glasshouse and field experiments, tissue samples from living clonal lines of *Eucalyptus marginata* (jarrah) were incubated immediately after sampling on agar (NARPH) selective for *Phytophthora*. *Phytophthora cinnamomi* was recovered 3-6 months after inoculation from 50% of samples with lesions and 30% of symptomless samples. However, up to 11% of samples with and without lesions and from which *P. cinnamomi* was not initially isolated contained viable pathogen. This was shown by removing tissue which had not produced any growth of *P. cinnamomi* on NARPH plates, cutting it into smaller sections, washing in sterile deionised water repeatedly for 9 days, and replating. Plating stem or bark tissue directly onto NARPH produced false-negative results for nine *P. cinnamomi* isolates and six jarrah clonal lines. The behaviour of the pathogen indicates that it could be present as dormant structures, such as chlamydospores, that need to be induced to germinate. Alternatively, fungistatic compounds in the tissue needed to be removed to allow the pathogen to grow. These results have important implications for disease diagnosis and management, disease-free certification and quarantine clearance.

Additional keywords: collar rot, dieback, hemibiotroph, necrotroph, recovery, root rot, fungistasis

Introduction

An important criterion in plant pathology is the ability to detect a pathogen in living, moribund or dead plant tissue. The presence of *Phytophthora cinnamomi* Rands is usually indicated by a lesion in the plant host, which is then confirmed by plating the tissue onto agar selective for *Phytophthora* (Tippett *et al*. 1983; Robin 1992; Bunny *et al*. 1995). Some reports indicate that *P. cinnamomi* is not confined to lesions as it has been isolated from symptomless tissue up to 30 mm in front of the lesion margin (Shea *et al*. 1982; Phillips and Weste 1984; Davison *et al*. 1994; Hüberli 1995). More important, however, is the recent isolation of *P. cinnamomi* from symptomless *Eucalyptus marginata* Donn. ex Smith (jarrah) stems inoculated with zoospores in two field trials (O'Gara *et al*. 1997; O'Gara 1998). In one of these trials, O'Gara (1998) demonstrated that *P. cinnamomi* could be isolated from a small proportion of mostly symptomless stems assessed as pathogen-negative by directly plating tissue onto agar selective for *Phytophthora*, after washing and baiting the stems with *Pimelea* leaves in soil extract solution.

In this study, we investigate the recovery of *P. cinnamomi* from inoculated jarrah stems with and without lesions from field and controlled environment experiments using direct plating and a washing technique. We tested the hypothesis that the standard direct plating technique used for isolating *P. cinnamomi* can give false-negative results.

Methods

Experimental design Recovery of a total of 68 *P. cinnamomi* isolates from live stem tissue was examined in nine clonal lines of jarrah (with or without lesions) that had survived for 3-6 months in three inoculation experiments (Table 1). These plants from environmentally controlled growth cabinet (Experiment 1), glasshouse (Experiment 2) and field (Experiment 3) experiments were harvested 86, 182 and 128 days after inoculation, respectively. Each experiment was a completely randomised factorial design.

Isolates A total of 68 *P. cinnamomi* isolates, all of A2 mating-type, were used in the three experiments. They were isolated from mine sites of Alcoa World Alumina Australia at Jarrahdale (n = 34) and Willowdale (n = 34) in the northern jarrah forest in the southwest of Western Australia (WA). Only isolate MP94-48 was used in all experiments.

Plant material and growth conditions All plants were supplied by the Marrinup Nursery (Alcoa World Alumina Australia, WA). Clonal lines of jarrah (line number given in Table 1) were propagated from seedlings that had been assessed for resistance/susceptibility to *P. cinnamomi* in the glasshouse using underbark wound inoculation of stems (McComb *et al*. 1990). The clonal lines were ranked according to lesion size, with large lesions and small lesions classed as susceptible (SS) and resistant (RR), respectively (McComb *et al*. 1990). Seedlings were raised from seeds collected from the northern jarrah forest. Numbers of surviving jarrah seedlings and clonal lines used in the recovery experiments are shown in Table 1.

