Phytophthora Contamination in a Nursery and Its Potential Dispersal into the Natural Environment

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Abstract

A detailed site investigation of a eucalypt nursery suffering disease losses revealed the causal agent to be *Phytophthora boodjera*. The pathogen was detected in vegetation surrounding the nursery production area, including the lawn, under the production benches during the growing season, and, most importantly, from plant debris in used trays. However, it was not found in the container substrate, water supplies, or production equipment or on the workers themselves. The sterilization methods used by the nursery were shown to be ineffective, indicating that a more rigorous method was required.

Boiling trays for 15 min or steaming at 65°C for 60 min eradicated *P. boodjera*. This pathogen was more pathogenic to the eucalypts tested in their early seedling stage than *P. cinnamomi*. Tracing of out-planting to revegetation sites showed that *P. boodjera* was able to spread into the environment. Dispersal via out-planting to native vegetation may affect seedling recruitment and drive long-term shifts in native plant species. Inadequate nursery hygiene increases the risk of an outbreak and can limit the success of biosecurity efforts as well as damage conservation efforts.

Increased plant trade facilitates the movement of invasive plant pathogens (Brasier 2008), and *Phytophthora* spp. are of particular significance (Jung et al. 2016; Pérez-Sierra and Jung 2013). *Phytophthora* spp. can move readily through the nursery supply chain when infected plants are asymptomatic (Parke and Grünwald 2012; Pérez-Sierra and Jung 2013). Diseases caused by *Phytophthora* spp. in nurseries are a worldwide problem (Bienapfl and Balci 2014) and are a direct threat to biodiversity.

In Western Australia, where there has been a longstanding concern about the presence of *Phytophthora* spp. Container-grown plants in 14 nurseries contained one or more *Phytophthora* spp. associated with rotted roots of 65 plant taxa (Hardy and Sivasithamparam 1988). Davison et al. (2006) found *Phytophthora* spp. in 10% of the samples of potting substrate in 15 consignments of nursery-grown plants imported into Western Australia from other states in Australia. One Western Australia nursery was the source of eight different *Phytophthora* spp. (Hardy and Sivasithamparam 1988). There are many examples of the spread of *Phytophthora* spp. to natural areas from nurseries in Europe, South America, and the United States. The extent of the problem in Europe was highlighted by Jung et al. (2016), who estimated that there is an average of 3.6 *Phytophthora* spp. in each infested tree nursery and planting site. Clearly, in Europe, current nursery hygiene and control practices are not sufficient to eliminate the risk of spreading this damaging pathogen.

The dispersal of *Phytophthora* spp. from nurseries to revegetation plantings in previously noninfested areas may result in serious threats to biodiversity in natural ecosystems. A recent outbreak of damping-off disease of young eucalypt seedlings, caused by *Phytophthora boodjera* A. V. Simamora & T. I. Burgess, has been observed in a eucalypt seedling production nursery in Tincurrin, in southern Western Australia. This nursery produces seedlings principally for conservation plantings in, or adjacent to, natural ecosystems, or to increase the diversity of plant species in remnant native bush stands on farms. Reinfestation was occurring on site despite extensive

preseason preparation to eradicate the pathogen. The *Phytophthora* database of the Department of Parks and Wildlife (DPaW) in Western Australia, which contains more than 44,000 records collected over 35 years, reveals only five recent records of *P. boodjera* not associated with nurseries. This, along with the findings of Simamora et al. (2015), implies that *P. boodjera* was probably introduced recently to Western Australia and, hence, is an ideal species for tracking from production nursery to the out-planting sites. This study presents a systematic sampling strategy to determine the possible sources of on-site contamination in the nursery to understand the epidemiology of *P. boodjera* and develop methods for its control within the nursery. In addition, targeted sampling was undertaken to determine the survival of *P. boodjera* in areas adjacent to the nursery and in the revegetation sites after the planting of potentially infected seedlings from the infested nursery.

Materials and Methods

The nursery is situated in the southeastern wheatbelt of Western Australia and has been operating since 1999, servicing the land care and revegetation needs of farmers, with a capacity of 4 million seedlings. The nursery (although not accredited) was designed along Nursery and Garden Industry Australia guidelines (NIASA 2013) and current best-practice management principles were followed.

A rigorous sampling regime was undertaken to determine potential sources of contamination. This was followed by an examination of pathogen survival within the nursery and within host tissue, the pathogen's ability to infect different plant species, and whether or not risk is heightened under waterlogged conditions. To help reduce ongoing infections, different sterilization techniques for reused trays were tested. The spread to the surrounding environment and tracing out-planting to the revegetation sites were also assessed.

