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Pathogenicity of *Phytophthora multivora* to *Eucalyptus gomphocephala* and *Eucalyptus marginata*

By P. M. Scott[1,2], T. Jung[1,2], B. L. Shearer[1,3], P. A. Barber[1,2,4], M. Calver[1,2] and G. E. St. J. Hardy[1,2,5]

[1]Centre for Phytophthora Science and Management, School of Biological Sciences and Biotechnology, Murdoch University, Australia; [2]Centre of Excellence for Climate Change, Woodland and Forest Health, School of Biological Sciences and Biotechnology, Murdoch University, Australia; [3]Science Division, Department of Environment and Conservation, Australia; [4]Arbor Carbon Pty. Ltd., Australia; [5]E-mail: G.Hardy@murdoch.edu.au (for correspondence)

Summary

Phytophthora multivora is associated with the rhizosphere of declining *Eucalyptus gomphocephala*, *Eucalyptus marginata* and *Agonis flexuosa*. Two pathogenicity experiments were conducted. The first experiment examined the pathogenicity of five *P. multivora* isolates and one *Phytophthora cinnamomi* isolate on the root systems of *E. gomphocephala* and one *P. multivora* isolate on the root system of *E. marginata*. In the second experiment, the pathogenicity of *P. multivora* to *E. gomphocephala* and *E. marginata* saplings was measured using under-bark stem inoculation. In Experiment 1, the *P. cinnamomi* isolate was more aggressive than all *P. multivora* isolates causing significant loss of fine roots and plant death. Two *P. multivora* isolates and the *P. cinnamomi* isolate caused significant losses of *E. gomphocephala* fine roots 0–2 mm in diameter and significantly reduced the surface area of roots 0–1 mm in diameter. One *P. multivora* and the *P. cinnamomi* isolate significantly reduced the surface area of roots 1–2 mm in diameter. Two of the *P. multivora* isolates significantly reduced the number of *E. gomphocephala* root tips. In *E. marginata,* the length and surface area of roots 0–1 mm in diameter and number of root tips were significantly reduced by *P. multivora* infestation. Rhizosphere infestation with the *P. multivora* isolates and *P. cinnamomi* isolate on *E. gomphocephala*, and one *P. multivora* isolate on *E. marginata*, did not significantly influence the foliar nutrient concentrations. In Experiment 2, under-bark inoculation with *P. multivora* caused significant lesion extension in *E. gomphocephala* and *E. marginata* saplings, compared to the control. We propose that *P. multivora* is inciting *E. gomphocephala* and *E. marginata* decline by causing fine root loss and subsequently interfering with nutrient cycling throughout the plant. The impact of fine root loss on the physiology of plants in sites infested with *P. multivora* requires further research.

1 Introduction

Decline and mortality of *Eucalyptus gomphocephala* (tuart) has occurred throughout its native range in the south-west of Western Australia since the early 1990s. In particular, the greatest decline has occurred in *E. gomphocephala* and *Eucalyptus marginata* (jarrah) since 2000 (Edwards 2004) and *Agonis flexuosa* (peppermint) since 2007 (Taylor et al. 2009) in the woodlands of the Yalgorup region on the Swan Coastal Plain south of Perth, Western Australia. Although a range of biotic and abiotic factors have been shown to contribute to tuart decline (Edwards 2004; Archibald 2006; Cai et al. 2010), no satisfactory aetiology has been established. The progressive canopy thinning and dieback, and the heterogeneous distribution of the decline, are similar to Phytophthora dieback of *E. marginata* involving fine and coarse root necrosis caused by *Phytophthora cinnamomi* (Shearer and Tippett 1989). In 2007, isolates of the recently described *P. multivora* (Scott et al. 2009) were recovered from rhizosphere soil of declining or dead trees of *E. gomphocephala*, *E. marginata* and *A. flexuosa*. A molecular re-evaluation of isolates from the culture collection of the Department of Environment and Conservation's Vegetation Health Service in Perth showed that over the past three decades *P. multivora*, previously identified as *Phytophthora citricola* based on morphological characteristics, has been isolated regularly throughout the south-west of Western Australia (Burgess et al. 2009) as part of regular soil and plant tissue testing for the wide scale quarantine of *P. cinnamomi* (Dell et al. 2005). *Phytophthora multivora* is the first *Phytophthora* species to be found in association with declining *E. gomphocephala* trees.

No studies have tested the pathogenicity of *P. multivora* on *E. gomphocephala*, but an isolate of *P. multivora*, designated as *P. citricola* DEC 236 from the VHS culture collection, had previously been used in an under-bark pathogenicity experiment on stems of *E. marginata* and *Banksia grandis* (Shearer et al. 1988). Isolate DEC 236, now confirmed as *P. multivora* (Scott et al. 2009), produced significantly larger lesions on stems of *E. marginata* than isolates of *P. cinnamomi*, but did not produce lesions in stems of *B. grandis* (Shearer et al. 1988). These results support the potential role of *P. multivora* in the decline of *E. gomphocephala* in Yalgorup National Park, as *E. marginata* trees are suffering substantial dieback whilst *B. grandis* trees remain comparatively healthy. *Banksia grandis* within Yalgorup are, however, associated with a range of *Botryosphaeriaceae* pathogens which have been shown to cause dieback of stems and branches (Taylor et al. 2009).