One-year-old potted clonal lines of jarrah were used in Experiments 1 and 2. In Experiment 3, the planted seedling and clonal lines of jarrah trees had grown in the Willowdale minesite for 5 years and ranged from 1.5-6 m in height prior to inoculation. Potted plants were grown in composted pine bark, coarse river sand and muck peat (2:2:1; Richgro Garden Products, Canning Vale, WA) with added basal fertiliser (O'Gara *et al.* 1996). Each pot was top-dressed with 12-13 g of a 4-9 month slow-release fertiliser (Scotts Osmocote Plus, Scotts Europe BV, Heerlen, The Netherlands) and watered daily to container capacity in free-draining pots. Plants in Experiment 1 were grown in environmentally controlled growth cabinets (Environ Air EA7BH; SRG Cabinet Sales, Greenacre, NSW, Australia) at 600-800 μ Einsteins /sec/m^2 irradiance on a day/night cycle of 14.5/9.5 h.

Inoculum production and Inoculation Watertight receptacles were constructed around the stems of plants in Experiment 1 and inoculated with zoospores as described by O'Gara *et al*. (1997). The 1 cm region of tissue immersed in the zoospore solution will henceforth be referred to as the region of inoculation (ROI). Plants in Experiments 2 and 3 were underbark wound-inoculated 5 cm and 15 cm from the soil surface with 1 cm and 5 cm diameter Miracloth (Calbiochem Corporation, La Jolla, CA, USA) inoculum discs, respectively, using the methods of O'Gara *et al*. (1996). The wounds in Experiments 2 and 3 were sealed with Parafilm and reflective silver Polyvinyl chloride duct tape (48 mm; Norton Abrasives Pty Ltd, Lidcombe, New South Wales, Australia), respectively. The tissue in contact with the inoculum discs is referred to as the site of inoculation (SOI). Plants in Experiment 3 were inoculated on 5 November 1997 (late spring).

Harvest At harvest, the presence or absence of stem lesions was recorded. Where stem lesions were absent, the periderm was carefully scraped back to determine if phloem lesions were present. In Experiments 1 and 2, stems were cut into 1 cm sections covering from 1 cm into the lesion front to 6 cm outside the lesion and up the stem, and cut longitudinally to expose the bark and wood to the selective medium. Symptomless stems were cut into 1 cm sections as above, but from the ROI or SOI for 7 cm up the stem. Stem sections were plated sequentially onto NARPH agar (see later) which is selective for *Phytophthora*. In Experiment 3, one piece (about 5x10 cm) of bark tissue (all the tissue outside the vascular cambium) was removed from either the lesion margin if present, or from the SOI if a lesion was absent. This was cut into smaller pieces (2 x 2 cm) and plated on NARPH medium. All plates were incubated in the dark at 24 ±1°C for 14 days and were examined every second day for the presence of *P. cinnamomi*.

The NARPH medium (modification of Shearer and Dillon (1995); J. Webster, Department of Conservation and Land Management, WA, personal communication) contained, per L deionised water, 17 g Oxoid cornmeal agar, 1 mL nystatin (Nilstat; Wyeth-Ayerst Australia Pty Ltd, Baulkham Hills, New South Wales, Australia), 100 mg ampicillin sodium (Fisons Pty Ltd, Sydney, New South Wales, Australia), 10 mg rifampicin (Rifadin; Hoechst Marion Roussel Australia Pty Ltd, Lane Cove, New South Wales, Australia), 100 mg PCNB (Terraclor; Uniroyal Australia Pty Ltd, Melbourne, Victoria, Australia), and 50 mg hymexazol (Tachigaren; Sankyo Company, Tokyo, Japan). The antibiotics were dissolved in sterile water and added to the cooled agar (about 50°C) prior to pouring into Petri plates.