Sample processing to detect *Phytophthora* spp. All plants, potting media, and debris were baited for the detection of possible *Phytophthora* spp. in 1-liter plastic containers (11.5 by 16.5 by 7.5 mm; GENFAC Plastics Pty Ltd.), with five replicate containers for each sample.

Soil and substrate baiting. Samples (100 to 150 g) were flooded with 350 ml of deionized (DI) water, and bait leaves of *Quercus suber*, *Q. ilex*, *Pimelea ferruginea*, *Poplar* spp., *Scholtzia involucreta*, *Hedera helix*, *Eucalyptus* spp. (juvenile leaves), or *Rosa officinalis*, or *Hibbertia scandens* petals (availability depending on the season) were placed on the water surface. After 4 to 10 days at room temperature (20 to 29°C), the baits with lesions were blotted on paper towels, cut into 1- to 3-mm pieces, and plated on NARH *Phytophthora* selective medium. Per liter of deionized water, NARH contains 17 g of oxoid commel agar, 1 ml

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*The e-Xtra logo stands for "electronic extra" and indicates that two supplementary tables are published online.

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nystatin (Nilsat, Wyeth-Ayerst Australia Pty Ltd, Baulkham Hills, NSW), 100 mg ampicillin sodium (Fisons Pty Ltd, Sydney, NSW), 10 mg rifampicin (Rifadin, Hoechst Marion Roussel Australia Pty Ltd, Lane Cove, NSW), and 50 mg hymexazol (Tachigaren, Sankyo Company, Toyota, Japan). The antibiotics were dissolved in sterile water and added to cooled agar (about 50°C) prior to pouring into Petri dishes. Plates were maintained in the dark at room temperature and regularly observed for colonies typical of *Phytophthora* spp. Water samples (500 ml) from the dams, rainwater tanks, and the potting shed drainage outlet were baited directly.

Filtering. Water samples (five samples of 1 liter each) were also filtered through 0.45-µm pore filters (Whatman) with three replicate filters per sample. Filter papers were plated directly onto NARH for 24 h and removed; then, the plates were incubated at room temperature and observed daily.

Direct plating. Seedling roots were washed in tap water to remove potting substrate and blotted dry, with 1- to 3-mm pieces plated onto NARH. Insects were collected, squashed, and plated directly onto NARH medium. Plates were incubated for 7 to 10 days at room temperature and observed for colony morphology and structures consistent with *Phytophthora* spp.

The identity of *Phytophthora* isolates was confirmed by sequencing of the internal transcribed spacer gene region, as described previously (Simamora et al. 2015).

Sampling for *Phytophthora* spp. in and around the nursery. Over 3 years, the following sets of samples were collected at the start of seeding, throughout the growing season, and when the nursery was fallow after the seedlings had been dispatched (Fig. 1; Table 1).

Water supply. Water was sampled from taps, the dam, rainwater tanks, and the drainage outlet from the concrete floor of the potting

shed. The water samples were tested in two ways. First, tap water and drainage outlet water was filtered. Second, water samples were collected from the surface of dams and rainwater tanks and from the potting shed drainage outlet and transported in 1-liter plastic containers, in cool boxes, to the laboratory for baiting and filtering within 24 h.

Material adjacent to nursery area. Tree roots which had grown into the water tank or pond and those of adjacent mallee eucalypts (native remnants) were collected for direct plating, as described above. Soil, leaves, and litter from the mallees planted as a windbreak and vegetation (weeds, moss, and grass) close to the nursery floor area were collected and baited, as described above.

Equipment utilized in potting, storage, and handling area. Used seedling trays stacked and ready for reuse, gloves, tires, wheel barrows, loader, potting machine, and other machinery, as well as worker's hands, were scraped to remove old potting substrate, soil, and residual plant debris. At least 100 g of material from each source was bagged and transported to the laboratory for baiting.

Nursery production area. Soil from beneath benches and from the adjacent windbreak trees, together with potting substrate from healthy and diseased seedlings on the nursery benches, was baited. In addition, seedling roots, ants from under or near benches, and gnat larvae from pots of plants showing symptoms were also collected and directly plated, as described above.

Tray and potting mix sterilization treatments. The efficacy of various treatment methods for sterilization of trays was examined in three experiments: (i) testing of used nursery trays with and without container substrate, (ii) testing of the nursery's standard tray treatment, and (iii) testing of trays with seedlings grown in different container substrates and watered with different sources of water.

Fig. 1. Sampling locations and potential sources of contamination of *Phytophthora boodjera* at a native tree nursery, Tincurrin, Western Australia. The numbers correspond to the sampling locations listed in Table 1.