The progressive and gradual nature of crown decline associated with *E. gomphocephala*, *E. marginata* and *A. flexuosa* dieback within the Yalgorup woodland, and the association of *Phytophthora* species in the rhizosphere, suggests that a fine feeder root pathogen may be involved in the decline. Compared to cankers, leaf and shoot diseases of above-ground parts of trees, fine root diseases are more difficult to diagnose because fine root losses are more challenging to measure than aerial symptoms and their impacts are usually expressed via non-specific symptoms of branch or crown dieback which may also be associated with other pathogens or insects (Hansen and Delatour 1999). In addition, diebacks incited by fine root damage often show a scattered spatial

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distribution (Jung et al. 1996, 2000). The assessment of fine root parameters has been successfully used in both adult forest trees and artificially infested seedlings to demonstrate the involvement of the fine root pathogen *P. quercina* in European oak decline (Jung et al. 1999, 2000; Jönsson et al. 2003, 2005; Jönsson 2004). The association between the reduction of fine feeder roots of *E. marginata* caused by *P. cinnamomi* and dieback has been difficult to substantiate. In the *E. marginata* forest, *P. cinnamomi* has been shown to only reach high inoculum densities in the soil for relatively short periods of time (Shea et al. 1979, 1980; Shearer et al. 2010) and *E. marginata* has a large turnover in fine roots throughout the year (Shea and Dell 1981), often in rapid association with rainfall events (Dell and Wallace 1983). The high tolerance of *E. marginata* to root loss was demonstrated by Crombie et al. (1987), when a significant reduction in stomatal conductance only occurred after removal of 50% of roots arising from the root collar. To explain the resilience in stomatal conductance of *E. marginata,* to fine root loss, Crombie et al. (1987) proposed that because of the dimorphic structure of *E. marginata*, the deep tap roots are predominantly involved in water relations and the fine roots have other primary functions including nutrient cycling. In the jarrah forest, *E. marginata* is predominantly killed by major root and collar damage (Shearer and Tippett 1989), on sites with lateritic hard pans, which favour *P. cinnamomi* infection of the major roots followed by rapid mortality (Shea et al. 1984). In contrast, more scattered slow declines of *E. marginata* may not be related to subsurface infection and the death of primary roots and the collar, but rather to a gradual chronic deterioration of the fine root system (Somerford et al. 1987).

Given the potential for fine root production in many tree species, a fine feeder root pathogen may only induce a decline if under the influence of other inciting factors such as changes to the water relations including water logging and or severe droughts (Dawson and Weste 1982; Ploetz and Schaffer 1989; Davison 1994; Brasier 1996) and stress related to insects and other pathogens (Brasier and Scott 1994). The seasonal variation in fine feeder root necrosis can result in a net reduction in root volume resulting in resource depletion (Jönsson 2006). Equally, fine root losses may increase susceptibility to severe droughts, secondary pathogens or secondary pests like bark beetles (Jung et al. 2000). On Western Australia's highly nutrient depauperate soils, fine feeder root loss and associated mycorrhizal loss could result in nutrient deficiency symptoms, contributing to decline (Podger 1972; Shea and Dell 1981). The condition of the fine root system of declining *E. gomphocephala* trees has not been assessed and extensive studies of mature trees in the field and pathogenicity trials are required to understand the aetiology of the decline and the possible association of *P. multivora*.

This research aims to determine the pathogenicity of *P. multivora* on *E. gomphocephala* and *E. marginata* tree species.

2 Materials and methods

Two experiments were conducted. In Experiment 1, a soil infestation tested the pathogenicity of *P. multivora* and *P. cinnamomi* in the root systems of *E. gomphocephala* and *E. marginata* seedlings. In Experiment 2, an under-bark inoculation measured the pathogenicity of *P. multivora* in the stems of *E. gomphocephala* and *E. marginata* saplings.

2.1 Experiment 1 – Soil infestation

Five isolates of *P. multivora*, and as a single comparison isolate of *P. cinnamomi*, were tested for their pathogenicity towards 4-month-old seedlings of *E. gomphocephala* in a soil infestation test over 12 months (Table 1). One of the five *P. multivora* isolates was also tested against 4-month-old *E. marginata* seedlings. Each isolate-host combination and the control treatments consisted of four replicate pots of six seedlings per pot, giving 24 seedlings for each treatment and a total of 168 *E. gomphocephala* seedlings and 48 *E. marginata* seedlings. Pots were arranged in a randomized block design. The isolates of *P. multivora* had been isolated in 2007 from the rhizosphere of declining *E. gomphocephala*, *E. marginata* and *A. flexuosa* at five sites in the tuart forest of Yalgorup National Park between 32.6° and 32.7° southern latitude (Scott et al. 2009).

Table 1. Phytophthora species, isolate numbers, their origin and the pathogenicity experiments in which they were used.

		Experiment 1 Soil infestation		Experiment 2 Under-bark inoculation	
		Host plant		Host plant	
Species and isolates	Origin of rhizosphere soils	*Eucalyptus gomphocephala*	*Eucalyptus marginata*	*E. gomphocephala*	*E. marginata*
P. multivora					
WAC 13201 (CBS 124094)	*E. gomphocephala* and *E. marginata* – mixed	✔	✔	✔	✔
WAC 13200	*E. gomphocephala*	✔		✔	
WAC 13202	*E. gomphocephala*	✔		✔	
WAC 13203	*Agonis flexuosa*	✔		✔	
WAC 13204 (CBS 124095)	*E. gomphocephala*	✔		✔	
P. cinnamomi					
MP 94-48	*E. marginata* forest	✔			

For experiments, ✔ = indicates host plants included in trials.
CBS, Centraalbureau voor Schimmelcultures Utrecht, Netherlands; WAC, Department of Agriculture and Food Western Australia Plant Pathogen Collection; MP, Murdoch University *Phytophthora* Collection.

Table 2. Nutrient status of the container medium used in the pathogenicity test (Experiment 1) prior to adding nutrients. Concentrations of base cations Mn, Fe and B were determined by inductively coupled plasma spectroscopy (Perkin Elmer®, Norwalk, CT, USA) after extraction of 20 g soil in 100 ml 0.1 $BaCl_2$ for 2 h. The total concentration of C was determined using an automatic combustion analyser (LECO® corporation instruments, St. Joseph, MI, USA).

