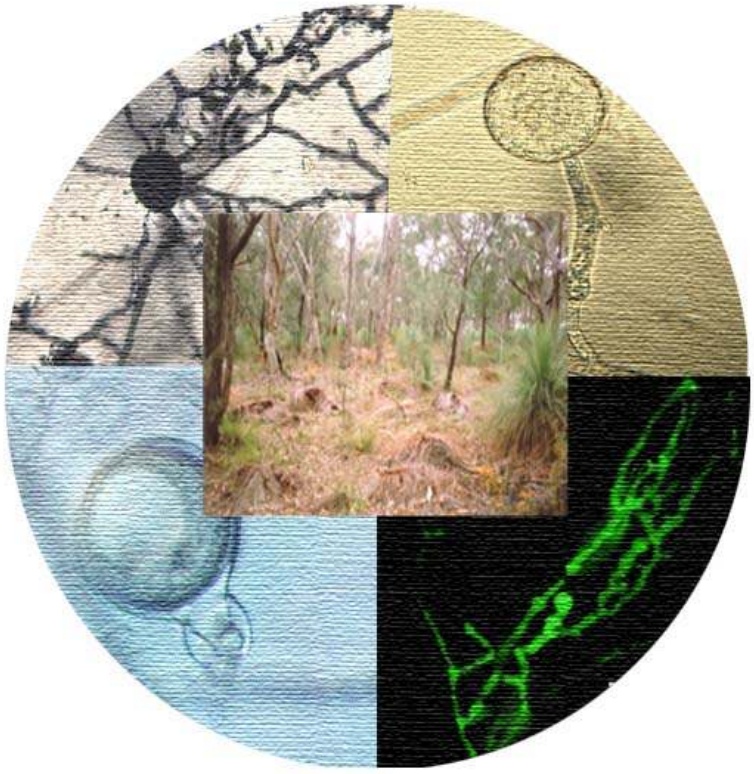
Saprophytic ability and the contribution of chlamydospores and oospores to the survival of *Phytophthora cinnamomi*



by

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DECLARATION

The work described in this thesis was undertaken while I was an enrolled student for the degree of Doctor of Philosophy at Murdoch University, Western Australia. I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution. To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged.

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ABSTRACT

Phytophthora cinnamomi has been recognised as a key threatening process to Australia's biodiversity by the Commonwealth's Environment Protection and Biodiversity Conservation Act 1999. Despite over 80 years of extensive research, its exact mode of survival is still poorly understood. It is widely accepted that thin- and thick-walled chlamydospores are the main survival propagules while oospores are assumed to play no role in the survival of the pathogen in the Australian environment, yet evidence is limited. The saprophytic ability of the pathogen is still unresolved despite the important role this could play in the ability of the pathogen to survive in the absence of susceptible hosts. This thesis aimed to investigate chlamydospores, oospores and the saprophytic ability of *P. cinnamomi* to determine their contribution to survival.

Phytophthora cinnamomi did not show saprophytic ability in non-sterile soils. The production of thick-walled chlamydospores and selfed oospores of *P. cinnamomi in vitro* was documented. Thick-walled chlamydospores were sporadically formed under sterile and non-sterile conditions *in vitro* but exact conditions for stimulating their formation could not be determined. The formation of thick-walled chlamydospores emerging from mycelium of similar wall thickness was observed, challenging the current knowledge of chlamydospore formation.

Selfed oospores were abundant *in vitro* on modified Ribeiro's minimal medium in one isolate. Three other isolates tested also produced oospores but not in large numbers. Although the selfed oospores did not germinate on a range of media, at least 16 % were

found to be viable using Thiozolyl Blue Tetrazolium Bromide staining and staining of the nuclei with 4′, 6-diamidino-2-phenylindole.2HCl (DAPI). This indicated the potential of selfed oospores as survival structures and their ability to exist dormantly.

The ability of phosphite to kill chlamydospores and selfed oospores was studied *in vitro*. Results challenged the efficacy of this chemical and revealed the necessity for further study of its effect on survival propagules of *P. cinnamomi* in the natural environment. Phosphite was shown to induce dormancy in thin-walled chlamydospores if present during their formation *in vitro.* Interestingly, dormancy was only induced by phosphite in isolates previously reported as sensitive to phosphite and not those reported as tolerant.

Chlamydospores were produced uniformly across the radius of the colony on control modified Ribeiro's minimal medium but on medium containing phosphite (40 or 100 µg ml^{-1}), chlamydospore production was initially inhibited before being stimulated during the log phase of growth. This corresponded to a point in the colony morphology where mycelial density changed from tightly packed mycelium to sparse on medium containing phosphite. This change in morphology did not occur when the pathogen was grown on liquid media refreshed every four days, and chlamydospores were evenly distributed across the radius of these colonies. This trend was not observed in selfed oospores produced in the presence of phosphite. Selfed oospore production was found to be inhibited by phosphite at the same concentrations that stimulated chlamydospore production.

Isolates of *P. cinnamomi* were transformed using a protoplast/ polyethylene glycol method to contain the Green Fluorescent Protein and geneticin resistance genes to aid in future studies on survival properties of the organism. Although time constraints meant the

stability of the transgene could not be determined, it was effective in differentiating propagules of the transformed *P. cinnamomi* from spores of other microrganisms in a non-sterile environment. Two different sized chlamydospores (approximately 30 μm diameter and < 20 μm diameter) were observed in preliminary trials of transformed *P. cinnamomi* inoculated lupin roots floated in non-sterile soil extracts and these were easily distinguished from microbial propagules of other species. The growth and pathogenicity was reduced in two putative transformants and their ability to fluoresce declined over ten subcultures but they still remained resistant to geneticin.

This study has improved our knowledge on the survival abilities of *P. cinnamomi in vitro* and has provided a useful tool for studying these abilities under more natural glasshouse conditions. Important implications of phosphite as a control have been raised.

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CHAPTER 1

General Introduction

Oomycetes are part of a branch of eukaryotic plant pathogens and saprophytic species (Kamoun 2001). They are grouped amongst the Stramenopiles that also include heterokont algae (Hardham 2005). The Oomycetes include the genus *Phytophthora* which holds many notorious plant pathogens, including the causes of potato late blight, cocoa black pod, dieback diseases of dry sclerophyll forests in southern Australia and sudden death of oaks in northern America (Garbelotto, 2001; Kamoun 2001). Natural ecosystems, pastoral, horticultural, ornamental and forestry industries may all be threatened by *Phytophthora* species (Table 1.1) which may cause major economical losses. The most important and widespread *Phytophthora* species in Australia is *Phytophthora cinnamomi* Rands (1922) (Weste and Marks 1987; Erwin and Ribeiro 1996; Colquhoun and Hardy 2000).

This review focuses on the lifecycle of *P. cinnamomi*, identifying the gaps in our knowledge on exactly what contribution the propagules sporangia, zoospores, chlamydospores and oospores have in the survival of this pathogen. The current control technique, phosphite and new molecular technologies that aid in the study of the biology of this pathogen are introduced.

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Table 1.1: Some *Phytophthora* species found in Australia and their hosts (Summarised from Irwin *et al.* 1995).

Phytophthora spp.	Plant species or group affected	State where recorded
P. cactorum	Horticultural and *Eucalyptus* spp.	Victoria (Vic), South Australia (SA), Western Australia (WA), Tasmania (Tas)
P. cambivora	Horticultural	Vic, SA, New South Wales (NSW)
P. cinnamomi	Natural ecosystems, *Eucalyptus* spp., Horticultural and Ornamental	All states
P. citricola	Natural ecosystems, Forestry, Horticultural and Ornamental	SA, WA, NSW, Queensland (Qld), Tas
P. citrophthora	*Citrus* spp.	Vic, WA, Qld, NSW
P. clandestina	Subterranean clover	Vic, NSW, WA
P. cryptogea	Natural ecosystems, forestry, Horticultural and Ornamental	All states
P. drechsleri	Natural ecosystems and Forestry	WA, Qld
P. erythroseptica	Potato	Vic, Tas
P. fragariae	Strawberries	SA
P. infestans	Potato	Vic, Tas
P. macrochlamydospora	Soybean	Qld, NSW
P. medicaginis	Chickpea and lucerne	Qld, NSW
P. megasperma	Natural ecosystems, Forestry, almond, white clover and Brassicas	Vic, SA, WA
P. nicotianae	Natural ecosystems, Forestry, Horticultural and Ornamental	WA, Qld
P. nicotianae var. *nicotianae*	Horticultural and Ornamental	WA, Northern Territory, Qld
P. nicotianae var. *parasitica*	Horticultural	WA, Qld
P. palmivora	Horticultural and Ornamental	Qld
P. parasitica	Horticultural and Ornamental	Vic, SA, Qld, NSW, WA
P. porri	Onion	NSW
P. sojae	Soybean	Vic, Qld, NSW
P. vignae	Cowpea	Qld, NSW

Phytophthora cinnamomi is a soil-borne pathogen and was first described by Rands in 1922 when the pathogen was isolated from cinnamon trees (*Cinnamomum burmannii*) suffering from stripe canker in Sumatra (Rands 1922; Zentmyer 1983; Erwin and Ribeiro 1996).

The A2 mating type of the pathogen has a worldwide distribution (Zentmyer 1980) whereas the A1 mating type has an extremely limited distribution (Zentmyer 1983). In 1949, Fraser first associated the pathogen with disease of Australian native plants when he isolated it from dead species (*Woollsia pungens, Epacris purpurascens, Xanthosia tridentata, Actinotus helianthi, Trachymene linearis, Dodenaea triquetra, Petrophila pulchella* and *Pultenaea* spp.) in eucalypt woodland in Sydney (Fraser 1956). The pathogen was not isolated from vegetation in Western Australia until 1964 by Zentmyer (Podger *et al.* 1965). In Australian forests, the impact of *P. cinnamomi* is mainly confined to the understorey with the exception of the tree species *Eucalyptus marginata* (jarrah) and *E. sieberi* (Davison and Shearer 1989). Affected plants include many species within the families Proteaceae, Epacridaceae, Dilleniaceae, Myrtaceae and Papilionaceae that are so species rich in Australia (Titze and Palzer 1969; Zentmyer 1980; Shearer and Tippett 1989; Shearer 1994). The greatest impact of the pathogen occurs in south-western Australia, mainly in the jarrah forest, but also in the Bassendean Dune system and Pinjarra Plain, west of the Darling Scarp, due to suitable environmental conditions (cool, wet winters and hot, dry summers) and human activity (Shearer and Tippett 1989; Shearer 1992; Shearer 1994).

Typical symptoms of plants infected by *P. cinnamomi* include root rot, lesions on roots and lower stems, leaf chlorosis and rapid dieback (Shearer and Smith 2000). Some infected plants suddenly collapse but others, particularly in cool, damp climates, may survive for several years (Erwin and Ribeiro 1996). *Phytophthora cinnamomi* can be recovered from

large roots and the collar regions of susceptible plants (Shea 1979; Blowes *et al.* 1982; Schild 1995).

Quarantine and hygienic practices are effective in controlling the dispersal of *P. cinnamomi* but once the organism invades an area, its impact on native vegetation can be disastrous. Application of fungicides and other control methods, effective on small areas or horticultural situations, have only limited capabilities in controlling the disease on a large scale in a natural ecosystem. For this reason, study of the pathogen's life cycle and physiology is important to discover ways to manipulate the environment to discourage its growth and survival (Shearer and Tippett 1989; Schild 1995). Currently, phosphite is the main control/ prevention method in use in Australia (Hardy *et al.* 2001) but the effects of this chemical on the environment and how it actually works on the pathogen are still largely unknown (Section 1.4).

1.1 Biology of *Phytophthora cinnamomi*

Infection by *P. cinnamomi* requires a temperature range between 15 and 34 °C while temperatures outside this range may limit infection (Hepting 1963). It tends to grow and sporulate better at soil temperatures higher (between 2 ºC and 21 ºC) than other *Phytophthora* species (Zentmyer 1983; Shearer and Tippett 1989). Optimum mycelial growth has been shown to occur *in vitro* within the range of 25 – 30 °C (Shearer *et al.* 1987) whereas optimal sporangial formation occurs between 22 and 28 °C (Chee and Newhook 1965a, b).

Soil moisture levels below field capacity can reduce infection, while soils that remain at wilting point for lengthy periods tend to reduce the survival chances of the pathogen outside host roots (Shea 1977). However, the ability to recover *P. cinnamomi* from sites varies with season suggesting it may be periodically dormant. Shearer and Shea (1987) found that the pathogen could not be recovered from surface soil during the dry summer months in jarrah forests and that recovery was greatest during the winter months when rainfall was highest and temperature lowest.

Growth of *P. cinnamomi* has been found to be suppressed by soils with high fertility including high organic matter levels, pH, nitrogen, cellulose and lignin content (Zentmyer and Bingham 1956; Zentmyer 1963; Gilpatrick 1969; Broadbent and Baker 1974; Nesbitt *et al*. 1979; Tsao and Zentmyer 1979; Tsao and Oster 1981; Malajczuk *et al.* 1983; Hoitink and Fahy 1986; You and Sivasithamparam 1994). High potassium levels have also been shown to suppress *P. cinnamomi* (Schmitthenner and Canaday 1983). *Phytophthora* species have an absolute growth requirement for thiamine (Vitamin B1) (Erwin and Ribeiro 1996).

Sterols are required for production of sporangia and chlamydospores (Hendrix 1965; Ponchet *et al.* 1999). However, Hendrix (1967) did observe that sporangia of *P. palmivora, P. capsici* and *P. parasitica* formed on media containing no sterols but these did not liberate zoospores. Sporulation in *P. cactorum, P. capsici, P. cinnamomi, P. citrophthora, P. palmivora* and *P. parasitica*, decreases with decreasing oxygen and increasing carbon dioxide (Mitchell and Zentmyer 1971).

For soils with low organic matter such as the lateritic soils of Western Australia, the pathogen may survive for years in dead host tissue as long as sufficient moisture is available (Weste and Ruppin 1977; Weste 1983a). These soils with lower organic matter have fewer soil microorganisms, low soil nitrogen and poor water-holding capacity (Weste and Vithanage 1978a) and therefore fewer antagonists.

1.2 Lifecycle of *Phytophthora cinnamomi*

Phytophthora can be moved into new areas in soil, water or infected root material. Chlamydospores have been observed to be the most persistent propagules, followed by sporangia and then zoospores in moist, artificially infested non-sterile soil (Hwang and Ko 1978).

1.2.1 Sporangia and Zoospores

Sporangiophores form under suitable conditions (warm, moist, aerated soils) and produce sporangia as temperatures rise above 12 °C (Zentmyer 1980). Sporangia production is influenced mostly by the water potential of the soil, many being produced when humidity approaches 100 % or water potential approaches 0 kPa (Ribeiro 1983). Many sporangia have been observed to form at -10 kPa but few at -300 kPa (Sneh and McIntosh 1974; Duniway 1975). Moisture, soil type, pH, percentage organic matter, oxygen, light and temperature have been identified as the most important factors affecting the production of sporangia (Waterhouse 1931; Reeves 1975; Malajczuk and Theodorou 1979). On liquid media, sporangia are usually more abundant on the surface indicating the necessity of oxygen for their production (Zentmyer and Erwin 1970).

Sporangia either germinate directly (Zentmyer and Erwin 1970) or, if a small drop in temperature occurs, they release zoospores that move by their flagella through water and apart from subsurface water flow, are the only way the pathogen can move through soil (Zentmyer 1980). Zoospore production is capable of intensifying the disease potential of the pathogen in a relatively short period of time (Zentmyer and Erwin 1970). The zoospores are attracted to soft tissues (chemotaxis) in the zone of root elongation where they attach and lose their flagella, forming cysts (Hardham 1987; Shearer and Tippett 1989; O'Gara *et al.* 1996). The cysts produce germ tubes that penetrate the root cells. Once inside the host cells, the germ tube branches forming mycelium that, unhindered, grows throughout the plant tissues, killing the plant through damage to the vascular tissues (Hardham 1987; Shearer and Tippett 1989).

Zoospores, the primary inoculum, have a short life that lasts not much longer than a few hours, requiring free water (Weste 1979). However, zoospore cysts of *P. cinnamomi* have been observed to survive up to 3 weeks under moist conditions at water potentials ≤-100 kPa and up to 10 weeks at -700 kPa under laboratory and natural environmental conditions (Hwang and Ko 1978; MacDonald and Duniway 1979). In soil extracts, 7 % of zoospore cysts survived 43 days in both suppressive and conducive soils (Malajczuk *et al.* 1983).

1.2.2 Chlamydospores

Chlamydospores of *P. cinnamomi*, were first described in 1922 (Rands 1922) and are frequently referred to as asexual reproductive survival structures (Zentmyer 1980). Since the A1 mating type of *P. cinnamomi* is uncommon in Australia, Weste and Vithanage (1979) concluded that chlamydospores are an important form of survival and the major inoculum source. The evidence for this widely accepted assumption is examined and

whether or not both thick and thin-walled chlamydospores are produced in nature or only in laboratory culture.

Chlamydospores of Phytophthora

Chlamydospores of *Phytophthora* are globose structures, 7 to 90 µm in diameter, separated from the mycelium by a basal septum (Blackwell 1949; Waterhouse *et al*. 1983; Erwin and Ribeiro 1996). Not all *Phytophthora* species produce chlamydospores and in some species, only certain isolates form them (Waterhouse *et al*. 1983; Stamps *et al.* 1990; Erwin and Ribeiro 1996). It is thought that asexual and sexual reproduction of *Phytophthora* is triggered by mycelium starvation (Bartnicki-Garcia and Wang 1983). Chlamydospores are considered more resistant structures than zoospores and sporangia as they have thicker walls and in older chlamydospores, vacuoles with lipids and dense inclusions similar to other resistant spores such as oospores (Hemmes and Wong 1975; Hemmes 1983).

Morphology and wall formation of chlamydospores

In vitro, chlamydospores of *P. cinnamomi* form in terminal or intercalary clusters of three to ten (Hemmes and Wong 1975; Gerrettson-Cornell 1983; Erwin and Ribeiro 1996). They form by expansion of the hyphal wall and cytoplasm flows into the structure (Hemmes and Wong 1975). Small chains of two chlamydospores may also form (Gerrettson-Cornell 1983). *In vitro,* initially the chlamydospore wall is thin (approximately 0.2 µm, similar to the hyphae), becoming thicker (0.5 µm – 0.6 µm) in 2-week-old spores after formation of the basal septum (Hemmes and Wong 1975). No equivalent measurements have been made

on chlamydospores in plant tissues, or when they have formed under the influence of antagonistic microbes.

Walls of chlamydospores of other *Phytophthora* species may be up to 5 µm thick (Erwin and Ribeiro 1996). The walls are comprised of β-linked glucose polymers (80 – 90 %), protein, lipids and other polysaccharides (observations based on *in vitro* cultures) (Bartnicki-Garcia and Wang 1983). They are similar to walls of oogonia and differ from sporangial walls (Hegnauer and Hohl 1978). It is curious that the outermost layers of chlamydospore walls are much thicker than the layers of the hyphal wall from which they develop (Hegnauer and Hohl 1978) although Hunsley (1973) observed that in hyphal tip growth, the outer layer also thickens with time. However, there is very limited information in the literature on exactly how chlamydospore walls thicken.

Production of thin-walled chlamydospores in soil

Evidence that chlamydospores are an important propagule for survival in Australia is limited, particularly for survival of chlamydospores not in plant material which would buffer them from some of the more extreme soil conditions. The evidence that chlamydospores may be produced in soil is sparse; the problem being that, when experimenting with non-sterile conditions, the observation of 'spores' is insufficient and the structures must be germinated to identify the species. For example, in experiments on mycelial lysis, chlamydospores were observed on colonised nylon discs in different soils within 5 to 15 days and persisted under different moisture regimes for up to 18 months (Kuhlman 1964; Mircetich and Zentmyer 1967; Reeves 1975). However, the viability and identity of these spores was not confirmed by germination.

The best evidence of thin-walled chlamydospore formation and short-term survival in non-sterile soil conditions comes from Weste (1983b). She buried mesh colonised by *P. cinnamomi* in a range of soil types and observed that under a constant temperature (22 °C) and moisture contents between -60 kPa to -1960 kPa, chlamydospore numbers increased for up to 28 days in the absence of a host or added food source. The colonisation of the mesh by *P. cinnamomi* also increased from 50 % after 10 days to almost 100 % after 28 days. The identity of the spores as *P. cinnamomi* was confirmed by use of a fluorescent brightener in the buried mycelium and germination of chlamydospores to indicate their viability. The chlamydospores germinated after 28 days. It would be interesting to know if there were any non-germinated chlamydospores that were viable and dormant, whether all chlamydospores were thin-walled and if they could survive for longer than the experimental period.

Production of thin-walled chlamydospores in plant roots

It has been stated that under both sterile and non-sterile conditions, chlamydospores form inter- or intra-cellularly in the root cortex (Mircetich and Zentmyer 1966, 1967; Reeves 1975; Weste 1983a), however evidence for non-sterile conditions is limited. The importance of this lies in the role root tissue could play in buffering the chlamydospores from unfavourable environmental conditions (Mircetich and Zentmyer 1967; Gilpatrick 1969; Weste 1983b; Schild 1995).

Chlamydospores have been reported in roots of susceptible plants such as *Eucalyptus sieberi* (Mackay *et al.* 1985; Cahill *et al.* 1989), *E. marginata*, *Acacia melanoxylon*,

Xanthorrhoea australis and *X. resinosa* (Cahill *et al.* 1989) and in field resistant species including *Corymbia calophylla* (syn. *E, calophylla*), *Corymbia maculata* (syn. *E. maculata*) (Malajczuk *et al.* 1977; Mackay *et al.* 1985; Cahill *et al*. 1989), *Juncus bufonius, Gahnia radula* and *Themeda australis* (Cahill *et al.* 1989). In contrast, *P. cinnamomi* did not produce chlamydospores in the roots of the resistant species *Acacia pulchella, Zea mays* and *Triticum aestivum* (Cahill *et al.* 1989). However, in all these cases (apart from Mackay *et al.* 1985), the observations were made under sterile conditions and it is important to know whether chlamydospore formation, wall thickness and dormancy would be the same under non-sterile field conditions. In contrast, chlamydospores were rare in forest inoculated stems and roots of *E. marginata*, over a 12 month period (Tippett *et al.* 1983). 'Relatively' thin-walled chlamydospores formed in *Pseudotsuga menziesii* roots buried in inoculated non-sterile soil in glasshouse experiments (Kuhlman 1964) but identification of chlamydospores was by microscopic examination only and not by the germination of spores. Further investigations are required to determine whether chlamydospores form in roots of a wide range of Australian species under non-sterile native community environments.

Production of thin-walled chlamydospores on root surfaces and within organic matter

Chlamydospores have been recorded to form in large numbers on the outer surface of infected plant roots under non-sterile and sterile conditions (Reynolds *et al*. 1985; Robinson and Cahill 2003). Further work is required in this area to discover the ability of chlamydospores to survive outside the buffered internal root environment.

Whether thin-walled chlamydospores are formed in dead organic matter is similarly not clear since it has not been proven that *P. cinnamomi* can survive as a saprophyte (Kuhlman 1964; Zentmyer and Mircetich 1966; Nesbitt *et al.* 1979). Experiments have shown that chlamydospores can be produced and survive in organic matter (Hwang and Ko 1978; Nesbitt *et al.* 1979; Weste 1983b) but these results have been obtained only from material that has been inoculated *in vitro* or infested whilst the host was still alive. It is surprising that for such an important pathogen, it is still unknown whether it can colonise dead organic matter and produce chlamydospores, thereby building up survival inoculum. Currently there is little evidence that chlamydospores are produced in non-sterile conditions in soil or in dead organic matter.

Thick walled chlamydospores of Phytophthora cinnamomi

It is often stated that *P. cinnamomi* chlamydospores may be thin or thick-walled (Royle and Hickman 1964; Marks *et al.* 1975; Weste and Vithanage 1979; Erwin and Ribeiro 1996). It is thus interesting that most literature concerns thin-walled chlamydospores. There is little evidence for the existence of thick-walled chlamydospores (walls greater than 1 µm thick) and this may be due to the difficulty of germinating the thick-walled structures.

Thick-walled chlamydospores occur *in vitro* for *P. cactorum* (1 – 1.5 µm thick), *P. macrochlamydospora* (1.5 – 4 µm thick), *P. palmivora* (>1.5 µm thick) (Kadooka and Ko 1973; Darmono and Parke 1990; Erwin and Ribeiro 1996; Irwin and Mackie 2000). When *P. palmivora* and *P. drechsleri* were tested under non-sterile conditions, both produced numerous thick-walled chlamydospores (>1.5 µm thick and 1 – 1.5 µm thick, respectively). Proof of the origin of these propagules was obtained by germination of the

thick-walled chlamydospores on a range of agar media (Cother and Griffin 1973; Kadooka and Ko 1973).

Thick-walled chlamydospores of *P. cinnamomi* have been observed in susceptible avocado roots in non-sterile soil (Mircetich and Zentmyer 1967). Actual wall thickness or spore diameter was not reported and spores were not germinated to prove identity. Chlamydospores formed within non-sterile avocado roots on cornmeal agar were not thick walled. This leads to the suggestion that adverse environments induce formation of thick-walls (Mircetich and Zentmyer 1967).

Cother and Griffin (1973) gave the most convincing evidence that thick-walled chlamydospores of *P. cinnamomi* are formed in plant roots in a soil environment. They observed spores 5.2 – 14.4 μm in diameter with walls 1.0 – 1.5 μm thick in *Lupinus augustifolius* L. roots buried in moist field soil. Identification of *P. cinnamomi* was confirmed by germinating chlamydospores first on water agar and then transferring the germinating spores to *Phytophthora* selective agar. Shew and Benson (1982), using a soil sieving technique, found thick-walled *P. cinnamomi* chlamydospores (walls 1.0 – 2.6 μm) in naturally infested soil and germinated the spores on selective medium to confirm their identity. The spores may have formed in soil but were more likely to have been in plant material broken down during the soil sieving.

Thicker-walled 'chlamydospores' (no mention of wall thickness) were also reported in *E. sieberi* colonised by *P. cinnamomi* in different soils (Old *et al.* 1984). The spores ranged from 12 – 20 μm in diameter, slightly larger than those reported by Cother and Griffin (1973). However, these structures were also found in roots of control plants and attempts

to germinate them using the methods of Cother and Griffin (1973) were unsuccessful so they may have been an invading fungus and not *P. cinnamomi.*

The lack of a reliable method for producing thick-walled chlamydospores in large numbers either *in vitro* or *in vivo* hampers research on these structures. It is important to produce real evidence of where and under what conditions thick-walled chlamydospores are produced. Without this knowledge we cannot quantify their importance in the life cycle of the pathogen or study aspects such as dormancy, survival and germination.

Dormancy and survival of chlamydospores

There is a conceptual link between thick-walled spores, dormancy and survival. It has been stated that a thick wall helps reduce the possibility of destruction of a dormant spore by soil microbes (Malajczuk 1983) although Hemmes and Wong (1975) point out that 'there is no available evidence that wall thickness alone is the primary factor in resistance against desiccation and antagonistic microorganisms'. It appears that although it is widely stated that *P. cinnamomi* may survive as dormant structures in soil (Blackwell 1949; Zentmyer and Erwin 1970; Erwin and Ribeiro 1996), there is little evidence for chlamydospore dormancy and interpretation of field studies conducted using chlamydospores produced *in vitro* has clear limitations.

There are several studies of survival of *P. cinnamomi* in plant tissues or organic matter where it has not been possible to identify chlamydospores as the survival structures. Shea *et al*. (1980) and Old *et al.* (1984) have observed *P. cinnamomi* surviving in organic matter in jarrah forest soil as well as *E. sieberi* seedlings. Neither study determined whether

mycelium, sporangia or chlamydospores contributed to survival. Similarly, Schild (1995), whilst studying the persistence mechanisms of *P. cinnamomi* in soil, was unable to determine the exact mode of survival as there was very little mycelium present and putative chlamydospores were only occasionally seen. False negative results have been obtained in detection of the pathogen from tissue sections plated onto selective agar (Hüberli *et al*. 2000). However, detection can improve if the tissues undergo wetting and drying cycles. This indicates that the pathogen is potentially surviving in a dormant state (Hüberli *et al*. 2000), but does not prove chlamydospores are involved.

The best evidence of chlamydospore dormancy is from Weste and Vithanage (1978b) who collected chlamydospores from naturally infested soil over a 2 year period by sieving and germinated them for identification. Chlamydospore numbers increased to a maximum in summer and autumn; few were detected in winter. This leads to the suggestion that the spores may have been present in winter but dormant and thus washed off the isolation plates and were not identified.

Mircetich and Zentmyer (1966) observed abundant chlamydospores (they did not record the wall thickness) surviving for up to 6 years in dead feeder roots of naturally infected avocado trees under field conditions. The pathogen was not recovered from the surrounding soil until the inoculated root material had disintegrated (Mircetich and Zentmyer 1967). The identity of these propagules could not be confirmed as they did not germinate on selective agar or after re-moistening the soil and baiting with a susceptible host. Zentmyer and Mircetich (1966) and Mircetich and Zentmyer (1967) therefore suggested that chlamydospores may not be important for survival under extreme drought conditions. This conclusion may need to be reassessed if the spores that have been

observed were dormant *P. cinnamomi* chlamydospores that required specific environmental conditions to germinate. Further experiments remain to be done to demonstrate the presence and survival time of dormant chlamydospores in soil and in plant material – this is particularly necessary in the case of thick-walled chlamydospores.

Chlamydospore germination

Due to difficulties in producing thick-walled chlamydospores, germination studies have only been carried out on thin-walled ones. *In vitro*, these germinate with germ tube walls linked to the layer immediately under the chlamydospore wall (Hemmes and Wong 1975). In order to germinate, chlamydospores require a source of exogenous nutrients such as amino acids, organic acids, casein hydrolysate and citric acid (Mircetich *et al.* 1968; Mircetich and Zentmyer 1969; Malajczuk and McComb 1977). Simple sugars alone are ineffective (Mircetich *et al.* 1968; Malajczuk and McComb 1977). Germination is relatively unaffected by pH between 3 and 9 (Mircetich *et al*. 1968) and root exudates may stimulate germination (Malajczuk and McComb 1977). However, all these experiments used artificially produced chlamydospores and tested them in mostly aseptic conditions.

Soil moisture affects germination. More chlamydospores germinated in non-sterile sand and clay soil at matric potentials between 0 and –10 kPa than at –25 kPa (Sterne *et al.* 1977). Similarly, Hwang and Ko (1978) found that in saturated soil, 23 % of chlamydospores germinated to produce sporangia on tips of germ tubes after 1 month of incubation, whereas only 18 % of chlamydospores in moist soil germinated.

Chlamydospore germination may differ under sterile and non-sterile conditions as the level of microbial competition may influence germination (Lockwood 1977). The only report on thick-walled chlamydospores is from Cother and Griffin (1973) who germinated these spores on water agar. Sterile water will not induce germination of thin-walled chlamydospores (Mircetich *et al.* 1968) suggesting that thick-walled and thin-walled chlamydospores germinate under different conditions.

In contrast to the results for *P. cinnamomi* thick-walled *P. palmivora* chlamydospores germinated poorly in distilled water, V8 agar and water agar whereas thin-walled ones showed 100 % germination (Kadooka and Ko 1973). They also showed thick-walled chlamydospores of *P. palmivora* were more tolerant to high temperatures (up to 46 °C) compared to thin-walled ones (Kadooka and Ko 1973). Comparisons between the two types have not been possible in *P. cinnamomi* due to the difficulty in producing large numbers of thick-walled spores.

Current status of knowledge on the role of chlamydospores

Despite over 80 years of work on *P. cinnamomi*, there is a surprising lack of recent research on *P. cinnamomi* chlamydospores, particularly from situations where we can be confident that the spores observed in experiments are formed by this pathogen. Of particular concern is the low number of studies on thick-walled chlamydospores. Despite this, *P. cinnamomi* is widely regarded as having the ability to produce both thick and thin-walled chlamydospores. The lack of research on thick-walled chlamydospores could limit the effectiveness of current control techniques and reduce our ability to detect the pathogen using existing isolation methods. Thick-walled chlamydospores may also have different

nutritional requirements or other environmental stimuli for germination than thin-walled ones (Kadooka and Ko 1973). Isolation techniques may also be inappropriate when the pathogen is in a state of dormancy and our knowledge regarding survival and dormancy is inadequate.

Other concerns regarding our knowledge of chlamydospore biology are that many studies have been performed in aseptic environments and the relevance to the natural environment must be treated with caution. At present, identification of species in a non-sterile environment requires loading the initial mycelium with fluorescent dyes or germination of the spores. Monoclonal and polyclonal fluorescent antibody techniques (Eren and Pramer 1966; Malajczuk *et al.* 1975; Malajczuk *et al.* 1978; MacDonald and Duniway 1979; Hardham 1986; Hardham 1989; Ferraris *et al.* 2004) or transformation of *Phytophthora* to incorporate fluorescent genes (Bailey *et al.* 1991, 1993; Bottin *et al* 1999; Si-Ammour *et al*. 2003; Vijn and Govers 2003) would aid in identification. The role of chlamydospores in the survival of *P. cinnamomi* requires considerably more research if we wish to control the pathogen effectively.