Where *P. cinnamomi* was not recovered after 14 days, one of each pair of plated stem sections (Experiments 1 and 2) or bark-pieces (Experiment 3) were removed from the NARPH plates and cut into smaller pieces (about 5-8 mm^2) to increase leaching. Cutting and replating of 2- to 12-month old lesions does not give additional recoveries of *P. cinnamomi* (G.E.St.J. Hardy, personal communication). Pieces were placed into sterile deionised water at room temperature and containers were rinsed and refilled with deionised water twice daily for 2 days and then daily for 7 days. The tissue samples were blotted dry, plated onto NARPH medium, incubated and scored for *P. cinnamomi* recovery as described previously. This technique is a modification of the method used by O'Gara (1998).

Results and Discussion

The absence of a visible lesion does not indicate that *P. cinnamomi* is absent in jarrah stems (Table 2). The pathogen was isolated from 61 out of 287 symptomless plants across all experiments. In Experiment 1, where lesions were not observed on any plants (Table 2), the pathogen could be recovered up to 5 cm beyond the ROI. If these plants were only visually assessed for disease, they would be rated as disease-free. It is important to recognise that symptomless plants having viable *P. cinnamomi* may, under conducive conditions, become diseased in the future and may provide a source of inoculum to contaminate otherwise disease-free sites. This concern has also been raised in root inoculation studies of *Abies fraseri* (Kenerley and Bruck 1983) and natural sedges (Phillips and Weste 1984), and in stem inoculations of jarrah (O'Gara *et al*. 1997).

The recovery of *P. cinnamomi* from beyond lesions or from symptomless plants indicates that the pathogen may function as a hemibiotroph. Other wound inoculation studies (Shea *et al*. 1982; Phillips and Weste 1984; Davison *et al*. 1994) have isolated *P. cinnamomi* in advance of lesions, although it was Davison *et al*. (1994) who first described this as a hemibiotrophic interaction. With time, this initial biotrophic interaction became necrotrophic. This type of hemibiotrophy is different from the symptomless plants which contained *P. cinnamomi* in our study and which were either wound- (Experiments 2 and 3) or zoospore-inoculated (Experiment 1) (Table 2). O'Gara *et al*. (1997) also produced infected, symptomless jarrah using zoospore inoculation.

Failure of some initial isolations to produce colonies on NARPH was probably not due to inhibition of *P. cinnamomi*. Any medium is inherently selective. The evidence suggests that NARPH constituents have minimal effect on the growth of *P. cinnamomi* (Tsao 1983). NARPH has no inhibitory effects on the growth of fastidious F1 progeny of *P. cinnamomi* (Tommerup and Catchpole 1997).

When inoculum levels in tissue with or woithout lesions are excessively low and/or are dormant, plating tissue directly onto agar selective for *Phytophthora* may produce some false-negative results. We found up to 11% of tissue, assessed as pathogen-negative by direct plating, contained live *P. cinnamomi* when washed in water. Importantly, up to 8.6% of symptomless stems were falsely negative, a level similar to the 9.8% observed in zoospore-inoculated plants by O'Gara (1998). Our results suggest that the pathogen is either present in some dormant, but viable, unit such as chlamydospores, selfed oospores or walled-off hyphae, or regrowth is being suppressed by fungistatic compounds such as phenolics. These false-negative results would be unacceptably high for diagnostic purposes, disease-free certification and quarantine clearance.