Elements	Concentration
Nitrate	1 μg/g
Ammonium	1 μg/g
Phosphorus	3 μg/g
Potassium	15 μg/g
Sulphur	1.7 μg/g
Copper	0.03 μg/g
Zinc	0.18 μg/g
Manganese	0.1 μg/g
Organic carbon	0.11%
Conductivity	0.007
pH_$CaCl_2$	6.2
pH_H_2O	6.7

Eucalyptus gomphocephala and *E. marginata* seeds were germinated and grown in a steam pasteurized mix (2 : 2 : 1) of composted pine bark, coarse river sand and coco peat in 5-cm-deep free-draining seedling punnets. Six-week-old seedlings were then transplanted into 20-cm-diameter free-draining polypropylene pots containing 4.3 l of coarse washed river sand (Table 2) (Richgro®, Jandakot, WA, Australia) pasteurized twice over 2 days at 65 ± 5°C for 60 min. Samples of the pasteurized river sand were baited with young oak leaflets according to Jung et al. (1996) to confirm the absence of *Phytophthora* species. No *Phytophthora* species were recovered. One 12-cm-long, 1-cm-diameter plastic tube was pushed into the container of river sand, adjacent to each of the six seedlings with an additional 2-cm-diameter tube placed into the centre of the pot at the time of transplanting. Every 2 weeks the pots were fertilized to container capacity with a water soluble, low phosphate fertilizer specifically formulated for Australian Natives (NPK 28-1.8-10) (Osmocote® Plus Native Gardens; Scotts Australia Pty. Ltd., Baulkham Hills, NSW, Australia), at the recommended application rate of 3.25 g/l, for 8 weeks until the soil was infested with the *Phytophthora* isolates. After infestation, the pots were fertilized every 4 weeks as described above. The plants were grown under controlled conditions in a glasshouse (6–27°C) (Murdoch University, Perth) and watered twice a day to container capacity with deionized water. Air temperature, soil temperature and relative humidity were measured every 15 min with a data logger, (HOBO®; Onset® Computer Corporation, Bourne, MA, USA).

Two different inoculum sources were produced for each isolate (Table 1); (i) colonized *Pinus radiata* (pine) plug inoculum, and (ii) a colonized mixture of vermiculite, oat grain and vegetable juice (VOV). The pine plug inoculum was modified after the study of Butcher et al. (1984). Briefly, live *P. radiata* branches were cut into plugs 1–2 cm in diameter and approximately 2 cm long, and the bark was removed. Plugs were soaked overnight in distilled water, rinsed and placed in a 1 l conical flask with 100 plugs per flask. Distilled water was added to the flasks to a depth of 1 cm and flasks were plugged with non-absorbent cotton wool and covered with aluminium foil. Flasks were autoclaved for 20 min at 120 kPa, then cooled to room temperature for 24 h and then re-autoclaved on two consecutive days. Individual cultures of isolates grown on 8-cm-diameter Petri dishes with vegetable juice agar (V8A) [100 ml/l vegetable juice (Campbells V8 vegetable juice; Campbell Grocery Products Ltd., Norfolk, UK), 900 ml/l demineralized water, 3 g/l $CaCO_3$ and 15 g A Grade Agar (Becton, Dickinson and Company, Sparks, MD, USA)] for 5 days at 20°C in the dark were cut into 1-cm squares and the whole culture transferred to the cooled flask, and incubated for 4 weeks at 20°C in the dark and shaken periodically. The VOV inoculum was prepared according to Matheron and Mircetich (1985) modified by Jung et al. (1999). Briefly the VOV inoculum was prepared by inoculating an autoclaved mixture of 250 cm^3 vermiculite, 20 cm^3 whole oat grains and 175 ml vegetable juice broth (consisting of 100 ml/l V8 juice, 900 ml/l demineralized water and 3 g/l $CaCO_3$) with five 1 cm^2 V8A discs of 5-day-old cultures. After 4 weeks incubation at 20°C in the dark, the inoculum of each isolate was rinsed with demineralized water to remove excess nutrients, immediately before soil infestation (Matheron and Mircetich 1985).

Plants were grown in the pots for 8 weeks before soil infestation. For inoculation, all seven tubes were removed from each pot. A colonized pine plug was inserted into each hole adjacent to the seedlings, which was left by the removal of the tubes adjacent to each plant. A total of 86.0 cm^3 (equivalent to 2% of container river sand volume) of the VOV inoculum was inserted into the central 2-cm holes left by the removal of the tube in the centre of each pot. The holes were then filled with sterile river sand. Control treatments received pine plug inoculum, and rinsed vermiculite, oat grains and vegetable juice broth inoculum that had been colonized with *P. multivora* and autoclaved for 20 min at 120 kPa and then cooled to room temperature. Sterility of the autoclaved control inoculum was confirmed using the oak leaf baiting method (Jung et al. 1996). To stimulate the production of sporangia and the release of zoospores from the inoculum source, the containers were flooded with deionized water to approximately 2 cm above the surface for 24 h, immediately after inoculation, and once again after 14 days to mimic woodland conditions after a heavy rainfall event. Flooding was achieved by placing each pot in a water-tight bag (Batini and Podger 1968). At each flooding event the water of each pot was baited with oak leaflets for the presence of *Phytophthora* inoculum, as described above.

Stem length and the above-ground condition of the seedlings were measured every 4 weeks for 12 months when the trial was harvested. The health of each seedling was based on wilting and estimated on a scale from 5 to 0 (5 = healthy; 0 = dead), assessed daily up to the time of death after which the plant was left in the pot, to avoid disturbing the root systems of surrounding plants.

One year after infestation, the remainder of the living plants was harvested. The stems were separated from the roots at the point where the cotyledon had been attached. Leaves were separated from the stem and branches and bagged in separate paper bags for each plant. Leaves and stems were dried at 70°C for 10 days and weighed. The root system of each plant was separated, and the river sand was sieved through a 2-mm sieve to collect any remaining pieces of the root system. Reisolation from the soil and a small subset of root fragments, covering the size classes of 0–2 mm diameter and above 2 mm diameter, was performed to confirm pathogen survival and root infection. Reisolation from the soil was performed using the oak leaf baiting method as described previously and root fragments were gently dried with paper towel and plated onto selective PARPNH agar, containing V8 juice (Campbell's®) 100 ml/1, agar 20 g/l, $CaCO_3$ 3 g/l, pimaricin l0 mg/l, ampicillin 200 mg/l, rifampicin 10 mg/l, pentachloronitrobenzene (PCNB) 25 mg/l, nystatin 50 mg/l and hymexazol 50 mg/l modified from (Tsao 1983).

