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In planta selfing and oospore production of *Phytophthora cinnamomi* in the presence of *Acacia pulchella*

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ABSTRACT

This paper provides the first evidence of A2 type 1 and type 2 isolates of *Phytophthora cinnamomi* producing selfed oospores *in planta* in an Australian soil and in a potting mix. Oospores were observed in infected lupin (*Lupinus angustifolius*) roots incubated for 7 d either in the substrate under potted *Acacia pulchella* plants, or in soils collected from under and near varieties of *A. pulchella* in jarrah (*Eucalyptus marginata*) forest. The A2 type isolates varied in their ability to produce selfed oospores and none were produced by A1 isolates. The gametangial association was amphigynous and spores were predominantly spherical with diameters from 13–40 μm. The oospores were viable but dormant. Two A2 type isolates produced small numbers of selfed oospores with amphigynous antheridia axenically in Ribeiro's liquid medium within 30 d, and one A2 type 2 isolate produced oospores after mating with an A1 strain. Evidence is presented that the presence of roots of *Acacia pulchella*, and particularly *A. pulchella* var. *glaberrima* and var. *goadbyi*, enhances the production of oospores.

Introduction

In vivo oospores of *Phytophthora cinnamomi* were first reported in avocado (*Persea americana*) roots and mycelial mats by Mircetich & Zentmyer (1966) but there has been only one subsequent report. Reeves & Jackson (1972) observed *P. cinnamomi* oospores on nylon mesh mats adhered to small pieces of *Castanea sativa* root, which had been buried for 6–8 d in soil. In contrast, there are many reports on *in vitro* production of oospores by *P. cinnamomi* generally from pairing A1 and A2 mating types (Chang *et al.* 1974; Zentmyer 1983). They can also be produced by chemical stimuli (Brasier 1971) or mechanical damage (Reeves & Jackson 1974). An unidentified volatile chemical produced by *Trichoderma viride* was shown to be an effective stimulus for *P. cinnamomi* (Brasier 1971, 1975) and other *Phytophthora* species (Reeves & Jackson 1972). Oleic acid present in root extracts of avocado initiated oospores in the A2 mating types of *P. cinnamomi*, *P. drechsleri* and *P. capsici*, but was not effective on the A1 type of *P. cinnamomi* (Zentmyer 1979, 1983). Lecithin extracted from soybean stimulated oospore production in *P. cactorum*, *P. parasitica* (Ko & Ho 1983), *P. capsici* (Ko 1985) and in *P. boehmeriae* and *P. sojae* (Jee *et al.* 2002; Wu *et al.* 2003).

P. cinnamomi oospores, produced in avocado roots and mycelial mats in soil (Mircetich & Zentmyer 1966) could have been stimulated by either the oleic acid found in the avocado roots or through chemical stimulus from *T. viride* in the soil. Reeves & Jackson (1972) suggested that the presence of *T. viride* and root material were imperative for *P. cinnamomi*

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to produce oospores in soil. *T. viride* and other oospore-inducing *Trichoderma* species are present in several Australian soils (Johnson & Heather 1982) including jarrah forest soils (Malajczuk & McComb 1979), but there are no reports in the literature of *P. cinnamomi* oospores either in jarrah forest soils or in soils of other native vegetation types of Australia. The only information on *P. cinnamomi* producing oospores in an Australian soil was obtained from studies conducted under laboratory conditions (Malajczuk & Theodorou 1979).

P. cinnamomi populations in Australia consist of three isozyme genotypes with low genetic variation, one A1 type and two A2 types (Old *et al.* 1984; Old *et al.* 1988). These genotypes represent three clonal lineages of *P. cinnamomi*. In Australia, the most commonly isolated is the A2 type 1 (Old *et al.* 1988; Dobrowolski 1999; Dobrowolski *et al.* 2003), whereas the A1 and A2 type 2 isozyme genotypes of *P. cinnamomi* are less frequently isolated and there is little information on their behaviour. There is no indication of the sexual interactions for *P. cinnamomi* in jarrah forest (Old *et al.* 1984, 1988; Dobrowolski 1999; Dobrowolski *et al.* 2003) or in other parts of Australia. In most areas only the A2 mating type is present, but both A1 and A2 mating types were isolated from the same site at Ourimbah and Kioloa in New South Wales (Old *et al.* 1984; Dobrowolski 1999), and Dobrowolski *et al.* (2003) also found the two mating types within a 1 m square at Gull Rock, Western Australia. Nevertheless, no sexual recombinant genotypes were recovered suggesting that the two mating types are incompatible due to differences in their ploidy or karyotype (Dobrowolski 1999).

