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Assessment of Australian native annual/herbaceous perennial plant species as asymptomatic or symptomatic hosts of *Phytophthora cinnamomi* under controlled conditions

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Summary

Phytophthora cinnamomi is a necrotrophic pathogen of woody perennials and devastates many biomes worldwide. A controlled perlite–hydroponic system with no other hyphae-producing organisms as contaminants present allowed rapid assessment of ten annual and herbaceous perennial plant species most of which have a wide distribution within the jarrah (*Eucalyptus marginata*) forest in Western Australia where this pathogen has been introduced. As some annuals and herbaceous perennials have recently been reported as symptomatic and asymptomatic hosts, laboratory screening of some of the field-tested annuals and herbaceous perennials and additional species was used to further evaluate their role in the pathogen's disease cycle. Nine of the species challenged with the pathogen were asymptomatic, with none developing root lesions; however, *Trachymene pilosa* died. The pathogen produced thick-walled chlamydospores and stromata in the asymptomatic roots. Furthermore, haustoria were observed in the roots, indicating that the pathogen was growing as a biotroph in these hosts.

1 Introduction

Since its identification in 1922, the number of plant species known to be susceptible to *Phytophthora cinnamomi* has constantly increased (Zentmyer 1980; Cahill et al. 2008). Within the context of Australia's native vascular flora, there are a large number of species with unknown host status to *P. cinnamomi* in particular among annual and herbaceous perennial species. This group of plants, characterized by short lifespans, is generally regarded as resistant due to their persistence or even increase in population size in ecosystems that are impacted by *P. cinnamomi* (McDougall et al. 2002). However, tolerant or resistant plant species could nevertheless host the pathogen (Phillips and Weste 1984; Sieler et al. 1999; McDougall 2005; Cahill et al. 2008), and we recently reported that some annual and herbaceous species in the jarrah forest were asymptomatic hosts (Crone et al. 2012). In these species, the pathogen produced a range of survival structures including stromata, thick-walled chlamydospores and selfed oospores allowing it to persist on infested sites (Crone et al. 2013). Asymptomatic root infections in susceptible species have also been recently reported for the primarily foliar pathogens *Phytophthora kernoviae* and *P. ramorum* (Fichtner et al. 2012). In horticultural settings, *Phytophthora* species such as *P. capsici* (French-Monar et al. 2006) or *P. ramorum* (Shishkoff 2012) may colonize asymptomatic weeds in addition to symptomatic hosts.

To screen for potential hosts, inoculation experiments are often conducted under sterile or controlled conditions. The most convenient way to assess the impact of the pathogen on a host is to measure the extent of necrosis it causes, as measured by lesion length (Hüberli et al. 2002). However, this approach would fail to recognize the presence of the pathogen in susceptible, but asymptomatic species.

Therefore, this study used a controlled perlite/hydroponic system for rapid assessment of potential asymptomatic *P. cinnamomi* infections and colonization of native annual and herbaceous perennials species and the propagules formed. As organisms with similar propagules were absent under these controlled conditions, the structures observed could be attributed to *P. cinnamomi* without the need of confirmation by germination or molecular identification.

2 Material and methods

2.1 Selection of plant species

Five annual and five herbaceous perennial plant species with previously unknown disease status or a 'field resistant' rating (McDougall 2005) were selected. We recently observed four of these species for their response to *Phytophthora cinnamomi* in the natural environment (Crone et al. 2012) while the remaining species were tested for the first time in the following experiment (Table 1).

No inoculation experiments on these species have been undertaken previously (McDougall 2005). All had germination percentages higher than 10% (Cromer 2007), and have a distribution that extends to other ecosystems such as the coastal plain and *Banksia* woodland (Western Australian Herbarium 1998).

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Table 1. Jarrah forest annual and herbaceous perennial species selected for inoculation experiments in the perlite/hydroponic system.