2.1.1 Root analysis

Roots were harvested by submerging the whole root ball from a pot in a water-filled 50 × 50 cm tub, and bubbling air for ca. 12 h from underneath through the root ball and then removing the loosened river sand by hand. Initially the root balls were completely submerged in deionized water in a plastic tub, 50 cm deep by 50 cm wide. Inside the plastic tubs the root balls rested on a plastic mesh with 3 cm^2 openings, raising the root balls 5 cm above the tub bottom. Beneath the plastic mesh, 6-mm-diameter plastic tubing was used to gently bubble air underneath the root balls. The bubbling air dislodged the river sand particles, which settled beneath the mesh, whilst leaving the root structure relatively intact. The root balls from each pot were exposed to the bubbling air for approximately 12 h. On removal, the residual container river sand was gently removed by hand and the excised root fragments were amalgamated and measured separately into the different size classes. All equipment was thoroughly washed and sterilized with 70% ethanol before re-use.

After washing, the roots were scanned on an Epson Expression 10 000XL light transmission scanner in a 10-mm-deep water bath, and root length, surface area and numbers of root tips were measured for five root diameter classes (0–0.5, 0.5–1, 1–2, 2–5, >5 mm) using the software WINRHIZO Pro V 2007d (Reagent Instruments, Québec, QC, Canada) (Jönsson et al. 2003). Root samples per plant were then sorted into diameter classes 0–2 mm (fine roots) and >2 mm (mother roots), and dried at 70°C for 10 days and weighed.

2.1.2 Chemical analysis

Eucalyptus gomphocephala leaves for each pot were bulked, to give four composite samples per treatment and analysed by CSBP Limited (Bibra Lake, WA, Australia). To determine the concentration of nitrogen (N), finely ground plant material was digested in nitric acid, combusted at 950°C in oxygen using a FP-428 Nitrogen Analyser (LECO® corporation instruments, St. Joseph, MI, USA) that measured the released nitrogen from the sample as it passes through a thermal conductivity cell. The concentrations of calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), sulphur (S) and phosphorus (P) were determined using an inductively coupled plasma analyser (Perkin Elmer®, Norwalk, CT, USA). Nitrate (NO_3^-) concentration was analysed by colorimetric measurements using flow injection analysis on a water extraction (Searle 1984).

2.2 Experiment 2 – Under-bark inoculation

In autumn (May) 2008, five isolates of *P. multivora* (Table 1) were tested for their pathogenicity towards self-seeded *E. gomphocephala* saplings using an under-bark inoculation experiment. One of the five *P. multivora* isolates was also tested on saplings of *E. marginata*. The pathogenicity experiment was conducted in a mixed *E. gomphocephala* and *E. marginata* stand (32.6822°S, 115.6417°E) in Yalgorup National Park, near the original isolation site of the *P. multivora* isolates (Table 1). The region has a Mediterranean climate and the study area receives approximately 649.4-mm rainfall annually, mainly over winter (June–August), and the mean monthly temperatures range from 10.6 to 29.2°C (2002–2010) (BoM 2011). Soils have a sandy texture and are derived from limestone (McArthur and Bartle 1980) with an average pH of 7.1 pH (H_2O) (Cai et al. 2010).

There were 10 replicate plants for each of the five isolates and the non-inoculated control treatments. Inoculation treatments were distributed with a randomized replicate block design. The saplings were between 0.5 and 1.5 m in height with stem diameters of 10–30 mm and were inoculated approximately halfway along the main stem at least 10 cm above ground level. An oblique incision was made with a sterilized, sharp scalpel through the outer bark into the phloem (O'Gara et al. 1996). A 9-mm-diameter inoculum plug was removed from 5-day-old *Phytophthora* cultures of each isolate grown on V8 agar and placed mycelial surface face down onto the exposed wounds. The bark flaps were replaced over the inoculum disc and the wound was covered with a 1 cm^3 piece of moist cotton wool, sealed with Parafilm® (Pechiney Plastic Packaging; Menasha, WI, USA) tape and aluminium foil (Shearer et al. 1988; O'Gara et al. 1996). Plugs of non-colonized V8 agar were used for the control inoculations. Lesion extension was measured after 9 weeks. Colonization above and below any visible lesion was assessed by plating 1-cm stem sections from 5 cm above and below the visible lesion onto PARPHN agar, giving a total of 10 sections per plant.

2.3 Statistical analysis

Data were analysed using parametric tests, and where required, non-parametric data were log transformed to achieve normal distribution and homogeneous variances before analysis. Significance was determined at $p \leq 0.05$. Soil infestation and under-bark inoculation experiments on *E. gomphocephala* and *E. marginata* were analysed separately. Experiment 1 (soil infestation)

was analysed as a mixed model nested multivariate analysis of variance (MANOVA), with the higher level factors being fixed (Bennington and Thayne 1994) or model I (Sokal and Rohlf 1995) and the lower level, nested factor of pot being random (Bennington and Thayne 1994) or model II (Sokal and Rohlf 1995). For experiment 2 (under-bark inoculation), a one-way ANOVA was used to test for significant differences, between the means of each treatment. Where treatments were significant, *post hoc* Fisher LSD tests were used to identify significantly different factor levels (Day and Quinn 1989). Analyses were carried out in STATISTICA software package Version 5 (Statsoft 1984–1999).

3 Results

3.1 Experiment 1 – Soil infestation

3.1.1 Above-ground condition and growth

No *E. gomphocephala* seedlings died in pots infested with *P. multivora* or in the control pots. Six *E. marginata* seedlings died in pots infested with *P. multivora* between 26 and 48 weeks after infestation. Four *E. gomphocephala* seedlings died in pots infested with *P. cinnamomi* during the experiment between 26 and 34 weeks after infestation. There were no significant differences in the above-ground condition, stem length or weight of dried plant parts when harvested including fine roots (diameters 0–2 mm), all roots (diameters >0 mm), stems or leaves, between plants inoculated with *P. multivora* or *P. cinnamomi* isolates and the control (data not shown).