1.2.3 Oospores

Phytophthora species may be heterothallic or homothallic (Shepherd 1978; Ko 1980). *Phytophthora cinnamomi* is mostly heterothallic, requiring the presence of opposite mating types (A1 and A2) (Galindo and Zentmyer 1964; Reeves and Jackson 1972; Zentmyer 1980). *In vitro*, sexual recombination has been observed to occur between A1 and A2 mating types of *P. cinnamomi* (Linde *et al.* 2001; Dobrowolski *et al.* 2002) but no evidence for sexual reproduction has been found in the Australian natural environment even though both mating types have been found in the same area (Dobrowolski *et al.* 2003). The mating

types are unrelated to gender and until recently, the species was thought to be solely amphigynous with mating occurring as the antheridium of one isolate penetrates the oogonium of another isolate (Savage *et al.* 1968; Stamps *et al.* 1990). However, Hüberli *et al.* (1997) showed the development of both amphigynous and paragynous antheridia in A1 and A2 paired cultures of *P. cinnamomi.* This was an unusual finding for a *Phytophthora* sp. as most heterothallic species of *Phytophthora* form amphigynous antheridia only while homothallic species tend to form paragynous and amphigynous antheridia when selfing (Savage *et al.* 1968; Stamps *et al.* 1990).

Selfed oospores

Oospores are mainly observed when both mating types are present in *P. cinnamomi* (Zentmyer 1980; Weste 1983a). Oospore production may be induced without physical proximity of opposite mating types due to isolate signals received during the exchange of volatiles (Zentmyer, 1979) or the presence of *Trichoderma* species (Brasier 1971; Reeves and Jackson 1972; Reeves 1975; Brasier 1978). The stimulation of oospores in *P. cinnamomi* by *Trichoderma* species is limited to the A2 mating type (Brasier 1978). The selfing response in *P. cinnamomi* to volatile compounds from *T. koningii* coincided with a decrease in mycelial growth rate, increased mycelial branching and increased hyphal density (O'Brien 1991). Mechanical or chemical damage to mycelium by *Trichoderma viride* or other microbes in the environment could potentially trigger oospore production in *P. cinnamomi* (Reeves and Jackson 1974). It has been suggested that selfing may be a response due to the accumulation of substances involved in sexual reproduction as a result of the activity or suppression of metabolic pathways during dormancy (Brasier 1972). However, although Brasier and Sansome (1975) showed there was regular meiosis in selfed

gametangia of *P. cinnamomi,* in all experiments where oospores have been observed in the absence of the opposite mating type, viability of the spore has not been determined by staining or germination (Table 1.2). For this reason, the ecological importance of the selfing response in *P. cinnamomi* is not known.

Table 1.2: Previous research regarding conditions required for production of selfed oospores of *Phytophthora cinnamomi.*

Stimulus	Sterile?	Oospores produced A1	Oospores produced A2	Viability tested?	Reference
Avocado root extracts	✓	Not given		X	Zentmyer 1952
45 day old cultures in V8 broth	✓	Not given		X	Mircetich and Zentmyer 1967
In soil on colonised fibreglass	X	Not given		X	Mircetich and Zentmyer 1967
Trichoderma viride	✓	X	✓	X	Brasier 1971
Mechanical damage to mycelium (scalpel, addition of H_2O_2 or diethyl ether)	✓	X	✓	X	Reeves and Jackson 1974
T. viride – only if this organism was present were oospores observed in root pieces of *Castanea sativa* buried in soil	X	Not tested	✓	X	Reeves 1975
A1 and A2 isolates grown on top of each other, separated by impenetrable membrane filter	✓	✓	X	X	Ko 1978
Avocado root extracts	✓	X	✓	X	Zentmyer 1979
Oatmeal agar after 4 – 5 months	✓	✓	X	X	Ho *et al.* 1983
V8 agar or oatmeal agar after 5 – 8 days *	✓	Not given		X	Ho *et al.* 1983

* Isolate was identified as *P. cinnamomi* but differences in this isolate compared to other *P. cinnamomi* isolates was recognised as it lacked the ability to produce large, botryose chlamydospores typical of *P. cinnamomi.* Molecular techniques are required to confirm this organism's identity. Using this culture, Zheng and Ko (1996) were able to germinate 85% of oospores produced on V8 agar within 12 days of incubation on S+L medium (Ruben *et al*. 1980), indicating the viability of these selfed oospores. ✓ = Yes; X = No.

Oospore germination

Germination percentages of both selfed and crossed oospores are always low (Pittis and Shattock 1994; Groves and Ristaino 2000). Maturity of oospores in *Phytophthora* spp. appears to be a determining factor in ability to germinate (Duncan 1985a; Jiang *et al.* 1989; Jiang and Erwin 1990). It has been reported that mature oospores occurred after the fusion of nuclei, after which germination percentages rose with age of the culture and therefore oospores (Ann and Ko 1988; Jiang *et al.* 1989; Jiang and Erwin 1990). Attempts by Stamps (1953) to germinate oospores produced in mixed cultures of *P. cryptogea* and *P. cinnamomi* were not successful and it was suggested that this might be due to oospore age, dormancy or failure of nuclear fusion.

There is a complex interaction between light and oospore maturation and germination. If oospores had been devoid of cool white fluorescent light in their maturation stage, germination decreased from 95 to 44 % and reduced to 15 % when light was omitted during germination (Ann and Ko 1988). When no light was provided at any stage, oospores did not germinate (Ann and Ko 1988). Interestingly, continual incubation in the dark while producing and germinating oospores from the mating of *P. cinnamomi* occurred on 1.5 % water agar after 3 days (Linde *et al.* 2001). This is in contrast to Ann and Ko's (1988) experiment as germination of oospores did not occur in distilled water but did in nutrient agar. However, comparisons between this experiment and Ann and Ko's (1988) is difficult as Linde *et al.* (2001) did not determine percentage of oospores germinating.

Oospore as survival structures

Oospores are considered the most resistant structures for survival of *P. cinnamomi* but their contribution to survival is limited in Australia due to the absence, in most cases, of the A1 mating type (Weste and Vithanage 1979; Zentmyer 1980; Weste 1983a).

Oospores have a huge potential as survival structures with their thick walls providing protection against antagonistic microorganisms in the environment. Oospores of *P. infestans* (heterothallic) have been reported to survive temperatures from 35 ºC down to -80ºC for 48 hours in non-sterile soil pot and field trials (Drenth *et al.* 1995). For oospores of *P. infestans* stored in sterile soil and water at temperatures between 0 and 20 ºC, the percentage viability (determined by plasmolysis) and germinability was highest for those stored at 20 ºC (21 % viability, 18 – 20 % germination) (Pittis and Shattock 1994). Oospores remained viable for 7 months but oospores stored at 0 ºC were unable to germinate after 5 months while only a few were viable and none germinated after 10 months storage in non-sterile soil (from an initial viability of 17 – 22 %) (Pittis and Shattock 1994). Germination percentage of the oospores was low throughout the experiment (maximum 7.8 %), suggesting that oospores have endogenous dormancy. Similar studies are necessary for selfed and crossed oospores of *P. cinnamomi* to determine the potential contribution each may have to survival of the organism.

1.3 Saprophytic Ability of *P. cinnamomi*

To be termed a saprophyte of ecological significance, an organism has to be able to colonise and live on dead organic matter in the presence of other competing microorganisms (Garrett 1956). Pathogens have an initial advantage over saprophytic soil

microorganisms as they colonise the plant tissue before the host dies (Garrett 1970). Many *Phytophthora* species have little competitive saprophytic ability (Garrett 1956; Glenn *et al.* 1988). Whether *P. cinnamomi* is a saprophyte is still a source of debate.

Phytophthora cinnamomi invaded wheat straw and dead avocado roots in both sterile and non-sterile soil up to 3cm from the food base when the water content was maintained close to saturation (Zentmyer and Mircetich 1966). This was considered saprophytic ability but it is possible that the 'movement' observed was due to zoospores. Hwang and Ko (1978), looking at the biology of chlamydospores, sporangia and zoospores of *P. cinnamomi* in soil, found that hyphae originating from chlamydospores or zoospores had the ability to colonise dead plant tissue in non-sterile soil. Reeves (1975) observed rapid colonisation by *P. cinnamomi* of organic matter and root pieces in non-sterile soils. However, in the experiment *Castanea sativa* root pieces were placed on *P. cinnamomi* colonised nylon mesh in soil. This, therefore, does not necessarily indicate saprophytic ability as the initial inoculum source was large and the pathogen could directly colonise these root pieces without growing through the soil, reducing competition from other microorganisms. No indication of distances the pathogen moved through the soil was given.

Both Kuhlman (1964) and Lacey (1965) observed that mycelium of *P. cinnamomi* and *P. infestans* was quickly lysed by microbial antagonists in non-sterile soil and concluded neither organism was capable of surviving saprophytically. Shea (1979) found that the survival of the fungus was low in trees that had been dead for more than one year which was probably due to saprophytic microorganisms that colonised the lignified tissue and competed with the pathogen. *Phytophthora cinnamomi* was never observed to colonise wood plugs infected by *P. citricola* (Bunny 1996). However, Bunny (1996) considered that

P. citricola showed saprophytic ability as it colonised wood plugs in soil infected with *P. cinnamomi.*

Studies by DeBruyn (1922) have given examples of *P. syringae, P. erythroseptica* and *P. infestans* surviving as saprophytes in sterile soil. However, this cannot be extrapolated to natural soil due to competition and other factors that cannot be reproduced in a sterile system. *P. erythroseptica* grew a limited distance (6mm) in non-sterile moist soil but only when it was added as a colonised malt agar bait (Vujicic and Park 1964). When non-sterile soil containing inoculum of *P. erythroseptica* was used, no colonisation of dead plant material was observed and only potato tubers in direct contact with the inoculum developed pink rot, not tubers that were only 2mm away (Vujicic and Park 1964).

1.4 Phosphite Control of *P. cinnamomi*

Phosphite is a systemic fungicide used for controlling plant diseases including Oomycete diseases (Guest and Grant 1991). In the literature, it is often referred to as Fosetyl-Al, phosphorus acid, phosphonic acid, phosphonate and phosphite. However, all are derived from phosphorous acid [$(OH)_3P$] which, in aqueous solution, becomes phosphonic acid [$HPO(OH)_2$] (Guest and Grant 1991). According to IUPAC nomenclature, both phosphonate and phosphite (the term used in this thesis) are acceptable to refer to the partially neutralised salt of phosphonic acid used to minimise phytotoxicity in its application as a fungicide (Roos *et al*. 1999).

Phosphite can be translocated in both the xylem and phloem (Ouimette and Coffey 1990) but plants do not appear to be capable of metabolising phosphite, thus the compound

persists in plants until it is diluted as a result of plant growth, leaf fall or fruit harvest (Guest and Grant 1991). The chemical is used to both prevent invasion and also cure plant diseases as it inhibits pathogen growth and stimulates the host defence response (Guest and Grant 1991; Daniel *et al.* 2005).

As mentioned above, the mode of action of phosphite is both direct and indirect but exact mechanisms are still unclear. Phosphite can act directly on the pathogen, inhibiting growth and enhancing elicitor levels in the cell walls (Fenn and Coffey 1984; Khan *et al.* 1986; Perez *et al.* 1995). The elicitors stimulate plant defence mechanisms so that the compound also indirectly effects pathogen growth (Khan *et al.* 1986; Perez *et al*. 1995). Plant defence against pathogens is a combination of the accumulation of phytoalexins, soluble phenolics and increased activity of host defence enzymes (Guest 1984; Khan *et al.* 1986; Saindrenan *et al.* 1988; Afek and Sztejnberg 1989; Nemestothy and Guest 1990; Jackson *et al.* 2000). In this way, phosphite induces susceptible hosts to respond to pathogen invasion in a similar way to naturally resistant plants (Guest 1984; Khan *et al*. 1986; Nemestothy and Guest 1990).

Applications for Phytophthora

Morphological and metabolic changes (eg. respiration rates, increased production of elicitors and poly- and pyro-phosphates) occur in *Phytophthora* cultures treated with phosphite *in vitro* (Fenn and Coffey 1984; Fenn and Coffey 1985; Khan *et al.* 1986; Niere *et al.* 1990; Barchietto *et al.* 1992; Niere *et al.* 1994; Perez *et al.* 1995). The effect that phosphite has on *P. cinnamomi* survival propagules will be considered further in Chapters 4 and 5.

Phosphite and phosphate compete for the same transporter binding sites (Barchietto *et al.* 1989; Guest and Grant 1991; Darakis *et al.* 1997). However, mM levels of phosphite are required to inhibit phosphate uptake, whereas μM levels of phosphate inhibit phosphite uptake (Griffith *et al*. 1989). For this reason, the effective levels of phosphite may differ in soils with different phosphate levels and the phosphite concentrations found to be inhibitory *in vitro* may differ *in planta* depending on soil type (Guest and Grant 1991).

Under low phosphate (~0.1 mM) *in vitro*, the dry weight of mycelium of *P. palmivora* and *P. cinnamomi* was reduced in the presence of phosphite (~1 mM) (Fenn and Coffey 1984; Niere *et al.* 1990). Mycelia growing on media with high phosphate (~10 mM) produced no significant changes in mycelial dry weight (Fenn and Coffey 1984; Niere *et al.* 1990). In contrast, under low phosphate (~0.1 mM) *in vitro*, the dry weight of mycelium of *P. palmivora* and *P. cinnamomi* was reduced in the presence of phosphite (~1 mM) (Fenn and Coffey 1984; Niere *et al.* 1990). Even at high concentrations (1-10 mM) phosphite is fungistatic rather than fungitoxic (Coffey and Joseph 1985; Guest and Grant 1991).

There have been some biochemical studies on the mode of action of phosphite on *Phytophthora*. Of interest is the accumulation of pyrophosphate and polyphosphate after phosphite treatment (0.1 mM) *in vitro* in mycelia of *Phytophthora* spp. (*P. citricola, P. infestans, P. melonis, P. mirabilis, P. nicotianae* and *P. palmivora*) (Niere *et al.* 1994). Those authors suggested that metabolism of pyrophosphate was the primary cause of phosphite inhibition in *Phytophthora* spp. These results were supported by similar results from studies on the effect of phosphite on *Saccharomyces cerevisiae* (McDonald *et al.* 2001).

Phosphite and the Environment in Western Australia

Phosphite is environmentally benign, with low phytotoxicity and mammal toxicity (Guest and Grant 1991). Foliar application rates between 1 – 10 g phosphite L^{-1} sprayed to run-off have been observed not to affect natural soil microorganisms (Wongwathanarat and Sivasithamparam 1991).

To control *Phytophthora* diseases in Western Australia, phosphite is currently applied as a foliar spray (5 g L^{-1}), aerial mist (400 g L^{-1}) or trunk injection (50 - 200 g L^{-1}) during autumn and spring, no more than once every two years or four years if trunk injecting (Hardy *et al.* 2001). Phosphite has been shown to remain effective after one trunk injection of *Banksia* species and *E. marginata* for at least four years (Shearer and Fairman 1997). However, the efficiency and longevity of the effect of phosphite varies depending on the interaction between plant species and *Phytophthora* isolate (Guest and Grant 1991; Wilkinson *et al.* 2001a). For example, foliar application rates of 5 – 10 g phosphite L^{-1}, inhibited the growth of *P. cinnamomi* for less than 6 months for *Hibbertia commutata* and less than 12 months for *Dampiera linearis* but at least 12 months in *Dryandra sessilis*, 18 months for *Banksia grandis* and *B. hookeriana* (Wilkinson *et al.* 2001a). Phosphite is most effective when it is used as a preventative rather than curative measure (Davis 1989).

Fairbanks *et al*. (2002) found that phosphite (2.5 – 10 g L^{-1}), applied by foliar spraying to run-off, reduced pollen fertility in many species found in the jarrah forest and sandplain of Western Australia. These included perennial species from families Proteaceae, Rhamnaceae, Sterculiaceae, Papilionaceae, Euphorbiaceae, Rutaceae, Dilleniaceae, Myrtaceae, Polygalaceae and Goodeniaceae. However, due to the longevity of these

species, this would only impact on long-term reproductive success if phosphite spraying regimes were increased to more than once a year (Fairbanks *et al*. 2002).

Phytophthora cinnamomi has shown no resistance to phosphite in the natural environment. However, Duvenhage (1994) found that isolates of *P. cinnamomi* taken from an avocado orchard in which trees had been injected annually for the past 13 years, had reduced sensitivity to phosphite *in vitro*. Reduced sensitivity of *P. capsici* and *P. parasitica* var. *nicotianae* has also been induced in the laboratory by chemical mutagenesis (Bower and Coffey 1985; Fenn and Coffey 1989). Thus, although no real resistance has been observed to phosphite in the field to date, continual monitoring of isolate response to phosphite is important, particularly in orchards or natural environments treated repeatedly with phosphite (Duvenhage 1994; Hardy *et al.* 2001).

1.5 Transformation of *Phytophthora*

Difficulties in differentiating propagules of *P. cinnamomi* from those of other microorganisms complicate studies of chlamydospore production and survival in *P. cinnamomi* or other propagules in a non-sterile environment. Direct plating onto selective media can allow identification of the pathogen but this is not appropriate for dormant propagules (Blackwell 1949; Zentmyer and Erwin 1970; Erwin and Ribeiro 1996) for which germination conditions are not always known (Shew and Benson 1982). For example, previous research has suggested that the pathogen produces thick-walled spores (Mircetich and Zentmyer 1967; Cother and Griffin 1973; Old *et al.* 1984) but difficulties in germination of these structures has limited research in this area and it is still unclear as to whether these are actually propagules of *P. cinnamomi.*

A method where the pathogen and its propagules can be readily distinguished through colour would be invaluable for following its survival and growth in complex environments such as non-sterile soil. One approach that has been used successfully for biological studies of *Phytophthora* spp. is transformation of the organisms with a GUS (β-glucuronidase) or GFP (Green Fluorescent Protein) gene as detectable markers (Chalfie *et al.* 1994; Jefferson *et al.* 1986).

This technique incorporates the Green Fluorescent Protein (GFP), originally isolated from the bioluminescent jellyfish (*Aequorea victoria*) (Chalfie *et al.* 1994), and the β-glucuronidase from *Escherichia coli* into the fungal chromosomal DNA (Jefferson *et al.* 1986). The GFP allows visualisation of the propagules of the organism containing this gene as it fluoresces under blue light (UV) excitation (480nm) (Chalfie *et al.* 1994; Lee *et al.* 2002; Si-Ammour *et al.* 2003). The GUS gene produces a blue product in the presence of X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) (Oliver *et al.* 1993). Both the GFP and GUS proteins have been shown to function in *Phytophthora* (Bailey *et al.* 1993; van West *et al*. 1999; Bottin *et al.* 1999; Cvitanich and Judelson 2003; Si-Ammour *et al.* 2003; Vijn and Govers 2003).

Transformation techniques

Many techniques such as biolistics, electroporation, polyethylene glycol (PEG) and *Agrobacterium tumefaciens*, have been used to transform fungi and oomycetes to include a gene of interest. Transformation has been shown to be successful with a number of *Phytophthora* spp. including *P. cinnamomi* (Table 1.3).

Table 1.3: Transformation of *Phytophthora* species using biolistics, PEG and *Agrobacterium*-mediated systems. * PEG = polyethylene glycol transformation

Technique	Organism transformed	Gene integrated	Stability of integration	Reference
Biolistics	*P. capsici*, *P. cinnamomi,* *P. citricola*, *P. citrophthora*	pCM54 & pBI426 containing GUS with Cauliflower mosaic virus 35 S promoter	pCM54 not stable but pBI426 stable	Bailey *et al*, 1993
Biolistics	*P. infestans*	GUS, neomycin phosphotransferase (*npt*) with *ham34* & *hsp70* promoter of *Bremia lactucae*	Stable	Cvitanich and Judelson *et al.* 2003
PEG*	*P. capsici,* *P. parasitica*	Hygromycin B phosphotransferase gene (*hph*) with *hsp70* promoters from *Ustilago maydis* (pCM54)	Transient expression. 25% less transformants in *Phytophthora* than in *U. maydis*	Bailey *et al.* 1991
PEG	*P. infestans*	*Hph* fused to *ham34* promoter of *B. lactucae* (pTH210 & pHAMT34H)	Stable	Judelson *et al*. 1991
PEG	*P. megasperma* f.sp. *glycinea*	Compared pCM54 to pTH210 & pHAMT34H	Not stable with pCM54 but stable with *hph* from other plasmids	Judelson *et al*. 1993
PEG	*P. parasitica* var. *nicotianae*	*Hph* & GFP using *hsp70* promoter and *ham34* terminator from *B. lactucae*	Stable	Bottin *et al.* 1999
PEG	*P. infestans*	GUSand *npt*II fused to *ipi*O1 promoter	Stable	van West *et al.* 1998
PEG	*P. palmivora*	GUS (*ham34* promoter from *B. lactucae*), *nptII* (*hsp70* promoter from *B. lactucae*) & GFP (*ham34* promoter from *B. lactucae*)	Stable	van West *et al.* 1999
PEG	*P. brassicae* (formerly *P. porri*), *P. infestans*	Vector p34GFN (*nptII*) & GFP with *ham34* promoter from *B. lactucae*)	Stable	Si-Ammour *et al.* 2003
Agrobacterium-mediated transformation	*P. infestans*, *P. palmivora*	GUS & *npt* fused to promoter *hsp70* from *B. lactucae*	Stable	Vijn and Govers 2003

Biolistics

Biolistics transformation involves the bombardment of a cell with microprojectiles (eg. gold) covered with the DNA to be integrated (Cvitanich and Judelson 2003). This technique can penetrate cell walls so protoplasts (which have low survival after treatment) are not needed (Judelson *et al.* 1991). Sporangia of *P. infestans* were successfully transformed using this procedure (Cvitanich and Judelson 2003). However, biolistics requires specialised equipment and produces heterokaryons (more than one type of nucleus), therefore requiring an added step to generate single zoospore cultures to obtain pure lines (Vijn and Govers 2003).

Electroporation

Electroporation involves the application of strong electrical fields to protoplasts to rearrange the cell membrane temporarily, developing pores to allow DNA to enter (Weaver 1995). Although electroporation appears to have a higher transformation efficiency than the PEG-mediated transformation system, it does require expensive, specialised equipment (Bailey *et al.* 1993; Latijnhouwers and Govers 2003; Weiland 2003; Vijn and Govers 2003).

Polyethylene glycol (PEG)

PEG transformation makes conditions more favourable to the fusion of introduced DNA to chromosomal DNA by reducing the medium volume, clumping protoplasts together to trap DNA as well as increasing membrane permeability (Fincham 1989; Kuhn 1991; Hood and Stachow 1992). A particular disadvantage of the PEG method is that polyethylene glycol is

highly toxic to *Phytophthora* and fungi and greatly reduces protoplast regeneration after transformation ($^{1}/10^{4}$ to $^{10}/10^{4}$ protoplasts regenerate after PEG transformation compared to $^{1000}/10^{4}$ in the case of electroporation) (Prell *et al*. 1991).

Many factors in protoplast transformation have to be optimised as these vary between species (Fincham 1989; Peberdy 1991). In particular, lytic enzymes, enzyme digestion time, temperature, pH, age and concentration of fungal material and type of osmotic stabiliser can influence protoplast release and subsequent regeneration (Fincham 1989). *Phytophthora* species differ from higher fungi in having cellulosic cell walls instead of chitin. Thus the cell walls of *Phytophthora* spp. are digested by cellulases that are normally used for plant protoplast production, allowing protoplasts to be released into an osmoticum (Kuhn 1991). Rupturing of the protoplast which lacks a cell wall is prevented due to the presence of the osmoticum (Kuhn 1991).

The PEG system only requires the gene of interest to be in cloned DNA fragments, so a binary vector is unnecessary (Vijn and Govers 2003). If all genes of interest are integrated on the same vector, then transformation has been observed to be 3 times more efficient (Si-Ammour *et al.* 2003).

Agrobacterium-mediated transformation

Agrobacterium tumefaciens is a soil bacterium that causes crown gall tumours in dicotyledonous plants at wound sites (Zambryski 1992). As *A. tumefaciens* infects plant tissues, it transfers a DNA segment (T-DNA) into the plant cell that can be incorporated into the plant genome, initiating plant cell proliferation and the subsequent formation of a

tumour (Bundock *et al.* 1995). Necessary for the process of T-DNA transfer are the virulence gene sequences (*vir*) also located on the Ti plasmid. The expression of these is stimulated by chemicals (eg. acetosyringone) secreted by the wounded plant cells (de Groot, 1998). For artificial transformation, genes with desired characteristics can replace the T-DNA as the virulence genes will still allow entry into the plant cells and integration of the genes into the genome. Artificial transformation can occur through the co-cultivation of *Agrobacterium* (containing the desired genes) and the oomycete or fungus to be transformed, in the presence of the chemical acetosyringone (de Groot *et al*, 1998). This method has previously been used to transform *P. infestans* and *P. palmivora* (Table 1.3).

The required DNA must be transformed into a binary vector (eg. pBINHL1; Figure 6.4) (Vijn and Govers 2003). Without this protection, DNA introduced into a recipient cell is likely to be lost as the plasmid needs to be able to independently replicate and also provide protection for the gene of interest as plasmids stop exonucleases attacking unprotected DNA. The *Agrobacterium*-mediated transformation system is less laborious than the PEG system (Vijn and Govers 2003). *Agrobacterium*-mediated transformation allows plasmids with the gene of interest to be transformed directly from *A. tumefaciens* into fungal fruiting bodies (Chen *et al*, 2000), spores or hyphal tissue (de Groot *et al.* 1998).

1.6 Thesis Aims

The survival of *P. cinnamomi* in Australian ecosystems is poorly understood. It is generally agreed that the main mode of survival for this devastating pathogen is through chlamydospores rather than through saprophyte survival or oospores but more evidence to support this is required. The overall aim of this thesis was to increase our understanding of *P. cinnnamomi* survival as chlamydospores, oospores or as a saprophyte. This was addressed by:

- determining whether *P. cinnamomi* is capable of surviving as a saprophyte (Chapter 2),
- studying the effects of environmental conditions on chlamydospore production *in vitro* and under non-sterile conditions and on the production of thick-walled chlamydospores (Chapter 3),
- determining the effect of phosphite on chlamydospore production, viability and germination, *in vitro* and *in planta* (Chapter 4),
- examining *in vitro*, the production, viability and germination of oospores produced by an A2 isolate of *P. cinnamomi* in the absence of the A1 mating type (Chapter 5),
- developing a transformed isolate of *P. cinnamomi* that incorporates the Green Fluorescent Protein (GFP) into its genome to allow any propagules of the pathogen to be distinguished from other organisms in a non-sterile environment (Chapter 6), and
- testing the stability of the GFP gene in transformed isolates of *P. cinnamomi* and the fitness of transformants (Chapter 7).

CHAPTER 2

Phytophthora cinnamomi as a Saprophyte

2.1 Introduction

Some soilborne pathogenic microorganisms may persist in soil without a living host plant, living as saprophytes or as dormant propagules. A saprophyte must be able to colonise new, independent pieces of dead plant tissue to survive, whereas dormant propagules survive by having an extremely low, or no metabolism or respiration (Griffin, 1972). Knowledge of the saprophytic ability of a pathogen is important in disease control as the organic matter in which it lives may act as a source of inoculum as well as increase the ability of the pathogen to survive unfavourable conditions (Weste 1983b; Keane *et al.* 2000).

Phytophthora cinnamomi has been previously classified as a 'saprophytic soil survivor' as it may survive in dead host tissue that was colonised when the plant was still alive (Griffin 1972; Marks *et al.* 1975). It is generally believed *Phytophthora* species are unable to compete and grow in soil with other microorganisms and are never secondary invaders (Erwin and Ribeiro 1996). However, there have been reports that contradict this and a critical review of the literature suggested that the question has not been satisfactorily resolved (Chapter 1.4).

It is important to resolve the question of the ability of *P. cinnamomi* to survive as a saprophyte in soil for effective management techniques to be developed. This study aimed to discover whether *P. cinnamomi* is capable of surviving as a saprophyte in soil and whether soil type, soil microbes and soil moisture influence this ability.

2.2 Materials and Methods

Experimental Design

Soils

Four different substrates were used, including conducive jarrah forest soil (a mixture of Havel soil types taken from the jarrah forest and stockpiled), conducive potting mix, suppressive red loam (Havel classified 'Q' jarrah vegetation type [Havel 1975; CALM 1990]) and conducive Bassendean sand (Shearer and Hill 1989) (Appendix 1). The jarrah forest soil was collected from Alcoa's Huntly mine site, approximately 75 km south east of Perth. Potting mix was pine bark : coarse river sand : coco peat in the ratio 2: 2: 1 (Richgro Garden Products, Western Australia), the jarrah vegetation Q red loam was collected from the top layer of soil at sites near Gleneagle, Western Australia (Map 50; 424107E, 6432155N) and the Bassendean sand was collected from the *Banksia* woodland at Murdoch University (Map 50; 389928E, 6450659N).

Substrates that required sterilisation were autoclaved at 121 °C for 30 minutes in approximately 1 kg lots on three consecutive days. To confirm that autoclaving was not detrimental to the soil, a lupin seedling bioassay showed there was no difference in germination, seedling development and health, between autoclaved and non-autoclaved soil.

Moisture content of each substrate was determined prior to all experiments by drying a known weight of each (approximately 100 g) in a 60 °C oven before again determining the mass. To determine maximum moisture holding capacity of each substrate, soils were

brought to container capacity (soil was flooded with water and allowed to freely drain for 24 hours) and their mass and dry mass were determined.

A moisture characteristic curve was determined for jarrah forest Q red loam and Bassendean sand using the method developed by Fawcett and Collis-George (1967) and Hamblin (1981). Soils were brought to container capacity and 100 g of each substrate was then weighed into 90 mm Petri dishes containing drainage holes, using a replication of 30 for each soil. Whatman No. 2 filter paper (Springfield Mill, UK) was cut into strips measuring 60 x 10 mm, then three strips were weighed and buried into each dish containing substrate. Over a 5 day period, the filter paper strips were removed and weighed along with each soil. Paper and soil was dried in a 45 °C oven for at least 12 hours and reweighed. Matric potential was determined using the calculations of Hamblin (1981) and Fawcett and Collis-George (1967) and graphed against moisture content of the soil to create a moisture characteristic curve for each soil type (Figure 2.1).

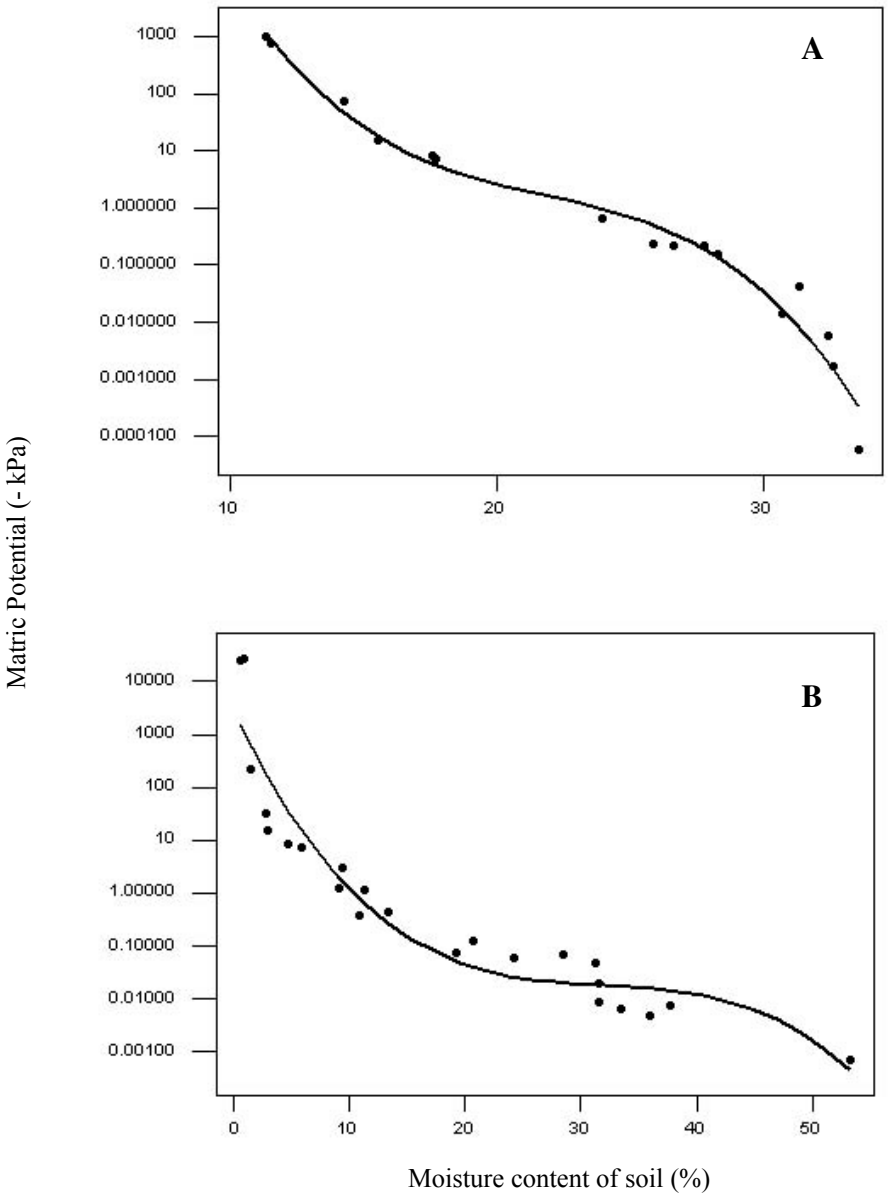

Figure 2.1: Moisture characteristic curves of jarrah vegetation Q red loam (A) and Bassendean sand (B). Vertical and horizontal scales differ on A and B.