Species	Lifeform	Former	New rating	Seed source
Amphipogon debilis	hp	?	Asymptomatic host	N
Austrostipa campylachne	hp	?	Asymptomatic host	A
Brachyscome iberidifolia	a	?	Asymptomatic host	N
Lagenophora huegelii	hp	FR	Asymptomatic host vs FR in nature*	A
Neurachne alopecuroidea	hp	FR	Asymptomatic host	A
Podotheca angustifolia	a	FR	Asymptomatic host/also in nature*	A
Rytidosperma caespitosum	hp	?	Asymptomatic host/also in nature*	A
Trachymene pilosa	a	?	Symptomatic host vs asymptomatic host in nature*	N
Waitzia nitida	a	FR	Asymptomatic host	N
Waitzia suaveolens var. *flava*	a	?	Asymptomatic host	N

a, annual; hp, herbaceous perennial; A, Alcoa Marrinup Nursery; N, Nindethana; former rating according to McDougall (2005): FR, field resistant;?, unknown, not rated.
*New rating are the results of the studies with 'asterisk' marking the findings from the field studies of Crone et al. (2012).
Species names are current in accordance with the Western Australian Herbarium (1998).

2.2 Selection of Phytophthora cinnamomi isolate

The isolate MUCC 773 (Murdoch University culture collection) was used for all inoculation experiments. It originated from the roots of *Hypocalymma angustifolia* harvested from a black gravel site in the jarrah forest in 2009. The identity of the isolate was confirmed as *P. cinnamomi* by DNA-sequencing of the ITS region as described by Aghighi et al. (2012) and lodged in GenBank (GenBank accession number JX 454789). Mating tests determined the isolate to be of the A2 mating type.

2.3 Experimental design

Seven 1000-ml polypropylene 'take-away' containers (167 mm × 108 mm) per plant species with a minimum of 15 plants per container were set up. Five replicate containers received *Phytophthora cinnamomi* inoculum and two of the containers were kept free from inoculum as a negative control. At harvest (maximum five harvests), five replicates of three randomly selected plants were taken from each container and gave 15 potentially infected plants and six control plants per harvest.

2.4 Seedling establishment and inoculation

Seeds were surface-sterilized for one minute with 70% ethanol and then soaked for 10 min in a 1% sodium hypochlorite solution. Before and after the sodium hypochlorite treatment, the seeds were washed for one minute under running deionized water. Where germination failed, new seeds were directly sown without surface sterilization. They were germinated in a thin layer of perlite (Grade: Fine and Extra Fine; The Perlite and Vermiculite Factory, Perth) and placed on a rectangular lid of a 'take-away' container whose centre was cut out, replaced with a fibreglass flyscreen mesh (approx. 140 × 90 mm; Cyclone, Dandenong South, Vic., Australia) and placed on its base, which was filled with perlite saturated with tap water. The mesh of the flyscreen held the seeds in position, while allowing the roots to grow through into the moist perlite below. Capillary action ensured that the perlite placed on top of the lid remained moist. To reduce evaporation, each container was covered with an 'upside down' 'take-away' base, and the layer of perlite was sprayed with distilled water each weekday until germination. To avoid light-inhibiting root growth, the container was placed inside a modified light-impermeable 'take-away' container. The containers were incubated at 21°C (±1°C) with a 1000-watt growth light (SS 1000 MH, Vossloh Schwabe) operating for 12 h/day. Once germinated, the plants were watered once a week with a dilute liquid native fertilizer solution (5 ml/l water; Australian Native Focus, Growth Technology, O'Connor, Western Australia, Australia).

When roots were 2–6 cm in length, usually when seedlings had produced their primary leaves, the flyscreen lid was lifted from the growth container, any perlite attached to the roots was gently shaken off and the flyscreen lid transferred to another container with a solution of *Phytophthora cinnamomi* zoospores.

2.5 Zoospore production

To produce zoospores, 7-day-old *P. cinnamomi*-colonized V8 agar was cut from the edge of the colony into 1-cm^2 blocks. Sporangial and zoospore production is enhanced by low nutrient levels (Ribeiro 1983), so the blocks were soaked twice, each for 1.5 h in distilled water. This was followed by two treatments of 3 h in fresh distilled water with 4% soil extract. Finally, five agar blocks were placed in each container and covered with 800 ml of distilled water and 50 ml of soil extract and left overnight (approx. 15 h). Agar blocks were examined the next morning to confirm sporangial production and zoospore release. There were approximately 750–3000 zoospores per inoculation treatment. However, it is likely that more sporangia were produced and zoospores released during the 24-h inoculation period. Soil extract was prepared by adding 100 g of potting mix (Coles Brand, Tooronga, Vic., Australia) to 1000 ml of distilled water and shaking for 3 h on a shaker at the speed of 159 rpm followed by filtering through a Whatman No. 1 filter paper.