In vitro oospore production is triggered where the two mating types come into contact (Chang *et al.* 1974). According to Brasier (1972) the gametangial interactions between A1 and A2 types can be manifold and the resulting *P. cinnamomi* oospores *in vitro* can be products of A2 oogonia and A1 antheridia or *vice versa*. They can also be selfs of either mating type (Linde *et al.* 2001). Zentmyer (1979) described *P. cinnamomi* as being functionally heterothallic but potentially homothallic under special conditions. Apomixis is also possible (Chang *et al.* 1974) as well as selfing.

Temperature, light, oxygen tension and composition of the medium have all been shown to influence sexual reproduction in *Phytophthora*, *in vitro* (Elliot 1983). However, very little information is available currently on *in planta* oospore production of *P. cinnamomi*. The discovery of *in planta* oospores of *P. cinnamomi* in infected lupin roots in soil under *Acacia pulchella* plants (Jayasekera 2006) is investigated further here. *A. pulchella* is a native legume of Western Australia and is resistant to *P. cinnamomi* attacks (Tippett & Malajczuk 1979; Cahill *et al.* 1989). Two experiments were conducted. The effect on oospore production within infected root material buried in soil of potted plants of three varieties of *A. pulchella* was investigated using one of A2 type 1 and type 2 isolates of *P. cinnamomi*. Second, the effect on oospore production of forest soils collected from under varieties of *A. pulchella*, was tested on A1, A2 type 1 and A2 type 2 strains of the pathogen.

Materials and methods

Isolates

All *Phytophthora cinnamomi* isolates were from the Murdoch University culture collection (Table 1) and were passaged through lupin roots, cleaned by growing on NARPH selective medium (Hüberli *et al.* 2000) for 3–4 d and subcultured on clarified V8 juice agar (Miller 1955). The clean cultures were maintained at 25 °C in the dark with regular sub-culturing. Cultures of each isolate have been deposited in the DAWA (Department of Agriculture, Western Australian, Baron Hay Court, Bentley, Western Australia) culture collection.

Plants and soils

Experiment 1. Oospore production in soils of potted plants of Acacia pulchella

Plants of the three varieties of *Acacia pulchella*, var. *pulchella*, var. *goadbyi* and var. *glaberrima*, were grown in jarrah forest soil and in a potting mix. The jarrah forest soil was from Alcoa

Table 1 – Details of the isolates of *Phytophthora cinnamomi* used in the two experiments of this study

Experiment		Isolate	Isozyme type	DAWA accession no.	Host	Location	Collector and year isolated
1	2						
	[a]	A15	A1	12873	*Eucalyptus marginata*	Kelmscot W.A.	*K. Old* (CSIRO) no date
	[a]	DP 55	A1	12874	*Banksia baxterii*	Fitzgerald River National Park	*Calm*, W.A. no date
	[a]	MP80	A2 type 1	12875	*E. marginata*	Jarrahdale, W.A.	*G. Hardy* 1993
	[a]	MP62	A2 type 1	12876	*E. marginata*	Jarrahdale, W.A.	*G. Hardy* 1993
[a]		97-16	A2 type 1	12877	*E. marginata*	Jarrahdale, W.A.	*N. D'Souza*, 1997
[a]	[a]	MP125	A2 type 2	12878	*E. marginata*	Jarrahdale, W.A.	*G. Hardy* 1993
	[a]	A26	A2 type 2	12879	*Casuarina cunninghamiana*	Barton ACT	*K. Old* (CSIRO) no date

DAWA, Department of Agriculture Western Australia; CSIRO, Commonwealth Scientific & Industrial Research Organisation; CALM, Conservation And Land Management; ACT, Australian Capital Territory.
a Used in the experiment.

World Alumina's Huntley mine site. It was baited with rose petal discs (Jayasekera 2006), and also plated onto NARPH selective agar and confirmed to be free of *Phytophthora*. The validity of the method was checked by infection of a soil sample with *P. cinnamomi* before baiting and its subsequent recovery. The potting mix was composted pine bark, coarse river sand and coco peat at the ratio of 2: 2: 1 (initially pH 5). No fertilizer was added to either substrate. All the plants were 12 months-old and were in 150 mm free-draining polyvinyl chloride pots, one plant per pot.