Determination of Phytophthora – free status

A baiting technique was used to check that the substrates were free of *P. cinnamomi.* To a 20 % (w/v) solution of each substrate, petals of a *Hibbertia* sp. were floated on the surface. After 3 days, petals were removed, blotted onto paper tissues to remove excess water and plated onto NARPH agar (Appendix 2). Plates were incubated at 20 °C for 3 days and then observed for any *Phytophthora* growth. Soils were found to be free from *P. cinnamomi*.

Soil Microbial Activity

The FDA (fluorescein diacetate – Sigma Chemical Co., USA) hydrolysis technique of Schnurer and Rosswall (1982) was used to determine soil microbial activity. To a McCartney bottle, 5 g of substrate and 10 ml of filter sterilised 60 mM potassium phosphate buffer (40 mM K_2HPO_4, 10 mM KH_2PO_4) was added. 400 µg of FDA was introduced to three replicates to begin the reaction, none being added to a 4th replicate which was a blank control. Bottles were shaken at 1.5 *g* at 28 °C for 20 minutes, then, 10 ml acetone was added to stop the reaction. The liquid was collected by filtration through Whatman No. 1 filter paper (Springfield Mill, UK) and the fluorescein concentration was subsequently determined by spectrophotometer (Heλiosα Thermo Spectronic; Australia) at wavelength 490 nm and compared to a standard curve. The blanks were used to correct for background absorbance.

To prepare standard curves, the method described by Chen *et al*. (1988) was used. This involved adding either 0, 80, 160, 240, 320 or 400 µg FDA to 5 ml of filter sterilised 60 mM potassium phosphate buffer in triplicate. The FDA was hydrolysed by heating in

boiling water for 60 minutes (Schnurer and Rosswall 1982). Approximately, 5 g of soil was added to the solution along with a further 5 ml of phosphate buffer, the bottles shaken at 1.5 *g* at 28 ºC for 20 minutes. 10 ml acetone was added and the liquid filtered and absorbance measured as described above.

Inoculum

Phytophthora cinnamomi isolates used included MP103 (A2 mating type isolated from *Corymbia calophylla* in Huntly, Western Australia, 1993), MP127 (A2 mating type isolated from *Eucalyptus marginata* in Jarrahdale, Western Australia, 1993) and MP94-15 (A2 mating type isolated from *E. marginata* in Willowdale, Western Australia). The *P. citricola* isolate used (MU1A) was isolated from *Pinus radiata* in Baudin plantation (1981). All isolates were passaged through sterile lupin roots prior to experimentation.

Perlite inoculum of *Phytophthora,* used for the soil sampling and soil baiting experiments, was produced using a similar method to that of Duncan and Keane (1996). This involved adding approximately 100 ml of pea broth (Appendix 2) to 250 ml conical flasks containing 15 g of perlite and sterilised through autoclaving (20 minutes; 121 ºC) on three consecutive days. Perlite was inoculated with three small blocks of pea agar (Appendix 2) colonised by a 3-day-old culture of either *P. cinnamomi* or *P. citricola*. The flasks were incubated for a minimum of 2 weeks at 25 °C, loosening the perlite every 3 days with shaking, before the colonisation of perlite was tested by plating on pea agar plates.

For the Miracloth colonisation experiment, lupin (*Lupinus angustifolius*) seeds were surface sterilised by immersion in 6 % (w/v) bleach for 30 minutes, followed by three washes for

two minutes in sterile water. Seeds were placed on moist filter paper in 90 mm Petri dishes and incubated for four days at 25 ºC. Lupin inoculum was developed by placing 1 cm excised sterile lupin roots, on a 3-day-old culture of *P. cinnamomi* and incubating for a week.

Experiments

Three techniques were used to assess saprophytic growth of *P. cinnamomi*. Growth through soil in a Petri dish from inoculum on one side was assessed by sampling the soil and comparing growth to a suspected saprophytic Oomycete (*P. citricola*) (Bunny 1996). Growth through soil in plastic containers (150 x 90 x 60 mm) was assessed from baiting the soil with lupin roots. Finally, growth from a central inoculum on Miracloth (Calbiochem, Australia) through soil in a Petri dish was measured by plating the Miracloth on NARPH medium. Variables of soil type, organic matter, water potential and the presence of soil microbes were investigated.

Effect of soil microorganisms and organic matter on growth of P. cinnamomi *and* P. citricola *through soil at container capacity as assessed by soil sampling.*

Petri dishes (140 mm) were set up with 250 g of either sterile or non-sterile jarrah forest soil that had previously been brought to container capacity with sterile water (Figure 2.2). To one side of the plate, 4 g of perlite inoculated with *P. cinnamomi* (isolate MP127) or *P. citricola* (isolate MU1A) was placed, separated by a sterile cardboard barrier while being set up. Additional organic matter was buried in half the plates (Table 2.1). Additional organic matter added was in the form of approximately 100 pieces of sterile *Banksia*

grandis leaves and branches measuring no more than 2 cm in length or width. There were three replicates of each treatment.

To encourage the growth of *P. cinnamomi,* leachates from *Banksia grandis* were added to the plates. Leachates were collected by saturating pots containing 1-year-old *B. grandis* plants overnight, then collecting the leachates. The leachates were sterilised by twice filtering through two layers of Whatman No. 1 filter paper in a Büchner Funnel before passing the filtered extracts through a sterile 0.22 µm Millipore filter (Schleicher and Schuell, Australia). Ten ml of sterile exudates were added evenly to each Petri dish. The divider was removed, lids were replaced, then plates sealed with plastic (Gladwrap, Australia). All plates were incubated at 20 °C on a slight angle so that the inoculum was at the lower edge of the slant to reduce the speed and therefore distance the zoospores could travel during the experiment.

Every seven days for three weeks, approximately 5 g of jarrah forest soil was taken from a position approximately 1 cm from the source of inoculum and replaced with sterile jarrah forest soil. The sample was sprinkled over a NARPH agar plate and a pea agar plate and incubated at 20 °C for 3 days before the plates were observed for growth of *Phytophthora*. If results were positive for *Phytophthora*, the following week samples would be taken from a further 1 cm from the inoculum source.

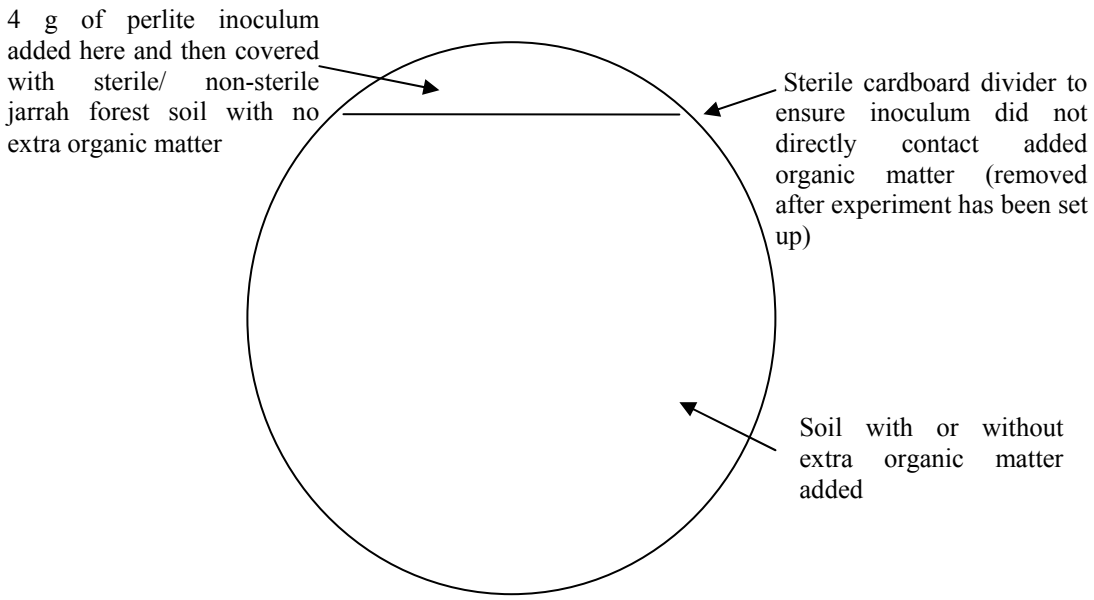

Figure 2.2: Diagrammatic presentation of experimental design to analyse the distance *Phytophthora cinnamomi* can grow through jarrah forest soil in a Petri dish.

The effect of different soil moisture conditions on the saprophytic ability of P. cinnamomi *in sterile and non-sterile jarrah forest soil and potting mix as assessed by soil baiting*

Plastic containers (150 x 90 x 60 mm) were filled with either sterile or non-sterile jarrah forest soil or potting mix. The amount of sterile water required to bring each substrate to the required percentage of container capacity was added (Table 2.1). Three replicate containers were inoculated with *P. cinnamomi* (isolate MP127) and one was not inoculated for each moisture level.

Miracloth bags containing approximately 10 g perlite inoculum of *P. cinnamomi* were buried in the substrate at the end of the inoculated container (Figure 2.3). Miracloth bags containing non-inoculated perlite were buried in control containers.

Containers were incubated in dark at 20 °C on a slight angle so the Miracloth bags were at the bottom of the slope to slow zoospore movement and therefore distance travelled by these propagules during the course of the experiment. Excised roots from 5-day-old sterile lupins were placed in rows 1 cm apart. After six days, the lupin roots from each row were removed and plated on NARPH. The lupin roots were replaced with fresh sterile lupin roots as described previously and the containers incubated for a further 6 days in the dark at 20 ºC and the lupin roots again removed, plated on NARPH and replaced in the soil. This process was repeated for 11 harvests.

NARPH plates were incubated at 20 °C and observed after 3 days for any growth of *P. cinnamomi*. This process of removal and replacement of lupin roots and scoring for *P. cinnamomi* was continued for the 11 weeks of the experiment. The Miracloth bags of inoculum were removed 2 weeks into the experiment and replaced with either jarrah forest soil or potting mix. Microbial activity of each sterile and non-sterile soil was tested prior to experimentation.

Table 2.1: Treatments and soil moisture capacities used in the experiment

Soil type	Sterile/ Non-sterile	Moisture Content (% of container capacity) *
Jarrah forest soil	Non-sterile	100
		85
		50**
	Sterile	100
		85
Potting mix	Non-sterile	100
		85
		67**
	Sterile	100
		85
		72**

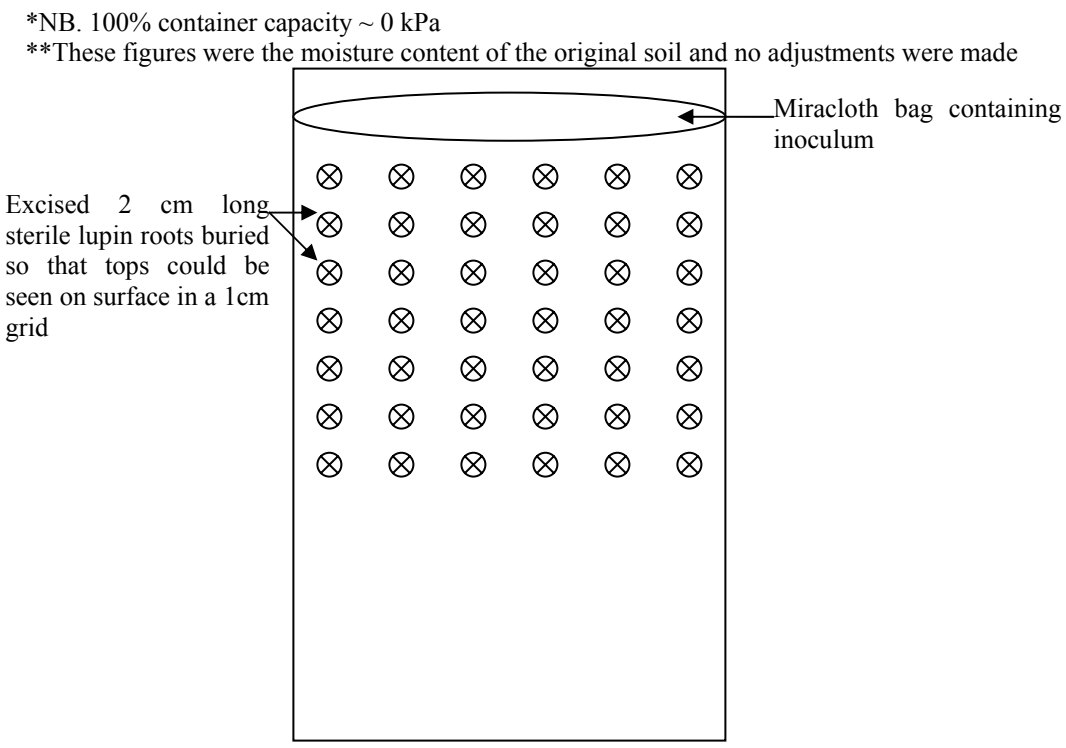

Figure 2.3: Experimental design to determine the distance *Phytophthora cinnamomi* could grow through soil and colonise fresh organic matter.

Effect of soil microbes and soil type on saprophytic ability of P. cinnamomi *as assessed by colonisation of Miracloth*

Sterile or non-sterile, jarrah vegetation Q red loam or Bassendean sand, were adjusted to a matric potential of -10 kPa (Figure 2.1) as this was the point where soils appeared moist with little free water. Each soil was added to a 90 mm Petri dish to fill it halfway, on which a sterile 60 mm^2 sheet of Miracloth was placed. Lupin inoculum was produced using *P. cinnamomi* isolates MP103, MP127 and MP94-15. One inoculated 10 mm lupin root was placed in the centre of each Miracloth sheet and covered with the corresponding soil (Figure 2.4). Each treatment was replicated 4 times and the experiment was repeated once. Plates were incubated at 20 ºC in the dark for 3 weeks.

After the incubation period, Miracloth sheets were removed, placed on NARPH medium and further incubated at 20 ºC in the dark for 3 days. Miracloth sheets were removed from plates and NARPH medium was examined for *P. cinnamomi.* Distance of observable growth from the original inoculum of *P. cinnamomi* was traced and measured. To eliminate the measurement of growth from zoospores, only growth related to the initial point of inoculation was recorded. During the 3 days incubation on NARPH, the pathogen would have grown further than the distance it grew on the dishes of soil. To account for this, plates were returned to the incubator for a further 3 days to enable estimation of the distance covered during the time on NARPH. Growth of the pathogen over this time (approximately 3 mm) was then subtracted from the original measurement. Soil microbial activity was tested prior to experimentation and after completion using FDA hydrolysis.

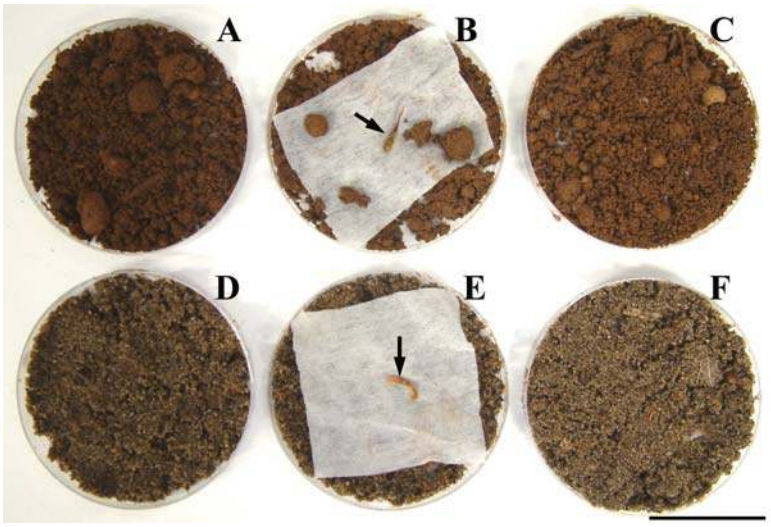

Figure 2.4: Experimental design. **A & D.** Petri dishes were filled halfway with soil; **B & E.** A sheet of Miracloth was placed on the soil. Lupin (with or without *Phytophthora cinnamomi* colonisation) inoculum was added to the centre (arrows); **C & F.** Miracloth was covered and the dish filled to the top with corresponding soil type. **A, B & C** = jarrah vegetation Q red loam; **D, E & F** = Bassendean sand. Bar = 45 mm.

Statistical Analysis of Data

To analyse the data from the first two experiments, a repeated measures ANOVA in Statistica (Statsoft, Oklahoma) was used. To test the effect of soil sterility, organic matter and the difference in behaviour between *P. cinnamomi* and *P. citricola*, factors included sterility of soil (sterile or non-sterile), species (*P. cinnamomi* or *P. citricola*), organic matter (extra added or not) and weeks (three harvests). To test the effect of sterility, different moisture contents and soil types on behaviour of *P. cinnamomi*, factors of soil type (jarrah forest soil and potting mix), sterility (sterile or non-sterile) and weeks (11 levels) with moisture as a covariate were used. The significance of the covariate was tested using a multivariate regression analysis. Using the appropriate Greenhouse-Geisser epsilon, degrees of freedom for main effects and interactions involving weeks were adjusted for each experiment to account for possible violation of the sphericity assumption.

To test the effect of sterility and soil type on the saprophytic ability of *P. cinnamomi* after 3 weeks incubation, square root transformation of data was necessary to satisfy Bartlett's test for equal variances. Data were then analysed using Minitab's (Minitab Release 13) General Linear Model and 2 sample t-test.

Microbial activity was compared within experiments using Minitab's 2-sample t-test.

2.3 Results

Effect of soil microorganisms and organic matter on growth of P. cinnamomi *and* P. citricola *through soil at container capacity as assessed by soil sampling.*

No *P. cinnamomi* or *P. citricola* were found in the control plates. After one week, bacteria were found in samples from all plates that were initially considered as 'sterile'.

Sterility of the soil had a significant ($P < 0.05$) effect on *Phytophthora* growth. In the sterile soil, regardless of whether or not additional organic matter had been added, both *P. cinnamomi* and *P. citricola* were detected 40 – 50 mm from the inoculation point after 3 weeks (Figure 2.5). Under non-sterile conditions for both *Phytophthora* species, the addition of organic matter affected ($P < 0.05$) their growth, the organisms only growing if organic matter had been added. The average growth was 17 mm (Figure 2.5). No significant ($P > 0.05$) difference was found between the behaviour of *P. citricola* compared to *P. cinnamomi* in either sterile or non-sterile soil.

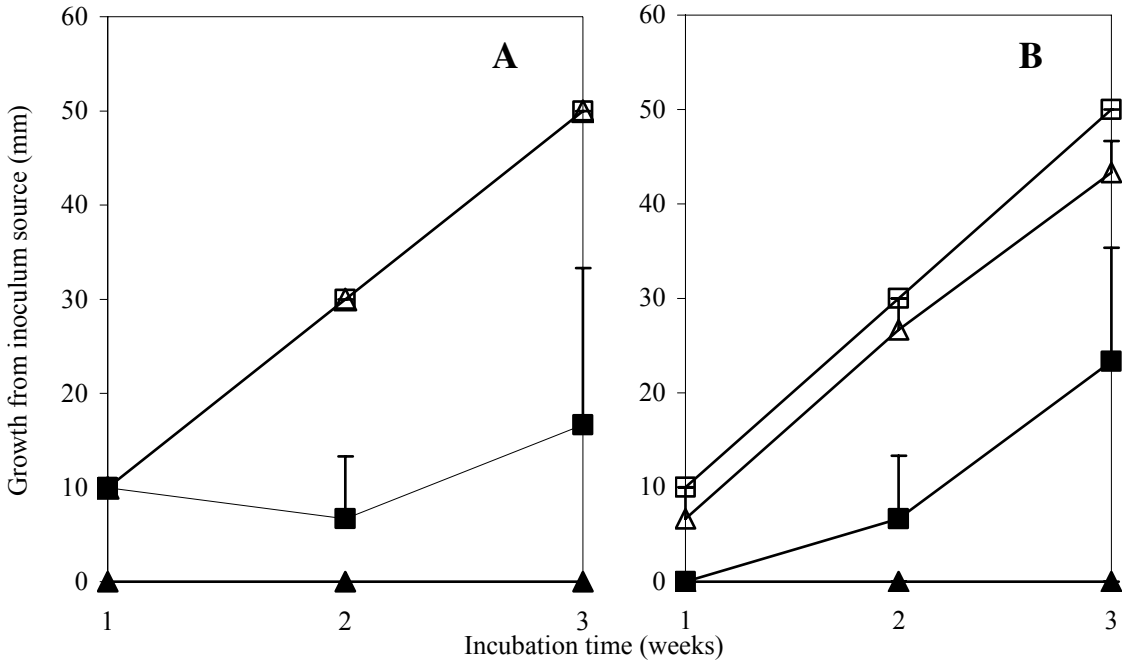

Figure 2.5: Growth of *Phytophthora cinnamomi* (A) and *Phytophthora citricola* (B) from an inoculum source through sterile or non-sterile jarrah forest soil, with or without additional organic matter. Pathogen detected by soil sampling. (Δ) Sterile soil, no organic matter added; (□) Sterile soil, organic matter added; (▲) Non-sterile soil, no organic matter added; (■) Non-sterile soil, organic matter added. Bars represent positive standard error of the means.

The effect of different soil moisture conditions on the saprophytic ability of P. cinnamomi in sterile and non-sterile jarrah forest soil and potting mix as assessed by soil baiting

Soil moisture levels between 50 and 100 % of container capacity had no effect on the saprophytic ability of *P. cinnamomi* (Table 2.2). Growth was also similar in potting mix and jarrah forest soil (Table 2.2), so data were combined for further analysis. However, in sterile soil, *P. cinnamomi* was detected up to 40 mm away from the inoculum source by week 11 while it was only detected up to 10 mm from the inoculum source in non-sterile soil at week 1 and by week 6, it was non-detectable (Figure 2.6). No *P. cinnamomi* was found in the control containers.

The initial microbial activity of sterile jarrah forest soil and potting mix was 7.15 (±0.09) and 7.96 (±0.11) µg FDA hydrolysed g^{-1} soil, respectively. Initial microbial activity of non-sterile jarrah forest soil and potting mix was 29.79 (±0.44) and 31.63 (±0.84) µg FDA hydrolysed g^{-1} soil, respectively. Although 'sterile' substrates were not completely sterile prior to experimentation, they were significantly ($P < 0.05$) lower in microbial activity than the non-sterile soils.

Table 2.2: ANOVA of data using repeated measures to determine the significance of soil type (jarrah forest soil and potting mix) and soil sterility over time (weeks) with moisture as a covariate on the saprophytic growth of *Phytophthora cinnamomi*. The covariate was found to be insignificant ($P > 0.05$) as tested using a multivariate regression analysis. Significant values are given in bold.

Effect	**$MS_{(\text{degrees of freedom, error degrees of freedom})}$**
Soil type	$7.74_{(1, 47)}$
Sterility	$\mathbf{228.68_{(1, 47)}}$
Weeks	$0.62_{(10, 480)}$
Soil type x sterility	$24.48_{(1, 47)}$
Soil type x weeks	$0.79_{(10. 480)}$ *
Sterility x weeks	$\mathbf{2.97_{(10, 480)}}$ *
Soil type x sterility x weeks	$0.25_{(10, 480)}$

* Degrees of freedom for main effects and interactions involving weeks that showed significance in original ANOVA were corrected using the Greenhouse-Geisser epsilon to account for possible violation of the sphericity assumption.

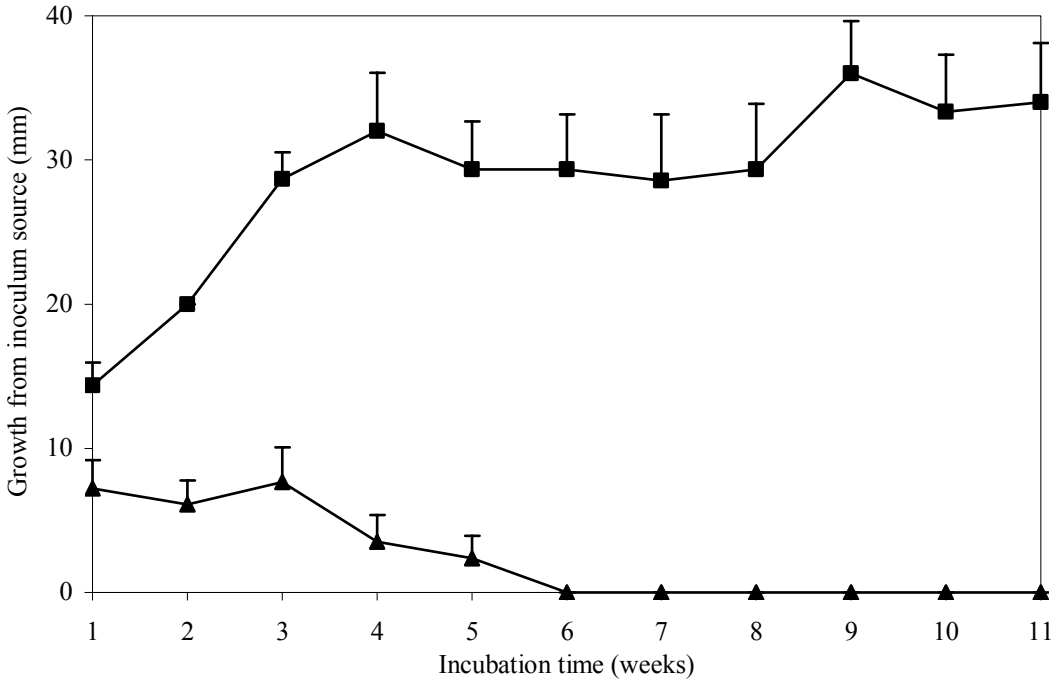

Figure 2.6: Growth of *Phytophthora cinnamomi* from an inoculum source through jarrah forest soil and potting mix (data combined). Sterile substrate (■) and non-sterile substrate (▲). Pathogen detected by soil baiting. Bars represent positive standard error of the means.

Effect of soil microbes and soil type on saprophytic ability of P. cinnamomi *as assessed by colonisation of Miracloth*

No *P. cinnamomi* was found in control plates. No significant difference was found between the original and the repeat experiments ($P > 0.05$) or the isolates used ($P > 0.05$) so data were bulked for further analysis. *Phytophthora cinnamomi* grew significantly ($P < 0.05$) further if soil was sterilised prior to inoculation (Figure 2.7). No significant ($P > 0.05$) difference was found between the soil types (Bassendean sand or jarrah vegetation Q red loam) on their own but a significant interaction ($P < 0.05$) was found between sterility of the soil and soil type. A 2-sample t-test comparing the two soil types under sterile and then non-sterile conditions found that *P. cinnamomi* was capable of growing significantly ($P < 0.05$) further in non-sterile sand compared to the non-sterile jarrah vegetation Q red loam but in sterile soils, there was no significant ($P > 0.05$) difference.

In sterile soils containing lupin inoculum of *P. cinnamomi*, mycelium observed growing across the surface and through each soil was traced back to the original inoculation point at the end of the incubation period (Figure 2.8). When measuring the maximum distance that mycelium could be microscopically observed from the initial inoculation site, it was found that this value always closely corresponded to the maximum distance as recorded on NARPH plates. No mycelium was observed in non-sterile soils at the end of the experimental period.

The presence of separately growing colonies with no obvious link to the initial inoculum were assumed to be from zoospores. These colonies were then eliminated from the

measurements on saprophytic growth when determining the distance of growth of *P. cinnamomi* through soil.

Two-sampled T-tests showed microbial activity of each soil at time 0 was not significantly ($P > 0.05$) different to that at Week 11 (Table 2.4). Microbial activity of sterile soils was significantly ($P < 0.05$) lower than that of non-sterile soil throughout the experiment (Table 2.4).

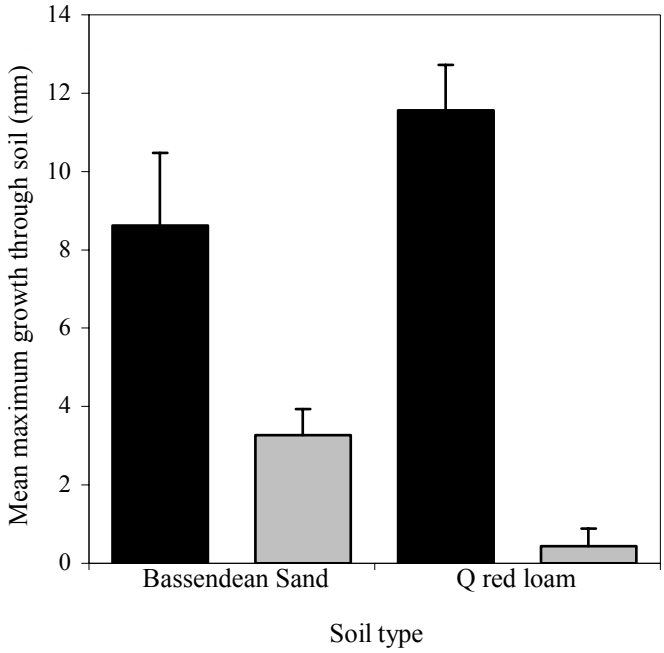

Figure 2.7: Growth of *Phytophthora cinnamomi* from original inoculated lupins after 3 weeks incubation at 20ºC in the dark in Bassendean sand and jarrah vegetation Q red loam. Black columns indicate sterilised soils and grey columns are non-sterile soils. Bars represent positive standard error of the means.

Figure 2.8: Growth of mycelium from lupin inoculum (lu) of *Phytophthora cinnamomi* through sterile soil. **A.** Bassendean sand, **B, C & D.** Jarrah vegetation Q red loam. Arrows indicate mycelial growth. Bars are 2 mm unless otherwise indicated.

Table 2.4: Microbial activity of Bassendean sand and jarrah vegetation Q red loam as measured by Fluorescein Diacetate hydrolysis. Microbial activity was measured prior (Week 0) and at the completion (Week 11) of experiments.

Substrate		Week 0	Week 11	Average
		FDA hydrolysed (µg FDA hydrolysed per gram of soil)		
Sand	Sterile	0.222 (± 0.047) ^{A}	0.745 (± 0.240) ^{A}	0.484 (± 0.160) ^{A}
	Non-sterile	10.065 (± 0.530) ^{B}	7.974 (± 2.152) ^{B}	9.020 (± 1.096) ^{B}
Q red loam	Sterile	0.405 (± 0.210) ^{A}	1.032 (± 0.190) ^{A}	0.719 (± 0.189) ^{A}
	Non-sterile	6.928 (± 0.780) ^{B}	8.000 (± 2.276) ^{B}	7.464 (±1.105) ^{B}

NB. Values followed by the same letter do not significantly (P>0.05) differ from each other.

2.4 Discussion

When colonisation of Miracloth was used to detect saprophytic growth, *P. cinnamomi* was not able to grow as a saprophyte in non-sterile soils. An exception was the Bassendean sand in which it grew approximately 3 mm from the initial inoculum source. However, when a soil baiting technique was used, *P. cinnamomi* moved further in jarrah forest soil and potting mix (up to 40 mm in sterile soils and 10 mm in non-sterile soils). It is probable that the presence of added organic matter (lupin roots) in the soil baiting technique may have contributed to this extra growth. The experiment in which soils were amended by the addition of extra organic material (sterilised *B. grandis* roots and branches) showed that *P. cinnamomi* could move further through the soils with additional organic material (17 mm compared to no growth). This may indicate that the lupin root material acted as an organic matter source, eliminating any differences that may have otherwise been observed between the growth of the pathogen in potting mix compared to jarrah forest soil.

It appears that *P. cinnamomi* was growing towards organic matter in both the soil baiting and soil sampling techniques, an indication of saprophytic growth. However, the organic matter was sterilised prior to incorporation, therefore *P. cinnamomi* would have had limited competition when colonising this material. The organic matter component of the potting mix, jarrah vegetation Q red loam and Bassendean sand was not determined but it would be interesting to find whether *P. cinnamomi* grew further through Bassendean sand than jarrah vegetation Q red loam due to differences in the organic matter component.

Interestingly, *P. cinnamomi* was not detected in soil after 6 weeks in the soil baiting experiment. The pathogen was not able to colonise the new lupin baits placed in the soil, indicating that it had either died or had converted to a survival state. Using a transformed

isolate of *P. cinnamomi* containing the Green Fluorescent Protein would aid future studies to determine whether the pathogen was still present in the soil and if so, in which form.

Under sterile conditions *P. cinnamomi* grew equally well on each soil type. Microbial activity of non-sterile jarrah forest soil was similar to non-sterile potting mix. Likewise, microbial activity in non-sterile jarrah vegetation Q red loam and Bassendean sand was similar. *Phytophthora cinnamomi* grew equally (although poorly) through the non-sterile jarrah forest soil and the potting mix. However, it grew significantly further in non-sterile Bassendean sand than it did in jarrah vegetation Q red loam (3.26 mm and 0.44 mm, respectively). This would suggest that the ability of *P. cinnamomi* to grow through soil saprophytically is a result of microbial composition rather than activity. Other soil physiological/ chemical characteristics may also play a role.

Natural soils are known to differ in their microbial composition as well as in nutrient availability (Malajczuk 1983). Specific isolates of *Bacillus, Streptomyces,* pseudomonads and others have been implicated in the suppression of *P. cinnamomi* (Malajczuk 1983; Murray *et al.* 1985) and it is possible that certain microbes were present in the Q type jarrah forest soil that were not present in the other soils tested. Sterilisation through autoclaving of the jarrah vegetation Q red loam would have diminished the microbial population (Broadbent and Baker 1974; Halsall 1978; Malajczuk *et al.* 1977b) and lead to the soil being conducive for growth of *P. cinnamomi.* Future work could look at isolating the organisms from each soil and screening *in vitro* for antagonism to *P. cinnamomi.*

No difference was found between the movements of *P. citricola* and *P. cinnamomi* through jarrah forest soil, sterile or non-sterile. Bunny (1996) suggested that *P. citricola* had

saprophytic ability as it was capable of colonising wood plugs previously colonised by *P. cinnamomi*. In non-sterile jarrah forest soil at container capacity, neither pathogen grew through the soil. This could indicate that this soil type is never favourable to saprophytic growth of either species. Further studies on different soil types and the use of more isolates are required.