Then, the seedlings were transferred into the inoculum containers. Leaf baits of *Quercus suber* (depending on seasonal availability) or *Scholtzia involucrata* were placed on the water surface, to confirm through leaf infections that zoospores were produced and infective at the time the roots were exposed to the inoculum. In addition, two growth containers for the control seedlings received the same treatment except that their roots were only exposed to V8 agar plugs free from *P. cinnamomi* during the inoculation step. These negative controls were also baited to detect for any potential contamination.

After 24-h exposure to zoospores, the plants were removed and transferred into containers with an aerated solution of 800 ml distilled water and 0.5 ml autoclaved seaweed extract (Seasol International, Bayswater, Vic., Australia). To minimize the risk of contamination and nutrient deficiencies, the liquid was replaced every 3 days.

2.6 Infection status and disease progression

In general, data for each species were obtained from the root systems of 15 individual plants. The majority of species were harvested at 21 and 28 days post-inoculation (21 and 28 dpi). The negative controls were harvested at the same time as the inoculated plants and consisted of two replicates, each of three plants.

Podotheca angustifolia and *Lagenophora huegelii* were used to observe disease progression over time and were harvested more frequently (*P. angustifolia* at 1, 2, 4, 7, 14, 21 and 28 dpi; *L. huegelii* at 1, 7, 14, 28 dpi). These two species also had a larger sample size at each harvest. Briefly, there were five replicate trays for the control, and in the case of *P. angustifolia*, five instead of three plants per replicate were examined.

The root system of each harvested plant was surface-sterilized for 20 seconds in 70% ethanol, rinsed with deionized water, dried and plated onto a *Phytophthora* selective medium (NARPH containing Nilstat, Ampicillin, Rifadin, PCNB=Terraclor and Hymexazol) (Hüberli et al. 2000). However, PCNB (1,2,3,4,5 Pentachloro-6-nitrobenzene) as an ingredient was only used for plating *P. angustifolia, L. huegelii* and *Rytidosperma caespitosum* as it was withdrawn from the market during the period of the experiment. Roots infected by *P. cinnamomi* were then placed in a 0.05% w/v trypan blue/lactoglycerol solution for 3–4 h and destained for a minimum of 3–4 h or overnight in lactoglycerol (88% lactic acid and glycerol 1 : 1) before mounting permanently on microscope slides (Brundrett 2008). Examination used a BX51 Olympus microscope and a MicroPublisher 3.3RTV Q Imaging camera. The experiment was a completely randomized design.

2.7 Data analysis of observed structures

For each species, the total root system of at least one individual per replicate was examined microscopically for the presence of *P. cinnamomi* structures. Data on the presence of each structure were primarily qualitative with following categories used to describe differences in frequency:

1 Very rare – Structure present but was only observed once or twice across all replicates.
2 Occasional – Structure was only found in low numbers but more than twice, sometimes not present in all replicates.
3 Frequent – Structure usually detected in all colonized areas across the replicates
4 Abundant – As category 3, but in some colonized areas, structures were in loose aggregates to dense clusters.

2.8 Capacity of lesion formation by the isolate used for inoculation experiments

As all inoculated plants, apart from *Trachymene pilosa*, were asymptomatic, an experiment was conducted to test whether the lack of symptoms was a characteristic of the *Phytophthora cinnamomi* isolate or whether the plant species examined were tolerant hosts. *Lupinus angustifolius* (cv. 'Mandalup') a species known to develop root lesions when infected with *P. cinnamomi* was chosen as the host plant. For inoculation, the *P. cinnamomi* isolate MP 94.48 (GenBank Accession number JX113294), from the Murdoch culture collection was chosen as a positive control for a comparison with MUCC 773.

Lupin seeds were germinated in aerated water until a root length of approximately 1 cm was reached and then inoculated with either MUCC 773 or MP 94.48 using the same inoculation method as described previously. There were five replicate tubs each with three lupin plants per tub per isolate in a completely randomized design. A not-inoculated control with lupins exposed to sterile agar plugs was set up to examine whether root lesions are caused by the growing method rather than the pathogen.

3 Results

With two exceptions (*Brachyscome iberidifolia* 60% and *Waitzia nitida* 93%), 100% of the inoculated harvested plants across the replications were infected with *P. cinnamomi* at 28 days post-inoculation (Table 2).