For the chemical analyses, samples of 100 g soil were taken from the root zone from each replicate pot. All the samples for each *A. pulchella* variety were bulked and 500 g soil per variety were used for the analyses. Soil samples were oven dried at 40 °C for 24 h then analysed commercially by CSBP (Bibra Lake, Perth, Western Australia) for pH and conductivity (Rayment & Higginson 1992), organic carbon by the Walkley & Black method (Rayment & Higginson 1992), nitrate and ammonium nitrogen (Searle 1994), sulphur (Blair *et al.* 1991), phosphorus and potassium by the Colwell method and reactive iron by the Tamm method (Rayment & Higginson 1992). The analyses were conducted with a measurement accuracy of ±0.15, according to the ASPAC (Australasian Soil & Plant Analysis Council) standards.

Experiment 2. Oospore production in soils collected from natural stands of Acacia pulchella

Soils were collected from provenances of the three varieties of *Acacia pulchella* growing in jarrah forest of the southwest of Western Australia. The selected localities were: Boyanup state forest 33° 30′ S, 115° 40′ E (*A. pulchella* var. *pulchella*), Mt Barker 34° 40′ S, 117° 40′ E (*A. pulchella* var. *goadbyi*) and Mount Saddleback reserve in Boddington 32° 50′ S, 116°30′ E (*A. pulchella* var. *glaberrima*). Soil from Mt Barker was collected in spring, whereas the others were collected in summer. Soil was excavated to a depth of 15 cm under the *A. pulchella* stands and from approximately 5 m from the nearest *Acacia*, transported in polythene bags and stored at 4 °C. Samples of each soil were flooded and baited with petals (as in experiment one) and shown to be free of *Phytophthora cinnamomi*. Using a 4 mm sieve, rocks, gravel and plant material were removed from the soil. Chemical properties were analysed as above. Experimental containers were set up by placing 250 g of each soil into 1000 ml polypropylene containers (Bonson Industries, Auckland, New Zealand) and watering to container capacity.

Inoculum and inoculation

Lupin seeds were germinated under aseptic conditions. For experiment 1, mycelial discs (6 mm diameter) of *Phytophthora cinnamomi* bearing sporangia, produced using the axenic method of Byrt & Grant (1979), were placed in 3 cm diam Petri dishes containing 5 ml sterile distilled water. The dishes were chilled for 30 min at 4 °C for synchronized zoospore release and zoospore density was determined to be 10–20 zoospores μl^{-1}. Lupin seedlings with ~1 cm long radicals were placed on the rim of the Petri dishes so that the root tips were in contact with the zoospore suspension. After 2 h the lupin seedlings were removed and the roots were excised close to the seed. Infected root segments 1 cm long were plated on water agar (0.7 %) and incubated at 25 °C in the dark for 7 d for lesion development. For experiment 2, radicles from germinated lupins were excised near the cotyledons and plated on half strength potato dextrose agar (PDA). Agar squares (1 cm^2) cut from growing edges of *P. cinnamomi* colonies of each isolate were placed next to the lupin roots and the plates were incubated at 25 °C in the dark for 7 d until the colonies grew over the root pieces. Randomly selected roots were observed microscopically to confirm the presence of *P. cinnamomi* within the tissues.

Each infected lupin root (1 cm long) was enclosed in a sachet made from porous nylon and tied with a string for easy retrieval. Inoculum sachets were inserted in five places at 10 cm depth into the pots. In the first experiment, each *A. pulchella* variety in the potting mix or in soil was inoculated with lupin roots infected with isolate MP 125 or MP 97-16. Pots of soil or potting mix without plants were also inoculated with each isolate as controls. There were five replicates of each treatment. Pots were arranged in a randomized block design on the bench in the glasshouse at 25 °C and watered daily.

For the second experiment, two inoculum sachets of each isolate (Table 1) were buried in the containers with each soil type and replicated five times. Containers were placed in a growth cabinet in the dark with the temperature controlled at 22 ± 1 °C and watered daily to container capacity.

The inoculum was retrieved after 7 d. Root pieces were washed first in 0.25 % sodium hypochlorite solution for 1 min then rinsed repeatedly in three washes of distilled water. Each 1 cm root segment was mounted on a microscope slide in a drop of distilled water. Using two needles the root pieces were spread into a thin layer and examined under ×100 magnification. Numbers of oospores were counted at ×100 magnification in ten fields of view.

Viability of oospores

The tetrazolium bromide (Sutherland & Cohen 1983; Jiang & Erwin 1990) and the fluorescein diacetate (Widholm 1972) staining methods were used to assess the viability of the oospores of *Phytophthora cinnamomi* in lupin root tissue. For each staining method, two root pieces containing high numbers of oospores from each treatment were stained. A 100 µl drop of 0.1 % solution of tetrazolium bromide (MTT) (Sigma-Aldrich) was added to the mycelium and incubated at 35 °C in the dark for 48 h (Sutherland & Cohen 1983). A control of dead oospores was produced by autoclaving infected tissues as used by Pittis & Shattock (1994) for *P. infestans*.