3.1.2 Reisolation

During each flooding event, both *Phytophthora* species were recovered from the water/soil of *Phytophthora* infested pots. No *Phytophthora* species were isolated from the control pots. *Phytophthora cinnamomi* was isolated from the collars of all four *E. gomphocephala* seedlings that died during the infestation experiment by plating onto PARPHN selective medium. After harvesting, all *Phytophthora* isolates were recovered from washed root material by both baiting and direct plating from the infested pots. *Phytophthora multivora* was recovered from fine root material of *E. gomphocephala* and *E. marginata* seedlings up to 1 mm in diameter, but not from thicker roots, collars or stem. *Phytophthora cinnamomi* was recovered from all root diameters of *E. gomphocephala* and from bark lesions below the collar of the remaining live seedlings. *Phytophthora cinnamomi* was reisolated from the collars of dead *E. gomphocephala* seedlings. No *Phytophthora* was isolated from the soil samples or fine root fragments of the control seedlings.

3.1.3 Root growth

In all inoculated and control plants of *E. gomphocephala* and *E. marginata,* the fine feeder roots <1 mm in diameter represented 96.9 and 98.2% of the total root length, respectively.

Two of the five *P. multivora* isolates, WAC 13200 and 13201, and *P. cinnamomi* isolate MP 94-48, caused significant reductions of root lengths compared to the control for all root diameters combined (Fig. 1a), root diameters 0–1 mm (Fig. 1b) and root diameters 1–2 mm (Fig. 1c). The length of *E. gomphocephala* roots between 2 and 5 mm in diameter was not influenced by any of the *P. multivora* isolates, or the *P. cinnamomi* isolate (data not shown). The *P. multivora* isolates WAC 13200 and 13201 significantly reduced the number of root tips of *E. gomphocephala* (Fig. 1d). *Phytophthora multivora* isolates WAC 13200 and 13201 and *P. cinnamomi* isolate MP 94-48 caused significant reductions in the surface area of *E. gomphocephala* roots 0–1 mm in diameter (Fig. 1e). *Phytophthora multivora* isolate WAC 13201 and *P. cinnamomi* isolate MP 94-48 also significantly (Fig. 1f) reduced the surface area of *E. gomphocephala* roots 1–2 mm in diameter.

In *E. marginata*, the *P. multivora* isolate WAC 13201 significantly reduced the length of roots when all diameter classes were combined (Fig. 2a) and for the 0–1 mm diameter range (Fig. 2b), but not for the 1–2 mm (Fig. 2c) or 2–5 mm root diameters (data not shown) compared to the control. The *P. multivora* isolate WAC 13201 significantly reduce the number of root tips (Fig. 2d), and area of 0- to 1-mm roots (Fig. 2e) of *E. marginata* compared to the control. Isolate WAC 13201 did not significantly reduce the area of 1- to 2-mm diameter roots (Fig. 2f) when compared to the control.

3.1.4 Foliar nutrient analysis

There were no significant differences in the concentrations of all measured foliar nutrients between *E. gomphocephala* plants inoculated with *P. multivora* or *P. cinnamomi* isolates and the control and between *E. marginata* plants inoculated with *P. multivora* and the control.

3.2 Experiment 2 – Under-bark inoculation

3.2.1 Under-bark inoculation experiment

Under-bark inoculation with *P. multivora* isolates caused significant lesion extension in self-seeded *E. gomphocephala* and *E. marginata* saplings. The greatest extension was recorded on *E. marginata* saplings (Fig. 3b) averaging 90.2 mm, compared to the *E. gomphocephala* saplings (Fig. 3a), which ranged from 3.2 to 20.6 mm across all five *P. multivora* isolates tested.