Nursery trays with and without potting substrate. Ten control trays (untreated, used trays) were rinsed with DI water in a container and the water collected was baited for *Phytophthora* spp. (three replicate bait containers per tray). Nonsterilized container substrate from the nursery was also tested. Additional trays were pasteurized, with and without container substrate (10 replicates/treatment). Pasteurization was carried out at a maximum of 65°C for 1 h. After 2 to 4 days, bait leaves with necrotic lesions were plated on NARH.

Standard on-site tray treatment. Trays in which high disease incidence had been observed in the seedlings were used for this study. Trays were emptied of container substrate and allowed to dry, as was normal nursery practice, and the following methods of tray disinfection were tested: (i) immersion in water which was brought to the boil, then rapidly boiled for 15 min; (ii) exposure to dry heat up to 65°C; and (iii) soaking in biocide (Steri-maX [Agricorp] and didecyl dimethyl ammonium chloride at 120 g/liter) in a 0.1% solution for 10 min. After the treatments, all trays

were individually rinsed with DI water and the rinse water was collected and baited for *Phytophthora* spp. (three replicate bait containers per tray).

Testing of trays with seedlings grown in different container substrates and watered with different sources of water. This trial was conducted using three different types of trays (new trays, used trays known to have had high disease incidence, and boiled trays). The trays were sown with *Eucalyptus loxophleba* subsp. *gratae* Benth and *Bankia occidentalis* R. Br. in two different container substrates: composted pine bark medium supplied from the nursery and composted pine bark supplied by the DPaW. The trays were watered daily using water from three different sources: rainwater (outside water supply, located approximately 3 km from the nursery, untreated rainwater), nursery treated water tank, and nursery rainwater. There were five replicate trays for each treatment. After 6 weeks, seedlings were removed and trays were emptied. Seedlings and potting substrates were baited as explained above.

Table 1. Recovery of *Phytophthora boodjera* from different materials sampled from various locations across the nursery site^y

Site	Material sampled	Season ^z		
		Prep	Grow	Fallow
Water supply				
1	Dam water	0/5	...	...
2	Rain water tanks	0/5	...	...
10	Drainage water	0/5	3/5	0/5
11	Water from potting shed taps	...	0/15	
11	Drainage outlet from potting shed floor	...	...	0/5
Adjacent to nursery				
2	Roots from water tank	0/5	...	...
3	Roots from eucalypt near water tank	5/10	...	2/10
4	Roots of eucalypt near pond	...	...	0/5
5	Mud from pond	...	...	0/5
5	Roots from pond near nursery	...	...	0/5
6	Nursery lawn	5/5	...	2/5
7	Soil from under trees (close)	5/5	...	4/10
8	Leaves from adjacent long hedgerow	...	...	0/40
8	Litter from adjacent long hedgerow	...	0/5	0/20
8	Soil from corner of adjacent hedgerow	...	...	1/40
9	Litter from adjacent trees - short hedge	...	...	0/5
9	Soil from secondary hedge trees	...	...	2/5
11	Vegetation close to potting shed floor	...	...	0/20
12	Eucalyptus near used tray storage area	...	...	0/10
Potting, storage, and handling				
11	Seed	–ve	...	...
11	Used trays (untreated)	20/20	...	...
11	Container substrate off delivery truck	0/5	...	...
11	Mud in front of soil shed	0/5	...	...
11	Soil from loader	0/5	...	...
11	Container substrate (different batches)	0/20	...	...
11	Steamed soil (65°C)	0/20	...	...
11	Fingers of nursery workers	0/5	...	...
11	Gloves of nursery workers	0/5	...	...
Debris from				
11	Container substrate shed after cleaning	0/5	...	...
11	Tray filling machine after cleaning	0/5	...	...
11	Adjacent to stored container substrate	0/5	...	...
11	Potting shed floor	0/5	...	...
11	Wheelbarrow shed	0/5	...	...
11	Bench top in potting shed	0/5	...	...
11	Tray racks	0/5	...	...
Nursery production area				
13	Soil under benches from nine locations	0/15	5/50	0/45
13	Cotyledon stage seedlings (2 weeks old)	...	–ve	...
	<i>E. loxophleba</i> subsp. <i>lissophloia</i>			
13	<i>E. loxophleba</i> subsp. <i>lissophloia</i> (bulk sample)	...	+ve	...
13	Ants	...	0/15	...
13	Fungal gnat larvae	...	0/10	...

^y Number of positive recoveries is shown over the number of samples tested; +ve and -ve denote presence or absence, respectively, in multiple samples that were too numerous to count.