The growth of *P. cinnamomi* through soil as a saprophyte could be more easily studied through the use of fluorescently labelled antibodies (Malajczuk *et al.* 1975) or through the use of a transgenically altered *Phytophthora* culture containing a green fluorescent protein allowing the visualization of its growth using a fluorescent microscope (eg. Si-Ammour *et al.* 2003). In combination with this, the effect of organic matter (and the original organic component of the soils used) and more extremes in matric potential need to be tested for their effect on the growth of the pathogen as a saprophyte. Investigation is also needed into the effect of a stimulus (eg. root exudates) on the organism's movement and to further our understanding of the impact of different microbes on the ability of *P. cinnamomi* to grow through soil. These laboratory studies then need to be applied to the natural environmental conditions.

Shearer and Smith (2000) believed that saprophytism in *P. cinnamomi* probably only occurs when microbial activity in soil is low. They deduced this from observations of colonies of *P. cinnamomi* growing from organic matter in *E. marginata* forest soils (Shea *et al.* 1980) which typically have low microbial activity (Podger 1972). The current experiments support this conclusion and have shown a reduction in microbial activity ('sterilisation' of soils) increases the ability of the pathogen to grow through soil.

However, results have shown that microbial composition is probably equally important as soil nutrients in suppression of growth of *P. cinnamomi* through soil.

The results open the question of whether an organism can be defined as a saprophyte if it can only live saprophytically in the absence of competition from other microorganisms. It seems that physiologically, *P. cinnamomi* may be able to grow as a saprophyte, but ecologically, its saprophytic ability is extremely limited, except possibly on soils of very low microbial activity.

CHAPTER 3

Production of Thick-walled Chlamydospores of *Phytophthora cinnamomi*

3.1 Introduction

Although it is often mentioned that *Phytophthora cinnamomi* is capable of producing thick-walled chlamydospores (Royle and Hickman 1964; Marks *et al.* 1975; Weste and Vithanage 1979; Erwin and Ribeiro 1996), there is little evidence to support this. In addition, little is known on how these spores form, what stimulates their production and how long they survive in the field. As discussed in Chapter 1.2.2, almost all experiments on the role of chlamydospores in the survival of *P. cinnamomi* have used thin-walled chlamydospores usually produced *in vitro*.

Since the demonstration of Cother and Griffin (1973) and Shew and Benson (1982) that *P. cinnamomi* was capable of producing thick-walled chlamydospores, there has been little research in this area. It is thought that the presence of a thick-wall around a chlamydospore would reduce its destruction by soil microbes or desiccation, thus allowing survival over extended periods (Malajczuk 1983).

Further investigations into these structures requires a reliable method for producing thick-walled chlamydospores in large numbers either *in vitro* or *in vivo*. This experiment aimed to produce thick-walled chlamydospores of *P. cinnamomi* and to determine the triggers for their production. Nutrients, matric potential, temperature, aeration, host tissues, root/ soil exudates and microbial competition were studied as potential factors that might stimulate production of thick-walled chlamydospores.

3.2 Materials and Methods

Experimental Design

Phytophthora cinnamomi isolates MP 97-8 (isolated from *Eucalyptus marginata* in Jarrahdale, Western Australia), MP 127 (Chapter 2), MP103 (Chapter 2) and MP 94-15 (Chapter 2) were used in all experiments. All isolates were passaged through sterile lupin roots and then grown for 3 days on pea agar (Appendix 2) prior to experiments. Five-mm^2 agar blocks were taken from the colony edge for subculturing into experimental conditions. Each experimental treatment was replicated four times in a completely randomised design and each experiment was repeated at least once. Unless otherwise stated, incubation of all cultures was at 25 ºC under cool white fluorescent light. Cellophane (Hallmark, Australia) was prepared by boiling 8 cm diameter circles in 1 L water for 2 hours with 0.5 g ethylenediaminetetra-acetic acid (EDTA) (AJAX, Australia) before rinsing and boiling for a further 2 hours in water. Cellophane was autoclaved for three consecutive days at 121 ºC for 20 minutes. Chlamydospores formed were observed under 200 – 400x magnification and chlamydospores were recorded as thick-walled if two layers were visible (Figure 3.1).

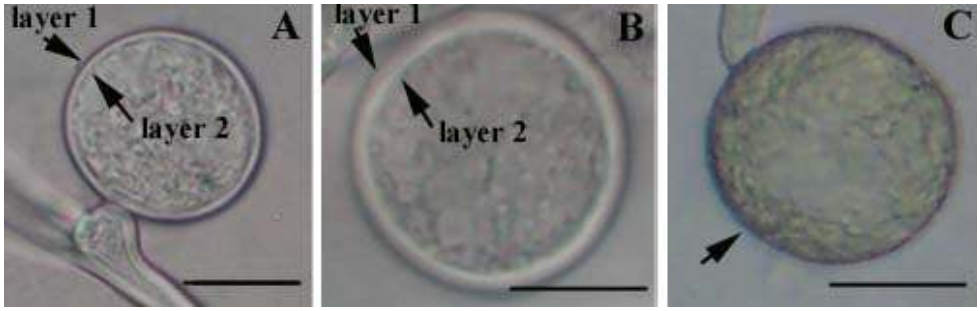

Figure 3.1: Chlamydospores of *Phytophthora cinnamomi.* **A & B.** Thick-walled: showing two distinct layers; **C.** Thin-walled: showing wall of a single layer. Bar = 15µm.

Experiments

The effect of nutrient deprivation

The four isolates of *P. cinnamomi* were grown at 20 ºC or 25 ºC on top of sterile cellophane on pea agar for seven days. To induce a sudden drop in nutrients, the cellophane containing the actively growing *P. cinnamomi* culture was then removed, its underside washed with sterile distilled water to remove nutrients and transferred to fresh, water-agar plates (1.5 % (w/v) agar (BBL)). Plates were incubated for a further week before the mycelium was examined for the presence of thick-walled chlamydospores.

In another experiment, cultures were exposed to a more gradual depletion of nutrients. This was done by subculturing isolates onto modified Ribeiro's minimal medium (Appendix 2) and incubating for 4 weeks. Cultures were then examined and wall thickness of chlamydospores observed (Figure 3.1).

The effect of osmotic potential

The four *P. cinnamomi* isolates were grown on cellophane on pea agar for 1 week. Cellophane was washed with sterile distilled water and transferred to fresh pea agar plates containing different amounts of agar (10, 15, 20, 25 g L^{-1}) to vary the osmotic potential. Plates were incubated for a further week before the wall thickness of the chlamydospores was observed.

The effect of temperature in the formation of thick-walled chlamydospores

The four isolates of *P. cinnamomi* were grown on pea agar at temperatures 20, 25, 30 and 37 ºC for two weeks in darkness before examination for the presence of thick-walled chlamydospores.

The effect of liquid cultures

In comparison to solid cultures, mycelium growing in liquid cultures were exposed to agitation. The four isolates of *P. cinnamomi* were grown in 250 ml conical flasks containing 100 ml pea broth. These were shaken (100 rpm) for 2 weeks at 25 ºC under cool white fluorescent light before mycelium was examined.

The effect of a host plant

Lupin (*Lupinus angustifolius*) seeds were surface sterilised (Chapter 2.2.1) and grown on sterile, moist filter paper in 90 mm Petri dishes for approximately one week before the roots were aseptically excised and placed on a pea agar plate with an actively growing culture of each isolate of *P. cinnamomi*. These were incubated at 25 ºC for one week before the roots were squashed under a coverslip on a microscope slide and chlamydospores observed in five roots for each replicate of each isolate.

The effect of sterile root leachates from susceptible and resistant hosts

Acacia pulchella (resistant host (Cahill *et al.* 1989) and *Banksia grandis* (susceptible host (Shearer and Hill 1989)) were grown in free draining pots (150 mm diameter free-draining polyeurythane pots) filled with potting mix (pine bark : coarse river sand : coco peat in the ratio 2: 2: 1) in the glasshouse for 10 months. To collect leachates surrounding the roots of the two hosts, the pots were flooded with tap water and then allowed to drain for 24 hours. The leachates (approx 500 ml) were collected and filtered through Whatman No. 1 filter paper (Springfield Mill, UK), followed by a 0.22 µm Millipore filter (Schleicher and Schuell, Australia). 1 ml of each of the sterile suspensions was spread onto separate pea agar plates. For each *P. cinnamomi* isolate, a cellophane disc with a 7-day-old culture grown on pea agar was placed on each plate. The plates were incubated for one week at either 20 or 25 ºC. Chlamydospore wall thickness was then observed (Figure 3.1).

The effect of microbial competition in soil leachates from susceptible and resistant plants

Exudates surrounding resistant *A. pulchella* and susceptible *B. grandis* roots were collected in the same way as described in the previous section but not filter sterilised. A 10 mm diameter hole was cut from the centre of pea agar plate and 1 ml of the exudates were added to fill the hole. Sterile water was used in place of exudates to act as a control. Plates were incubated for 48 hours before a 48-hour-old culture of each *P. cinnamomi* isolate growing on an 80 mm diameter cellophane disc on pea agar was transferred to cover the centre hole. Plates were incubated at 20 or 25 ºC for one week before chlamydospores were observed for wall thickness (Figure 3.1).

The effect of a combination of liquid culture, microbial activity and different root leachates

Three agar blocks from *P. cinnamomi* (isolate MP97-8) were transferred to 20 ml of sterile pea broth in a 100 ml conical flask. Leachates of *A. pulchella, B. grandis* and potting mix were collected as described earlier and filtered through Whatman No. 1 filter paper.

Approximately 1 ml of each filtered extract was added to each flask at time 0, day 3 and 7. One ml of filtered tap water was the control. Flasks were shaken (1oo rpm) at 25 °C under light for two weeks.

A small mycelial sample was then aseptically removed from each flask, and examined for chlamydospore wall thickness. The experiment was repeated 5 times for isolate MP97-8 and once for the three other isolates.

Chlamydospore formation in lupin roots and the effect of the soil environment

Leachates from conducive Bassendean sand (Shearer and Hill 1989) and suppressive jarrah vegetation Havel classified 'Q' red loam (Havel 1975; CALM 1990) (Appendix 1) were collected as described earlier and filtered through Whatman No. 1 filter paper. Ten ml of each leachate was added to separate 45 mm Petri dishes, using sterile water for control plates. Sterile lupin roots were inoculated with *P. cinnamomi* isolates MP103 and MP127 by wounding and then placing an agar block of *P. cinnamomi* on the wound site. Inoculated lupins were incubated for a further week. Roots were aseptically excised into 10 mm lengths and one was placed in each Petri dish containing leachates. Non-inoculated lupin roots were used as controls in the experiment. Plates were incubated for 4 weeks in the dark at 20 ºC. Roots were removed from plates, squashed on a microscope slide and

observed microscopically for chlamydospore wall thickness. Any squashed roots containing suspected thick-walled chlamydospores were transferred to NARPH medium and incubated for 1 week at 25 ºC. Germinating spores were assessed for mycelium characteristic of *P. cinnamomi.* Data were tested for equal variances and analysed using Minitab's General Linear Model (Minitab Release 13).

The experiment was repeated using Green Fluorescent Protein (GFP) transformed *P. cinnamomi* isolates MP103 (1/44) and MP127 (17/27) produced in Chapter 6 as a preliminary trial. Spores observed were firstly viewed under white light and then under blue excitation (490 nm) at 1000x magnification. Spores could be distinguished as *P. cinnamomi* by the production of a green fluorescence.

3.3 Results

Thin-walled chlamydospores were seen in all experiments both *in vitro* and *in planta*, the only exception being the aseptic inoculation of lupin roots where only mycelium and no chlamydospores were observed (Table 3.1). Thick-walled chlamydospores were seen in three out of ten different experiments (Table 3.1).

Thick-walled chlamydospores were observed in all isolates when mycelium was exposed to gradually depleted nutrients on modified Ribeiro's minimal medium over 4 weeks (Figure 3.2) but not when cultures of *P. cinnamomi* were transferred from a rich medium (pea agar) to water agar. However, thick-walled chlamydospore production was sporadic, not being present on all plates. Thick-walled chlamydospores for all isolates were always associated with mycelium of similar wall thickness (Figure 3.2). Chlamydospores appeared to begin as swellings on the hyphae into which cytoplasm flowed before a septum was laid (Figure

3.2). The width of walls for the thick-walled chlamydospores corresponded with the thickness of the hyphal wall and ranged from 1 – 5 μm.

Soil leachates from *A. pulchella, B. grandis* or potting mix also induced production of thick-walled chlamydospores and thick-walled mycelium (Figure 3.3) but this was only observed when these leachates had been added to isolate MP97-8 cultured in pea broth and incubated on a shaker (Table 3.1). This was not observed when these leachates were placed in the centre of a pea agar plate without shaking. Thick-walled chlamydospores were not observed in water controls. Thick-walled chlamydospores were not observed in repeat experiments or in other isolates.

Many thick-walled chlamydospores produced in leachates of *A. pulchella, B. grandis* and potting mix appeared devoid of cytoplasm (Figure 3.3). In thick-walled chlamydospores with cytoplasm, the cytoplasm often appeared quite granulated. This was in contrast to those thick-walled chlamydospores produced on modified Ribeiro's minimal medium which were mostly intact and with full cytoplasmic contents (Figure 3.2).

Table 3.1: Chlamydospore formation of *Phytophthora cinnamomi* under different experimental conditions. ✓ = Yes; X = No

Experimental Condition	Chlamydospores present?	
	Thin-walled	**Thick-walled**
Nutrient deprivation :		
▪ Pea agar – water agar	✓	X
▪ Ribeiro's minimal medium for four weeks	✓	✓
Osmotic potential	✓	X
Temperature (20 – 37ºC)	✓	X
Liquid culture	✓	X
A. pulchella and *B. grandis* sterile leachates	✓	X
Microbial competition from *A. pulchella* and *B. grandis* leachates	✓	X
Microbial competition from *A. pulchella* and *B. grandis* leachates in liquid culture	✓	✓
Within aseptic roots (*L. angustifolius*)	X	X
Within *L. angustifolius* roots in presence of soil leachate	✓	✓

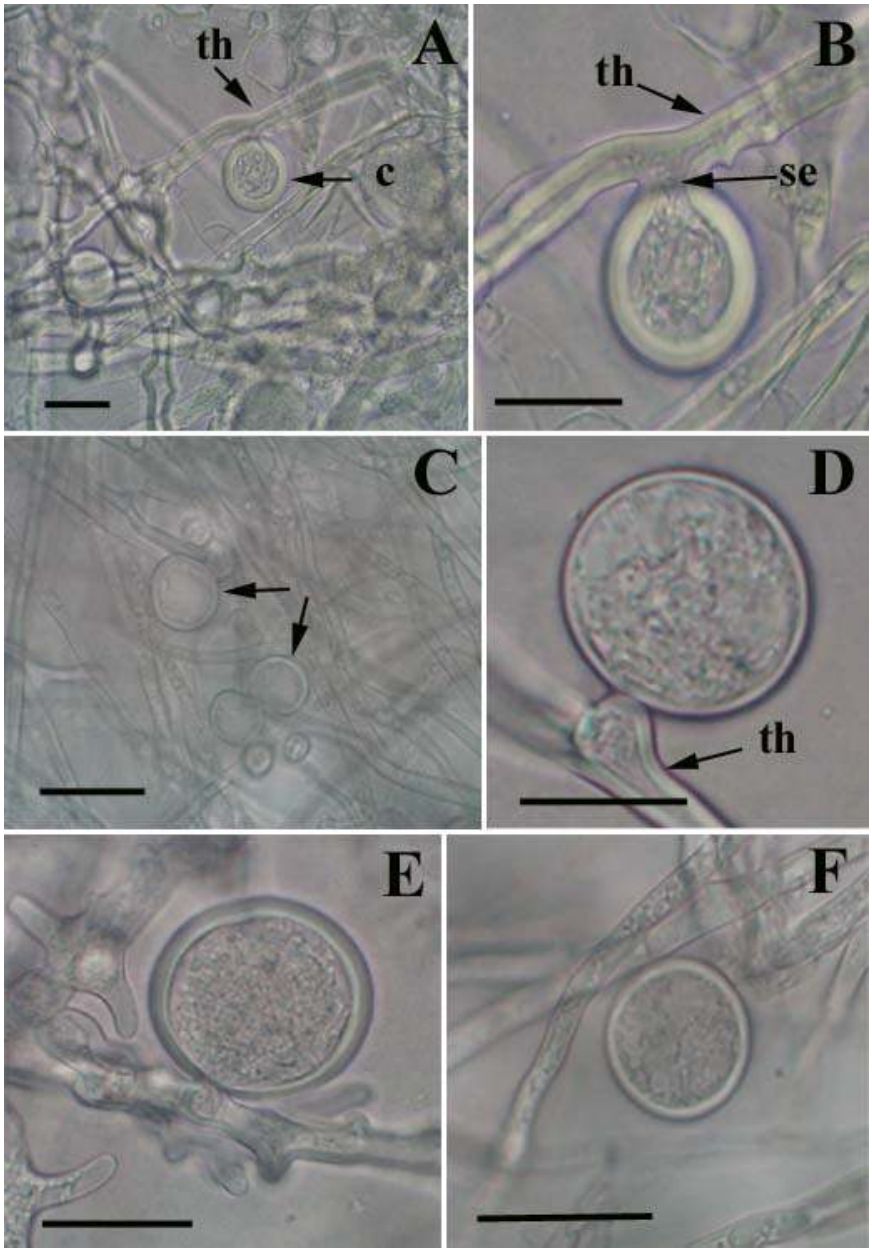

Figure 3.2: Thick-walled chlamydospores of isolates of *Phytophthora cinnamomi* produced after 4 weeks on modified Ribeiro's minimal medium. **A.** Thick-walled chlamydospore (c) forming from thick-walled mycelium (th). **B.** Higher magnification of A showing the cell lumen filling with cytoplasm and a septum (se) being laid to complete chlamydospore. **C, D, E & F.** Fully formed thick-walled chlamydospores, D attached to thick-walled mycelium (th). (Bar = 30 μm).

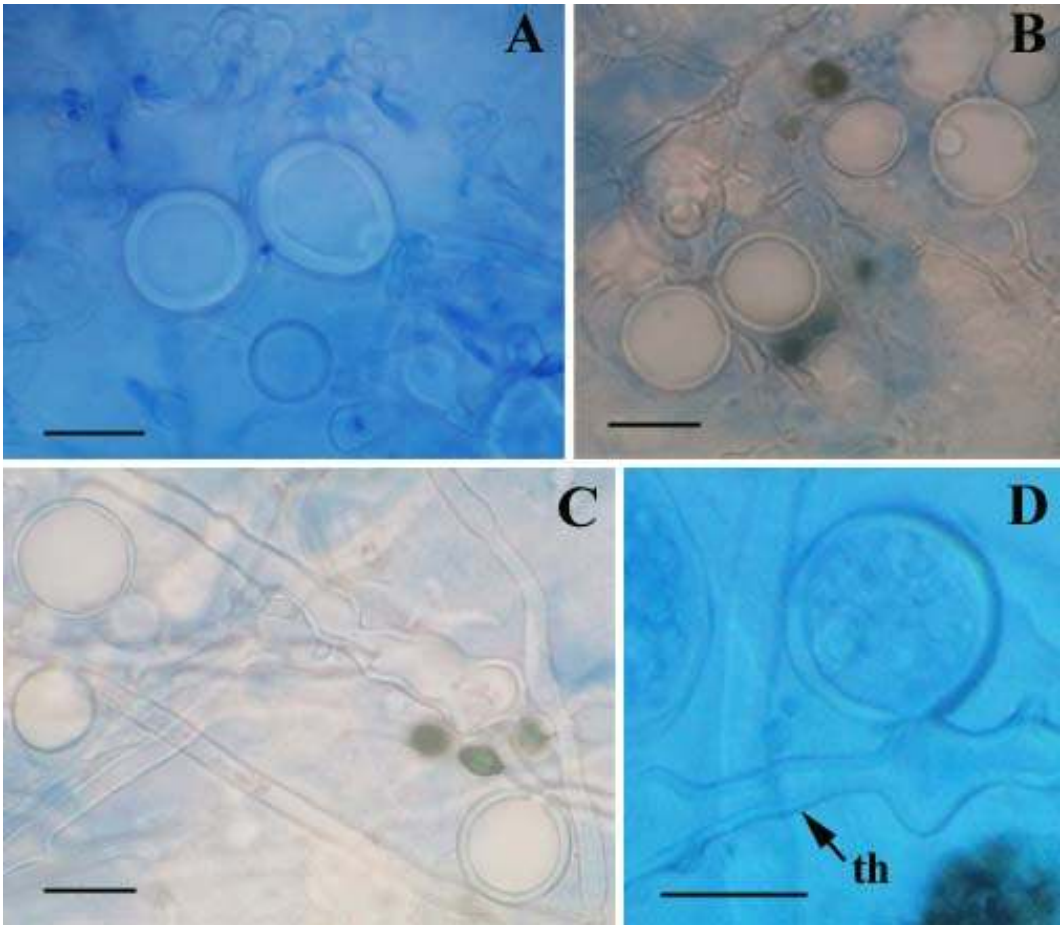

Figure 3.3: Thick-walled chlamydospores of *Phytophthora cinnamomi* produced in the presence of exudates from *Acacia pulchella.* **A, B & C.** Appear to be devoid of cytoplasm. **D.** Cytoplasm present but granulated. Note presence of thick-walled hyphae (th) (Bar = 30 µm).

Chlamydospore formation in lupin roots and the effect of the soil environment

Thick-walled chlamydospores were observed in the excised lupin roots floated in non-sterile soil extract or sterile water for both isolates (Figure 3.4). Many 'thick-walled' chlamydospores appeared to be lysing (Figure 3.4). Many chlamydospores were smaller than 30 μm in diameter.

The total number of chlamydospores within the lupin roots after 5 weeks incubation in leachates from the two different soil types (jarrah vegetation Q red loam and Bassendean sand) did not differ ($P > 0.05$) and were similar to the number observed in sterile roots (a mean of 93 (± 21)). The percentage of total chlamydospores that were thick-walled significantly ($P < 0.05$) differed depending on the interaction between isolate and substrate (Figure 3.5). Few were thick-walled in solution for jarrah vegetation Q red loam and sterile water stimulated an equal proportion of thick-walled chlamydospores as did Bassendean sand for isolate MP103. However, isolate MP127 produced 100 % thick-walled chlamydospores on Bassendean sand leachate.

Positive identification of thick-walled chlamydospores of *P. cinnamomi* within a non-sterile environment was difficult as spores from other microorganisms might be mis-identified as *P. cinnamomi*. Spores of a similar appearance were often observed in non-inoculated controls. Attempts to germinate spores on NARPH were unsuccessful.

A preliminary repeat of the experiment was performed using GFP-transformed cultures of *P. cinnamomi*. Fluorescing spores differed in appearance, some producing many patches of green fluorescence in the one spore whereas others produced only one patch of

fluorescence and these tended to be smaller in size (Figure 3.6). Spores that had been classified in the previous experiment as thick-walled were never observed to fluoresce (Figure 3.6).

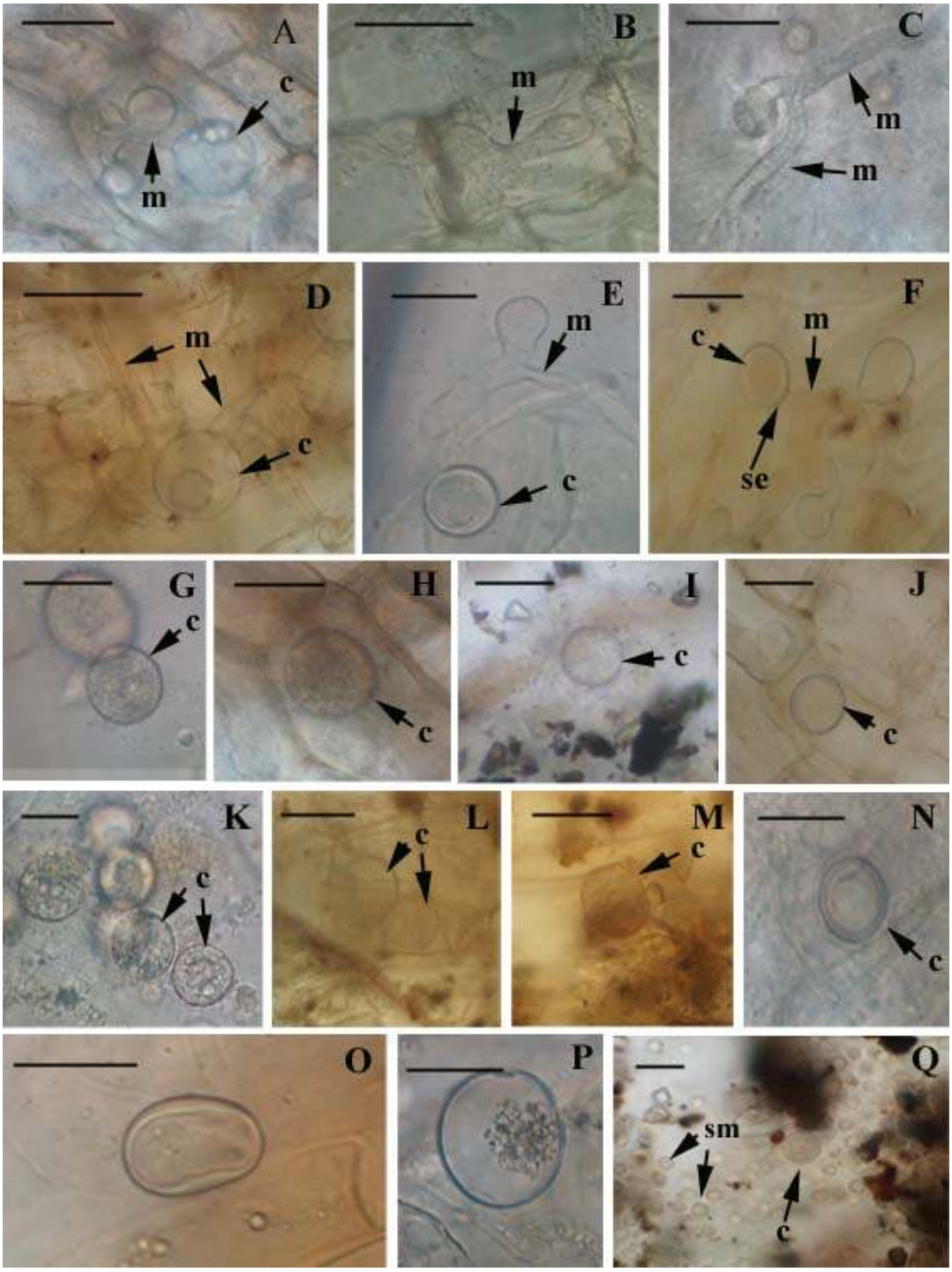

Figure 3.4: Chlamydospores of *Phytophthora cinnamomi* within inoculated lupin roots in jarrah vegetation Q red loam and potting mix leachates. **A.** Mycelium (m) with forming thin-walled chlamydospores (c); **B & C.** Thin-walled mycelium within lupin roots; **D.** Germinating thin-walled chlamydospores within root cell; **E.** Mycelium and thick-walled chlamydospore; **F.** Mycelium and forming thick-walled chlamydospores, septa (se) being laid; **G - M.** Thin-walled chlamydospores; **N & O.** Could be thick-walled chlamydospore or lysing chlamydospore; **P.** Lysing thin-walled chlamydospore; **Q.** Chlamydospore-like spores (sm) and chlamydospore. Bars = 30 µm.

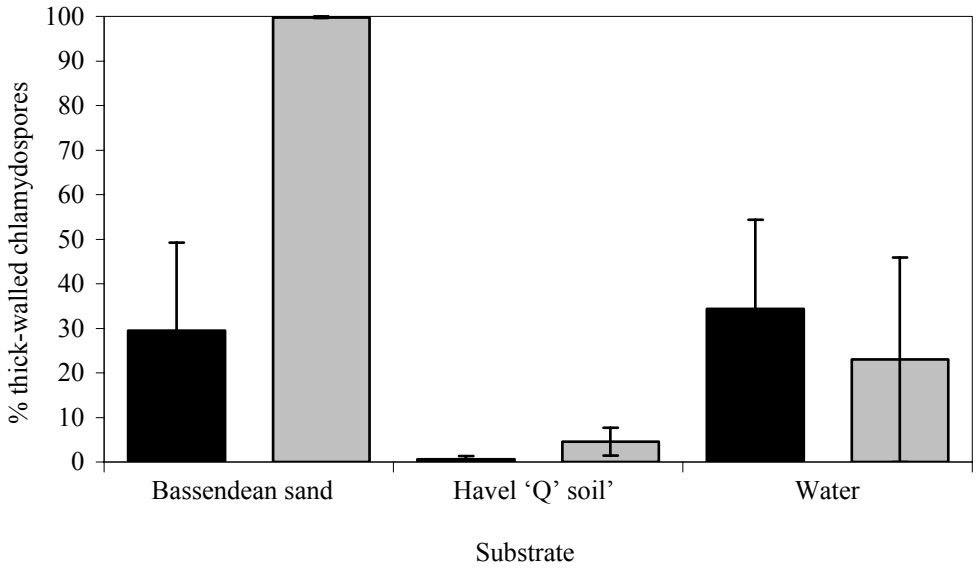

Figure 3.5: Percentage of thick-walled chlamydospores in lupin roots infected with *Phytophthora cinnamomi* isolates MP103 (black columns) and MP127 (grey columns) and floated on soil leachates from Bassendean sand, jarrah vegetation Q red loam and water. Bars represent the standard error of the means.

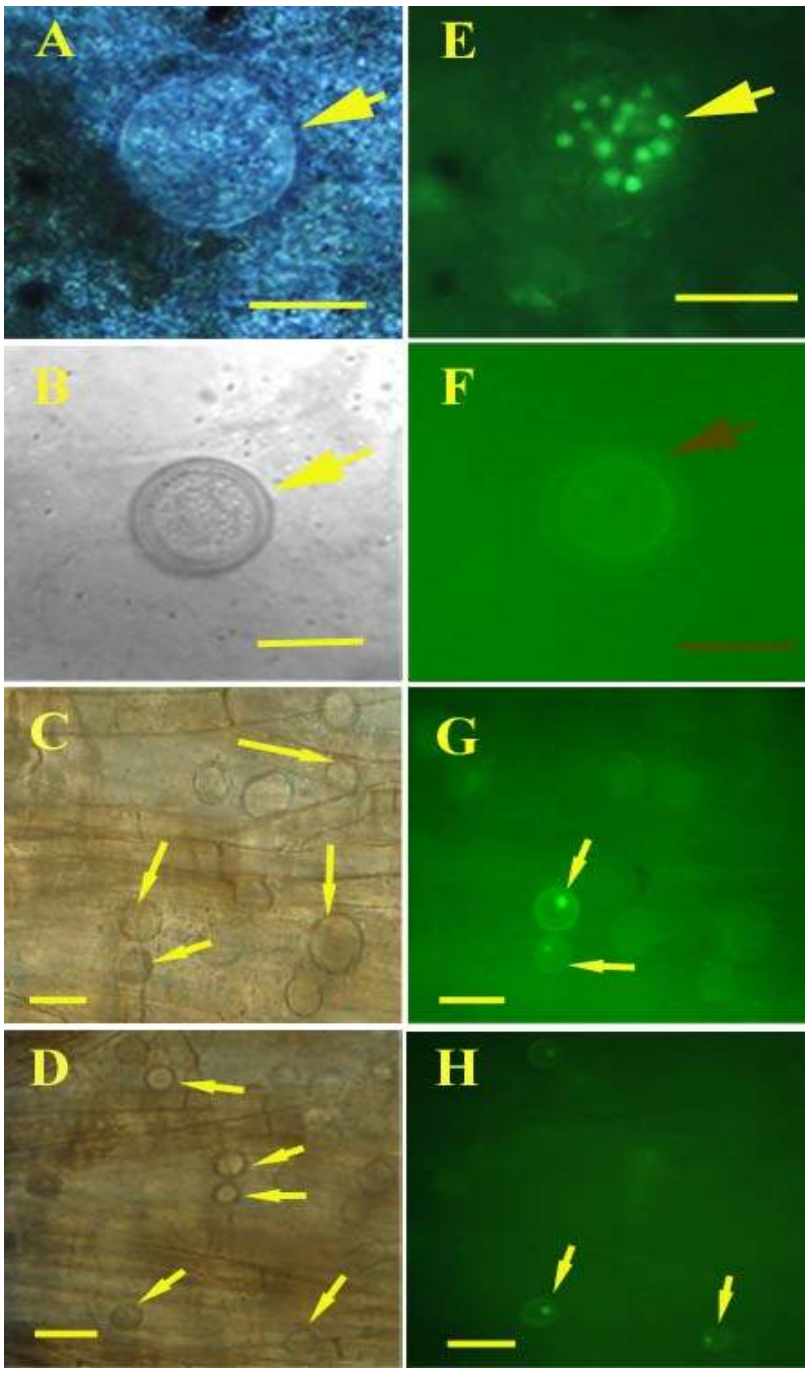

Figure 3.6: Spores formed in lupin roots under non-sterile conditions viewed under white light (**A, B, C, D**) could be confirmed as *Phytophthora cinnamomi* or of other fungal origin by the presence or absence of a green fluorescence under blue excitation (**E, F, G, H**). **A & E.** Thin-walled chlamydospore of *Phytophthora cinnamomi*; **B & F.** Thick-walled spore with non-fluorescing cytoplasm. Possibly not from *Phytophthora cinnamomi*; **C & G, D & H.** Many spores present in root material of which some small spores are confirmed as *Phytophthora cinnamomi* by single fluorescent areas in the cells. Bars = 30 µm.