No negative controls yielded the pathogen. All plant species except *Trachymene pilosa* were symptomless with no root lesions, and there were no noticeable macroscopic differences in roots or shoots between pathogen-free controls and inoculated plants. Control plants of *T. pilosa* were healthy, but the majority of inoculated plants were dead after 6 dpi. Inoculated and not-inoculated *Waitzia suaveolens* var. *flava* roots were 'unhealthy' in appearance and brown in colour, but this was the case for the control and inoculated treatments alike. In all plant species, except *W. nitida* where the pathogen was restricted to small areas, *P. cinnamomi* produced a range of structures in the inoculated roots, although the abundance of these structures varied in frequency between species (Table 3).

Table 2. Percentage of *Phytophthora cinnamomi* infected plants at each harvest to 28 days post-inoculation (dpi). There were 15 plants at each harvest (*n = 6 due to lack of material).

Species	1 dpi	2 dpi	4 dpi	7 dpi	14 dpi	21 dpi	28 dpi
Amphipogon debilis							100
Austrostipa campylachne							100
Brachyscome iberidifolia						40	60
Lagenophora huegelii	87			100	100	100	100
Neurachne alopecuroidea							100
Podotheca angustifolia	64	92	100	100	100	100	100*
Rytidosperma caespitosum						100	100
Trachymene pilosa				100			
Waitzia nitida							93
Waitzia suaveolens var. *flava*						100	100

Table 3. Phytophthora cinnamomi structures formed in roots of annual and perennial plant species grown and inoculated in the perlite/hydroponic system. *P. cinnamomi* was recovered from all species.

Plant species	Hyphae, thin walled	Chlamydospores thin walled	Hyphae, thick walled	Chlamydospores thick walled	Stromata	Haustoria	Lignituber
Amphipogon debilis	3	3	2	2	1	1	
Austrostipa campylachne	3	2				1	
Brachyscome iberidifolia	3	2	1	2	1	1	
Lagenophora huegelii	3	1	3	3	1	2	1
Neurachne alopecuroidea	2	2	2	2	1	2	
Podotheca angustifolia	4	4	4	4	1	3	
Rytidosperma caespitosum	4	3	2	2	1	2	
Trachymene pilosa	4	3	1		1	2	
Waitzia nitida	1			1			
Waitzia suaveolens var. *flava*	3	3	1	1	1	1	

Presence and frequency are shown as 1, very rare; 2, occasional; 3, frequent; 4, abundant at 28 dpi (except *Trachymene pilosa* at 10 dpi) according to criteria given in the text.

For example, thick-walled hyphae and chlamydospores were commonly observed in *L. huegelii* and *P. angustifolia* 28 dpi (Table 3, Fig. 1 c, e, f). In both species, only thin-walled chlamydospores were formed within 7 dpi, but formation of thick-walled hyphae and thick-walled chlamydospores started at 14 dpi. At the same time, the first stromata were observed in several species (Fig. 2, Table 3).

Hyphal strands with globose structures at regular intervals (Fig. 3 a–b) were observed in the asymptomatic hosts. Some remained globose and were regarded as haustoria while the tips of others resumed growth, and these are referred to as haustoria-like structures (Fig. 3c).

3.1 Lesion formation by the isolates used for inoculation experiments

Both isolates caused root lesions on *Lupinus angustifolia* (cv. 'Mandalup') of similar severity consistently in all plants, whereas no root lesions were observed in the non-inoculated control plants.

4 Discussion

With exception of the wilted *Trachymene pilosa*, all the annual and herbaceous perennial species were asymptomatically infected by *Phytophthora cinnamomi*. This indicates that annual and herbaceous perennial species contribute to the increase in inoculum without developing symptoms, and the survival propagules (chlamydospores and stromata) that are produced contribute to the continued persistence of *P. cinnamomi* in the jarrah (*Eucalyptus marginata*) forest. As differences in the numbers of survival propagules were noted between and within plant species, this indicates that not all annual and herbaceous perennial plant species equally contribute to the persistence of the pathogen in the jarrah forest, given that biotic and abiotic factors may further lead to alterations in host–pathogen interactions. However, in general, it is likely that many other annual and herbaceous perennial species rated 'field resistant' or with unknown status might be 'tolerant' and prone to asymptomatic infection. The black gravel isolate used for the inoculation experiment did not differ in aggressiveness to *Lupinus angustifolius* compared to the 'reference isolate' MP 94.48, and this strongly suggests that the asymptomatic growth of the pathogen in the roots of the newly identified host species was not an isolate-specific feature or a result of the environmental conditions of the perlite/hydroponic growth system. Extensive studies on 73 *P. cinnamomi* jarrah forest isolates derived from either jarrah or marri trees demonstrated that despite marked differences in aggressiveness; almost all iso-