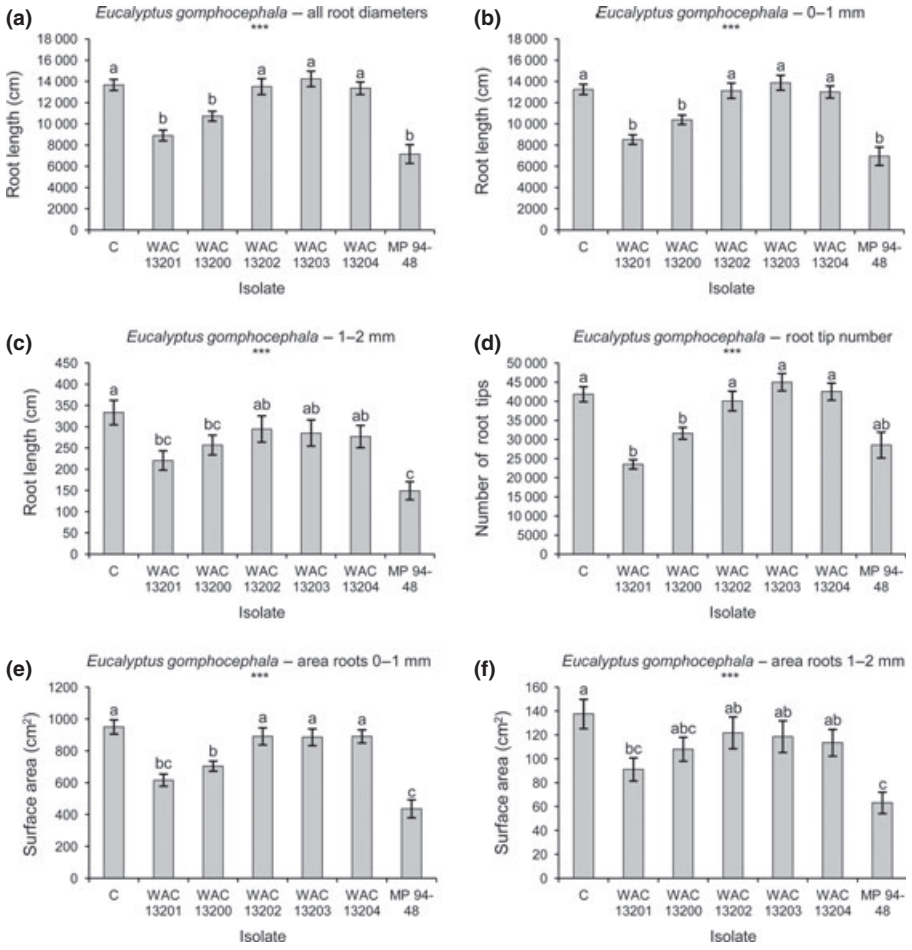

Fig. 1. Mean root parameters (±SE) of *Eucalyptus gomphocephala* seedlings after 12 months growth in soil infested with *Phytophthora multivora* isolates (WAC13200 – WAC13204), *P. cinnamomi* isolate MP 94-48 or non-infested control soil (C). Root parameters include: mean root length for (a) all root diameters combined, and roots 0–1 mm (b) and 1–2 mm (c); number of root tips; and the surface area of diameter root classes 0–1 mm (e) and 1–2 mm (f). Statistics are for univariate tests where the multivariate main effect is significant. ***$p \leq 0.001$. Small letters denote the results of the *post hoc* test (Fisher LSD) and different letters indicate significant ($p \leq 0.05$) differences.

4 Discussion

Phytophthora multivora was pathogenic to *E. gomphocephala* and *E. marginata* in soil and under-bark inoculations. *Eucalyptus gomphocephala* was susceptible to *P. multivora* because two of the five isolates caused significant fine root loss; with the most substantial loss for roots <1 mm in diameter and all isolates caused significant stem lesions. Soil infestation and under-bark inoculation with *P. multivora* did not, however, cause *E. gomphocephala* death. In contrast, *E. marginata* was shown to be more susceptible to *P. multivora* than *E. gomphocephala* because of greater fine root loss and the death of six plants in the soil infestation trial and greater lesion extension in the under-bark inoculation trial. Koch's postulates were satisfied as *P. multivora* was isolated from the rhizosphere of declining *E. gomphocephala* woodland trees suffering significant fine root loss. The rhizosphere of *E. gomphocephala* seedlings grown under controlled conditions were infested with *P. multivora* and subsequently suffered significant fine feeder root loss. Finally, *P. multivora* was reisolated from the rhizosphere and roots of these artificially infested seedlings.

Above-ground symptoms were not observed in the soil infestation trial on the living *E. gomphocephala* and *E. marginata* seedlings at the end of the trial, although symptoms may have developed if the trial was to continue for longer. Similarly, soil infestation of *Juglans regia* with five *Phytophthora* species did not correspond to a significant reduction in above-ground plant development, although four species caused significant necrosis of fine roots and a significant reduction of root weight compared with non-inoculated seedlings (Vettraino et al. 2003).

Eucalyptus gomphocephala is more susceptible to infection by the *P. cinnamomi* isolate than the *P. multivora* isolates, as indicated by greater fine root loss and the death of 17% of plants. However, it is unlikely that *P. cinnamomi* poses a threat to *E. gomphocephala*, which grows predominantly on the calcaric Spearwood and Quindalup soils (Ruthrof et al. 2002) which are not conducive to *P. cinnamomi* infestation (Shearer and Dillon 1996). It is also unlikely that *P. cinnamomi* is present throughout the *E. gomphocephala* Yalgorup woodland, as highly susceptible species including *B. grandis* and *Xanthorrhoea* spp., which are

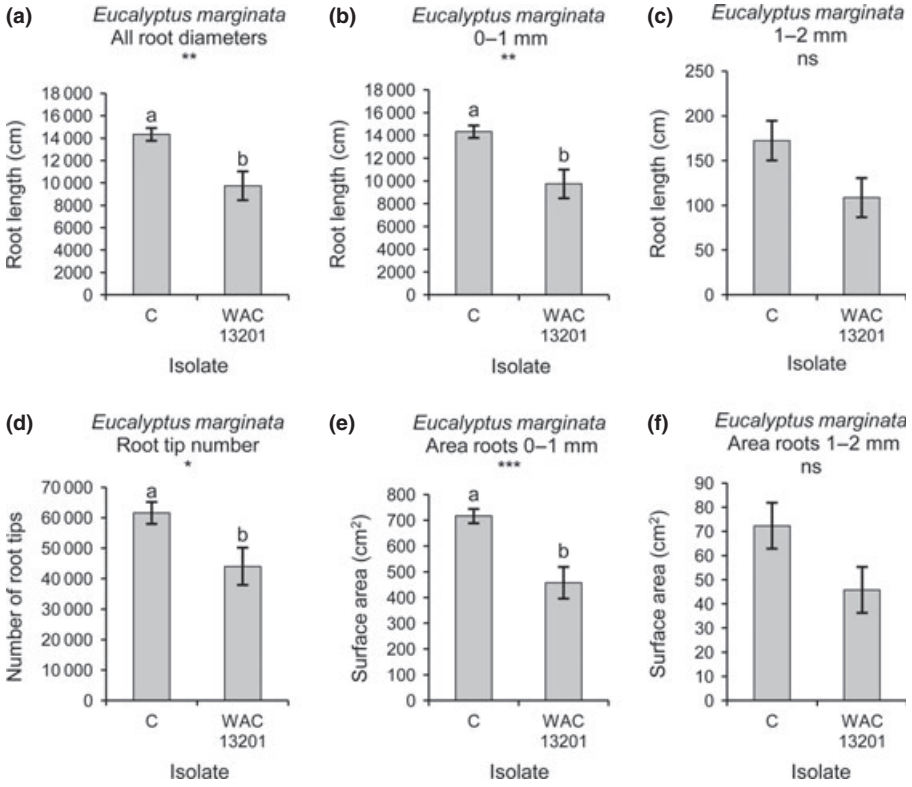

Fig. 2. Mean root parameters (±SE) of *Eucalyptus marginata* seedlings after 12 months growth in soil infested with *Phytophthora multivora* isolates (WAC13201) or non-infested control soil (C). Root parameters include: mean root length for (a) all root diameters combined, and roots 0–1 mm (b) and 1–2 mm (c); number of root tips; and the surface area of diameter root classes 0–1 mm (e) and 1–2 mm (f). Statistics are for univariate tests where the multivariate main effect is significant. *p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; ns, not significant. Small letters denote the results of the *post hoc* test (Fisher LSD) and different letters indicate significant (p ≤ 0.05) differences.