3.4 Discussion

Thick-walled chlamydospores can be produced *in vitro* on culture medium (Ribeiro's) or in plant material (lupin roots). Thick-walled chlamydospores were sporadically produced by *P. cinnamomi* on Ribeiro's medium possibly due to nutrient depletion in older cultures and this requires further investigation.

The developmental sequence of the thick-walled chlamydospores differed from previous reports in the literature. Hemmes and Wong (1975) found that, *in vitro*, the chlamydospore wall is thin (approximately 0.2 μm) becoming thicker (0.5 μm – 0.6 μm) with time (2 weeks) after the basal septum has been formed (Hemmes and Wong 1975). Hegnauer and Hohl (1978) also presented similar findings that the outermost layers of chlamydospore walls are much thicker than the layers of hyphal wall from which they develop. In the experiment using modified Ribeiro's minimal medium, the thickness of the chlamydospore walls appeared to be related to the thickness of the hyphae from which they developed. This has not been shown previously and further work is required on their formation.

Thick-walled chlamydospores of *P. cinnamomi*, often attached to thick-walled mycelium, were also seen on mycelium growing in pea agar with the addition of potting mix, *A. pulchella* and *B. grandis* leachates. The result was not repeatable so the factor that triggered this response could not be determined. However, imposition of conditions that were considered adverse to the growth of the pathogen such as a reduction in medium osmotic potential, temperature variations, a drop in medium nutrients (pea agar to water agar) and liquid culture did not contribute to the production of thick-walled chlamydospores under sterile conditions *in vitro*. It is thought that a sudden change in environment induced by the introduction of different biological or chemical compounds

may have resulted in production of thick-walled chlamydospores. This has been considered a response to adverse environmental factors (Mircetich and Zentmyer 1967) but further work is required to understand the environmental factors influencing thick-walled chlamydospore production.

Chlamydospores with thickened walls were abundant in lupin roots in Bassendean sand leachate but not in lupin roots in jarrah vegetation Q red loam. This could be due to microbial and chemical differences between the soils. Thick-walled spores could not be germinated on NARPH for identification. Subsequent use of the GFP-transformed isolates could not confirm that the chlamydospores recorded as 'thick-walled' were *P. cinnamomi*. It is possible that they were from *P. cinnamomi* but the Green Fluorescent Protein was not synthesised due to these thick-walled chlamydospores being lysed; alternatively, these spores may have been from another fungal species.

The production of two types of chlamydospores with different fluorescent patterns has not been previously recorded. Larger spores (around 30 μm in diameter) had numerous fluorescent patches whereas smaller spores (less than 20 μm in diameter) had only one fluorescent area. Further work is needed to investigate these different spores and how they contribute to survival of the pathogen. It is possible that the fluorescent patches observed were storage vacuoles (Hemmes 1983), chlamydospore size affecting the number present. Thin-sectioning is required to investigate whether the patches were storage vacuoles. Although the GFP-transformed isolate did not highlight any thick-walled chlamydospores of *P. cinnamomi*, their presence in the soil environment cannot be ruled out as this was only

a preliminary experiment into the search for solid evidence of thick-walled chlamydospores of *P. cinnamomi* in nature.

This study suggests that the environment has a major influence on the thickness of chlamydospore walls and spore diameter. It appeared soil type rather than the presence of leachates from a susceptible (*B. grandis* (Shearer and Hill 1989)) or a resistant host (*A. pulchella* (Cahill *et al.* 1989)) influenced chlamydospore wall thickness. However, these results should be treated with caution as spores were not germinated for confirmation of identity. Further work needs to be conducted in this area to determine the effect of microbial composition or nutrient availability of different soil types and how this relates to the suppressive/ conducive nature of a soil as natural soils are known to vary widely with these characteristics (Malajczuk 1983).

Previous experiments observing thick-walled chlamydospores have shown their presence in roots of inoculated plants or soil under non-sterile conditions (Mircetich and Zentmyer 1967; Cother and Griffin 1973). This is the first report of thick-walled chlamydospores being produced under laboratory conditions in the absence of plant roots and soil. The current study also contradicts what has previously been reported regarding the development of thick-walled chlamydospores from thick-walled hyphae. The best prospects for producing thick-walled chlamydospores under native conditions appear to be dependent on soil type. Viability and dormancy of such thick-walled chlamydospores needs to be determined to assess their actual contribution to survival of the pathogen.

CHAPTER 4

In vitro Influence of Phosphite on Chlamydospore Production and Viability of *Phytophthora cinnamomi*

4.1 Introduction

Phosphite is an effective fungicide for the control of *Phytophthora* spp. (Guest and Grant 1991). However, different *Phytophthora* species vary in sensitivity to phosphite and within a species there is a range of sensitivity amongst isolates (Coffey and Bower 1984; Niere *et al.* 1994). For example, Coffey and Bower (1984) showed the ED_{50} for mycelial growth of nine different *Phytophthora* species to range between 5.2 to 224.4 μg ml^{-1}. The *Phytophthora* species that have been found to be most sensitive to phosphite *in vitro* include *P. clandestina, P. cinnamomi* and *P. citricola* (Coffey and Bower 1984). Variation in phosphite sensitivity as assessed by ED_{50} values ranges from 1.7 to 148 μg ml^{-1} on modified Ribeiro's medium within *P. cinnamomi* isolates (Coffey and Joseph 1985; Wilkinson *et al.* 2001b), with A1 mating-type isolates more tolerant than A2 mating-type isolates (Wilkinson *et al.* 2001b).

Phosphite has been shown to inhibit sporangia production (Coffey and Joseph 1985; Wilkinson *et al.* 2001c), zoospore release (Coffey and Joseph 1985; Wilkinson *et al.* 2001c), oospore (Coffey and Joseph 1985) and chlamydospore (Coffey and Joseph 1985; Aberton *et al.* 1999) production in *Phytophthora*. The fungicide inhibits sporulation of *P. cinnamomi* and *P. citricola* at levels (< 10 μg ml^{-1}) too low to affect mycelial growth (Coffey and Joseph 1985; Aberton *et al.* 1999). *In vitro,* less than 3 μg phosphite ml^{-1} was required for 50 % inhibition of sporangial production while 6 μg phosphite ml^{-1} was

necessary to inhibit zoospore release by 50 % of sporangia (produced in the absence of phosphite) (Coffey and Joseph 1985). Wilkinson *et al.* (2001a) observed a significant reduction in zoospore production from infected *Banksia grandis* and *Eucalyptus marginata* seedlings after treatment with 5 – 10 g phosphite L^{-1}.

Chlamydospore production by *P. cinnamomi* was reduced to 50 % with 15 – 44 µg phosphite ml^{-1} (Coffey and Joseph 1985). However, chlamydospores of *P. cinnamomi* and *P. parasitica* produced in the absence of phosphite were remarkably resistant to phosphite as germination was only reduced by 20 % in 1000 µg phosphite ml^{-1} (Coffey and Joseph 1985). Not known was whether chlamydospores produced during phosphite treatment were viable and whether such chlamydospores were able to germinate on media containing phosphite.

In the current study, preliminary experiments on the effect of phosphite on numbers of chlamydospores *in vitro* gave varying results (data not shown). In these experiments, the number of chlamydospores was assessed by examining mycelium about half way between the inoculation point and the edge of the 90 mm Petri dish as this was where unusual mycelial growth in plates occurred at higher phosphite concentrations. Colony morphology, rather than being uniformly tightly packed across the plate, changed to sparse growth on plates with phosphite. The change over was closer to the inoculation point in plates with higher levels of phosphite (Figure 4.1). Variability in chlamydospore numbers was found to be due to a high number of spores in this sparse growth of mycelium. The reason for an increase in chlamydospore numbers across the plate and a change in mycelial density was hypothesised to be due to a change in the medium and its relative phosphite concentration due to the aging of the medium at 25 ºC.

Understanding the efficacy of control using phosphite requires knowledge of how the reproductive propagules of the fungus respond to phosphite. Experiments in this chapter test the hypothesis that chlamydospores produced on a medium with and without phosphite have the same level of viability and percentage germination. It also reports the peak of chlamydospore production at approximately 15 – 25 mm from the inoculation point in a 90 mm Petri dish and tests the further hypotheses that:

- Chlamydospore production is a function of distance of mycelial growth from the inoculum. In this case, the location of the chlamydospores should be the same whether cultures are grown in a 90 mm or 140 mm culture dishes.
- Chlamydospore production is a result of the aging of medium. In this case:
    - i) Mycelium cultured on medium kept at 25 ºC for 32 days before inoculation should develop chlamydospores evenly across the plate rather than concentrated in a band,
    - ii) Cultures grown in liquid medium which is changed frequently should develop chlamydospores distributed evenly through the mycelium.

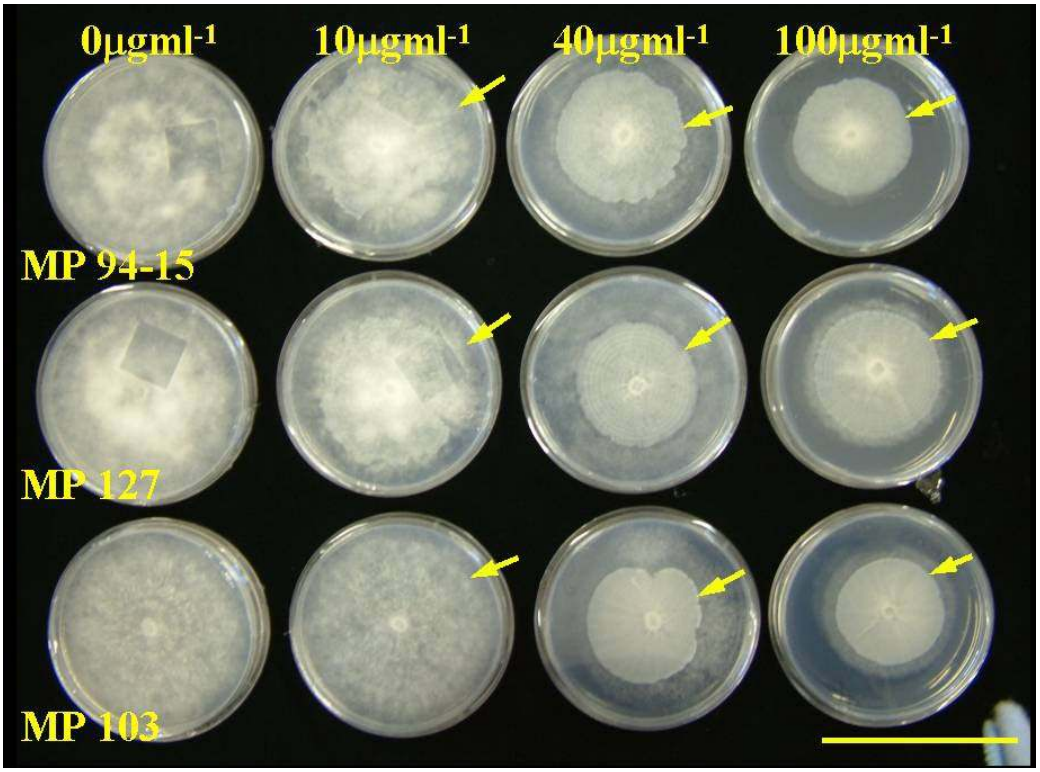

Figure 4.1: Growth of isolates of *Phytophthora cinnamomi* on Ribeiro's medium with 0 – 100 µg phosphite ml^{-1} after 2 weeks growth. Inoculation point is plate centre; arrows indicate where mycelium becomes sparse. Bar = 90 mm

4.2 Materials and Methods

Experimental Design

Phytophthora cinnamomi isolates sensitive (MP94-15, MP127) or tolerant (MP103, MP128) to phosphite (Wilkinson *et al.* 2001b) were used. All were A2 mating type and had been isolated from Western Australia with MP127 and MP94-15 from *Eucalyptus marginata* located in Jarrahdale and Willowdale, respectively. MP128 had previously been isolated from *Xanthorrhoea preisii* in Jarrahdale and MP103 from *Corymbia calophylla* in Huntly. All isolates were passaged through sterile lupin roots and then grown for 2 weeks on modified Ribeiro's minimal medium (Appendix 2) prior to experiments. Phosphite in the experiments was added to the medium prior to inoculation using Fosject 600 (600 g phosphite L^{-1} present as mono-di-potassium phosphite, Unitec Group, Australia) filter

sterilised through a 0.22 µm Millipore filter. Phosphite concentrations used were 0 – 100 µg ml^{-1} after examining results of Wilkinson *et al*. (2001b) who showed the EC_{50} for 11 isolates of *P. cinnamomi* to be 5 – 50 µg ml^{-1} phosphite with almost 100 % inhibition occurring at 160 µg ml^{-1} phosphite. Plates were sealed with plastic (Gladwrap, Australia) for the incubation period. Incubation conditions were 25 ºC in darkness. Preliminary experiments showed no significant ($P > 0.05$) difference between cultures incubated in dark and light for four weeks (data not shown). Chlamydospores were observed under the light microscope at 200x magnification.

Experiments

Germination of chlamydospores produced in the presence of phosphite

Chlamydospores produced on modified Ribeiro's minimal medium with 0 and 100 µg phosphite ml^{-1} from isolates MP103, MP128, MP94-15 and MP127 were used for germination studies. Once formed, chlamydospores were collected by adding sterile water to cover the mycelium before using a scalpel and glass rod to loosen and break up the mycelium to release chlamydospores. The solution was filtered through sterile cheesecloth to remove most of the mycelium. Additional sterile water was used to wash through any remaining chlamydospores. To concentrate the chlamydospores, the solutions were collected in 50 ml Falcon tubes (TPP; Switzerland) and centrifuged at 3800 *g* for 8 minutes. All but approximately 300 µl of the supernatant was discarded and the chlamydospores resuspended in the residual. Chlamydospore concentration was determined by counting chlamydospores in a 5 µl sample of the suspension.

To test the germination of the chlamydospores, three separate 100 µl aliquots of modified Ribeiro's minimal medium containing 0, 100 or 160 µg phosphite ml^{-1} were placed on

microscope slides and approximately 50 chlamydospores from each suspension added. There were three replicates for each isolate for each concentration of phosphite. A preliminary experiment determined that chlamydospore germination percentages can be accurately measured between 10 – 20 hours after incubation at 25 ºC. The experiment was repeated three times using incubation times of 12, 16 and 18 hours.

On the third repeat of the experiment, after determining numbers of germinating and non-germinating chlamydospores, spores were stained with Thiozolyl Blue Tetrazolium Bromide (MTT) (Sigma, Germany). A 0.1% solution of MTT was added to cover the chlamydospores and incubated at 37 ºC for 24 hours in a moist chamber. Control, dead chlamydospores were obtained by autoclaving at 121 ºC for 20 minutes and included. Non-germinated chlamydospores were assessed as viable (black/ blue stained) and dead (magenta or clear).

Distribution of chlamydospore production on the colony

To determine the number of chlamydospores produced in different areas of cultured mycelium, a 5 mm^2 colonised agar block of *P. cinnamomi* was placed in the centre of 12 ml solid modified Ribeiro's minimal medium agar in 90 mm Petri dishes containing either 0, 40 or 100 µg phosphite ml^{-1} of medium; 5 replicate plates were used for each isolate and phosphite concentration. Incubation was 3 weeks at 25 ºC in the dark. To record chlamydospore numbers over the radius of the culture, a strip of agar approximately 5 x 45 mm from the point of inoculation to the edge of the 9 cm Petri dish was removed and placed on a microscope slide. The agar was compressed under a coverslip and the strip was divided into 8 segments (approximately 4 mm wide), noting which end had been closest to

the inoculum source (Figure 4.2). Chlamydospore numbers were counted in each segment twice by scanning along the width of the segment in a straight line before taking an average. In this way, the chlamydospore numbers across the culture radius could be determined. The experiment was repeated twice.

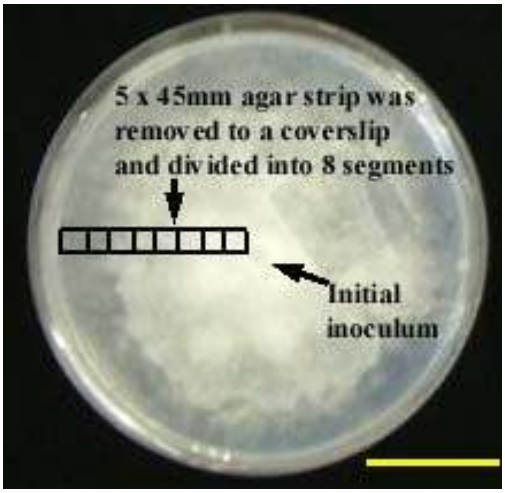

Figure 4.2: Sampling for chlamydospore distribution across a growing culture of *Phytophthora cinnamomi*. Bar = 30 mm.

Effect of medium age on chlamydospore production

Plates of modified Ribeiro's minimal medium with 0, 40 or 100 µg phosphite ml^{-1} were stored for 4 weeks in dark at 25 ºC to create 'aged' medium. Aged plates, along with fresh medium plates, were inoculated and incubated for a further 3 weeks under the same growth conditions. Chlamydospore numbers across the radius were counted as described previously and compared to chlamydospore numbers on plates that had not been aged. The experiment was repeated twice.

Colony growth rate

To determine if the growth rate of *P. cinnamomi* was uniformly affected over the incubation period, colony diameters were recorded over time. A 5 mm^2 cube of mycelium

of *P. cinnamomi* isolate number MP94-15 or MP103 was placed in the centre of a 140 mm Petri dish containing 30 ml of either 0 µg or 100 µg phosphite ml^{-1} modified Ribeiro's minimal medium. Colony diameter was measured every few days until colonies had reached the edge of the plate. Five replicate plates per phosphite concentration and isolate were used.

At the end of the experiment a section of agar, representing the radial length of the colony, was removed from the plate and chlamydospore numbers in different regions of the culture radius was determined as described above. The experiment was repeated.

A comparison of liquid and solid media

To determine if changes in the medium over the period of culture affected chlamydospore numbers, MP103 and MP94-15 were grown on 15 ml of liquid modified Ribeiro's minimal medium with 0 or 100 µg phosphite ml^{-1} without shaking and solid modified Ribeiro's minimal medium with 0 or 100 µg phosphite ml^{-1} in 90 mm Petri dishes and incubated for 4 weeks. Every four days, the liquid from some of the Ribeiro's plates (five for each phosphite concentration and isolate) was removed under aseptic conditions and replaced with 15 ml of fresh modified Ribeiro's minimal medium. The remaining liquid plates and all solid plates were used as controls in the experiment. At the end of the experiment, chlamydospore numbers were counted across the radius of the colonies using a 5 x 45 mm wide strip of mycelium. pH across each of the solid media was determined by removing a 10 mm^2 agar block at 10, 20, 30 and 40 mm distances from the point of inoculation, centrifuging each for 10 minutes at 10600 *g* and adding a 0.1 ml drop of pH Indicator

(range 6.0 – 7.6) (Aquasonic, Australia). This was also performed for non-inoculated control plates. pH of liquid media was also tested as described above.

Statistical Analysis

Germination percentages obtained for each treatment were converted by arcsine transformation into degrees and analysed using Minitab's General Linear Model (Minitab Release 13).

Other data were analysed using Minitab's MANOVA (Multivariate Analysis of Variance) function. Prior to analysis, data were transformed where necessary by square root to satisfy Levene's test for equal variances to determine significance between individual points.

4.3. Results

Germination of chlamydospores produced in the presence of phosphite

Chlamydospores produced in the presence of 100 μg ml^{-1} phosphite in Ribeiro's medium and placed on fresh modified Ribeiro's minimal medium containing 0, 100 or 160 μg phosphite ml^{-1}, showed significantly ($P < 0.05$) lower percentages of germination than control chlamydospores from medium without phosphite (Figure 4.3).

The extent of the response varied between repeat experiments ($P < 0.05$), therefore each experiment was statistically analysed separately (Table 4.1). For isolates MP94-15 and MP127 (all experiments), MP103 (experiment 1 and 3) and MP128 (experiment 2), statistical analysis of the data revealed that phosphite concentration in the germination medium had no affect on the ability of chlamydospores to germinate (Table 4.1). However, the reduction in germination of chlamydospores that had been produced on medium

containing 100 µg phosphite ml^{-1}, compared to those produced in the absence of phosphite, was significant at $P < 0.05$ (Table 4.1).

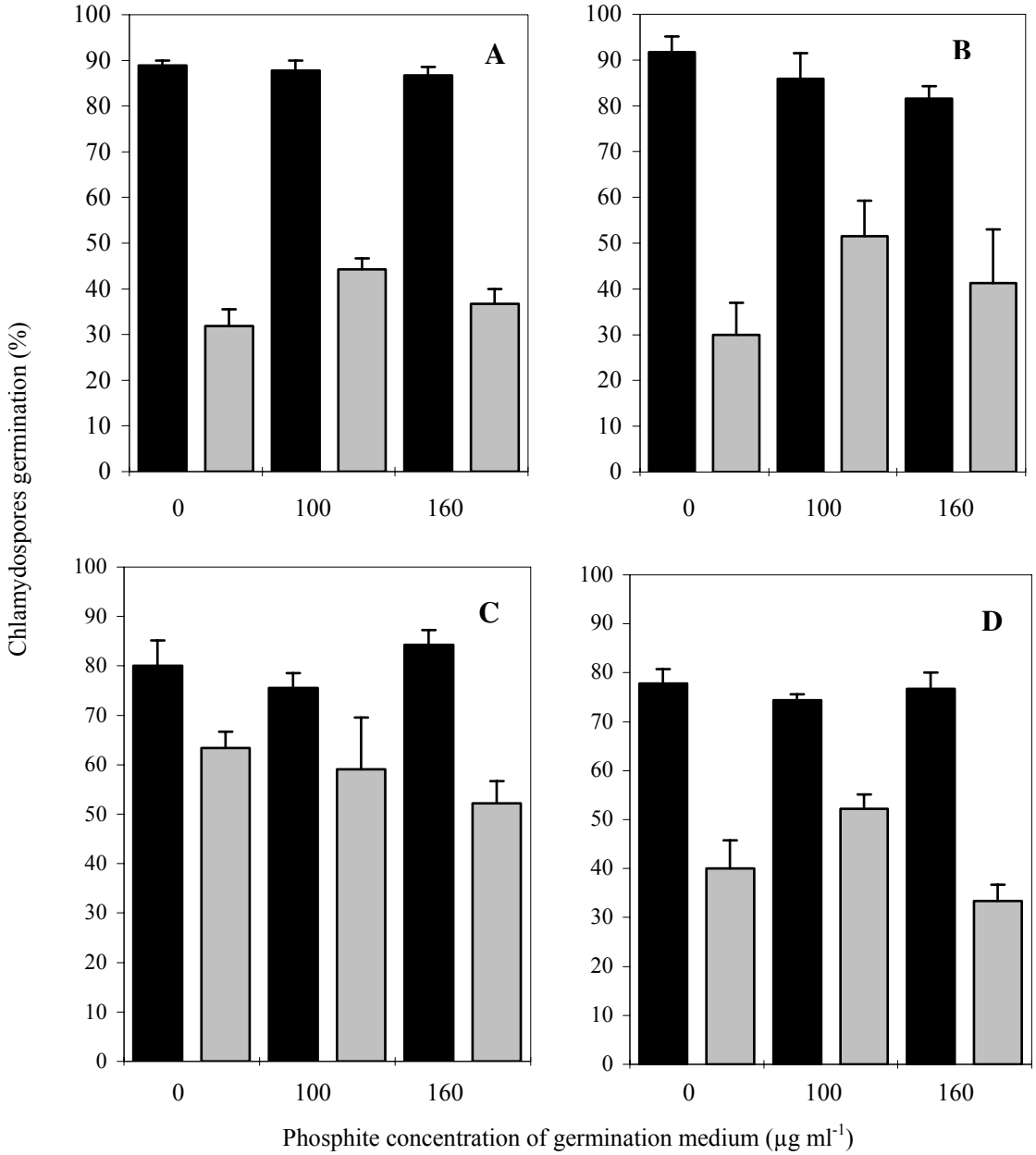

Figure 4.3: Germination of chlamydospores of *Phytophthora cinnamomi* produced on modified Ribeiro's minimal medium with no phosphite (black columns) or 100 µg phosphite ml^{-1} (grey columns) and then exposed to different (0, 100 or 160 µg phosphite ml^{-1}) phosphite concentrations for germination. (A) MP94-15, (B) MP103, (C) MP128, (D) MP127. Data are the means of three experiments. Bars represent positive standard error of the mean.

Table 4.1: ANOVA comparing the effect of phosphite concentration in modified Ribeiro's minimal medium containing 0, 100 or 160 µg phosphite ml^{-1} used to germinate chlamydospores of *Phytophthora cinnamomi* (Germination). Phosphite concentration in germination medium did not affect chlamydospore germination so data was bulked to identify the effect on germination if 100 µg phosphite ml^{-1} had been added to modified Ribeiro's minimal medium when producing chlamydospores (Production). Adjusted Mean Square values are given; degrees of freedom in brackets. Significant values are in bold.

	Experiment 1		Experiment 2		Experiment 3	
Isolate	**Germination**	**Production**	**Germination**	**Production**	**Germination**	**Production**
MP94-15	$MS_{(1,12)}$ = 10849.4	**$MS_{(2,12)}$ = 270.8**	$MS_{(1,12)}$ = 4582.2	**$MS_{(2,12)}$ = 18.26**	$MS_{(1,12)}$ = 629.9	**$MS_{(2,12)}$ = 41.7**
MP103	$MS_{(1,12)}$ = 3965.6	**$MS_{(2,12)}$ = 45.1**	*	*	$MS_{(1,12)}$ = 418.3	**$MS_{(2,12)}$ = 28.0**
MP128	*	*	$MS_{(1,12)}$ = 858.9	**$MS_{(2,12)}$ = 11.8**	**$MS_{(1,12)}$ = 63.6**	**$MS_{(2,12)}$ = 28.3**
MP127	$MS_{(1,12)}$ = 7494.0	**$MS_{(2,12)}$ = 153.7**	$MS_{(1,12)}$ = 1936.3	**$MS_{(2,12)}$ = 33.7**	$MS_{(1,12)}$ = 772.6	**$MS_{(2,12)}$ = 26.1**

* A significant ($P < 0.05$) interaction between produced and germinated found.

Viability of chlamydospores produced on medium containing phosphite

Autoclaved control chlamydospores which were presumed dead, did not stain or rarely stained magenta/ pink (Figure 4.4A). Spores that had already germinated always stained black, often with a trace of blue (Figure 4.4H). Occasionally, germinated chlamydospores stained magenta/ pink while their mycelium was black (Figure 4.4E & I). Spores that had degenerating cytoplasm always stained magenta or pink (Figure 4.4C & F). From these observations, it was concluded that chlamydospores that appeared intact and full of cytoplasmic contents but stained magenta were non-viable. Non-germinated chlamydospores were thus categorised as either viable (black or blue) or non-viable (clear, pink or magenta) (Figure 4.4).

As 70 – 95 % of chlamydospores produced on the control medium germinated, there were few non-germinated spores remaining from which viability was assessed. For two isolates

(MP103 and MP128), there was no significant ($P > 0.05$) difference in viability of non-germinated spores from media with or without phosphite (Figure 4.5), but from isolates MP94-15 and MP127, there was a much higher ($P < 0.05$) percentage of viable non-germinated spores amongst those produced on phosphite medium (Figure 4.5).

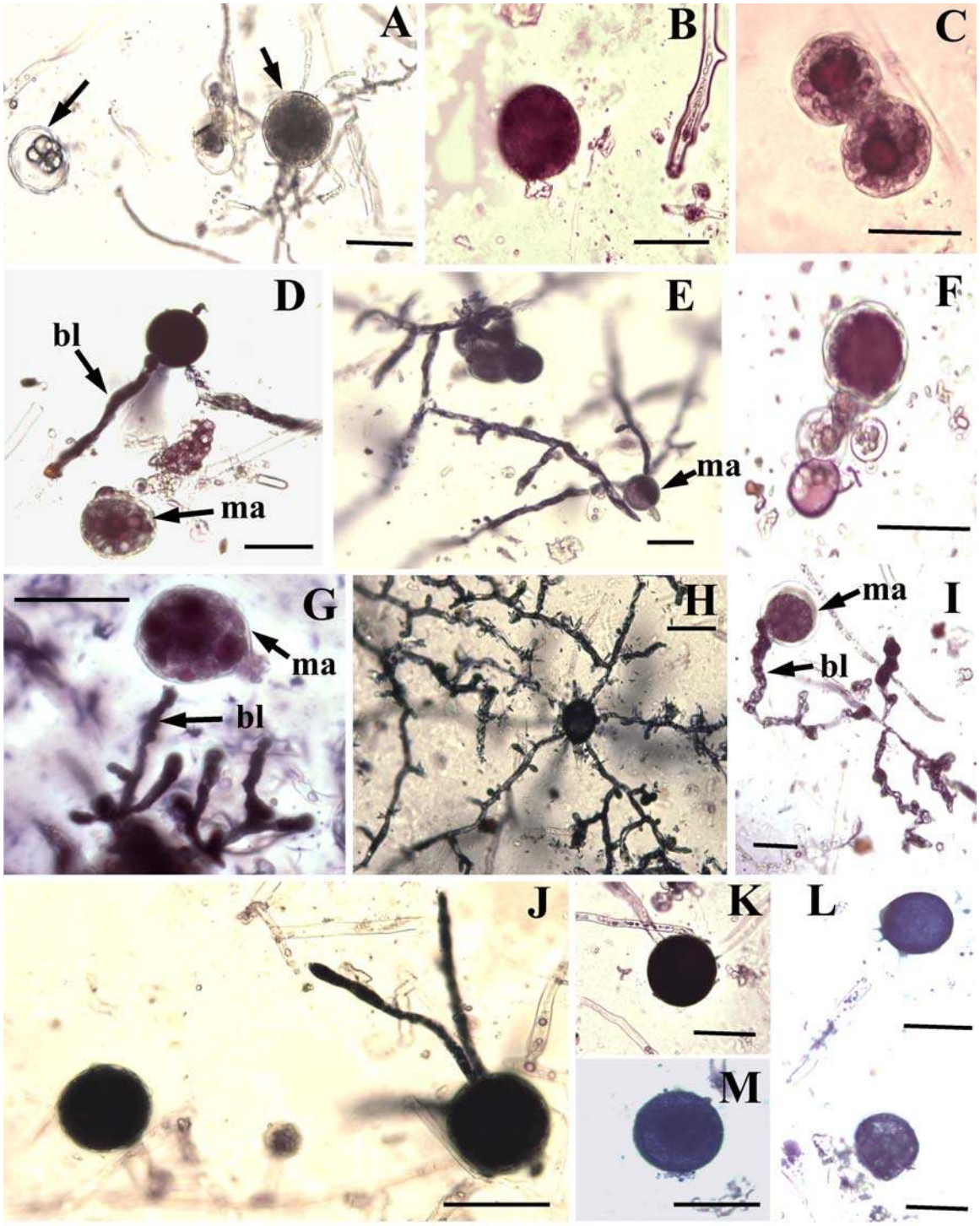

Figure 4.4: Spores and hyphae of *Phytophthora cinnamomi* stained with Thiozolyl Blue Tetrazolium Bromide (MTT). **A.** Autoclaved control, no stain uptake (arrows); **B.** Intact chlamydospore with full cytoplasmic contents stained magenta; **C & F.** Collapsing chlamydospores stained magenta/ pink; **D & G.** Pink, non-viable chlamydospore (ma) and black viable hyphae (bl); **E & I.** Black stained viable hyphae (bl) with magenta stained chlamydospore (ma); **H.** Black stained germinated chlamydospore; **J.** Black non-germinated chlamydospore next to germinated, black chlamydospore; **K.** Black, non-germinated chlamydospores; **L & M.** Blue, non-germinated chlamydospores. Bar = 40µm.