^z Season of sample collection. Prep = preparation for planting (October to November), Grow = growing season (October to June), and Fallow = July to October.

Persistence of naturally produced oospores. A trial of heat treatments to eradicate oospores of *P. boodjera* living inside the root debris in used trays was undertaken. Inoculum was prepared as follows: 400 ml of vermiculite substrate (400 mg of vermiculite, 4 g of millet seed, and 240 ml of V8 broth) was placed into a 500-ml Erlenmeyer flask, sealed with nonabsorbent cotton wool, and covered with aluminum foil. The flasks were autoclaved three times at 121°C for 20 min over three consecutive days, then inoculated once the substrate had cooled. Inoculum per flask consisted of one Petri dish (9 cm in diameter) of V8 agar plugs colonized for 7 days by *P. boodjera* isolate (VHS 26806; recovered from soil in 2012, Tincurrin, Western Australia). Flasks were shaken, placed inside ziplock plastic bags, and incubated at 20°C in the dark. The flasks were shaken every 3 days for the first 2 weeks to evenly spread the inoculum. Five-week-old inocula were rinsed with DI water to remove excess nutrients (Matheron and Mircetich 1985) immediately before inoculation of the container substrate. Successful colonization of the inocula was confirmed by placing 3-g subsamples onto NARH agar, and also into a Petri dish containing DI water, before incubation at room temperature in the dark.

Seed of *E. loxophleba* subsp. *gratae* were grown in 10-cell trays (90 ml/cell; Garden City Plastics) containing sterilized, washed river sand. At the time of sowing, seed were placed in random cells of the trays and two holes were made in the sand at the center of each cell, one for seed and one for a 10-mm plastic tube placed for later inoculum delivery. After 2 months, the seedlings were inoculated with 2.5 g of inoculated vermiculite and covered with a thin layer of sand. They were harvested at 4 months old, allowed to dry at room temperature, and checked microscopically for the presence of oospores inside the roots using staining. Briefly, the root samples (1 to 2 g)

fixed in 50% EtOH were rinsed with water, cleared by heating in 10% (wt/vol) KOH in an autoclave, stained with 0.05% (wt/vol) trypan blue, and destained with lactoglycerol (Brundrett et al. 1996).

Root pieces were also plated on NARH. Root pieces (5 to 10 mm) containing oospores were placed in glass Petri dishes, then heated in an oven at 20, 37.5, 65, 80, and 95°C for 30, 60, 120, and 240 min. Dry conditions were simulated by leaving Petri dishes unsealed, while moist conditions were created by placing moist filter paper in the bottom of the dish and sealing with Parafilm. After treatment, root pieces were plated on NARH and incubated in the dark at 20°C. There were three replicates of 10 root pieces per treatment. Plates were observed daily for colony growth.

Susceptibility of plant species to *P. boodjera*. To determine host susceptibility to *P. boodjera*, seed of *E. polybractea* R. Baker, *E. kochii* subsp. *plenissima* (C. A. Gardiner) Booker, *E. loxophleba* subsp. *gratae*, *Melaleuca atroviride*, and *Cytisus proliferus* (Tagasaste) were germinated in used container substrate in used 90-cell trays (utilizing the middle section only, 15 cells) taken from benches where mortality had been observed. This glasshouse trial was conducted at Murdoch University with six replicate trays per plant species containing 20 to 25 seeds/cell. Seed of all species germinated between 7 and 20 days. After 12 weeks, plants were harvested, roots were examined for necrotic lesions and plated on NARH, and disease incidence and severity were recorded. Disease incidence for each plant species was calculated as the percentage of cells with diseased seedlings, and disease severity in all seedlings within each cell was rated as 0 = 0, 1 = 1 to 25, 2 = 26 to 50, 3 = 51 to 75, and 4 = 76 to 100% of cells affected.

Susceptibility and effect of waterlogging on disease expression. Simamora et al. (2017) showed that *P. boodjera* is a damping-off

Fig. 2. Distribution of *Phytophthora boodjera* from the tracing study of infected plants, out-planted from a nursery, and as detected by targeted soil sampling from natural vegetation remnants.

pathogen of mallee eucalypts under freely draining conditions. In the nursery, seedlings showed more severe damping-off in trays where the cells were not free draining (shelving bars here blocked drainage holes in the trays). To determine whether waterlogging increased the potential for disease, duplicate trials to those presented by Simamora et al. (2017) were carried out, where the trays were placed in larger trays permanently containing water, with the remainder of trays free draining.