To confirm viability, small root segments containing *P. cinnamomi* oospores and autoclaved root segments containing dead oospores were stained with 0.1 ml fluorescein diacetate solution (Widholm 1972) and examined using a compound microscope with fluorescence illumination (Olympus BX-1, Tokyo, Japan). The wavelength of the excitation filter was 460–490 nm. The emission filter transmitted light >520 nm.

Oospore germination and dormancy

In an attempt to germinate oospores, they were dislodged from the root tissues under a dissecting microscope using a scalpel and needles. A drop of distilled water was added to

the separated oospores of isolate MP 125 produced in experiment one, and using a micropipette ten oospores from each treatment were transferred onto water agar and incubated at 25 °C in the dark for 30 d. After assessment, the remaining slide specimens of root segments containing oospores were placed in 15 cm diam Petri dishes lined with moist filter paper, sealed with Para film and placed in the dark at 10–15 °C for 60 d. The slides were inspected weekly to examine any changes in the oospores.

Axenic oospore production

To produce oospores axenically, 6 mm diam Miracloth discs (Calibochem, La Jolla, CA, USA) were autoclaved three times then inoculated with a 1 cm square of *Phytophthora cinnamomi*, and cultured for 7 d on V8 agar. The mycelial mats on the 6 mm Miracloth discs were then incubated in 20 mL Ribeiro's liquid medium (Ribeiro 1978) at 25 °C in the dark for 30 d. The mycelia were washed three times in sterile distilled water and macerated using a hand-held macerator. The suspensions were strained through four layers of sterile gauze and examined by microscopy.

In vitro *mating of A1 and A2 type isolates*

Mating of the two types were carried out according to Chang *et al.* (1974) with modifications. Disks of 6 mm diam were cut from colonies of the A1 isolates (DP 55 and A 15) and A2 isolates (MP-80, MP-62, MP 125 and A 26) grown on half-strength PDA. One disc of each A2 type isolate was placed 3 cm away from a disc of the A1 type on V8 juice agar medium and incubated at 25 °C ± 1 °C in the dark. Plates were observed microscopically after 96 h, then every second day for four weeks. Further observations were made weekly for four more weeks.

Statistical analysis

The experiments were repeated twice. Data were analysed using SPSS 12.0.1 for windows (SPSS, Chicago, Illinois, USA). A univariate analysis of variance (ANOVA) between subjects was performed and statistical differences were expressed at the 95 % confidence level. Multiple comparisons between means were done using Tukey HSD.

Results

In planta *oospore production in soil and potting mix under potted* Acacia pulchella

Isolates MP125 and MP 97-16 of *Phytophthora cinnamomi* produced oospores with distinctive golden-brown, thick walls within lupin root pieces after 7 d in the soil and potting mix under *Acacia pulchella* plants (Fig 1A–E). Isolate MP 125 produced a greater number of oospores than isolate 97-16 (Fig 2). A univariate ANOVA showed a significant ($df = 7,64$ $f = 9.35$ $P = 0.00$) difference in oospore production between the two isolates. No oospores were produced by isolate MP 97-16 in the lupin root tissues under *A. pulchella* var. *goadbyi*. In contrast, oospore production by isolate MP 125 occurred under all three varieties and in the soil and potting mix without plants. The highest oospore producer of all the treatments