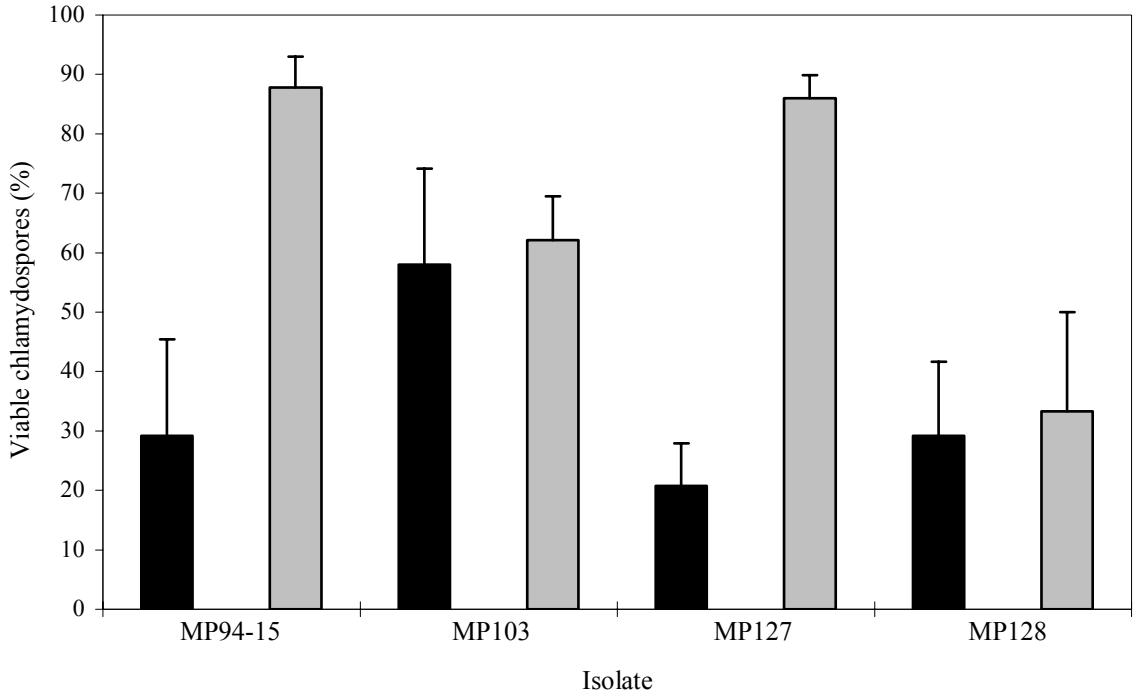

Figure 4.5: Percentage of viable chlamydospores amongst non-germinated chlamydospores of *Phytophthora cinnamomi* on modified Ribeiro's minimal medium with 0, 100 and 160 µg phosphite ml^{-1} as assessed by Thiozolyl Blue Tetrazolium Bromide (MTT) staining. Phosphite concentration (0, 100 or 160 µg ml^{-1}) of the germination medium had no significant ($P > 0.05$) effect so data were bulked to compare only viability of non-germinated spores that had been produced on no phosphite (black columns) to viability of non-germinated spores that had been produced on 100 µg phosphite ml^{-1} (grey columns). Bars represent the standard error of the mean.

Distribution of chlamydospore production on the mycelium and the effect of medium age

Control 90 mm diameter plates (with no phosphite) had an even distribution of chlamydospores across the radius of their growth, and no significant ($P > 0.05$) difference in chlamydospore numbers was found at any point across the radial length of the colony.

Phosphite initially inhibited chlamydospore production. There was a significant ($P < 0.05$) reduction for isolates MP94-15 and MP103 but although the trend was evident, the reduction was not significantly ($P > 0.05$) different for isolates MP127 and MP128. However, where the mycelium changed its growth morphology from tightly packed mycelia to sparse (approximately 20 mm; Figure 4.1), chlamydospore numbers

significantly ($P < 0.05$) increased on both concentrations of phosphite until the edge of the colony where numbers in most isolates dropped. Chlamydospore numbers increased closer to the initial inoculum source on 100 μg phosphite ml^{-1} than on 40 μg phosphite ml^{-1} (Figure 4.6). Media containing phosphite and stored for 4 weeks prior to use produced a significantly ($P < 0.05$) higher number of chlamydospores across the plate for all phosphite treatments compared to the media inoculated immediately after it was made (Figure 4.6). However, there was no consistent difference from fresh plates in the distance from the inoculum to the region of high chlamydospore production.

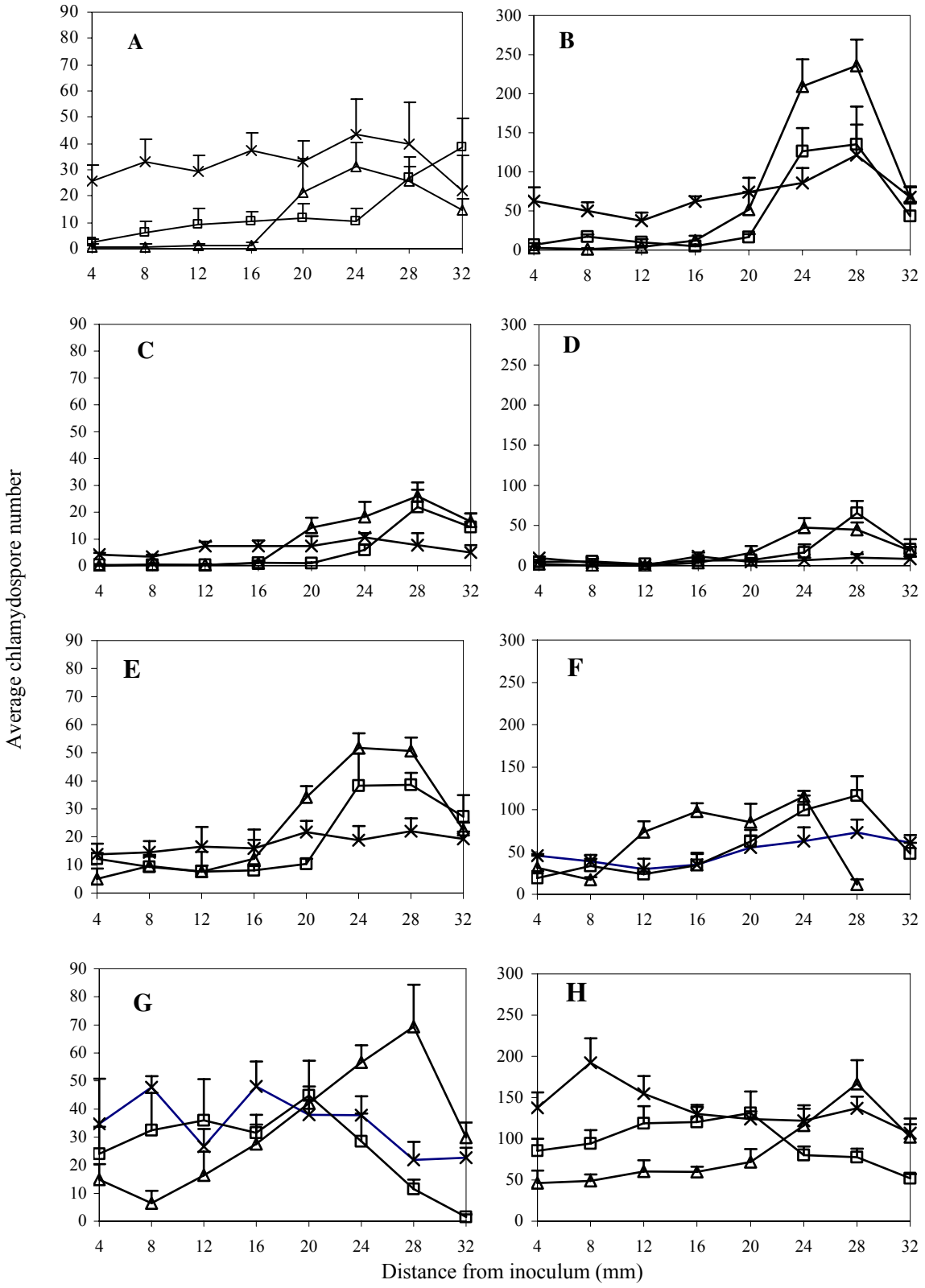

Figure 4.6: Mean chlamydospore numbers of *Phytophthora cinnamomi* across the radius of cultures grown on 0 (x); 40 (□) and 100 (Δ) µg phosphite ml^{-1}. Isolate MP94-15 (**A-B**), MP103 (**C-D**), MP127 (**E-F**), MP128 (**G-H**). **A, C, E, G.** Fresh media; **B, D, F, H.** Stored media (Vertical scales differ between graphs for fresh and stored media). Bars represent the positive standard error of the mean.

Large numbers of oospores were consistently produced by isolate MP128 on no phopshite under the conditions of the experiment but were rare in the other isolates. Oospore production is discussed further in Chapter 5.

On 140mm Petri dishes, the production of chlamydospores on 100 µg phosphite ml^{-1} showed a similar pattern to that seen in the smaller 90 mm plates with a peak 52 – 56 mm from the inoculum (Figure 4.7). The MP94-15 control also showed a slight increase in chlamydospore numbers at 56 mm but the increase was not significant ($P > 0.05$) compared to the increase seen in plates containing phosphite. The distance from the inoculum at which a significant ($P < 0.05$) increase in chlamydospore numbers was observed was greater on the larger plates, with isolate MP103 responding at a greater distance than isolate MP94-15.

The growth rate of the two isolates in response to phosphite was the same in the two repeat experiments ($P > 0.05$) so the data were combined for further analysis. Growth of *P. cinnamomi* on 100 µg phosphite ml^{-1} was slower than on the no phosphite medium (Figure 4.8). Cultures on the medium with phosphite grew slowly until day 2 or 3 when the growth rate began to accelerate until approximately day 30 or 32 when the mycelium reached the edge of the plate and growth rate stopped (Figure 4.8). Colony morphology was similar to that shown in Figure 4.1 for 0 and 100 µg phosphite ml^{-1}.

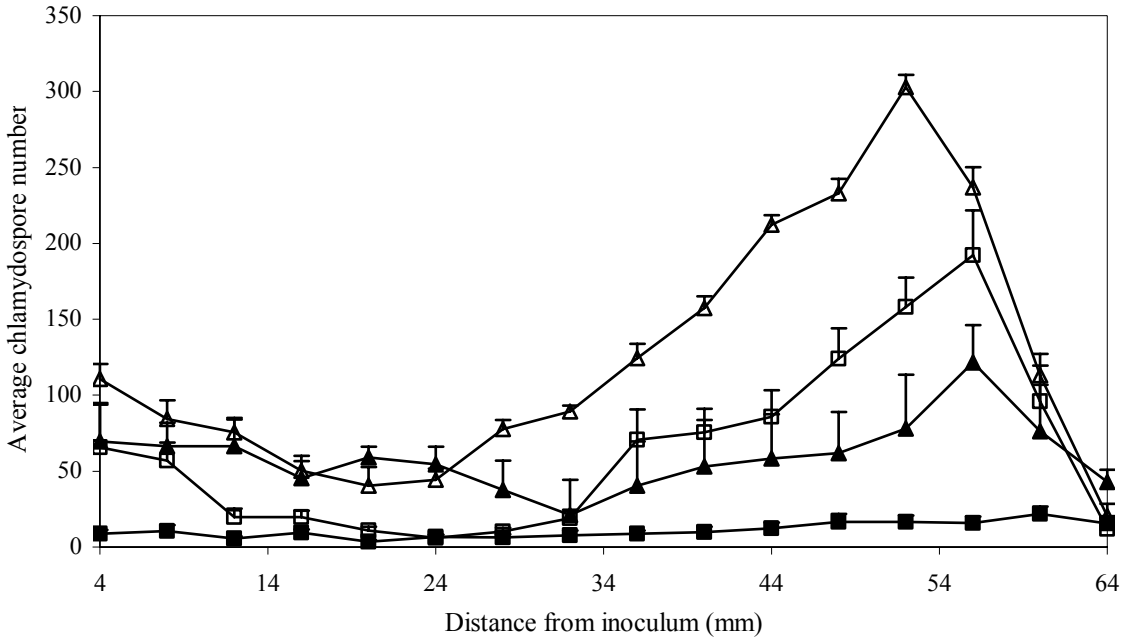

Figure 4.7: Effect of 0 and 100 µg phosphite ml^{-1} on average chlamydospore numbers of *Phytophthora cinnamomi* across the radius of cultures on 140 mm plates of modified Ribeiro's minimal medium. (▲) MP94-15 Control; (Δ) MP94-15 100 µg phosphite ml^{-1}; (■) MP103 Control; (□) MP103 100 µg phosphite ml^{-1}. Bars represent positive standard error of the mean.

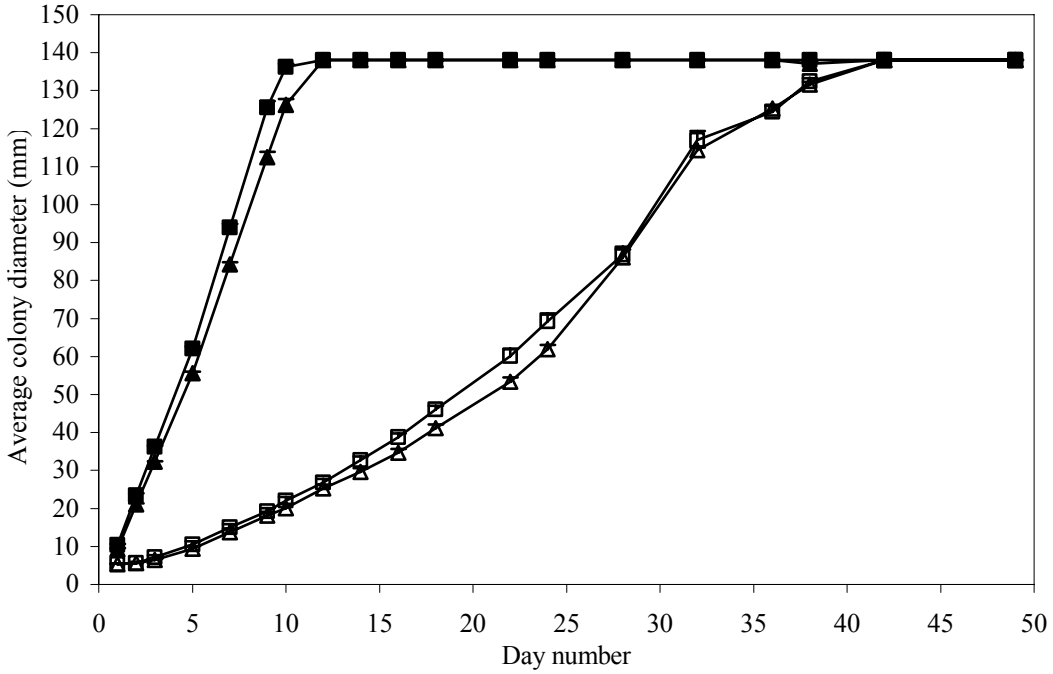

Figure 4.8: The effect of 0 and 100 µg phosphite ml^{-1} on colony growth of *Phytophthora cinnamomi* over time. (▲) MP94-15 Control; (Δ) MP94-15 100 µg phosphite ml^{-1}; (■) MP103 Control; (□) MP103 100 µg phosphite ml^{-1}. Bars represent positive standard error of the mean.

A comparison of liquid and solid media

Chlamydospore numbers for cultures that were incubated on modified Ribeiro's minimal liquid or solid medium without replacement differed across the radius of the plate depending whether 0 or 100 µg phosphite ml^{-1} was used (Figure 4.9; Table 4.2). The trends observed were similar to those described earlier. Briefly, from approximately 20 mm from the initial inoculation point, chlamydospore numbers on modified Ribeiro's minimal medium containing 100 µg phosphite ml^{-1} increased rapidly to numbers higher than plates containing no phosphite. This point corresponded to a change in colony morphology for cultures on the medium containing 100 µg phosphite ml^{-1}.

When the liquid medium was changed every 4 days, no significant ($P > 0.05$) difference between chlamydospore numbers at 0 and 100 µg phosphite ml^{-1} was found at any point of the radius of the culture at the end of 4 weeks incubation for both MP94-15 and MP103. Numbers of chlamydospores remained similar across the radius of each culture (Figure 4.9). Plates which received new medium every 4 days had continuous dense mycelium in plates with 100 µg ml^{-1} phosphite. pH of the medium was between 6.4 and 7.0 for all plates, irrespective of the presence of 100 µg phosphite ml^{-1}.

Table 4.2: MANOVA of control (non-replenished) medium for Figure 4.9. Differences are compared between chlamydospore numbers of *Phytophthora cinnamomi* produced on 0 and 100 µg phosphite ml^{-1}, liquid and solid Ribeiro's medium. (A) MP94-15 and (B) MP103. Adjusted Mean Square values given, treatment and error degrees of freedom in brackets. Significant values are in bold.

Distance from inoculum (mm)	MP94-15: Solid media. Comparing 0 and 100 µg phosphite ml^{-1}	MP94-15: Liquid media. Comparing 0 and 100 µg phosphite ml^{-1}	MP103: Solid media. Comparing 0 and 100 µg phosphite ml^{-1}	MP103: Liquid media. Comparing 0 and 100 µg phosphite ml^{-1}
4	**$MS_{(1,5)} = 73.31$**	$MS_{(1,5)} = 2.37$	$MS_{(1,7)} = 3.45$	**$MS_{(1,6)} = 15.66$**
8	**$MS_{(1,5)} = 82.52$**	**$MS_{(1,5)} = 38.78$**	$MS_{(1,7)} = 0.00$	**$MS_{(1,6)} = 40.67$**
12	$MS_{(1,5)} = 37.13$	$MS_{(1,5)} = 1.75$	$MS_{(1,7)} = 0.67$	**$MS_{(1,6)} = 57.44$**
16	**$MS_{(1,5)} = 91.01$**	$MS_{(1,5)} = 7.71$	$MS_{(1,7)} = 0.27$	**$MS_{(1,6)} = 27.03$**
20	$MS_{(1,5)} = 7.14$	**$MS_{(1,5)} = 48.09$**	$MS_{(1,7)} = 8.35$	$MS_{(1,6)} = 7.02$
24	**$MS_{(1,5)} = 110.35$**	**$MS_{(1,5)} = 63.19$**	$MS_{(1,7)} = 16.30$	$MS_{(1,6)} = 8.87$
28	**$MS_{(1,5)} = 180.37$**	**$MS_{(1,5)} = 63.45$**	**$MS_{(1,7)} = 34.66$**	$MS_{(1,6)} = 17.83$
32	**$MS_{(1,5)} = 27.46$**	**$MS_{(1,5)} = 137.61$**	$MS_{(1,7)} = 11.53$	**$MS_{(1,6)} = 155.82$**

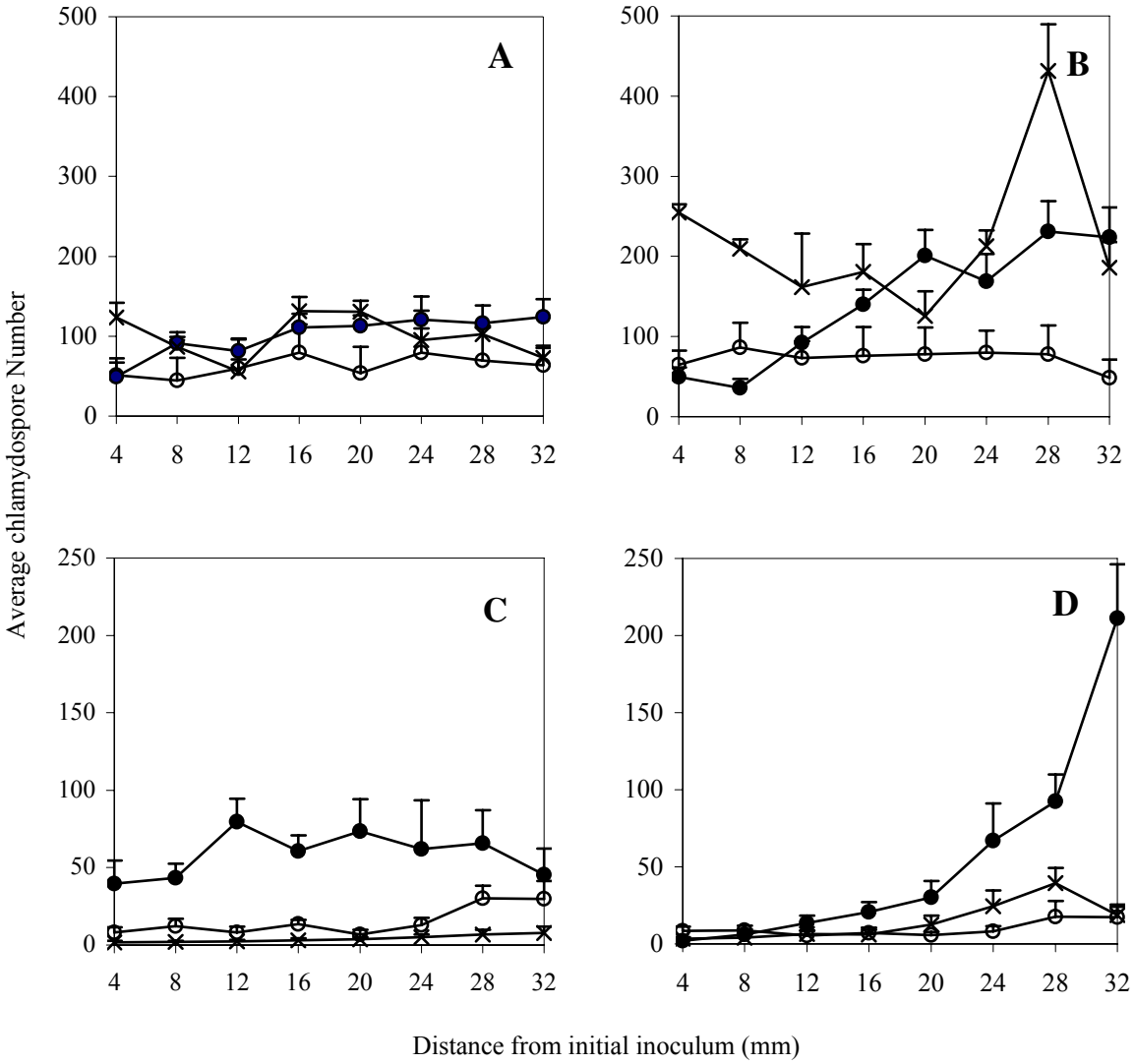

Figure 4.9: The effect of medium age and phosphite on the position of chlamydospore production on *Phytophthora cinnamomi* mycelium. **A & C.** No phosphite; **B & D.** 100 µg phosphite ml^{-1}. **A & B.** Isolate MP94-15; **C & D.** Isolate MP103. (○ – Liquid medium replaced with fresh medium every 4 days, ● – Liquid medium control, x – Solid medium control). NB. Vertical scales differ. Bars represent the standard error of the mean.

4.4 Discussion

The presence of phosphite in the germination medium had no affect on the percentage germination of chlamydospores as has been reported by Coffey and Joseph (1985). However, on germination medium amongst non-germinated chlamydospores that had previously been produced on medium containing phosphite, there was a high level of viability indicating that phosphite induces dormancy in chlamydospores. A wider range of isolates need to be screened to determine the frequency of isolates in which this occurs. Management of *P. cinnamomi* in diseased areas by use of phosphite must consider the possibility of induction of dormant chlamydospores.

Phosphite was found to cause significant dormancy in chlamydospores for isolates previously reported as sensitive to phosphite (MP94-15 and MP127; Wilkinson *et al.* 2001b) and not those reported as tolerant (MP128 and MP103; Wilkinson *et al.* 2001b). The induction of dormancy could be due to the accumulation of pyrophosphate and polyphosphate which has previously been found to be contributing to the inhibition of mycelial growth of *Phytophthora* spp. in the presence of phosphite (Niere *et al.* 1994). This growth inhibition may vary across isolates depending on their sensitivity to phosphite. Further investigations should determine the relationship between phosphite sensitivity and the induction of chlamydospore dormancy. The growth rate of mycelium from chlamydospores germinating on the medium containing phosphite was not determined and future studies should assess this and whether chlamydospores produced in the presence of phosphite, produce mycelium less inhibited by phosphite.

Phytophthora cinnamomi chlamydospores were not produced evenly over the mycelial mat *in vitro.* Chlamydospore development was initially inhibited by the presence of 40 or 100

µg phosphite ml^{-1} but numbers were significantly higher than on the control medium without phosphite at about the point when the mycelia changed in density from tightly packed to sparse. This point was approximately 20 – 24 mm from the initial site of inoculation for isolates on the medium with 40 µg phosphite ml^{-1} and 8 – 20 mm for isolates on the medium with 100 µg phosphite ml^{-1} in 90 mm Petri dishes containing 12 ml medium. On 140 mm Petri dishes that contained 30 ml medium and 100 µg phosphite ml^{-1}, chlamydospore production was stimulated at approximately 28 - 32 mm from the initial inoculation point. This indicated that the increase in chlamydospore number was not a function of length of growth from the initial inoculum and that it only occurred in the medium containing phosphite. There have been previous reports of inhibition of chlamydospore production in the presence of phosphite (Coffey and Joseph 1985; Aberton *et al.* 1999) but the stimulation of chlamydospore production by phosphite has not been previously observed. It is possible that with time, phosphite is stressing the pathogen which then enters a survival mode (chlamydospores).

The inhibitive effect of phosphite on mycelial growth is clearly indicated by the comparison of growth curves for cultures with and without phosphite and has been recorded previously (Coffey and Bower 1984; Coffey and Joseph 1985; Niere 1994; Komorek *et al.* 1997; Wilkinson *et al.* 2001b). Stimulation of chlamydospore production was observed to occur during the log phase where growth was accelerating. The decline in chlamydospore production towards the outer edge of the plate could be due to the media staling where particular nutrients necessary for chlamydospore production become limiting or waste products have accumulated (Griffin 1994). High concentrations of staling products have been observed to result in cessation of activity and autolysis, reducing mycelium density in *Fusarium oxysporum* (Park 1961) and this might explain why mycelial

density of *P. cinnamomi* on plates with phosphite dropped towards the outer part of the colony.

The typical growth curve observed indicates that phosphite was not breaking down over time. This observation was supported by the fact that when plates with phosphite were stored for 4 weeks at 25 ˚C in the dark prior to inoculating the agar with the pathogen, the distribution of chlamydospores was similar to that recorded on 'fresh' plates. The higher chlamydospore production that was observed occurring on old media versus fresh was probably due to a reduction in water content of the medium resulting in an increased phosphite concentration.

Although pH affects the utilisation of some compounds (Griffin 1994), the pH range of the medium did not vary greatly across the agar plate (6.4 – 7.0) and so it is unlikely this contributed to the variation in chlamydospore production across the colony.

Phosphite is known to induce the production of elicitins in *Phytophthora* spp. (Fenn and Coffey 1984; Khan *et al.* 1986; Perez *et al.* 1995). Elicitins are 'small hydrophilic cysteine-rich proteins' that are able to carry sterols (Ponchet *et al.* 1999). Many biological activities of oomycetes rely on the ability of the proteins to bind sterols (Ponchet *et al.* 1999). Sterols are important in asexual reproduction of *Phytophthora* spp. (Hendrix 1965; Ponchet *et al.* 1999). It is possible that at the point where the mycelium becomes sparse, there is increased elicitin production, trapping available sterols in the medium resulting in a stress on the culture leading to sparse mycelium and increased chlamydospore production. This could be tested by growth of cultures in liquid medium for a period followed by a change in medium to one lacking sterols.

Although phosphite has been shown in these experiments to stimulate chlamydospore production *in vitro*, it only occurred when the medium containing phosphite was allowed to 'stale'. When the medium was refreshed every four days, chlamydospore production was inhibited. In applying this to the natural environment, it is most likely that chlamydospore production would be inhibited by phosphite in situations where leaching and recycling in the soil environment prevents the build-up of staling products and the replacement of nutrients. However, in phosphite treated plants, phosphite accumulates in root tips (Seymour *et al.* 1994; Carswell *et al.* 1996; Fairbanks *et al.* 2000; Jackson *et al.* 2000) and the invading *Phytophthora* may be surrounded by metabolic products that may stimulate chlamydospore production. For example, lowered availability of sterols due to elicitin production may trigger chlamydospore production.

Chlamydospore production, germination and dormancy *in planta* as a response to phosphite needs to be checked against these *in vitro* observations. The concern is that if dormant spores are induced, they may provide a large inoculum source. When conditions become favourable for pathogen growth, or when phosphite is depleted in the host, they may provide the means for a disease outbreak under warm and wet conditions known to be optimal for *P. cinnamomi*. However, the ability of the dormant spores to withstand desiccation and attack from other soil microorganisms and how dormancy of these spores is broken needs to be determined.

CHAPTER 5

Production and Viability of Selfed Oospores by *Phytophthora cinnamomi*

5.1 Introduction

Phytophthora cinnamomi is generally thought to be heterothallic, requiring both mating types for sexual reproduction (Galindo and Zentmyer 1964; Reeves and Jackson 1972; Zentmyer 1980; Weste 1983). Single isolate cultures of *P. cinnamomi* have been observed to produce selfed oospores *in vitro* (Zentmyer 1952; Stamps 1953; Brasier 1971; Reeves and Jackson 1972; Reeves and Jackson 1974; Brasier 1978; Ko 1978) and in non-sterile situations (Mircetich and Zentmyer 1967; Reeves 1975; Zentmyer 1979). However, in all these cases, viability of the oospores was not determined; so their contribution to survival of *P. cinnamomi* remains unknown. Selfing may be a reason for the variation observed in pathogenicity, morphology and physiology in A2 isolates as a result of the rarity of the A1 type in Australia (Reeves and Jackson 1972; Hüberli *et al.* 1997).

Fungicides including Ridomil 2E (metalaxyl; phenylamide), Ridomil Gold EC (mefenoxam; phenylamide), Maneb (maneb; dithiocarbamate), Manzate (mancozeb; dithiocarbamate), Curzate (cymoxanil + mancozeb; acetimide/ dithiocarbamate) and Acrobat Mz (dimethomorph + mancozeb; morpholine/ dithiocarbamate) have been observed to induce oospores in single-isolate cultures of the normally heterothallic *P. infestans,* with viability percentages ranging between 4.9 – 52.3 % (Groves and Ristaino 2000). Strawberry roots containing oospores of *P. fragariae* were exposed to 1000 μg ml^{-1} solutions of fungicides including captafol, dichlofluanid, phosphite and metalaxyl for 60 days (Duncan 1985b). Only phosphite killed oospores (14.4 %) within roots. In addition,

reduced oospore germination on agar was observed amongst oospores from phosphite and metalaxyl treated roots (Duncan 1985b). *Phytophthora fragariae* oospores were clearly capable of surviving fungicidal treatment in nursery stock and the residue of the fungicides on roots tested for infection could suppress oospore germination resulting in false negative results (Duncan 1985b).

In vitro, almost complete inhibition (89 – 97 %) of oospore production (produced using opposite mating types) was observed for *P. citricola* in as little as 1 µg phosphite ml^{-1} whereas *P. cinnamomi* tolerated 50 µg ml^{-1} before similar inhibition in oospore development occurred (Coffey and Joseph 1985; Bunny 1996). Since phosphite is a widely used control technique for *P. cinnamomi* in Australia (Hardy *et al.* 2001), it is important to determine the effect of this fungicide on selfed oospores.

During phosphite trials (Chapter 4), selfed oospores were observed in single isolate cultures of *P. cinnamomi* on modified Ribeiro's minimal medium. Experiments were then set up to study the effect of light and dark, liquid versus solid media and phosphite on selfed oospore production and viability of *P. cinnamomi*.

5.2 Materials and Methods

Experimental Design

Oospore formation was observed in single isolate cultures of *P. cinnamomi* (MP103, MP94-15 and MP128) on modified Ribeiro's minimal medium (Chapter 4). Oospores were consistently produced in high numbers for isolate MP128 and this was used for further study. Before use, the culture was passaged through sterile lupin roots. Sterile lupins were prepared as described in Chapter 2.2. To inoculate seven-day-old lupins, a small cut was made in the root tip on which a 5 mm^2 cube of mycelium from a one-week-old culture of *P. cinnamomi* growing on modified Ribeiro's minimal medium (Appendix 2) was placed. After one week incubation at 25 ºC under cool white fluorescent light, the edge of the lesion that had formed was excised and placed on a NARPH plate (Appendix 2) before transferring to a fresh modified Ribeiro's minimal medium plate after 3 days. The isolate was confirmed as *P. cinnamomi* by PCR and sequencing (data not shown). Cultures were always incubated at 25 ºC. General observations of oospores were made at 400x magnification, including number and type of antheridia.

Experiments

Light and dark influences on selfed oospore production

Modified Ribeiro's minimal medium agar plates were inoculated with MP128 and incubated in darkness or under cool white fluorescent light for 3 and 6 weeks using a replication of 5 for each treatment in a completely randomised design. The methods described in Chapter 4 were used to record the differences in oospore numbers across the radius of the plate. Numbers were bulked for each plate. Oospore diameters were also recorded. The experiment was repeated twice.

Liquid versus solid media on selfed oospore numbers

Phytophthora cinnamomi (MP128) was grown on either liquid or solid modified Ribeiro's minimal medium in the dark for 6 weeks using a replication of 5. Oospore numbers were counted as described previously along the radius of the culture. To collect mycelium from liquid cultures, a 5 mm wide strip of mycelium was excised along the length of the radius. The experiment was repeated once.

Phosphite effect on selfed oospore numbers

P. cinnamomi isolates (MP94-15, MP128, MP127 and MP103) were grown on solid modified Ribeiro's minimal medium containing 0, 20, 40, 60, 80 and 100 µg phosphite ml^{-1} and incubated in darkness for 4 weeks, using 5 replicate plates for each isolate and phosphite concentration. Oospore numbers were counted as described in the previous section. The experiment was repeated.

Determining viability of selfed oospores

Selfed oospores of *P. cinnamomi* were produced on solid modified Ribeiro's minimal medium that had been incubated in darkness for 6 weeks. An agar square (approximately 15 mm^2) was removed from cultures growing on modified Ribeiro's minimal medium with 0, 60 or 100 µg phosphite ml^{-1}, approximately 5 mm away from the original inoculation site. Four replicate plates were used for each isolate and phosphite concentration. The agar square was squashed on a microscope slide and covered by a 0.1 % solution of Thiozolyl Blue Tetrazolium Bromide (MTT) (Sigma, Germany) combined with 2.8 mM CoCl (Meier and Charvat 1993). Oospores were incubated at 37 ºC for 48 hours in a moist chamber.

The control consisted of dead oospores that were obtained by autoclaving at 121 ˚C for 20 minutes. Oospores were assessed as alive (magenta), dead (black or clear without a visible ooplast) or indeterminate (clear with a visible ooplast).