Five species of mallees (*E. polybractea*, *E. kochii* subsp. *plenissima*, *E. kochii* subsp. *borealis*, *E. loxophleba* subsp. *lissophloia*, and two seedlots of *E. loxophleba* subsp. *gratae*) were inoculated with four *Phytophthora* isolates (*P. boodjera* isolates VHS 26806, VHS 27382, and PAB 11.56 and *P. cinnamomi* Rands MP94-48).

The sand container substrate and trays (90 ml/cell; Garden City Plastics) were pasteurized with steam for 1 h at 65°C. The trays containing seedlings were arranged in a randomized complete block design and watered daily with DI water and fertilized weekly with water-soluble nutrients (Thrive; Yates Company) at 2 g per 5 liters. At the time of sowing, seed were placed in random cells of the trays and two holes were made in the sand at the center of each cell. Into one cell, 20 to 25 seeds were placed, and the vermiculite inocula (2.5 g = 1% of sand volume) were placed into the second hole and covered with a thin layer of sand. For a second experiment, seedlings were inoculated at 14 days via a tube placed at sowing time next to the seed, which was removed for inoculation.

The number of surviving seedlings was recorded at the termination of the trial. For each experiment, a one-way analysis of variance was performed in SPSS to analyze the response of each eucalypt species to infestation by the different *Phytophthora* isolates for each inoculum time and each watering treatment. Roots were examined for necrotic lesions and plated on NARH.

Tracing of infected plants from the nursery. To determine whether the pathogen can be recovered from established environmental plantings of seedlings from this nursery, in 2014, roots and soil were collected from five areas planted in the 2011 and 2012

seasons: Toolibin Lake (north of Wagin), 12 samples; Lake Bryde (east of Pingrup), 12 samples; Morawa (north of Gin Gin), 9 samples; Bullfinch (northeast of Kondinin), 4 samples; and Badgebup (northeast of Katanning), 3 samples (Fig. 2). Roots and attached soil were sampled from the rhizosphere of both dying and healthy plants. Each sample consisted of four to seven subsamples (300 to 500 g) from adjacent, separate plants taken at a depth of 20 to 30 cm. Subsamples were bulked together and kept dry and cool (4 to 12 samples/site). To detect *Phytophthora* spp., a double baiting method was used. If no recoveries were made after the first round of baiting, the water was decanted and the soil dried for 3 weeks; then, baiting was repeated to induce germination of dormant spores to increase the likelihood of detection.

Targeted sampling in natural remnants. Further sampling targeted areas of environmental plantings and eucalypt plantations in the wheatbelt region of Western Australia. Seventy-one soil samples from asymptomatic plants within natural vegetation remnants were collected (Fig. 2). All soil and root samples were collected from at least 20 m away from any obvious disturbance (such as roads or drains), except for one isolate collected from roadside remnant vegetation in Northam. In addition, samples were taken from other nurseries in the same region. The same double-baiting method was used as described above.

Results

On-site sampling. All water supplies used in the nursery were clean; therefore, *P. boodjera* was already present in the production area because it was detected in the drainage water (site 10; Table 1), and further confirmed as present in soil samples from under the production benches (but only during the growing season). *P. boodjera* was also detected in all material sampled adjacent to the nursery area (sites 3, 6, 7, 8, and 9). The only equipment used in potting, storage, and handling with positive recoveries of *P. boodjera* was the used untreated trays. Some batches of eucalypt seedlings at different ages (2 weeks and 2 months old) were infected by *P. boodjera*. *P. boodjera* was not isolated from all other plant genera, ants, or fungal gnat larvae (Table 1).

Tray sterilization. Used untreated trays were infested with *P. boodjera* but the unused potting substrate was pathogen free (Table 2). One run of pasteurization was enough to eliminate *P. boodjera* in empty used untreated trays, and in trays containing potting mix.

The treatments used by the nursery (dry heat for 2 days, high-pressure water cleaning, and soaking in biocide) were not as effective as tray cleaning treatments because *P. boodjera* was recovered from them as often as from untreated trays. In contrast, boiling of the trays for 15 min was effective in destroying *P. boodjera*.