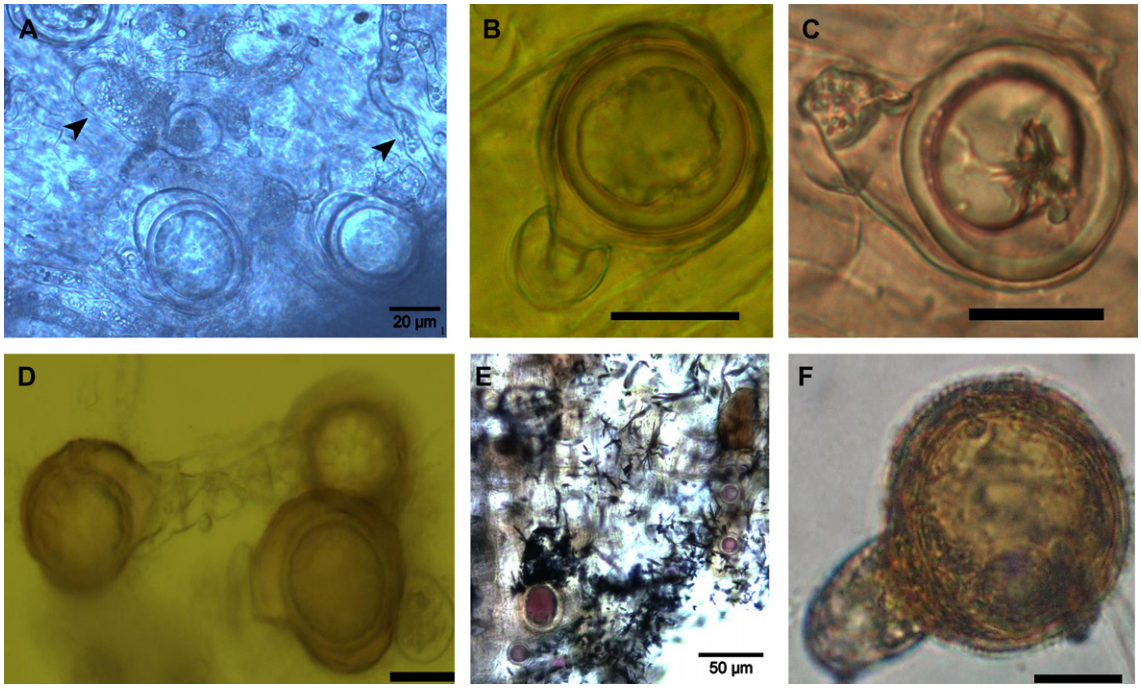

Fig 1 – Selfed oospores of *Phytophthora cinnamomi*. A–E. Oospores produced *in planta*. A. Many amphigynous oospores concentrated into an area with vacuolated hyphae (►). B. Spherical oospore. C. Tapering oogonium. D. Paired oospores. E. Dormant and viable oospore stained with tetrazolium bromide. F. Axenically produced oospore of isolate 97-16. Bars: A–D and F = 20 µm, E = 50 µm.

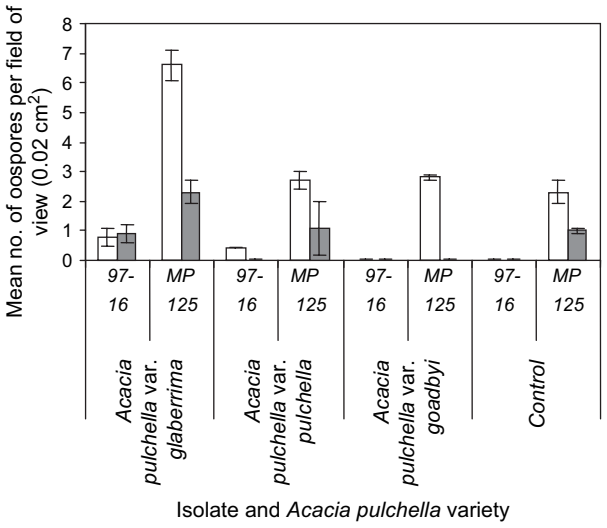

Fig 2 – Mean number of oospores per field of view for A2 isolates MP 97-16 (A2 type 1) and MP-125 (A2 type 2) of *Phytophthora cinnamomi* within lupin roots buried under three varieties of *Acacia pulchella* plants growing in potting mix (□) or jarrah forest soil (■) in the glasshouse. Controls are potting mix or jarrah forest soil without plants. Means are of five replicates and bars represent S.E. of means where large enough to be shown.

was MP-125 under *A. pulchella* var. *glaberrima* in potting mix. Tukey multiple comparison test showed significant ($P < 0.05$) differences of the means between MP-125 in *A. pulchella* var. *glaberrima*/potting mix treatment and the other combinations.

Oospores of *P. cinnamomi* isolate MP 97-16 were mainly spherical, 30–40 μm diam and all had smooth thick (~2 μm) walls and amphigynous antheridia (Fig 1A–B). In contrast, the oospores produced by isolate MP-125 varied in size from 13–39 μm and not all were at the same level of maturity. They were either spherical or elongated. Apart from the fully formed oospores with the amphigynous antheridial attachment (Fig 1AB–D), there were thick-walled spores lacking a visible antheridium. Some oogonia had a tapered base (Fig 1C). Among the fully formed ones some antheridia were comma-shaped. Some were with slightly to highly ornamented or loosely organized oogonial walls. There were oospores that were markedly aplerotic. Paired oogonia were present infrequently (Fig 1D).