Germination of selfed oospores

Nuclei numbers in each oospore were assessed using the fluorescent stain 4′, 6-diamidino-2-phenylindole.2HCl (DAPI) (product number D9542, Sigma, Australia) dissolved in McIlvane's buffer (0.1M citric acid, 0.2M dibasic sodium phosphate, pH 5.5) (Crane *et al.* 2000). Stained material was then observed for nuclei using a fluorescence microscope (Olympus BX51; Olympus, Australia) at excitation wavelength 350 nm. If nuclear fusion had occurred, oospores were considered ready to germinate (Ann and Ko 1988; Jiang *et al.* 1989; Jiang and Erwin 1990).

Oospores were collected from cultures 28, 84 and 91 days old on modified Ribeiro's medium by grinding the mycelium and agar in a sterile mortar and pestle with approximately 5 ml sterile water. The mixture was filtered through sterile cheesecloth and washed with a further 5 ml of sterile water. The filtrate was centrifuged at 2700 *g* for 5 minutes, the supernatant discarded and the oospores resuspended in 200 µl sterile water. Four replicates were used for each culture age. Oospores and mycelia were stained for 30 minutes with several drops of a 0.2 µg DAPI ml^{-1} as above.

To assess whether oospores were capable of germinating on different media, oospores not used in the DAPI staining nuclei test were used. The solution was frozen at -20 ºC for 45 minutes to kill any mycelial fragments or chlamydospores remaining in the suspension

which has previously been shown not to kill oospores (Cohen 1984). The solution was brought back to room temperature and oospore number determined in 1 µl of solution under light microscopy. Approximately 100 oospores were spread across plates containing either NARPH (Appendix 2), Malt Extract agar (2 % Malt extract [Bacto, Australia], 1.5 % [w/v] agar [BBL, Australia]), ½ Potato Dextrose agar (1.95 % [w/v] Potato dextrose agar [BBL, Australia], 0.75 % [w/v] agar [BBL, Australia]), clarified V8 agar (Appendix 2), modified Ribeiro's minimal medium (Appendix 2), water agar (1.5 % [w/v] agar [BBL]) or S+L medium (Ruben *et al.* 1980) (Appendix 2). In a repeat experiment, prior to placement of oospores on S+L medium, oospores were treated with 0.2 % (w/v) $KMnO_4$ for 15 minutes in an attempt to stimulate germination (Ann and Ko 1988). Before treatment with $KMnO_4$, oospores undergoing this treatment were checked for nuclear fusion using DAPI staining. Numbers of nuclei using DAPI staining were counted after $KMnO_4$ treatment to assess nuclear fusion. All plates were incubated under cool white fluorescent light for 60 days and oospores assessed weekly for germination.

Statistical Analysis of Data

Minitab's (Minitab Release 13) 2-sampled t-test was run to compare separately, the effect of incubation time and light and dark on oospore production. To compare the effect of phosphite concentration, an ANOVA was run for all isolates before 2-sampled t-tests were used to determine individual significance. To determine the significance of percentage of viable oospores at different phosphite concentrations, data were arcsine transformed and an ANOVA was run to compare between experiments.

A binary logistic regression was run to determine whether any difference was observed between nuclei present in differently aged cultures. An ANOVA (general linear model)

was conducted to compare the differences in number of nuclei present in oospores before and after treatment with $KMnO_4$.

5.3 Results

General oospore morphology

All oospores had amphigynous antheridia (Figure 5.1). Paragynous as well as amphigynous antheridia were present on 0.05 % of oospores (Figure 5.1).

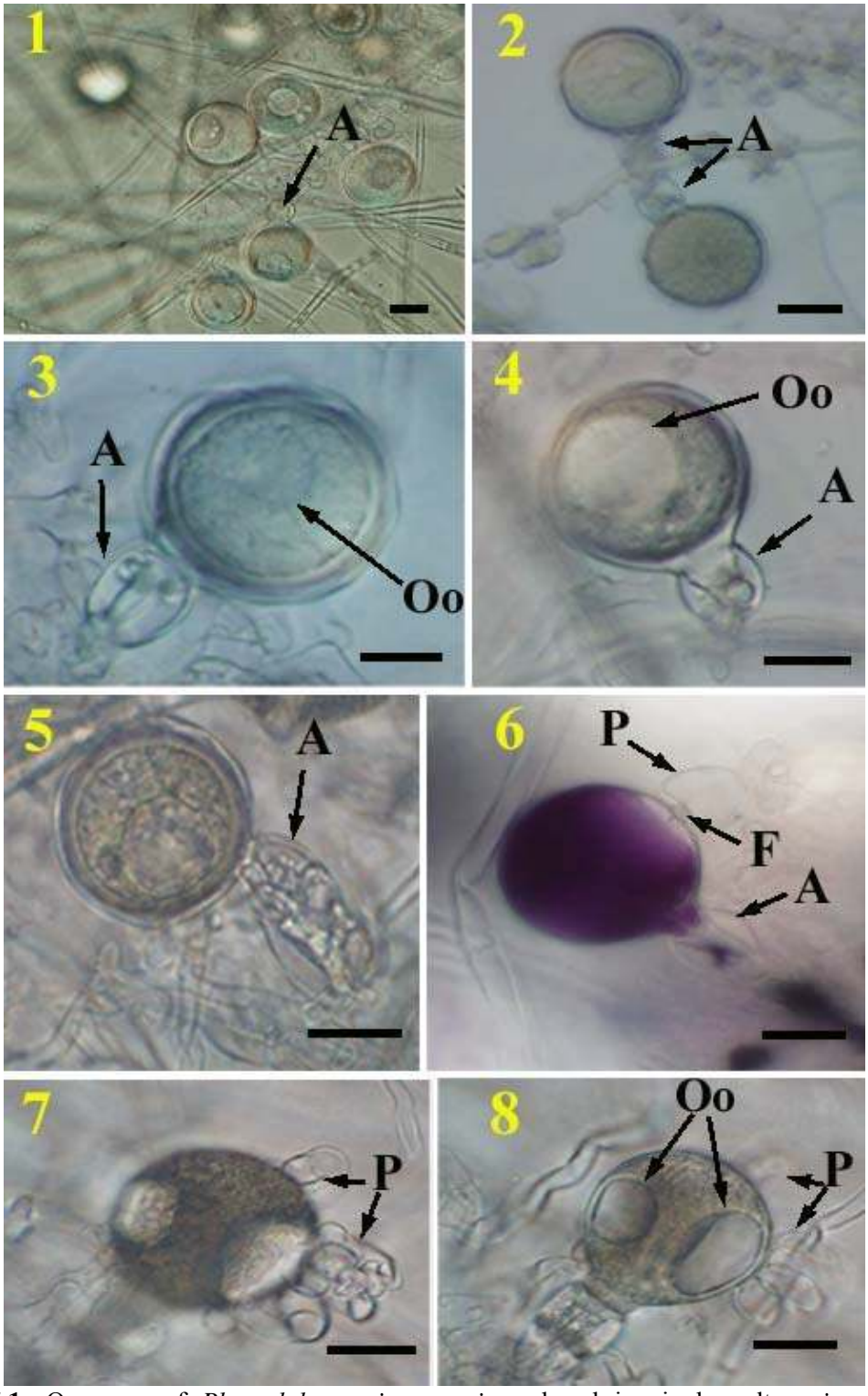

Figure 5.1: Oospores of *Phytophthora cinnamomi* produced in single culture *in vitro*. A, Amphigynous antheridium; F, Fertilisation tube; Oo, Ooplast; P, Paragynous antheridium. **1 - 5.** Oospores with amphigynous antheridium; **6.** Developing viable oospore (magenta stained with MTT), amphigynous and paragynous antheridia present. Note fertilisation tube from paragynous antheridium; **7 - 8.** Same oospore, different focus. At least 5 paragynous antheridia present with amphigynous antheridium. Bar = 20µm.

Light and dark influences on selfed oospore production

No significant ($P > 0.05$) difference was found in oospore numbers when the three isolates were incubated for 3 weeks compared to 6 weeks under light in modified Ribeiro's minimal medium. However, when cultures were incubated in the dark, there was a significant ($P < 0.05$) increase in oospore numbers between 3 and 6 weeks (Mean 7.6 (± 2.3) and 35.5 (± 7.7) oospores cm^{-2}, respectively). It was found that cultures incubated in darkness for 6 weeks, produced significantly ($P < 0.05$) more oospore numbers than those incubated under light (Mean 35.5 (± 7.7) and 2.1 (± 0.9) oospores cm^{-2}, respectively). No significant ($P > 0.05$) difference was found between diameter size of oospores incubated in darkness compared to those cultures incubated under light. Oospore diameter ranged between 28 – 52 μm, mean oospore diameter being 41.0 μm (± 0.2).

Liquid versus solid media on selfed oospore numbers

No oospores were observed on liquid modified Ribeiro's minimal medium in either experiment. An average of 43.8 oospores cm^{-2} (± 5.2) was observed on plates containing solid media.

Phosphite effect on selfed oospore numbers

Selfed oospore numbers were low on the medium at all phosphite concentrations for isolates MP94-15, MP127 and MP103. No significant ($P > 0.05$) difference was found between numbers of oospores formed at different phosphite concentrations, the average number being 0.15 oospores cm^{-2} (± 0.08). Selfed oospores were produced on all phosphite concentrations for MP128. Low levels of phosphites (20 and 40 μg ml^{-1}) appeared to result

in a small increase in the number of oospores while production was inhibited at higher levels (Figure 5.2). However, these differences were not statistically significant except for the inhibition at 100 µg phosphite ml^{-1} ($P < 0.05$).

The distribution of oospores across the radius of the colony did not significantly ($P > 0.05$) differ for each phosphite concentration except for 100 µg phosphite ml^{-1} (Table 5.1). The average number of oospores cm^{-2} was initially high (approximately 5 – 22 cm^{-2}) at the point of inoculation for all phosphite concentrations (except 100 µg ml^{-1}), but fell with increasing distance from the inoculum and there were none found at 2.8 cm or further (Figure 5.3).

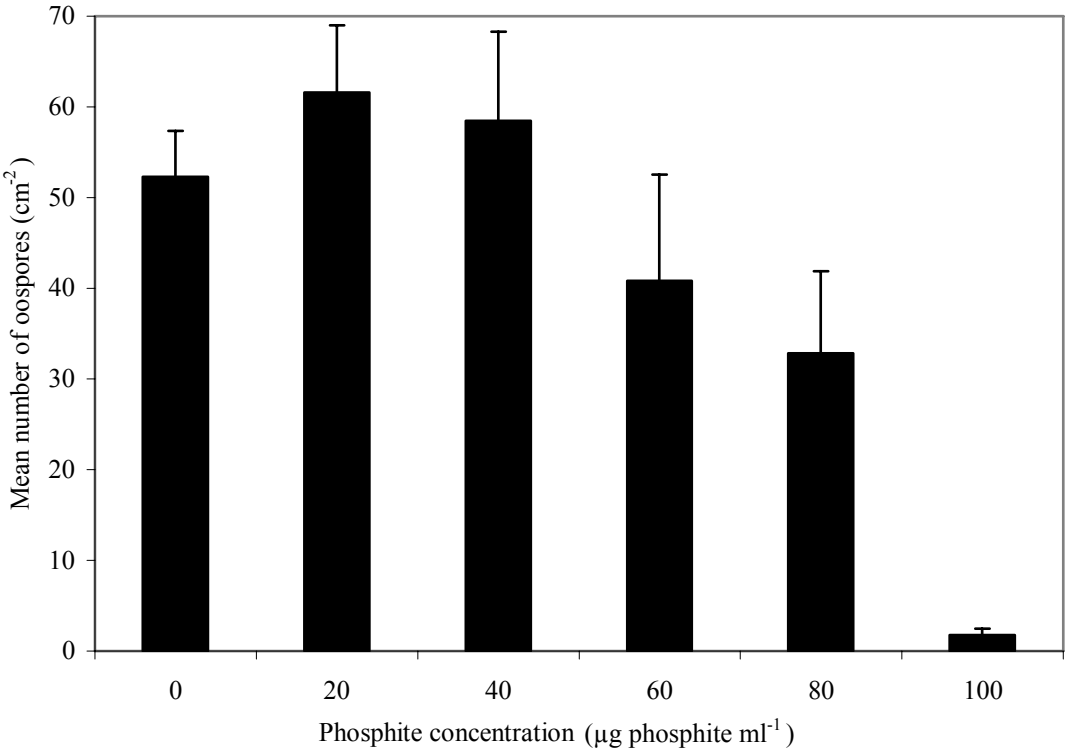

Figure 5.2: Average oospore production on modified Ribeiro's minimal medium containing different concentrations of phosphite by *Phytophthora cinnamomi* isolate, MP128. Bars represent positive standard error of the mean.

Table 5.1: MANOVA to compare oospore numbers across the colony radius of *Phytophthora cinnamomi* isolate, MP128, growing on Ribeiro's media with varying phosphite concentrations (0, 20, 40, 60, 80, 100 µg phosphite ml^{-1}). Analysis was performed on data with and without the inclusion of data from 100 µg phosphite ml^{-1}. Mean Square (MS) values are given (with degrees of freedom for the effect and error in brackets. Significant values are in bold.

Distance from inoculum (cm)	Phosphite concentration effect when 100 µg ml^{-1} included	Phosphite concentration effect when 100 µg ml^{-1} not included
0.4	**$MS_{(5, 23)}$ = 18.81**	$MS_{(4,20)}$ = 7.71
0.8	**$MS_{(5, 23)}$ = 10.73**	$MS_{(4,20)}$ = 2.98
1.2	**$MS_{(5, 23)}$ = 13.79**	$MS_{(4,20)}$ = 2.64
1.6	**$MS_{(5, 23)}$ = 12.78**	$MS_{(4,20)}$ = 5.41
2.0	**$MS_{(5, 23)}$ = 8.06**	$MS_{(4,20)}$ = 2.52
2.4	**$MS_{(5, 23)}$ = 2.44**	$MS_{(4,20)}$ = 1.88
2.8	$MS_{(5, 23)}$ = 0.65; P = 0.362	$MS_{(4,20)}$ = 0.62
3.2	All values 0, no analysis made	All values 0, no analysis made

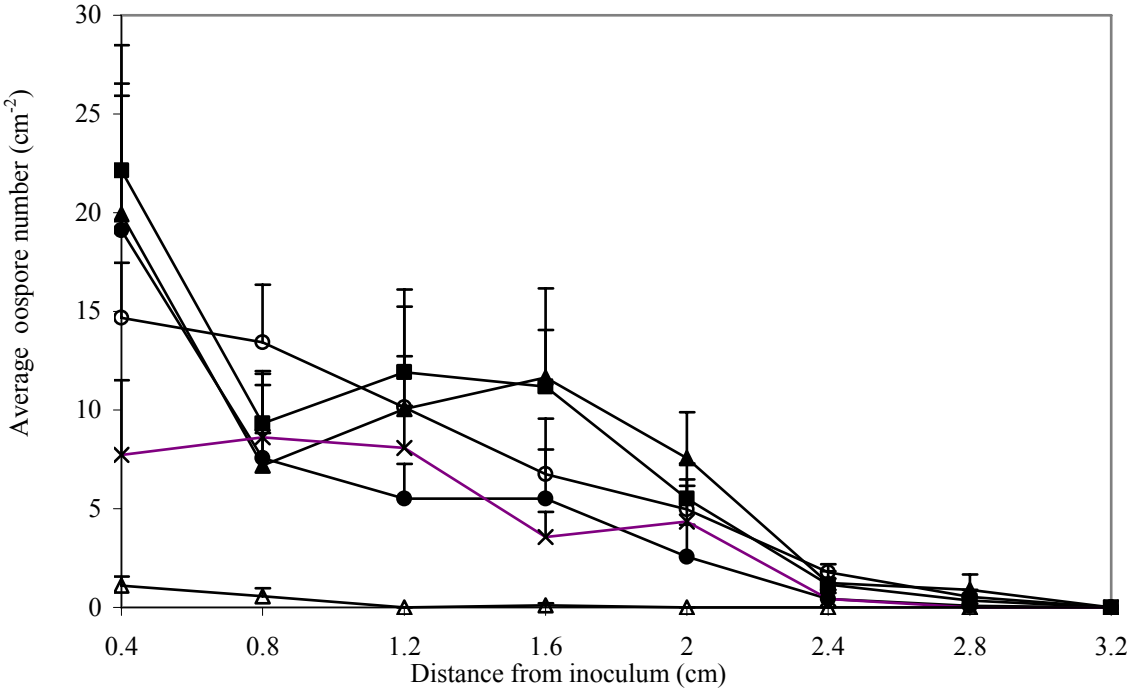

Figure 5.3: Effect of phosphite concentration (○ 0 µg ml^{-1}; ■ 20 µg ml^{-1}; ▲40 µg ml^{-1}; ● 60 µg ml^{-1}; x 80 µg ml^{-1}; Δ100 µg ml^{-1}) on mean oospore numbers cm^{-2} across the radius of *Phytophthora cinnamomi* (isolate MP128) on modified Ribeiro's minimal medium. Bars represent positive standard error of the mean.

Determining viability of selfed oospores

Autoclaved oospores (presumed 'killed') lacked a visible ooplast and when stained with Thiozolyl Blue Tetrazolium Bromide (MTT), the cell contents were black or remained clear (Figure 5.4). Non-autoclaved oospores stained magenta or black or remained clear (Figure 5.4). Non-autoclaved oospores lacking a visible ooplast did not stain magenta. For these reasons, oospores stained magenta were considered viable. When MTT was used without adding 2.8mM CoCl, staining was non-specific with the cytoplasm of autoclaved controls also producing a pink or magenta colouration.

Phosphite in the medium at concentrations 0, 60 or 100 μg phosphite ml^{-1}, did not affect oospore viability (Table 5.2). The percentage of non-viable oospores did not significantly ($P > 0.05$; Table 5.2) differ across the two experiments so the data were bulked showing that the percentage of definitely non-viable oospores (as assessed by MTT staining) was 32.3 % (± 3.9 %). The percentage of viable oospores differed (Table 5.2) across the two experiments, 27.3 % (± 4.7 %) and 47.4 % (± 5.4 %) for experiment 1 and 2, respectively. The percentage of oospores for which viability could not be determined (ie they did not stain but had a visible ooplast) also differed being 45.7 % (± 4.2 %) and 15.5 % (± 2.7 %) for experiments 1 and 2, respectively.

Table 5.2: ANOVA of data. Percentage of oospores of *Phytophthora cinnamomi* staining magenta (Viable), staining black or clear without a nucleus (Non-viable) and clear with a visible nucleus (Unclear) is analysed by comparing phosphite concentration across the repeat experiments. Adjusted Mean Squares (MS) are given with degrees of freedom for the effect and error in brackets. Significant values are in bold.

Effect	% Viable	% Non-viable	% Unclear*
Phosphite Conc.	$MS_{(2, 17)} = 311.6$	$MS_{(2, 17)} = 15.2$	$MS_{(2, 17)} = 89.1$
Experiment No.	**$MS_{(1, 17)} = 917.4$**	$MS_{(1, 17)} = 476.6$	**$MS_{(1, 17)} = 2497.0$**
P. Conc. x Exp. No.	$MS_{(2, 17)} = 99.7$	$MS_{(2, 17)} = 345.0$	$MS_{(2, 17)} = 129.5$

* Oospores that did not stain could not be classified as viable or non-viable as staining could be affected by wall thickness and/ or dormancy of the spore.

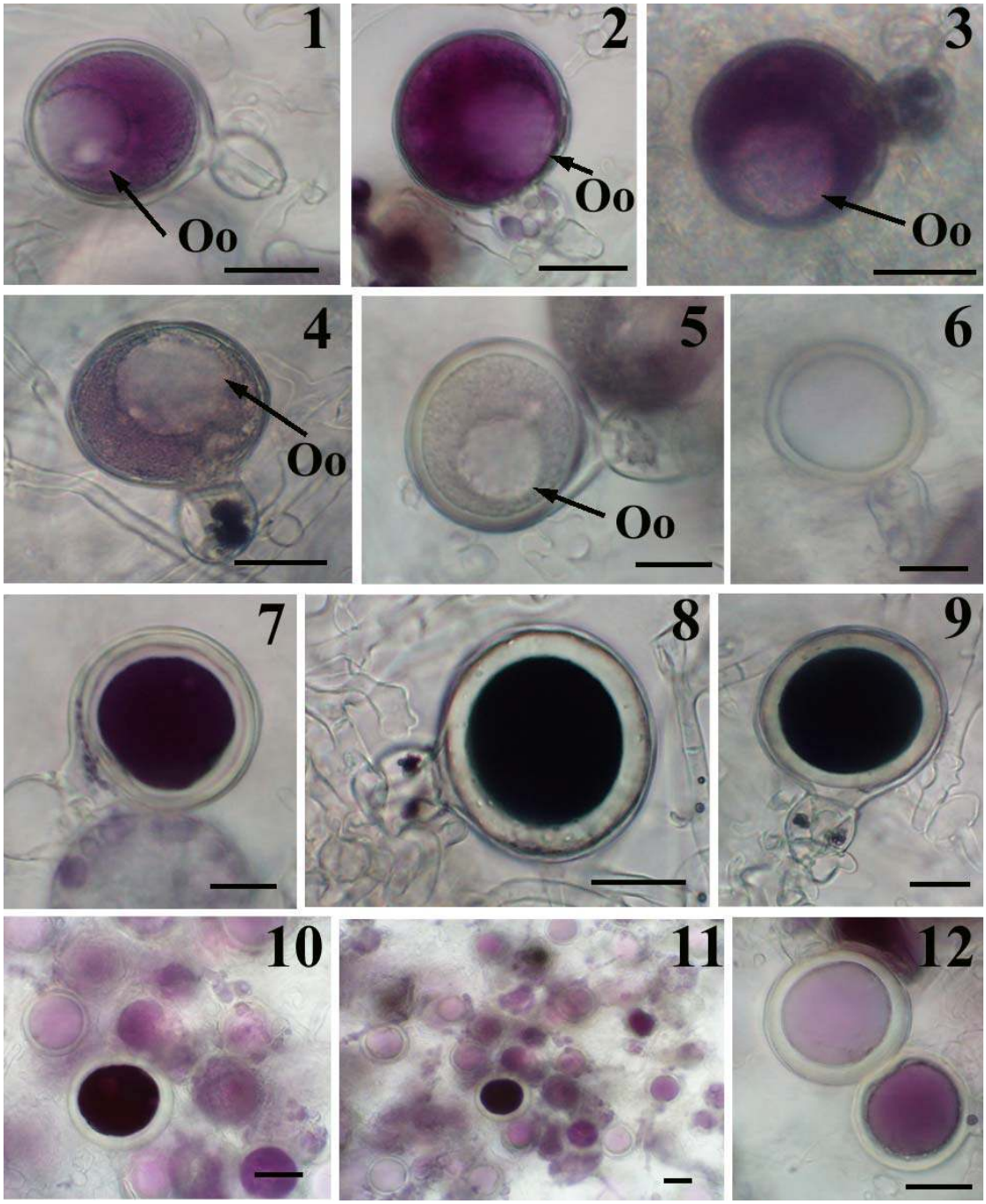

Figure 5.4: 1 – 4. Magenta/ pink stained oospores with ooplast (Oo), classified as viable; **5**. Clear, unstained oospore with ooplast (Oo) classified as non-viable; **6.** Clear, unstained oospore with no ooplast; **7 - 9**. Black stained oospore – non-viable; **10 – 12**. Pink stained oospores with no ooplast found in autoclaved controls without the addition of CoCl. Bar = 20 µm.

Germination of selfed oospores

Culture age (28, 84 and 91 days) had no effect ($P > 0.05$) on whether nuclei were present or absent in oospores as analysed by a binary logistic regression. No significant ($P>0.05$) difference was found in number of nuclei present in oospores from aged cultures compared to oospores from younger cultures. Treatment of oospores with $KMnO_4$ did not enhance nuclear fusion and cause a drop in nuclei number in the hour after treatment as DAPI staining revealed that the nuclei number of oospores did not significantly ($P > 0.05$) differ with or without treatment. Approximately, 16 % of oospores contained nuclei and of those, an average of 4 (± 0.3) nuclei was found per oospore (Range 1 - 17) (Figure 5.5).

Oospores, after freezing, did not clearly germinate on any medium tested (NARPH, V8 agar, Malt Extract agar, ½ Potato Dextrose agar, modified Ribeiro's minimal medium and Water agar). Occasionally, oospores were observed to have what appeared to be new mycelial growth that appeared to originate from either the oospore wall or amphigynous antheridium (Figure 5.6). However, observations of the same oospores after further incubation for 2 weeks under light at 25 ºC did not show any further growth. On S + L medium, with or without treatment with $KMnO_4$, oospores did not germinate.

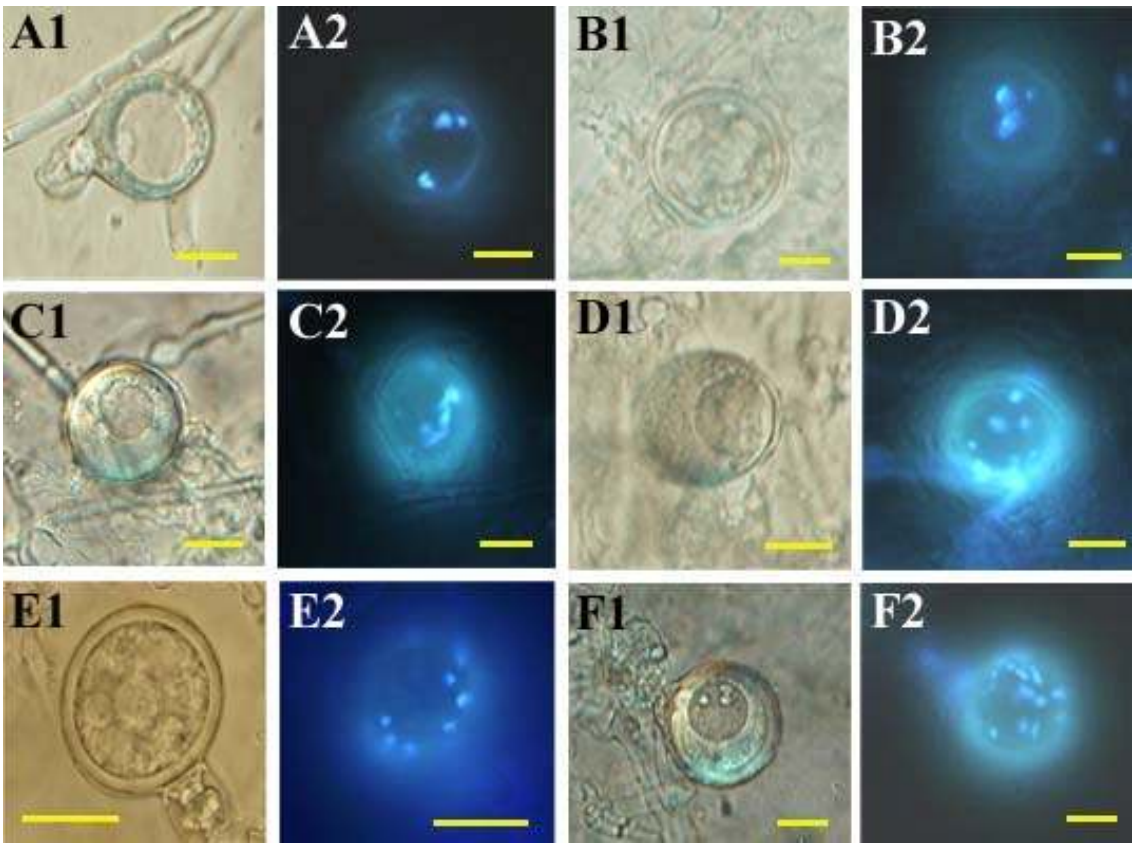

Figure 5.5: 4′, 6-diamidino-2-phenylindole.2HCl (DAPI) stained oospores of *Phytophthora cinnamomi* showing nuclei under white light (**1**) and at wavelength 350 nm (**2**). **A & B.** Three nuclei; **C.** Five nuclei; **D.** Six nuclei; **E.** Seven nuclei; **F.** Eleven nuclei. Bar = 20 µm.

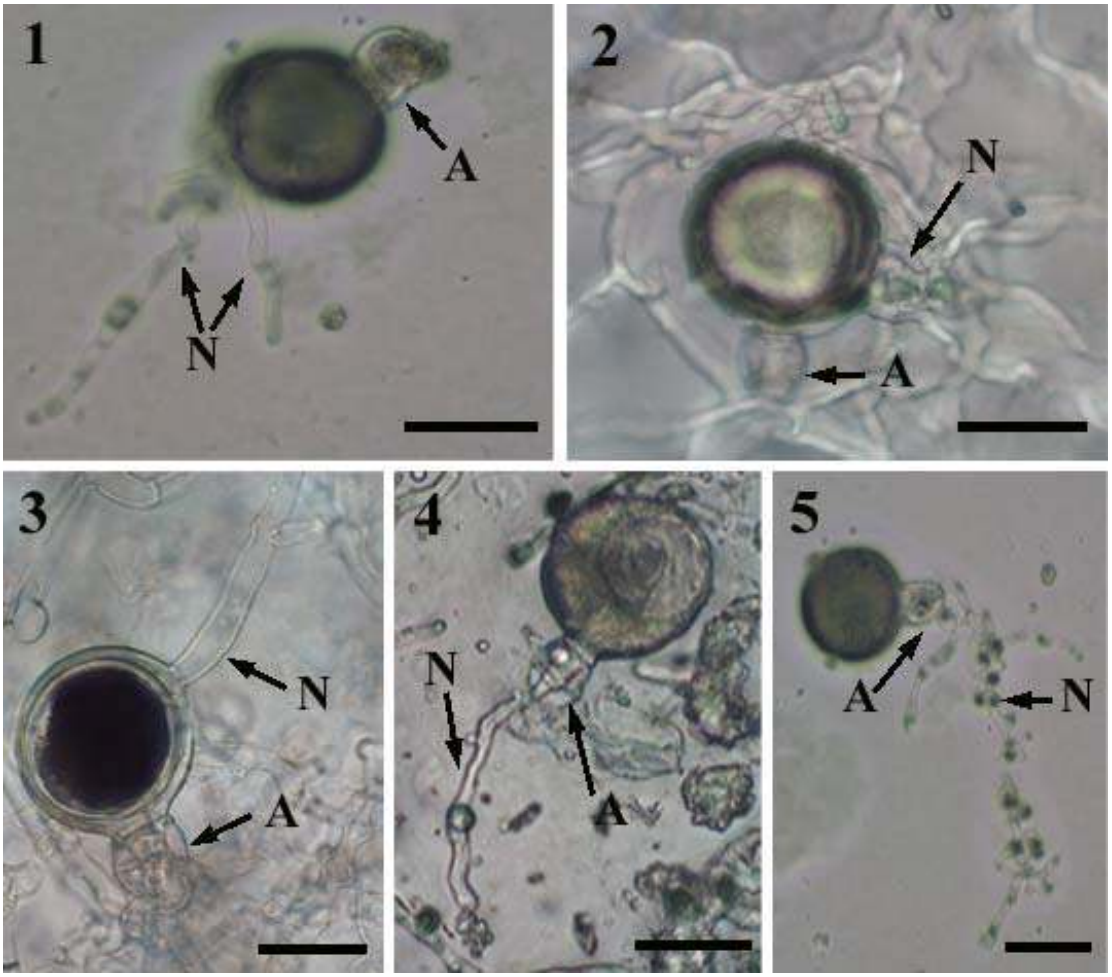

Figure 5.6: Possible germination of selfed oospores of *Phytophthora cinnamomi* on NARPH medium. A, Amphigynous antheridium; N, New mycelial growth. **1 - 3.** New mycelial growth may be from oospore wall; **4 & 5.** Oospores may be germinating through amphigynous antheridium. Bar = 40 µm.

5.4 Discussion

Four isolates of *P. cinnamomi* demonstrated homothallic responses *in vitro,* producing selfed oospores on modified Ribeiro's minimal medium. The frequency of oospore production ranged from 0.15 oospores cm^{-2} (MP127, MP94-15 and MP103) to 36 oospores cm^{-2} (MP 128). Oospore diameters (28 – 52 μm) were similar to those observed by Ho and Zentmyer (1977) who found selfed oospores of *P. cinnamomi* induced in the presence of avocado extracts were 28 – 44 μm (average 35.7 μm) in diameter. More isolates and media need to be screened to determine if oospore production *in vitro* is restricted to only some isolates and only one medium type.

An amphigynous antheridium was always involved in the formation of an oogonium. The presence of paragynous antheridia was seen on two separate occasions, once with only one paragynous antheridium present, the second with at least five present. This is consistent with previous research of homothallic cultures of *Phytophthora* spp. where either amphigynous or paragynous antheridia predominate with both rarely seen in the same culture (Savage *et al.* 1968; Stamps *et al.* 1990). It has been shown for *P. boehmeriae* that medium nutrient conditions influence whether amphigynous or paragynous antheridia will predominate (Gao *et al.* 1998). However, paragynous antheridia in oospores of *P. cinnamomi* produced in selfed cultures, have not been observed (Ho and Zentmyer 1977). Hüberli *et al.* (1997) and Daniel *et al.* (2003) showed the presence of both paragynous and amphigynous antheridia in *P. cinnamomi* A1 x A2 pairings although proportions of oospores with paragynous antheridia were mostly low (0.2 – 10 %). This may have been due to the use of a high nutrient medium which has been found to favour amphigynous antheridia in *P. boehmeriae* (Gao *et al.* 1998). The rare paragynous antheridia in the

current study were also associated with amphigynous antheridia as observed in the mating study of Hüberli *et al.* (1997) for *P. cinnamomi*.