New trays and boiled trays could be used for growing seedlings in the nursery because they were free of *P. boodjera* (Table 3). Different types

Table 2. Detection of *Phytophthora boodjera* following steam pasteurization of seedling trays with and without container substrate

Sample	Pasteurization runs	Number infested/number treated
Tray	0	9/10
Container mix	0	0/5
Tray	1	0/10
Tray + container mix	1	0/10
Tray	2	0/10
Tray + container mix	2	0/10

Table 3. Recoveries of *Phytophthora boodjera* from seedling trays containing different container substrates and using different sources of water

Seed used	Tray type	Potting media ^y	Water source ^z	<i>P. boodjera</i> recovery
<i>Eucalyptus loxophleba</i> subsp. <i>gratae</i>	New	Pine bark	RW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	New	Pine bark	TW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	New	Pine bark	NRW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	New	DPaW PM	RW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	New	DPaW PM	TW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	New	DPaW PM	NRW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	Boiled	Pine bark	RW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	Boiled	Pine bark	TW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	Boiled	DPaW PM	RW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	Boiled	DPaW PM	TW	Negative
<i>E. loxophleba</i> subsp. <i>gratae</i>	Used	Pine bark	RW	Positive
<i>E. loxophleba</i> subsp. <i>gratae</i>	Used	Pine bark	TW	Positive
<i>E. loxophleba</i> subsp. <i>gratae</i>	Used	DPaW PM	RW	Positive
<i>E. loxophleba</i> subsp. <i>gratae</i>	Used	DPaW PM	TW	Positive
<i>Banksia occidentalis</i>	New	Pine bark	TW	Negative
<i>B. occidentalis</i>	New	DPaW PM	TW	Negative
<i>B. occidentalis</i>	Used	Pine bark	TW	Positive
<i>B. occidentalis</i>	Used	DPaW PM	TW	Negative

^y DPaW PM = standard container substrate used by the Department of Parks and Wildlife for glasshouse trials.

^z RW = rain water obtained from off-site, TW = nursery tank water, and NRW = nursery rain water.

of container substrate (pine bark from the nursery and DPaW) were not the source of *P. boodjera* inoculum in the nursery (Table 3). Off-site rain water, nursery tank water, and nursery rainwater were also free of *P. boodjera* and were safe to be used (Table 3). Disease was only observed, and *P. boodjera* only recovered, from used untreated trays.

Heat treating infected roots (oospores). *P. boodjera* was not recovered from infected roots subjected to all exposure times above 65°C (moist or dry), while moist heat of 65°C for at least 1 h eradicated the pathogen (Table 4).

Host susceptibility. The *Eucalyptus* spp. were significantly more affected by *P. boodjera* than the *Melaleuca* sp. and *C. proliferus*. Damping-off symptoms were first seen at day 15, when some seedlings started to wilt, then yellowed and died within 5 days (Table 5). *E. polybractea* seedlings had significantly lower mortality and severity ratings than *E. kochii* subsp. *plenissima* and *E. loxophleba* subsp. *gratae*. All *M. atroviride* and *C. proliferus* seedlings remained healthy for the duration of the trial (Table 5).

Susceptibility and waterlogging. *Eucalyptus* seed germinated within 7 to 14 days, with an average rate of 80% for all eucalypt species tested. When inoculated before emergence, there was no difference in seedling survival between the constant water and free-draining treatments but, for all treatments, seedling survival was significantly ($P < 0.05$) lower than in noninoculated controls (Supplementary Table S1).

Tracing of *P. boodjera* from the nursery. *P. boodjera* has been recovered from three nurseries (Fig. 2). For tracing studies, *P. boodjera* was recovered in 2014 from three samples from trees out-planted in 2011 or 2012 around Toolibin Lake and one sample from Badgeup. It was not recovered from plantings at Lake Bryde, Bullfinch, or Morawa (Fig. 2).

Detection of *P. boodjera* from remnant vegetation. *P. boodjera* was recovered from asymptomatic plants in only 1 of the 71 sampled sites. This recovery was made in Northam, in a remnant stand of eucalypt trees on the edge of the highway (Fig. 2). It had previously been recovered from a dying *Banksia* specimen on a road verge near Ravensthorpe, near the south coast (Simamora et al. 2015), but additional sampling in the region did not result in any more recoveries. *P. boodjera* has been detected in routine sampling of declining trees in urban parks and from reserves in the peri-urban areas around Perth (Simamora et al. 2015) (Fig. 2). A complete list of isolates of *P. boodjera* from Western Australia is provided in Supplementary Table S2.

Discussion

During a detailed analysis of the infection sources and pathways in a single nursery where production of eucalypt seedlings had been debilitated by *P. boodjera* infestation, we were able to demonstrate that this pathogen was not killed by the nursery's standard hygiene practices. *P. boodjera* caused higher mortality in seedlings of these eucalypt species than a highly pathogenic isolate of *P. cinnamomi*. Also, *P. boodjera* appears to have survived out-planting in the revegetation sites. These findings are of great concern for nurseries, plantations, revegetation projects, and urban areas as the host range and survival capability of *P. boodjera* have not been investigated.