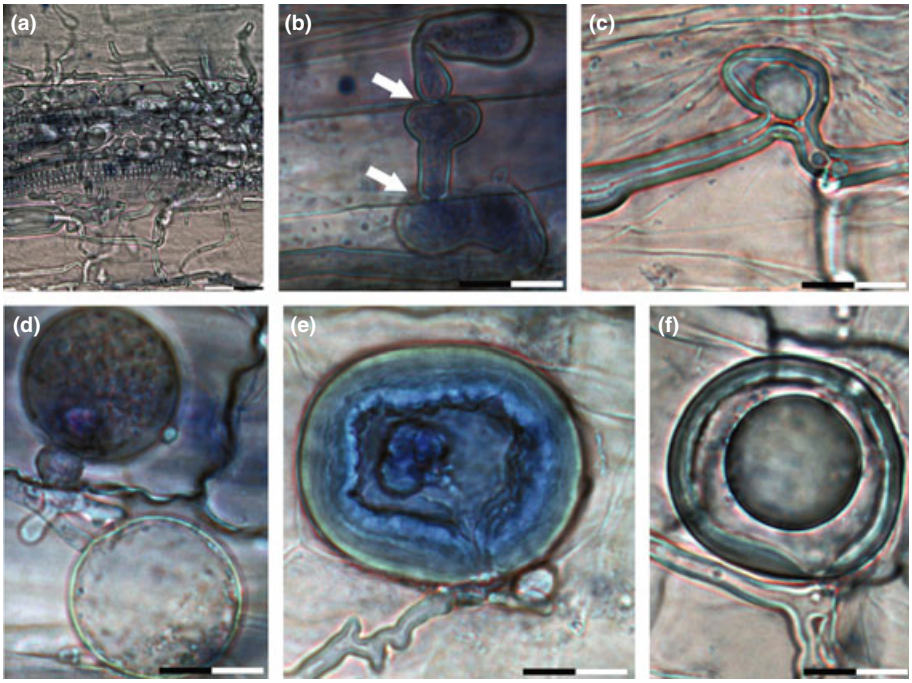

Fig. 1. Structures of *Phytophthora cinnamomi* produced in the roots of annual and herbaceous perennial plants grown in the perlite–hydroponic system. (a) Extensive asymptomatic colonization within roots of *Podotheca angustifolia*, 28 dpi; (b) proliferation through root material with two infection pegs (arrows) (*Trachymene pilosa*, 7 dpi); (c) detail of thick-walled hyphae (*Podotheca angustifolia*, 28 dpi); (d) thin-walled chlamydospores, the lower spore has germinated (*Trachymene pilosa*, 7 dpi); (e) and (f) thick-walled chlamydospores (*Podotheca angustifolia*, 28 dpi). Scale bars: (a) = 80 μm, (b) to (f) = 20 μm.

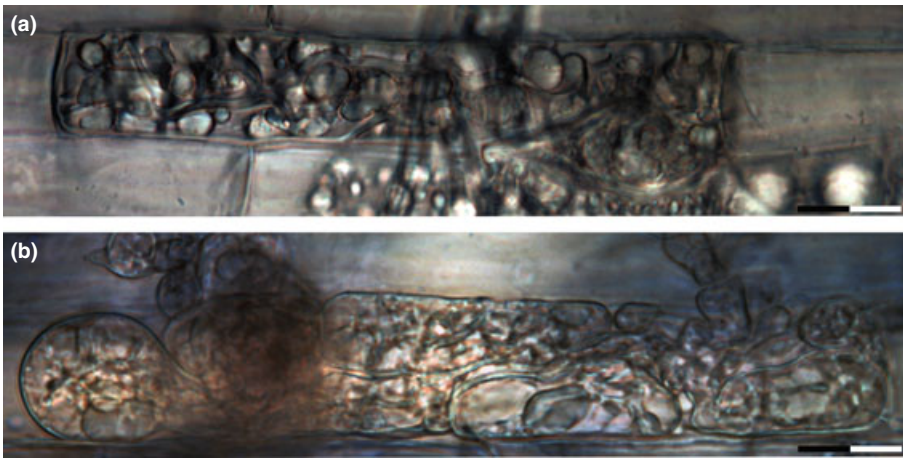

Fig. 2. Stromata of *Phytophthora cinnamomi* in the roots of plants grown in the perlite–hydroponic system. Stroma in root cells of (a) *Amphipogon debilis*, 28 dpi; (b) *Neurachne alopecuroidea*, 28 dpi. Scale bars: 20 μm.