Phenolic compounds have been shown to act as inhibitory substances to pathogens (Christie 1965; Alfenas *et al*. 1982) and may contribute to dormancy or fungistasis. They may be released by washing stem tissue, enabling propagules to regrow. The survival of *P. cinnamomi* in plant tissue is poorly understood. The pathogen has been recovered from static jarrah root lesions up to 2 years after inoculation (Tippett *et al*. 1985) and more than 1 year in dead *Banksia grandis* collars (Shea 1979). Chlamydospores have been observed in inoculated plants (Malajczuk *et al*. 1977; Tippett *et al*. 1983) and in survival trials where excised *Eucalyptus* roots were buried for up to 100 days after inoculation (Old *et al*. 1984; Mackay *et al*. 1985). In previous trials assessing fungistasis or survival, it is possible that the pathogen persisted longer than stated as those studies used direct plating rather than tissue-washing and plating. Currently, we are investigating the factors involved in dormancy, fungistasis and long term survival of *P. cinnamomi* in jarrah and *Banksia* tissue.

The recovery of the pathogen after tissue-washing does not appear to be a phenomenon of any particular *P. cinnamomi* isolate, seedling or jarrah clonal line. It has been found for nine out of 68 isolates of *P. cinnamomi*, seven out of nine jarrah clonal lines and a seedling. The experiments were done under controlled and field conditions. Therefore, our findings are not confined to individual isolate-host interactions or environmental conditions, but are a widespread phenomenon in inoculated stems.

This study is the first to report the use of tissue-washing to increase positive isolations of *P. cinnamomi*. It also raises issues about symptomless tissue and false-negative isolations with important implications for disease management in natural vegetation, forests, plantations and horticulture. It also has important implications for disease-free plant certification in all nursery stock and quarantine clearance of plant material for *P. cinnamomi* and possibly for other *Phytophthora* species.

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Table 1 Experimental treatments using different *Phytophthora cinnamomi* isolates, jarrah seedlings and clonal lines, and numbers of surviving *Eucalyptus marginata* (jarrah) plants harvested from three inoculation experiments

Exp.[A]	Temperature (°C)	Isolate	Jarrah clonal line[B]	No. of surviving plants harvested
1	15, 20, 25 or 30	MP94-48	1J30 (RR)	32
			121E47 (RR)	5
			11J402 (SS)	16
2	20 (min), 29 (max)[C]	67 isolates[D]	77C40 (RR)	176
3	6 (min), 46 (max)[C]	MP99	1J30 (RR)	21
		MP94-48	5J336 (RR)	20
			11J379 (SS)	23
			11J402 (SS)	23
			12J96 (RR)	24
			121E293 (RR)	20
			seedling	20

[A]Controlled growth cabinets (1), cooled glasshouse (2) and field (3) experiment.
[B]Jarrah clonal lines susceptible (SS) and resistant (RR) to *P. cinnamomi* as determined by McComb *et al*. (1990).
[C]Ambient temperature in the shade.
[D]Includes isolate MP94-48, but not MP99.

Table 2 Percentage recovery of *Phytophthora cinnamomi* from stem tissue of *Eucalyptus marginata* (jarrah) with and without lesions harvested from three inoculation experiments. Recovery was scored after plating tissue directly onto NARPH agar selective for *Phytophthora*, and after washing in deionised water repeatedly for 9 days. Actual numbers are in parentheses.

Exp. [A]	Stems with lesions			Stems without lesions		
	% recovery		No. plant samples washed	% recovery		No. plant samples washed
	Direct plating[B]	After washing[C]		Direct plating[D]	After washing[C]	
1	-[E]	-	-	34.0 (18)	8.6 (3)	35
2	48.4 (30)	3.1 (1)	32	28.1 (32)	8.5 (7)	82
3	51.0 (76)	11.0 (8)	73	50.0 (1)	0	1
Exp. total	50.2 (106)	8.6 (9)	105	30.2 (51)	8.5 (10)	118

[A]Controlled growth cabinets (1), cooled glasshouse (2) and field (3) experiment.
[B]Recovery of *P. cinnamomi* from the lesion margin and/or beyond the lesion.
[C]Previously measured as free of *P. cinnamomi* by direct plating, then washed.
[D]Recovery of *P. cinnamomi* from the region or site of inoculation and/or beyond the inoculation area.
[E]- No plants had lesions.