All the oospores within root tissues were viable as they stained magenta pink after 48 h incubation in 0.1 % solution of tetrazolium bromide (MTT) (Sutherland & Cohen 1983; Bunny 1996) (Fig 1E) whereas the autoclaved oospores stained black. The live oospores in the root tissues also stained with fluorescein diacetate, fluoresced with the characteristic yellow–green, whereas autoclaved oospores did not. Isolated oospores did not germinate, with only one oospore of MP-125 remaining uncontaminated for 30 d, by which time it had deteriorated. The *in planta* oospores stayed intact with ooplasts largely unchanged during the 60 d incubation period. The antheridia were observed to be diminishing and a few oospores had contracted or plerotic ooplasts.

In planta oospore production in soils collected from under field-grown Acacia pulchella

When lupin roots infected with A1, A2 type 1 and A2 type 2 strains of *Phytophthora cinnamomi* were incubated in soils collected from field grown *Acacia pulchella* varieties, only the A2 type 2 isolates, MP 125 and A 26 produced oospores within root tissues (Fig 3). The oospores produced by both isolates had amphigynous antheridial attachments. The two A2 type 2 isolates, operated similarly in the soils collected from under all three varieties of *A. pulchella*. A univariate ANOVA between subjects failed to find a significant ($df = 1,48$ $f = 3.09$ $P = 0.08$) difference between the two A2 type 2 isolates in oospore production. However, a significant ($df = 5,48$ $f = 6.25$ $p = 0.00$) difference was shown between the soils at 95 % confidence level. The highest numbers were recorded from soils from *A. pulchella* var. *goadbyi*, and some oospores were also observed in soil collected 5 m from the nearest *A. pulchella* var. *goadbyi* (Fig 3). The Tukey multiple comparison test based on the means showed a significant ($P = 0.05$) difference in the *in planta* oospore numbers between the soils under *A. pulchella* varieties *goadbyi* and *pulchella* for both isolates.

Soil chemical properties

The soil chemical properties under the three *Acacia pulchella* varieties grown in the glasshouse showed, on the whole, higher levels of inorganic nutrients than in the pots without plants (Table 2). Nutrient levels were generally highest in soil and potting mix under *A. pulchella* var. *glaberrima* in which there were higher levels of oospores than in soils under the other two varieties. Nutrient levels were more

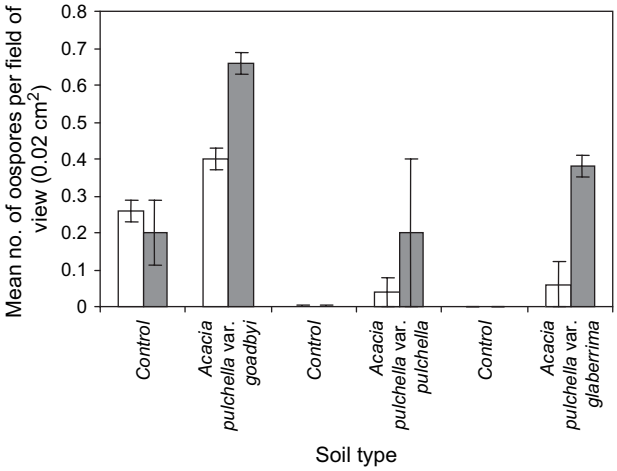

Fig 3 – Mean number of oospores per field of view for A2 type 2 isolates of *Phytophthora cinnamomi* MP 125 (□) and A 26 (■) produced within lupin roots after 7 d in the jarrah forest soils from under the three varieties of *Acacia pulchella*: var. *goadbyi*, var. *pulchella* and var. *glaberrima*. Controls were the soil 5 m away from the *A. pulchella* plants. Means are of five replicates and bars represent the S.E. of the means. Isolates of A1 and A2 type 1 strains produced no oospores so data are not shown.