P. boodjera persisted on site in the nursery and reinfected young seedlings in subsequent years. Extensive on-site sampling recovered the pathogen from the lawn and the roots of eucalypt hedgerow plantings adjacent to the nursery benches, and from soil under the benches (which was presumably contaminated by the *P. boodjera* zoospores from trays and pots on the benches during irrigation). However, the source of reinfestation between years was root debris containing oospores adhering to the sides of the used potting trays. Although used trays were always sterilized in 5% chlorine (calcium hypochlorite) solution, due to residual organic material, the active agent would not have been maintained for the necessary retention period and, thus, was unable to eradicate the pathogen.

Oospores generally are resistant to desiccation, cold temperatures, and other extreme environmental conditions, and can survive in the soil for several years in the absence of a host plant (Erwin and Ribeiro 1996; Hausbeck and Lamour 2004). This study found that oospores of *P. boodjera* were profusely formed in infected roots of the

eucalypt seedlings, and it has been suggested that this mechanism promotes long-term survival in harsh natural environments (Crone et al. 2013; Mircetich and Zentmyer 1966). *P. boodjera* did not survive during the nursery's fallow or dry season in the untreated soil under the production benches, probably due to the absence of host material. However, the inoculum did survive over the fallow period on root fragments attached to used seedling trays. Oospores were completely inactivated after 60 min at 65°C in a moist heat treatment, and after 30 min at 80°C in a dry heat treatment. A dry treatment of 50°C for 120 min was required for inactivation of *P. ramorum* in soil medium (Schweigkofler et al. 2014) whereas *P. cinnamomi* required 30 min at 75°C in a steam treatment (de Jager and Kotzé 1994), indicating the need for comprehensive testing of sterilization techniques for a range of species to determine the optimal standards necessary for industry.

P. boodjera in the nursery lawn, and in soil under the nearby trees and hedgerow trees, presents a continual risk to the nursery, as well as the chance of workers spreading the pathogen offsite. Thus, it is important to consider strict hygiene methods or quarantine of these areas. Monitoring areas outside the current infection range to detect any expansion of the known infestation area (Tkacz et al. 2006), determine inoculum loads (Hulvey et al. 2010; Reeser et al. 2011), and evaluate the success of any eradication activities (Filipe et al. 2012; Kanaskie et al. 2011) are of utmost importance to reduce the spread of the pathogen and ensure the future of the business.

Waterlogging in the glasshouse did not increase the infection rate of *P. boodjera* on eucalypt seedlings. This is contrary to the studies on *P. cinnamomi*, where waterlogged situations increase the potential for infection by enabling zoospores to move freely while creating stressful hypoxic conditions for plant roots (Burgess et al. 1999; Davison 1988; Hardy et al. 1996; O'Gara et al. 1997). Seedling survival postinoculation with *P. boodjera* was better for waterlogged seedlings than those in free-draining conditions; under waterlogged conditions, some seedlings survived with damaged root systems, whereas, if free draining, more seedlings wilted and died. However, these results may not extrapolate to the natural environment or hold for older plants; thus, further testing of this pathogen is required to understand the effect of water availability on the infection process.

P. boodjera infected all *Eucalyptus* spp. tested, and they were more susceptible when inoculated prior to emergence rather than

Table 4. Recovery of *Phytophthora boodjera* from infected root pieces after treatment with steam pasteurization (moist) or dry heat (+ = positive recovery and – = no recovery)

Temperature (°C)	Time of exposure (min)							
	30		60		120		240	
	Moist	Dry	Moist	Dry	Moist	Dry	Moist	Dry
20	+	+	+	+	+	+	+	+
37.5	+	+	+	+	+	+	+	+
65	+	+	–	+	–	+	–	+
80	–	–	–	–	–	–	–	–
95	–	–	–	–	–	–	–	–

Table 5. Percentage of seedling tray cells with dead seedlings (Mortality) and the disease severity (DS) of affected cells in nursery seedlings inoculated with *Phytophthora boodjera*^a

Plant species	Mortality (%) ± SE	DS (rating) ± SE
<i>Eucalyptus kochii</i> subsp. <i>plenissima</i>	22.67 ± 3.61 a	1.83 ± 0.30 a
<i>E. loxophleba</i> subsp. <i>gratae</i>	15.33 ± 3.45 a	1.74 ± 0.35 a
<i>E. polybractea</i>	8.67 ± 2.00 b	1.44 ± 0.35 b
<i>Melaleuca</i> spp.	0 c	0 c
<i>Cytisus proliferus</i>	0 c	0 c

^a Disease severity was rated as 0 = all healthy and 1 = 1 to 25, 2 = 26 to 50, 3 = 51 to 75, and 4 = 76 to 100% of cells affected. Numbers in columns followed by the same letter are not significantly different. SE = standard error.