lates were more aggressive to jarrah than marri (Hüberli et al. 2001). This shows that despite possible differences between *P. cinnamomi* isolates, host species have a stronger influence on the plant–pathogen interaction compared to between isolates. We have shown that *P. cinnamomi* is able to colonize some annual and herbaceous perennial species as either a biotroph or an endophyte and not as a necrotroph. The haustoria observed here (Fig. 3 a, b) and also reported from naturally infected roots of asymptomatic annuals and herbaceous perennials from the jarrah forest (Crone et al. 2013) suggest a biotrophic mode of growth. This is supported by field observations of annuals and herbaceous perennials from germination to natural senescence or cease of growth during summer. Weekly sampling from winter to spring confirmed that *P. cinnamomi* infected many of these during winter, remained viable in the root system of the hosts, yet the majority of species remained asymptomatic (Crone et al. 2012).

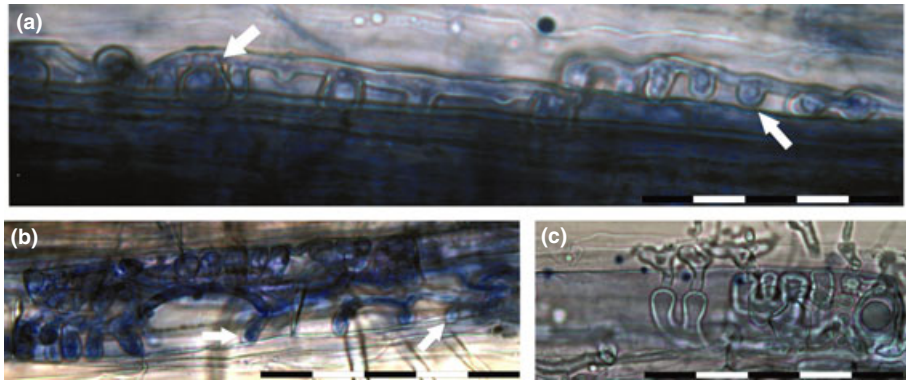

Fig 3. Haustoria or haustoria-like structures of *Phytophthora cinnamomi* in the roots of annual and herbaceous perennial plants grown in the perlite–hydroponic system. (a), (b) Haustoria in *Rytidosperma caespitosum,* 21 dpi (arrows show examples); (c) haustoria-like structures in *Podotheca angustifolia*, 28 dpi, whose distal ends continue to grow. Scale bars: (a), (c) = 100 μm; (b) = 200 μm.

The experimental conditions also induced stromata, structures recently reported for *P. cinnamomi* (Crone et al. 2013). Even though the system was not sterile, almost all the examined roots were free of other hyphal-producing organisms. This allowed the conclusion that these stromata were produced by *P. cinnamomi*.

However, in contrast to the regular and abundant appearance of autogamous (selfed) oospores in naturally infected annual and herbaceous perennials on black gravel sites of the jarrah forest (Crone et al. 2013), none were observed in this experiment. As selfed oospores (autogamy) of the heterothallic *P. cinnamomi* are reported to be occasionally formed by the A2 mating type as a stress response such as due to the presence of antagonists (Zentmyer 1980), this controlled system most likely lacked the necessary stimuli. Furthermore, there was a significant difference of *Trachymene pilosa* in response to infection between the current controlled experiment and those observed under natural conditions (Crone et al. 2012). While the majority of seedlings collapsed (without root lesions) in the current study, infected individuals on the black gravel sites remained asymptomatic. Thus, while there were some differences between host–pathogen interactions between the controlled conditions and the natural environment, the perlite/hydroponic assessment system proved to be a rapid and reliable method to screen for potential hosts of *P. cinnamomi*. It was shown that the pathogen is capable of increasing its inoculum potential in these newly identified asymptomatic hosts and that thick-walled chlamydospores and stromata are formed within the root tissues. Future research could utilize this semi-sterile perlite/hydroponic system to screen large numbers of annuals and herbaceous perennials as potential hosts of *P. cinnamomi* and to expand studies to other ecosystems. Furthermore, other classical necrotrophic or hemibiotrophic *oomycetes* could be assessed for their behaviour in annual and herbaceous perennial plant species with this system.

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