Table 2 – Chemical properties of the soil and potting mix under the three *Acacia pulchella* varieties grown in the glasshouse

Soil and species	pH	Conductivity ($dS\,m^{-1}$)	Organic carbon (%)	Nitrogen as nitrate ($mg\,kg^{-1}$)	Nitrogen as ammonium ($mg\,kg^{-1}$)	Sulphur ($mg\,kg^{-1}$)	Phosphorus ($mg\,kg^{-1}$)	Potassium ($mg\,kg^{-1}$)	Iron ($mg\,kg^{-1}$)
Jarrah forest soil									
Control (no plants)	7.2	0.062	2.08	1	1	7.9	1	25	857
Acacia pulchella var. *pulchella*	7.7	0.088	2.48	1	3	11.7	1	28	1069
A. pulchella var. *goadbyi*	7.7	0.127	2.90	6	2	15.6	2	29	1039
A. pulchella var. *glaberrima*	7.6	0.091	2.71	30	6	16.0	12	63	1166
Potting mix									
Control (no plants)	6.9	0.291	4.10	1	2	9.2	5	8.5	516
A. pulchella var. *pulchella*	5.4	0.113	2.71	1	8	19.0	2	111	1209
A. pulchella var. *goadbyi*	5.5	0.844	6.60	7	6	39.5	5	95	979
A. pulchella var. *glaberrima*	5.5	1.395	6.81	5	6	53.1	14	105	919

variable in soils collected from the forest, even from the same site (Table 3). It was noted that in all forest soils with sulphur content above $5.4\,mg\,kg^{-1}$ oospores were observed in the A2 type 2 isolates, A 26 and MP125 (Fig 3).

Axenic oospore production

Isolates MP 97-16 and MP 125 of *Phytophthora cinnamomi* incubated in Ribeiro's medium produced amphigynous oospores infrequently (Fig 1F), therefore a statistical analysis was not conducted. However, these observations confirmed the ability of the two isolates to self in axenic culture.

In vitro *mating of A1 and A2 type isolates*

Only one pairing of A1 and A2 strains produced oospores after four weeks. The A15–A26 pairing had fully formed oospores with amphigynous antheridial attachments. The oospores were concentrated into an area (2 cm diam) towards the edge of the Petri dish, at the junction of two colonies on the A26 side. There was a clear zone in the middle of the two colonies, and it was not certain whether the two opposite types intermingled and fused. None of the other pairings produced oospores after incubation for four weeks or longer.

Discussion

The ability of *Phytophthora cinnamomi* to produce selfed oospores *in planta* in a number of Western Australian soils and in a potting mix under controlled conditions has been demonstrated for the first time. Two A 2 Type isolates of *P. cinnamomi* were shown to be able to produce small numbers of selfed oospores *in vitro*, and together with an additional two A2 Type 2 isolates, also produced higher numbers of oospores *in planta*. Previous work has shown that several extrinsic factors can induce selfing of the A2 mating type of *P. cinnamomi*. Stress response, a *Trichoderma* effect (Brasier 1975) and plant effect were regarded as likely to be the contributing factors towards initiating this phenomenon in the present study, and may act singly or in synergy. Mycelium within the lupin root pieces, buried for 7 d under the potted *Acacia* plants or in soil, was largely deteriorated and the root pieces highly macerated. The diminishing food base and/or the dying mycelium may

Table 3 – Chemical properties of the soil from field collections of *Acacia pulchella* and 5 m from the closest *A. pulchella* plant (controls)

Species	pH	Conductivity ($dS\,m^{-1}$)	Organic carbon (%)	Nitrogen as nitrate ($mg\,kg^{-1}$)	Nitrogen as ammonium ($mg\,kg^{-1}$)	Sulphur ($mg\,kg^{-1}$)	Phosphorus ($mg\,kg^{-1}$)	Potassium ($mg\,kg^{-1}$)	Iron ($mg\,kg^{-1}$)
Acacia pulchella var. *goadbyi*	5.2	0.088	3.99	1	5	8.7	50	67	367
Control	6.1	0.070	5.56	1	4	8.0	4	210	3177
A. pulchella var. *glaberrima*	6.1	0.055	4.96	1	14	6.3	12	174	1346
Control	6.0	0.070	5.36	1	2	5.2	5	153	953
A. pulchella var. *pulchella*	5.6	0.078	2.95	1	5	7.4	5	58	330
Control	6.2	0.040	3.98	1	6	5.4	8	75	1268

have prompted the oospore formation as a stress response. Although the presence of *Trichoderma* spp. was not tested in this study, the *Trichoderma* effect can not be ruled out in the experiments in soil, potting mix and forest soils as *Trichoderma* species are common in the jarrah forest soil (Malajczuk & McComb 1979). However, although stress and *Trichoderma* may be involved in stimulating oospore production, it is likely that some factor associated with *A. pulchella* is also important.