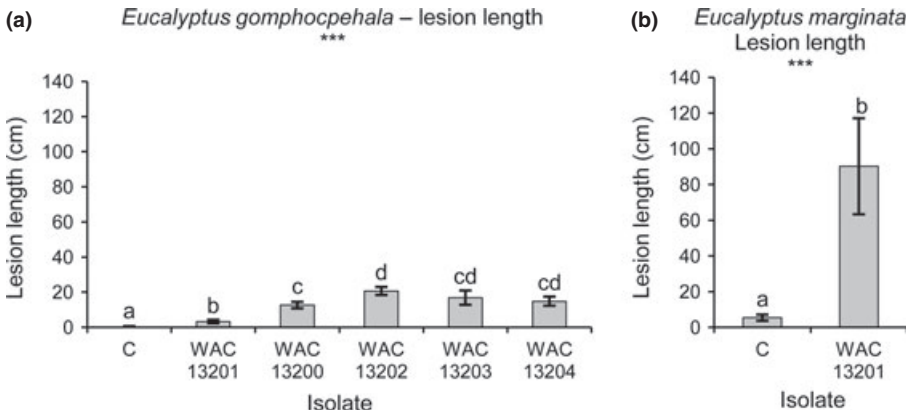

Fig. 3. Mean lesion lengths (±SE) from under-bark inoculation (a) *Eucalyptus gomphocephala* saplings inoculated with *Phytophthora multivora* isolates WAC13200 – WAC13204 and (b) *E. marginata* saplings inoculated with *P multivora* isolate WAC13201. C indicates blank inoculated control. Statistics are for one-way ANOVA. ***p ≤ 0.001. Small letters denote the results of the *post hoc* test (Fisher LSD) and different letters indicate significant (p ≤ 0.05) differences.

used as 'indicator species' to map *P. cinnamomi* distribution, do not exhibit typical symptoms associated with *P. cinnamomi* infested sites. Previous surveys for *Phytophthora* soil-borne pathogens associated with *E. gomphocephala* decline have not isolated *P. cinnamomi* (Scott et al. 2009). However, *P. cinnamomi* has been reported near Yalgorup National Park in areas of State forest east of the Old Coast Road, on Karrakatta and the Bassendean soil complexes (Portlock et al. 1993), which are conducive to *P. cinnamomi* infestation and high disease impact (Shearer and Dillon 1996).

Molecular re-evaluation of historic *Phytophthora* isolates (Burgess et al. 2009) has found that *P. multivora* kills *E. marginata* growing in restored mine sites and causes large stem girdling cankers on *E. marginata* growing on restored bauxite mines in

the jarrah forest (G. E. St. J. Hardy, personal communication). Therefore, *P. multivora* is probably also associated with the decline of *E. marginata* at Yalgorup. In the Yalgorup woodland, where *E. marginata* is only found in scattered pockets, it may be more susceptible to *P. multivora* than throughout the remainder of its range where it is often the dominant keystone species.

Conditions for the soil infestation pathogenicity trial in the glasshouse may not directly relate to natural environments. Whilst some isolates of *P. multivora* and the *P. cinnamomi* isolate were able to induce significant fine root loss, it is not known if the observed root damage would result in *E. gomphocephala* decline in natural stands.

Under-bark stem inoculation with *P. multivora* caused greater lesion extension on *E. marginata* than on *E. gomphocephala*. Whilst under-bark inoculation experiments demonstrate pathogenicity, the inoculation procedure bypasses early infection processes. Further work is required to determine if under natural conditions in the *E. gomphocephala* woodland, *P. multivora* can invade the bark and phloem of *E. gomphocephala* and *E. marginata* and cause expanding lesions throughout the thicker root systems and stems. *Phytophthora multivora* may only infect the major roots and collar in the presence of specific environmental conditions which enable the pathogen to bypass outer defence mechanisms, as shown for collar rot of mature beech trees by *P. cambivora* in the presence of boring insects (Jung 2009) or *E. marginata* seedlings by *P. cinnamomi* in the presence of prolonged flooding (O'Gara et al. 1996). Infection of the collar and major roots by *P. multivora* would likely result in greater damage, than if it is only a fine root pathogen.

Quantification and replication of stresses affecting naturally growing mature trees is difficult to achieve. For example, the chemical and biological characteristics of natural woodland soil may significantly interact with *Phytophthora* species, affecting their pathogenicity. An understanding of the epidemiology/aetiology of *E. gomphocephala* decline is required to demonstrate the impact that fine feeder root and collar disease may have. This may be determined by quantifying the relationship between the inoculum density of *P. multivora*, fine root condition and tree health under natural conditions. To accurately quantify these, measurements of the whole root system and inoculum dynamics are required, without disturbing the fine root structures. This may be conducted through the use of technologies such as ground penetrating radar and the air spade.