postemergence. In the nursery, the seedlings became infected and died when they reached the four- to six-leaf stage (approximately 2 months old). This is probably because the inoculum load in the trays was initially very low and only immediately after the germination of seedlings did the infection process commence, leading to increased inoculum levels in the trays over time. *Eucalyptus* spp. were the main host of *P. boodjera* in the nursery and the pathogen was not isolated from other plant genera. However, it has been isolated in association with declining *Agonis flexuosa*, *Banksia* spp., *Corymbia calophylla*, *C. ficifolia*, and *Xanthorrhoea preissii* from other locations (Simamora et al. 2015). Understanding what determines a pathogen's host range and the potential for host shifts is of critical importance in controlling its introduction into new environments (De Vienne et al. 2009). Recently introduced exotic species are the greatest concern in the nursery trade because they may escape detection, resulting in an expanded geographical range when out-planted (Parke et al. 2014). More information is required on the potential Australian hosts of *P. boodjera* and its ability to survive in the environment.

P. boodjera survived in two locations where eucalypt seedlings from this nursery had been planted 3 to 4 years earlier. The low frequency of its recovery is probably due to low levels of inoculum present. This pathogen functions as a pre- and postemergent damping-off pathogen of young seedlings (0 to 4 weeks old), where it reduces root biomass, and the damage it causes decreases with the age of the plant (Simamora et al. 2017). Because the trees were greater than 3 years old, it is likely that inoculum levels at these sites were low by this time. Some newly introduced *Phytophthora* spp. initially have a patchy distribution of inoculum but can have a large effect on the subsequent pattern of disease when conditions for proliferation are conducive (Ristaino and Gumpertz 2000). Because *P. boodjera* is a damping-off pathogen, its continued presence postplanting may affect regeneration and seedling recruitment and drive long-term vegetation shifts (Ibáñez et al. 2015; Suarez and Kitzberger 2008).

Currently, our knowledge of the geographic distribution and host range of *P. boodjera* is limited, and the consequence of this new introduction is unknown. Because *P. boodjera* has the potential to be devastating to eucalypt production and revegetation projects, we must improve our diagnostic and management capability. The survival mechanisms of *P. boodjera* in both the glasshouse and the natural environment and oospore survival under different soil water potentials need to be understood, along with the survival ability of the various propagules at different soil depths and within host material across seasons. The optimum baits need to be identified as well as the development of specific molecular probes with primers to identify *P. boodjera* in soil, regardless of its environmental conditions and dormancy. The susceptibility of *P. boodjera* to chemicals such as phosphite and the optimum treatment for decreasing damage caused by *P. boodjera* also need to be verified.

In Europe, the plant trade has been identified as a key introduction pathway for invasive pathogens (Santini et al. 2013). In the United States, there are many reports of *Phytophthora* spp. in nurseries. For instance, Bienapfl and Balci (2014) reported finding 16 species in Maryland, while Parke et al. (2014) found 28 different species or taxa in Oregon nurseries. Bienapfl and Balci (2014) provided an additional 15 references from the United States and Australia, demonstrating the potential for the ongoing introduction of *Phytophthora* spp. into the environment from these nurseries. In an appraisal of nursery industry protocols for the control of *Phytophthora* spp. in Australia, Hancock (2015) concluded that current nursery industry advice is not sufficient to address the threat to Australian flora posed by *Phytophthora* spp. from nursery stock, particularly for the supply of plant material to conservation areas. There needs to be a higher standard for *Phytophthora* control and there should be a system developed by which nurseries can be cleared after positive *Phytophthora* spp. testing (Hancock 2015). Supporting this, a dual-stage cleaning process was recommended for used containers, including steam sterilization and hot washes (80°C). This recommendation is supported by the current study.

This extensive examination of sources of nursery contamination for *P. boodjera* has made a major contribution to our understanding

of this newly recorded pre- and postemergent damping-off disease of eucalypts in a plant production nursery, including its pathogenicity and epidemiology. These findings will help managers and scientists to understand aspects of damping-off disease in other eucalypt and plant species in nurseries and are likely to be applicable to all soil-borne oomycetes and other pathogens. Early detection, in addition to identification of the pathways of *Phytophthora* infection and spread, are of high importance to minimize the threat posed to natural ecosystems, because emerging pathogens can result in disease epidemics which can be ecologically and economically difficult to manage (Hyun and Choi 2014; Scott et al. 2013). In all countries, the risk of any outbreak can result from poor hygiene in a single nursery, limiting the success of biosecurity efforts (Bate et al. 2016) as well as damaging conservation efforts.

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