Soil nutrient levels under the *A. pulchella* var. *glaberrima* plants in pots, where abundant oospores were formed, were markedly higher than the other two varieties and soil. Presence of an organic nitrogen source was a requirement for sexual reproduction of some *Phytophthora* species (Ribeiro 1978). Chang *et al.* (1974) demonstrated that a high concentration of V8 juice (50 $g\,l^{-1}$) resulted in more abundant oospores of *P. cinnamomi in vitro* than a low one (20 $g\,l^{-1}$). The highest level of sulphur was recorded under *A. pulchella* var. *glaberrima* plants in potting mix, where highest numbers of oospores were recorded. Although sulphur levels in soils collected from the field were lower than in glasshouse pots, the highest levels of sulphur were associated with the most abundant oospores. Two volatile sulphur compounds have been identified from the steam distillate of *A. pulchella* roots, and the strong sulphurous aroma of the *A. pulchella* roots were believed to be associated with them (Whitfield *et al.* 1981). It is possible that the high levels of sulphur in the soil are a result of *A. pulchella* root exudates that contain water-soluble sulphur compounds. A preliminary experiment that involved adding sulphur to a sand substrate showed that for isolate MP-125, selfing and oospore formation *in planta* was initiated by the addition of elemental sulphur to the substrate (Jayasekera 2006).

Root extract of avocado stimulated oospore production but reduced sporangial production in *P. cinnamomi* (Zentmyer 1979). Similarly leachates from soils of the potted *A. pulchella* that stimulated oospore production have been shown to reduce sporangial production, and cause collapse of chlamydospores of *P. cinnamomi* (Jayasekera 2006). Hence, Zentmyer's (1979) suggestion of vascular plants providing the stimulus for the pathogen to produce oospores as a host defence mechanism is supported in this study with *A. pulchella*.

The diameters of the *in planta* oospores produced were consistent for *P. cinnamomi* (Stamps *et al.* 1990). The measurements and descriptions provided in the key of Stamps *et al.* (1990) were from oospores produced by mating two compatible types on agar. This is the first information on dimensions for the selfed oospores for *P. cinnamomi in vitro* and for *in planta*-formed oospores of *P. cinnamomi*. The variability in oospore sizes and the gametangial development observed for isolate MP-125 within root tissue is suggestive of selfing and oospore formation occurring at various times over the 7 d of soil incubation. For isolate MP 97-16, the uniformity of the oospore sizes suggests that selfing might have occurred at the beginning of soil incubation, and that the resulting oospores had reached their maximum diameter by the seventh day.

The most common oogonial–antheridial association observed *in planta* was amphigynous. Some authors have associated paragyny with selfing and amphigyny with heterothallism (Savage *et al.* 1968), but our results showed that *P. cinnamomi* is an exception to this rule. A mix of gametangial associations has been reported from *in vitro* studies of compatible mating types (Hüberli *et al.* 1997) and such studies cannot exclude the possibility of selfing alongside heterothallism.

Staining with tetrazolium bromide or fluorescein diacetate indicated that oospores were viable but dormant because germination was not observed. Oospores of oomycetes are, in general, endogenously dormant and capable of long-term survival (Ribeiro 1983). According to Mircetich & Zentmyer (1966) oospores of *P. cinnamomi* can survive many years. To our knowledge there is no information on survival and infectivity of *P. cinnamomi* oospores produced *in planta*. Difficulties encountered in germinating the oospores in the present study hampered further investigations such as determining whether the oospores produced by 'mating' were in fact selfs.

This is the first evidence of *P. cinnamomi* oospores formed *in planta* in an Australian soil and also in the presence of a resistant plant species. Although the experiments were conducted under controlled conditions, it is likely that appropriate conditions are encountered in natural forest situations. Thus, despite the apparent absence of sexual reproduction of *P. cinnamomi* in Australian forest soils (Old *et al.* 1984, 1988; Dobrowolski *et al.* 2003), it is likely that oospores can be formed in Australian soils in the presence of plant species such as *A. pulchella*. Other *P. cinnamomi*-resistant plant species might also provide conditions conducive for oospore production. The potting mix contained pine bark, which is widely used in nursery mixes, stimulated the oospores. The other commonly used hardwood bark products in nursery industry of Western Australia are marri (*Corymbia calophylla*) and karri (*Eucalyptus diversicolor*) and all have been shown to suppress *P. cinnamomi* (Sivasithamparam 1981; Sivasithamparam *et al.* 1981; Hardy & Sivasithamparam 1991). It is a possibility that pine bark or other plant material present in nursery mixes also can provide the stimulus for selfing and oospore formation of *P. cinnamomi* in Australia and elsewhere. Although the infectivity of the oospores produced *in planta* was not ascertained, it is possible that they can function as survival structures.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mycres.2006.11.003.

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