The fine feeder root damage observed for *P. multivora* on *E. gomphocephala* seedlings suggests that the pathogen is acting as a fine feeder root pathogen. The failure to reisolate *P. multivora* from roots of more than 1 mm diameter demonstrates its specificity as a fine feeder root pathogen. In addition, *P. multivora* caused only small lesions on *E. gomphocephala* in the under-bark stem inoculation experiment, which is in accordance with the absence of bark lesions on stems of *E. gomphocephala* in the field. On *E. marginata*, *P. multivora* caused larger stem lesions than on *E. gomphocephala*. Interestingly, *Phytophthora* isolates that were later shown to be *P. multivora* based on molecular re-evaluation (Scott et al. 2009) were also shown to be aggressive pathogens of *E. marginata* and *Corymbia calophylla* (Shearer et al. 1987) and in *E. marginata* (Bunny 1996). Further molecular re-evaluation of other *Phytophthora* isolates originally identified as *P. citricola,* based on morphological and isozyme identification, may likely reveal that *P. multivora* isolates have a wider host range.

In general, the pathogenicity of the *P. multivora* isolates in the soil infestation experiment negatively correlated to the pathogenicity observed in the under-bark inoculation experiment. For example, *P. multivora* isolate WAC1302 caused the greatest fine feeder root loss, but caused the smallest stem lesion during the under-bark inoculation experiment. Whilst *P. multivora* isolate WAC 13203 isolated from *A. flexuosa* (Scott et al. 2009), caused little root damage but large stem lesions. As yet, pathogenicity of *P. multivora* to *A. flexuosa* remains to be tested. Previous recoveries of *P. multivora*, then designated as *P.* sp 4, show that *P. multivora* is associated with dying plants from a range of species including *E. marginata*, five *Banksia* species, a *Conospermum* sp., *Leucopogon verticillatus, Xanthorrhoea gracilis, Podocarpus drouyniana,* a *Patersonia* sp., a *Bossiaea* sp., *Gastrolobium spinosum* and *Pinus radiata* (plantation) (Burgess et al. 2009). It is likely that *P. multivora* may also be associated with the decline and/or death of other plant species. Future research could screen the pathogenicity of *P. multivora* isolates obtained from specific hosts (including *A. flexuosa*) against other host species to determine if there is any host specificity.

A greater understanding of the epidemiology of *E. gomphocephala* decline may be determined by measuring the association between host-pathogen dynamics at different scales. The temporal and spatial inoculum dynamics of *P. cinnamomi* have been measured in the *E. marginata* forest (Shea et al. 1980, 1983; Blowes et al. 1982; Shearer and Shea 1987; Kinal et al. 1993) and between the *Banksia* woodland and *E. marginata* forest of the Swan Coastal Plain (Shearer et al. 2010). Shea and Dell (1981) and Dell and Wallace (1983) measured the interaction between inoculum levels and the dynamics of *E. marginata* root cycling under natural conditions. Jung et al. (2000) demonstrated highly significant correlations between the presence of *Phytophthora* spp. in the rhizosphere with fine root degradation and canopy decline of oaks. Similar spatial and temporal approaches are required to determine the epidemiology of *E. gomphocephala* and *E. marginata* declines, specifically in the *E. gomphocephala* woodland on calcareous soils. Currently, little is known about the physiology of *E. gomphocephala* roots and their dynamics and turnover in the presence or absence of pathogens and other damaging factors.

Further work is required to understand how *P. multivora* pathogenicity is influenced by climate and environmental characteristics including pH, calcium content and soil hydrology. Brasier et al. (1993) proposed that in Iberian oak decline, *P. cinnamomi* was a more aggressive pathogen on moist sites which allowed greater fine root infections than soils that are only seasonally wet, where infestation only resulted in partial root loss which could be replaced by vigorous root growth except when combined with drought, flooding or other decline pressures. Brasier et al. (1993) proposed that a similar interaction was responsible for the disease expression of *P. cinnamomi* on *Castanea* spp. (chestnut) in the USA, which often began on poorly drained sites, and spread onto drier hills causing chronic disease (Crandall et al. 1945). Similarly, Vannini et al. (2010) found that Ink disease of *Castanea sativa* caused by *P. cambivora* in a chestnut forest in Italy occurred preferentially along natural drainage routes. The relationship between unseasonal rainfall and drought periods and *Phytophthora* diseases associated with *Fagus sylvatica* (Beech) decline was also demonstrated in central Europe (Jung 2009). Similar disease and climate interaction may be involved in *E. gomphocephala* and *E. marginata* decline within the Yalgorup National Park. The Yalgorup woodland is

within a Mediterranean climate with variable soil drainage. The relationship between disease expression and soil hydrology may help indicate the role of *P. multivora* in *E. gomphocephala* decline.

Increased climatic changes within south-western Australia (Hope et al. 2006) may be a significant factor influencing *P. multivora* pathogenicity in the future. Within the current study, plant roots were initially flooded with deionized water for 24 h after inoculation, again after 14 days and then continuously kept moist with regular watering. Greater fine root loss may have resulted by treating seedlings with periods of restricted watering intermixed with flooding. Cycles of soil flooding and drying may have increased plant stress and preferentially favoured the growth of *Phytophthora* within the soil. Plants were not droughted or flooded for longer than 24 h, because there was concern that this may unintentionally kill the plants. The dry Mediterranean south-west climate may particularly favour pathogens specialized in tolerating extended dry periods and rapidly exploiting wet periods. The thick-walled oospore resting structures of *P. multivora*, and its ability to sporulate within 24 h, may allow it to tolerate infrequent rainfall.

In conclusion, *P. multivora* causes significant fine root loss of *E. gomphocephala* under controlled conditions which may be an indication of its capacity to do the same in natural stands. However, more work is required to confirm pathogenicity with greater replication and under natural conditions. Healthy woodlands may be artificially infested with *P. multivora* to determine Koch's postulates, as demonstrated for *P. cinnamomi* (Podger 1972). *In situ* soil infestation is not an option as areas of woodland free from decline are of high conservation value and the time necessary for disease establishment and expression may be decades. Alternatively, the impact of *P. multivora* as a fine feeder root pathogen may be quantified by measuring the interaction of the pathogen, the root systems and disease progression of affected trees over time, and determining if the significant interactions *in situ* result in a net decrease in plant resources and subsequent decline. Trees infected with *Phytophthora* may also eventually die as a consequence of secondary pathogens and pests, and these relationships need to be determined.

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