Length of incubation and presence of light were significant influences in oospore production with a longer incubation period in darkness producing more selfed oospores. The use of solid Ribeiro's medium was also important as no oospores were obtained when liquid Ribeiro's medium was used. Liquid media have previously been found unsuitable for oospore production of several pairs of *P. infestans* isolates (Fabritius *et al.* 2002).

Phosphite only inhibited oospore production at high levels (100 μg phosphite ml^{-1}). Oospore production across the radius of the culture was similar for phosphite concentrations between 0 and 80 μg ml^{-1}, with most oospores being produced close to the inoculation site, numbers dropping as the outer colony edge was reached, perhaps a factor of incubation time. 80 μg ml^{-1} phosphite is a higher concentration than that observed by Coffey and Joseph (1985) for oospores of *P. cinnamomi* produced by mating but this difference could be due to isolate variation.

Phosphite concentration in the medium on which oospores were produced had no effect on their viability as assessed by Thiozolyl Blue Tetrazolium Bromide staining. At least 27 % of oospores produced with or without phosphite present, were found to be viable using Thiozolyl Blue Tetrazolium Bromide staining. Staining of the nuclei of oospores with 4′, 6-diamidino-2-phenylindole.2HCl (DAPI) showed that approximately 16 % of oospores contained nuclei. An average of 4 nuclei were found in each of these oospores which is similar to previous studies on the homothallic species *P. megasperma* f. sp. *medicaginis, P. megasperma* f. sp. *glycinea* and *P. cactorum* (Jiang *et al.* 1989). No drop in nuclei number

occurred in the hour after treatment with $KMnO_4$ suggesting nuclei fusion did not occur within this timeframe and germination of oospores on S + L medium did not occur after this treatment. This is in contrast to studies by Ann and Ko (1988) who found that germination of oospores produced by mating *P. parasitica* improved to 90 % by treatment with $KMnO_4$ and plating on S+L medium. V8 agar, potato dextrose agar and water agar only resulted in the germination of 28 – 46 % oospores (Ann and Ko 1988) and in the current experiment, no obvious germination of *P. cinnamomi* oospores occurred on these media either.

It appeared that a few oospores germinated on NARPH, producing a germ tube from the oospore wall or amphigynous antheridium. No further mycelial growth occurred in these 'germinating' oospores. This could indicate that these selfed oospores of *P. cinnamomi* germinated into 'unfit' cultures, not capable of further growth. Viability of oospores of *P. infestans* has previously been shown by the tetrazolium bromide assay to range between 4.9 – 52.3 % viability with less than 5 % of these germinating and not all of those germinating resulting in viable progeny (Groves and Ristaino 2000).

Oospore germination of *P. cinnamomi* has always been difficult (Stamps 1953; Duncan 1985a; Jiang *et al.*1989; Jiang and Erwin 1990). Oospore age, dormancy or failure of nuclear fusion are suggested reasons for these difficulties (Stamps 1953; Ann and Ko 1988; Jiang *et al.* 1989; Pittis and Shattock 1994; Groves and Ristaino 2000). Different light regimes have been found to influence germination ability of oospores of *P. parasitica*, germination dropping from 95 % to 44 % if cool white fluorescent light was not provided during the maturation stage (Ann and Ko 1988). Varying light availability during oospore

production and length of freezing the oospore suspension needs to be further investigated to determine if this plays a role in germination of selfed oospores of *P. cinnamomi.*

Brasier and Sansome (1975) found oospores forming in single cultures of 12 isolates of the normally heterothallic culture of *P. palmivora*. This was observed in 50 % of cultures taken from an oil stock for the first three subcultures, but oospore numbers decreased with subculturing (Brasier 1972). It was suggested that this phenomena was due to the accumulation of substances involved in sexual reproduction as a result of the activity or suppression of metabolic pathways during dormancy (Brasier 1972). This is not the case in the current study as all isolates were passaged through lupins prior to experimentation and repeat experiments showed similar oospore production.

Whether these A2 isolates of *P. cinnamomi* are capable of producing selfed oospores in nature is yet to be determined. Previously, selfed oospores have been observed in naturally infected avocado roots and on colonised fibreglass or nylon mesh that had been buried in soil (Mircetich and Zentmyer 1967; Reeves and Jackson 1974). Identity was not confirmed nor was viability of the spores. The ability to produce oospores in the A2 mating type provides an efficient survival mechanism (Zentmyer 1979). However, it is necessary to determine the pathogenicity and fitness of cultures arising from these selfed oospores and compare these to other propagules of *P. cinnamomi*. Oospores from the mating of *P. cinnamomi* (A1 and A2) cultures have been shown to be less aggressive colonisers of *E. smithii* than their parents (Linde *et al.* 2001). Also, oospores from the interspecific cross between *P. sojae* and *P. vignae* showed less aggressiveness than their parents (May *et al.* 2003).

Although the viability of these selfed oospores produced *in vitro* is low, they still have the potential to contribute to survival inoculum. A simple, repeatable *in vitro* technique has been described to produce selfed oospores from several isolates of *P. cinnamomi*. From this, future research will be able to investigate factors affecting oospore dormancy and germination to allow better application of isolation techniques when assessing areas for infestations of the pathogen. By understanding dormancy, recovery of the pathogen from infested sites will be improved, reducing false negative results.

CHAPTER 6

Transformation of *Phytophthora cinnamomi*

6.1 Introduction

A particular disadvantage of studying microorganisms in a non-sterile system has always been the difficulty in distinguishing the organism of interest from other organisms present in the system. For fungi, mycelium and spores can be particularly difficult to identify. Traditional methods using morphology or isolating and growing propagules are time consuming, require knowledge of fungal taxonomy and may not always be possible due to contamination or dormancy preventing growth.

Hyphal staining (Newall *et al.* 1987) and the analysis of the distribution of ergosterol (Bruzzese and Hasan 1983) have been used to detect fungal propagules. Molecular techniques such as DNA hybridisation or Polymerase Chain Reaction (PCR) have been used to detect *P. cinnamomi* (Dobrowolski and O'Brien 1993) but these methods only allow the detection of the presence of the organism and do not allow the visualisation of the propagules or distinguish live from dead *P. cinnamomi* propagules. All these methods tend to be time consuming and require special equipment (Oliver *et al.* 1993). An immunofluorescence technique to detect *P. cinnamomi* using rabbit antibodies to the pathogen was not completely species specific (Malajczuk *et al.* 1975; MacDonald and Duniway 1979). 'Calcofluor White M2R' (disodium salt of 4,4'-bis(4-anilino-6-diethylamino-*s*-triazin-2-ylamino)-2,2'-stilbene-difulfonic acid) has been used as an effective vital stain in distinguishing propagules of *Phytophthora* spp. in soils, but propagules lose fluorescence over time due to dilution of the stain as the organism grows

(Tsao 1970). Transformation of the organism to express a distinctive trait is another possibility for identification. Ideal traits for this purpose are the green fluorescent protein (GFP) or β-glucuronidase (GUS) gene (Chapter 1.5).

The GFP gene has many advantages over the GUS gene. In particular, no addition of chemicals are required to visualise the fluorescence produced under UV light for GFP. However, GUS can be observed macroscopically, a key advantage over GFP (van West *et al.* 1999). However, the addition of X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) required to produce the blue product by the GUS gene is inappropriate for fragile tissues such as zoospores as these may collapse during staining and in hyphae and spores, the blue crystals are only located in particular areas of the cell (van West *et al.* 1999).

The GUS gene has been successfully incorporated into many *Phytophthora* species such as *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora, P. infestans, P. megasperma* f. sp. *glycinea* and *P. palmivora* (Judelson *et al*. 1992, 1993; Bailey *et al.* 1993; van West *et al.* 1999; Vijn and Govers 2003). GFP has been integrated into *P. brassicae, P. infestans*, *P. palmivora* and *P. parasitica* var. *nicotianae* (Bottin *et al.* 1999; van West *et al.* 1999; Si-Ammour *et al.* 2003). Transformation methods have been further discussed in Chapter 1.5.

The aim of the following experiments was to transform *P. cinnamomi* with GFP and GUS. For comparison, other *Phytophthora* species, *P. cambivora, P. citricola, P. cryptogea, P. drechsleri, P. megasperma* and *P. palmivora*, were used. Different methods such as the use of *Agrobacterium tumefaciens* and protoplast transformation with plasmid DNA were compared.

6.2 Materials and Methods

6.2.1 Plasmid Preparation

Plasmids

For *Agrobacterium*-mediated transformation, a plasmid containing the hygromycin resistance gene (*hph*) with either the Green Fluorescent Protein (GFP) gene or β-glucuronidase (GUS) gene was developed. Plasmid pVW2 (van West *et al.* 1999; Figure 6.1) and plasmid pVBK1 (O'Brien and Kelly unpublished; Figure 6.2) provided two different sources of the GFP gene. Plasmid pHAMT35G (van West *et al.* 1999) provided the GUS gene (Figure 6.3). Plasmids pVW2 and pHAMT35G were constructed in the vector pUC19, which contains an ampicillin resistance gene while pVBK1 was constructed in the vector pBIN19 which contains a kanamycin resistance gene. The binary plasmid pBINHL1 containing a selectable hygromycin resistance gene was also used for *Agrobacterium* transformation (Wu 2004; Figure 6.4).

The construct used for protoplast transformation was kindly supplied from F. Mauch's laboratory in Switzerland and was developed by Si-Ammour *et al*. (2003). This plasmid, p34GFN (Figure 6.5), contained the Geneticin (G418) resistance gene (*npt*II) as a selectable marker and the GFP gene, each flanked by the HAM promoter and terminator (Si-Ammour *et al.* 2003).

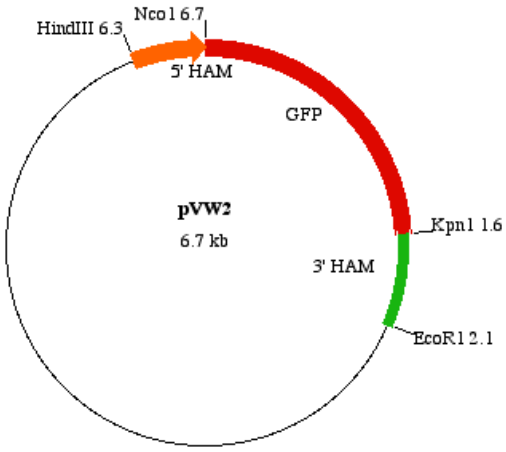

Figure 6.1: Map of plasmid pVW2 containing the Green Fluorescent Protein (GFP) gene flanked by HAM promoter and terminator (van West *et. al* 1999).

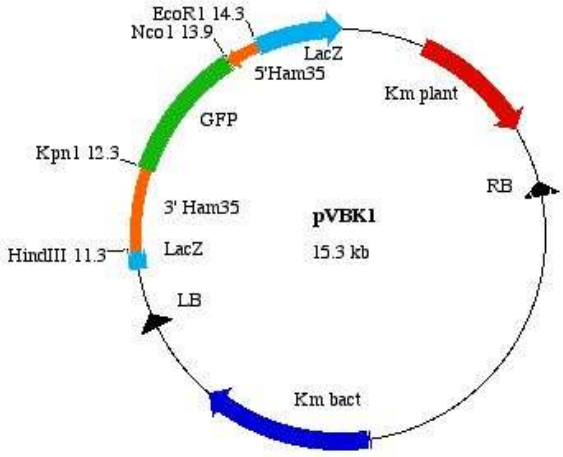

Figure 6.2: Map of plasmid pVBK1 containing the Green Fluorescent Protein (GFP) gene flanked by HAM promoter and terminator (O'Brien and Kelly unpublished).

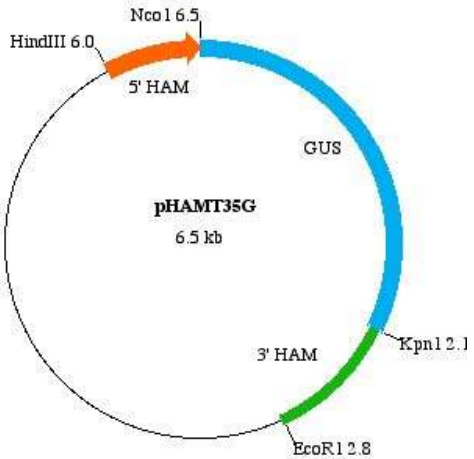

Figure 6.3: Map of plasmid pHAMT35G containing the β-glucuronidase (GUS) gene flanked by HAM promoters and terminators (van West *et. al* 1999).

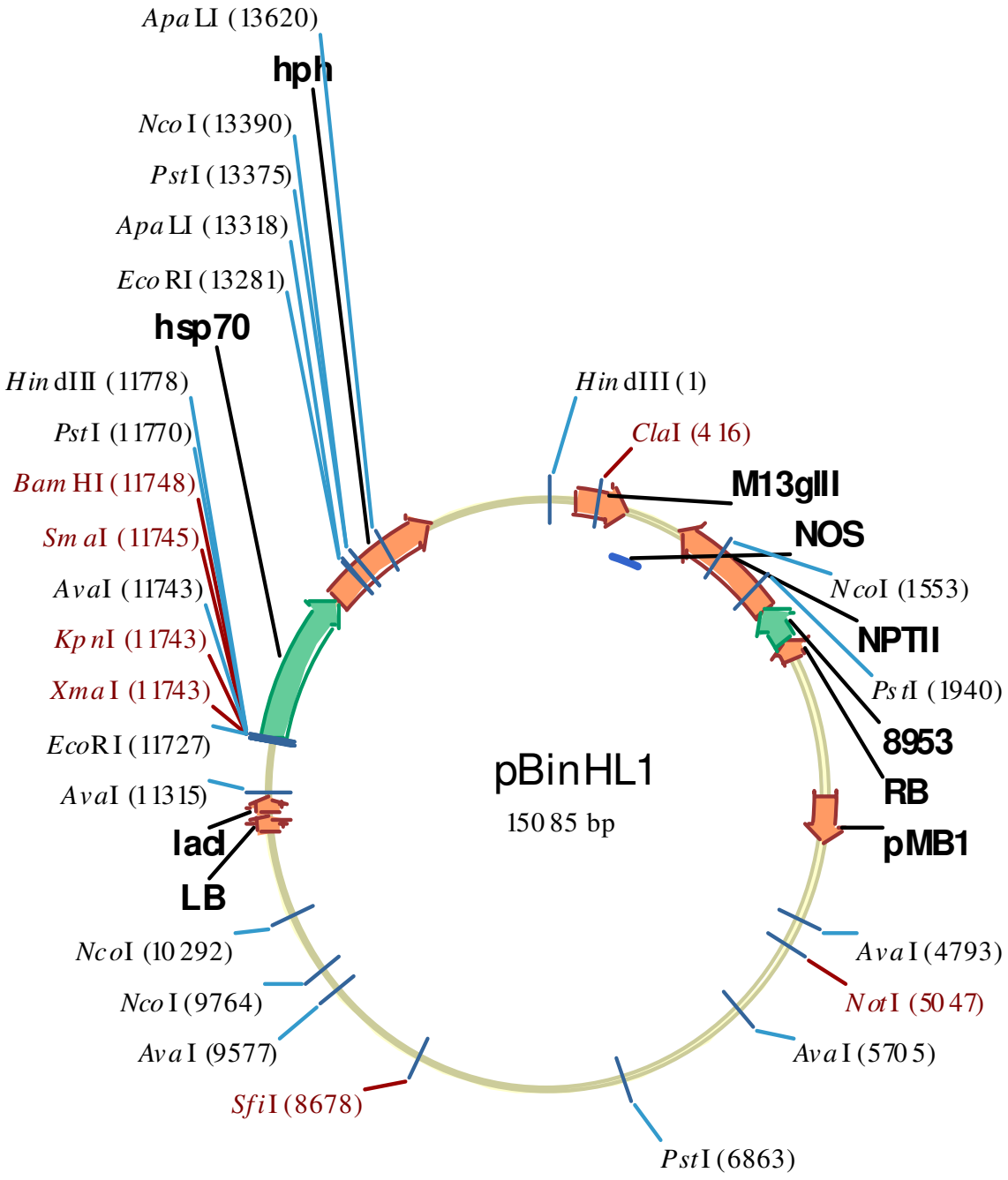

Figure 6.4: Map of binary pBinHL1 plasmid containing the hph-gene for Hygromycin resistance as well as left (LB) and right (RB) border for *Agrobacterium*-mediated transformation (Wu 2004).

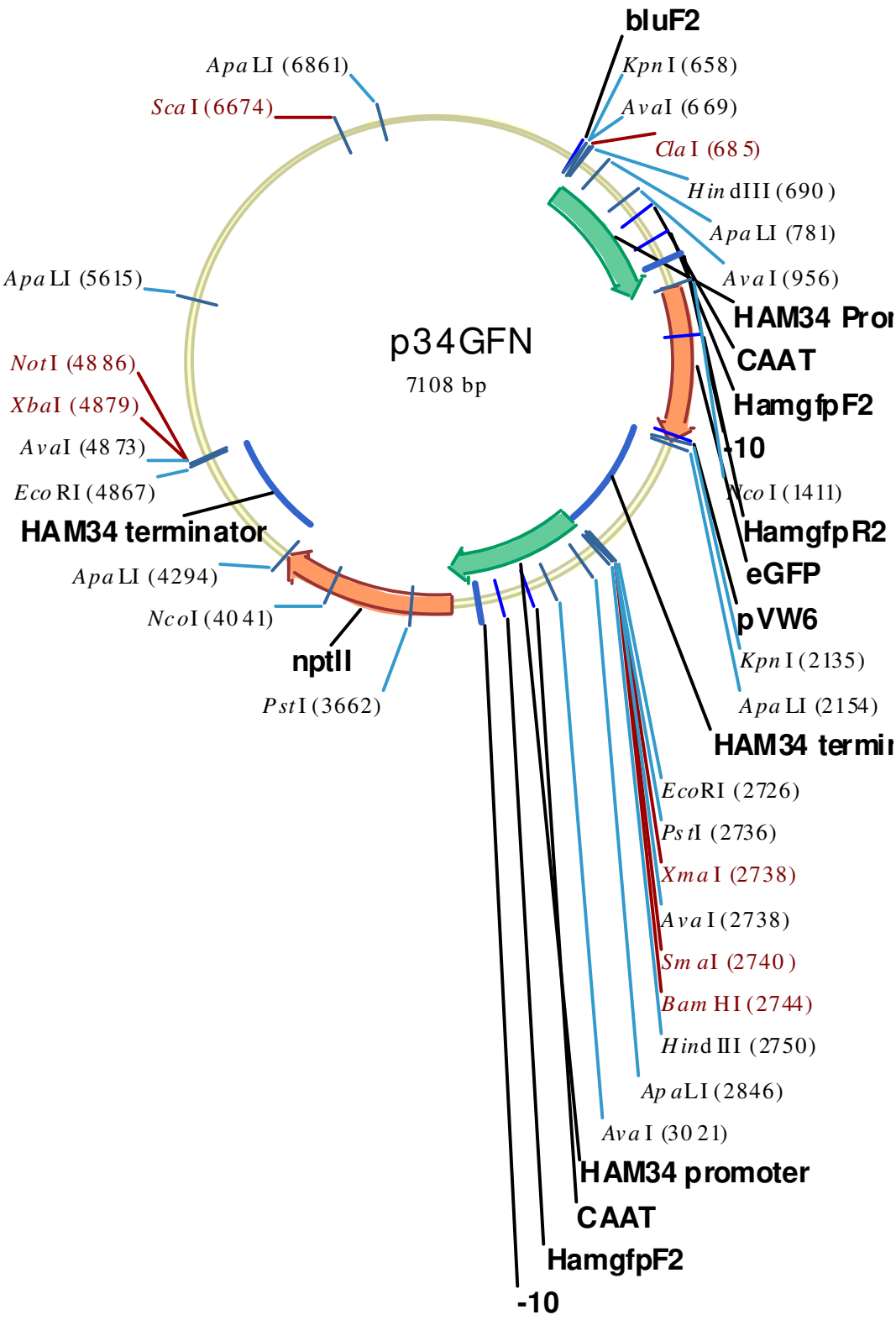

Figure 6.5: Map of plasmid p34GFN. P34GFN contains the Green Fluorescent Protein (GFP) gene and the geneticin resistance (nptII), both flanked by the HAM promoters and terminators (Si-Ammour *et al*. 2003).

Plasmid extraction

Following the instructions from the manufacturer, the QIAprep® Spin Miniprep Kit (QIAGEN; Australia) was used for small scale preparations of plasmid DNA (pHAMT35G, pVW2 and pVBK1).

The method described by Birnboim and Doly (1979) was used for large-scale extraction of pBINHL1 and p34GFN from their *Escherichia coli* hosts. *Escherichia coli* containing pBINHL1 was grown in 250 ml LB Broth (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl) containing kanamycin (50 μg ml^{-1}) on a rotary shaker at 37 °C overnight. *Escherichia coli* containing p34GFN was grown under the same conditions but with the replacement of kanamycin with ampicillin (100 μg ml^{-1}).

Cells were centrifuged at 1 700 *g* for 10 minutes and the supernatant discarded. Cells were resuspended in 5 ml of ice-cold buffer 1 (50 mM glucose; 10 mM EDTA Na_2; 25 mM Tris-HCl pH 8.0) and left for 10 minutes.

10ml of freshly made buffer 2 (200 mM NaOH; 1 % (w/v) SDS) was added to the bacterial suspension, shaken and incubated on ice for 10 minutes. 7.5 ml of buffer 3 (3 M potassium acetate; 11.5 % glacial acetic acid) was added, the mixture shaken vigorously and incubated on ice for 10 minutes. The precipitate was collected by centrifugation at 1 300 g for 10 minutes, and the supernatant poured into a fresh tube.

Two volumes of 100 % ethanol was mixed with the solution and allowed to stand for two minutes. The tube was centrifuged for 15 minutes at 2 700 *g*, the supernatant discarded and

the tube inverted to allow the last of the ethanol to evaporate. Pellets were dissolved in 1 ml sterile distilled water containing 200 µg RNAse and incubated at 37°C for 15 minutes.

To clean the plasmid preparations, 100 µl of a 1:1 solution of phenol:chloroform was mixed into the solution and the mixture was centrifuged for 5 minutes at 10600 *g*. The top aqueous layer was transferred to a fresh tube to which 100 µl of chloroform was mixed. The tube was centrifuged (10 600 *g,* 5 minutes) and the upper aqueous layer transferred to a fresh microcentrifuge tube.

The plasmid preparations were purified using the ethanol precipitation method. 500 µl of the extracted plasmid was mixed by shaking with 50 µl of 3 M sodium acetate and 1 ml of 100 % ethanol (stored at –20 °C). This was incubated in the freezer for 2 hours. After this time, the mixture was centrifuged at 10 600 *g* for 10 minutes and the supernatant decanted. The tube was inverted over tissue to allow excess ethanol to drain off 15 minutes. The inside top of the tube was then wiped with a tissue to remove any remaining ethanol before resuspending the plasmid in 100 µl sterile water.

The concentration of all plasmids was then measured using the Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech; USA) and checked on a 1 % (w/v) electrophoresis gel.

Restriction Digestion of Binary Plasmid

Digestion reactions were carried out in 80 µl volumes containing pBINHL1 DNA (approximately 20 ng), 5 µl of enzyme (10 units μl^{-1}), 10 µl buffer and 55 µl sterile water. Enzymes including those from *Klebsiella pneumoniae* (*Kpn*1) (Promega; Australia), *Bacillus amyloliquefaciens* H. (*Bam*H1) (Promega; Australia), *Streptomyces phaeochromogenes* (*Sph*1) (Promega; Australia) and *Streptomyces stanford* (*Sst*1) (Promega; Australia) were used to separately digest pBINHL1. Buffer used for each enzyme varied so that *Kpn*1 and *Sst*1 enzymes used Multicore buffer (Promega; Australia) (250 mM Tris acetate, 1 M Potassium acetate, 100 mM Magnesium acetate, 10 mM dithiothreitol (pH 7.9)), Buffer E (Promega; Australia) (60 mM Tris.HCl, 60 mM $MgCl_2$, 1 M NaCl, 10 mM dithiothreitol) was used for *Bam*H1 enzyme and Buffer K (Promega; Australia) (100 mM Tris.HCL, 100 mM $MgCl_2$, 1.5 M KCl, 10 mM dithiothrietol) was used for *Sph*1 enzyme. The reaction was incubated in a 37 ºC waterbath for 3 hours and the digestion confirmed by electrophoresis.

Removal of Phosphate Group from end of DNA

The phosphate group on the ends of the digested pBINHL1 plasmid were removed by mixing approximately 20 ng of digested plasmid DNA with 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 16 units Shrimp Alkaline Phosphatase (SAP) (Promega; Australia) in a total volume of 80 µl. This mixture was incubated at 37 ºC for 30 minutes. A further 10 µl (10 units) of SAP were added and the incubation continued for a further 30 minutes at 37 °C. To purify the product the QIAquick PCR purification kit was used, following the manufacturer's instructions (QIAGEN, Australia).

PCR Amplification of Gene of Interest

The GFP and GUS genes were amplified from plasmids pVW2 and pHAMT35G by PCR. PCR amplifications were carried out in 10 µl reactions containing 10.7 mM Tris.HCl (pH 8.8), 2.7 mM $(NH_4)_2SO_4$, 0.45% (w/v) Triton X-100, 0.2 µg μl^{-1} gelatin, 0.03 mM dNTP's, 10 pmol μl^{-1} of each primer, 0.5 units *Tth* Plus DNA polymerase (Biotech; Australia) and 1.5 mM $MgCl_2$.

Amplification of the GUS and GFP genes in pVW2 and pHAMT35G and the attachment of a *Kpn1* site at each end was carried out using the primer pair

5' CGGGGTACCAGGGTTTTCCCAGTCACGAC 3' (Proligo),

5' CGGGGTACCTGACCATGATTACGCCAAGC 3', designed using the Vector NTI program. The primers were synthesized by Proligo Ltd. The *Kpn*1 site in each primer is underlined. Since *Kpn*1 does not cut efficiently at sites located at the end of a molecule (Fermentas 2005) a CGG triplet was inserted 5' to the *Kpn*1 site in each primer.

The cycling conditions for these primers consisted of:

1 cycle	95°C	2 minutes
30 cycles	94°C	1 minute
	63°C	1 minute
	72°C	2 minutes
1 cycle	72°C	10 minutes

PCR products from pVW2 and pHAMT35G were digested with the *Kpn*1 enzyme as described earlier. The digested PCR products of plasmids pVW2 and pHAMT35G were extracted by adding an equivalent volume of chloroform: isoamylalcohol (24:1) solution and mixing. This mixture was then centrifuged at 10 600 *g* for 5 minutes. The upper layer

was then carefully transferred to a new microcentrifuge tube. The product was then cleaned using the ethanol precipitation method as described earlier.

Ligation of PCR Products to pBINHL1

The final concentration of DNA for pBINHL1, pVW2 and pHAMT35G was determined using the Hoefer DyNA Quant 200 Fluorometer (Amersham Pharmacia Biotech; USA). The concentration of the PCR digested products of pVW2 and pHAMT35G was adjusted with sterile water to measure 10 ng μl^{-1}. Twenty-four ng of digested pBINHL1 was used for the ligations.

Nine ligation reactions were conducted. Three different concentrations (10, 20 and 40 ng μl^{-1}) of *Kpn*1 digested amplicons from pVW2 and pHAMT35G were used in combination with 24 ng μl^{-1} pBINHL1, 20 units T4 DNA Ligase (Invitrogen; Australia), 25 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT and 2.5 % (w/v) Polyethylene Glycol 8000. Control reactions did not include pVW2 or pHAMT35G but used digested pBINHL1 or 25 ngμl^{-1} undigested pBINHL1 only for positive controls and no DNA was included in a reaction for a negative control. Each reaction was mixed and incubated at 15 °C overnight.

6.2.2 Transformation

Bacterial transformation

Plasmids from successful ligation reactions were transformed into *E. coli* using the *E. coli* Competent Cells kit according to the manufacturer directions (Product No. 9FB035; Promega, Australia). Growing colonies were subcultured to fresh selective plates (LB agar + 50 μg ml^{-1} kanamycin). To confirm successful plasmid formation and bacterial transformation, colonies of *E. coli* growing on selective plates were tested for successful bacterial transformation. Amplification of the desired fragment was achieved using PCR amplification as described earlier with the exception that the initial denaturation step was adjusted to 95 ºC for 10 minutes. An electrophoresis gel was run and the band produced was excised. DNA was extracted using the QIAquick Gel Extraction Kit, following the manufacturer's instructions (QIAGEN, Australia). Digestion of 3 μl of each product using the enzyme *Nco*1, from *Nocardia corallina* (Promega; Australia), was performed in 15 μl as described earlier. An electrophoresis gel was run and products producing bands of the desired length were traced back to the original bacterial colony and labelled as pBINHLGUS. The colony was grown overnight in 10 ml L-Broth (50 μg ml^{-1} kanamycin). 1.8 ml of the broth was added to a freezer vial with 0.2 ml DMSO (dimethylsulphoxide) for long-term storage at –80 °C.

Conjugal transfer of plasmids to Agrobacterium tumefaciens

Plasmids pBINHLGUS (GUS + *hph*), pVBK1 (GFP and kanamycin resistance gene) and pBINHL1 (*hph* only) were introduced into the *Agrobacterium tumefaciens* strains LBA4404 and AGLO by conjugation (Hooykaas, 1988). These strains were confirmed as

Agrobacterium using the ketolactose test (Hooykaas 1988). This involved streaking the organism to be tested onto lactose medium (1 % (w/v) lactose, 0.1 % (w/v) yeast, 2 % (w/v) Bacto agar), incubating at 28 °C for 24 hours before flooding the plate with Benedict's reagent (25.6 % (w/v) $Na_2CO_3.10H_2O$, 13.2 % (w/v) sodium citrate, 1.32 % (w/v) $CuSO_4.5H_2O$). Colonies were confirmed as positive for *Agrobacterium* if they produced a yellow colour around the colony in the lactose medium.

LBA4404 and AGLO were grown overnight at 28 °C in LBMG broth (L-Broth mixed with an equal quantity of MG broth – 1 % (w/v) Mannitol, 0.23 % (w/v) sodium glutamate, 0.05 % (w/v) KH_2PO_4, 0.02 % (w/v) NaCl, 0.02 % (w/v) $MgSO_4.7H_2O$, 0.2 % (w/v) Biotin). *Escherichia coli* cultures containing plasmids pRK2013 (mobilising plasmid), pBINHLGUS, pVBK1 and pBINHL1 were grown overnight in L-Broth at 37 °C.

Cultures were centrifuged at 1 700 *g* for 5 minutes and the supernatant discarded. Cells were washed with 5 ml MG Broth and centrifuged (1 700 *g*, 5 minutes), the supernatant decanted. Cells were resuspended in 0.1 ml MG broth. 20 µl of the *E. coli* suspensions were then mixed separately with 20 µl *Agrobacterium* (LB4044 or AGLO), with or without *E. coli* pRK2013 (controls), in the centre of an LBMG agar plate.

Plates were left uncovered in the laminar flow until the liquid containing bacteria had dried. Plates were incubated at 28 °C overnight. The next day, bacteria was scraped from each plate and streaked onto Hooykaas minimal medium (Appendix 2) with 50 µg ml^{-1} kanamycin. Plates were incubated at 28 °C.

To confirm successful transformation, a PCR was run on colonies growing on selective medium as described earlier with some exceptions. To test for pBINHLGUS incorporation, the primer pair 5'-GGTGGAAAGCGCGTTACAAG-3' and 5'-GTTTACGCGTTGCTTCCGCCA-3' (50 pmol μl^{-1}; Proligo) were used. Primer pair 5'-TGTCACTACTTTCTCTTATGG-3' and 5'-CCATCTTTAATGTTGTGTCT-3' (50 pmol μl^{-1}; Proligo) were used for identifying incorporation of pVBK1 and the primer pair 5'-CCATGGAAAAGCCTGAACTCACCGCG-3' and 5'-CCATGGTCCATGGCCTCCGCGACCGG-3' (10 pmol μl^{-1}; Proligo) were for identification of successful transformation of pBINHL1. The PCR products from the pVBK1 and pBINHL1 amplifications were run on a 2.5 % high resolution gel.

Transformation of Phytophthora by Agrobacterium

A loop of each transformed *Agrobacterium* culture was grown overnight in 5 ml Hooykaas minimal medium (50 µg kanamycin ml^{-1}) at 28 ºC on a shaker (1.5 *g*). 1 ml of the culture was then transferred to 100 ml Hooykaas minimal broth (50 µg kanamycin ml^{-1}) and incubated under the same conditions. Bacteria were collected by centrifugation at 420 *g* for 5 minutes. Bacteria were resuspended in induction medium (Appendix 2) diluted to OD_{600nm} 0.5 – 0.8 and incubated between 3 – 6 hours with shaking (1.5 *g*) to induce virulence. A dilution of 1:100 was then made using induction media. 1 ml of the non-diluted culture and 1 ml of the culture that had been diluted 1:100 was added to 19 ml induction media and these were the mixtures used for transformation of *Phytophthora*.

Intact and damaged mycelium was used for transformation. To prepare intact mycelium, five plugs of mycelium from clarified V8 agar (Appendix 2) from *P. cinnamomi* isolates

MP94-15, MP127, MP128 and MP103, were grown on cellophane on clarified V8 agar for 48 hours at 25 ºC under cool white fluorescent light. For preparing damaged mycelium, the same isolates were grown in clarified V8 broth (Appendix 2) at 25 ºC for 72 hours before being macerated using either a scalpel or a mortar and pestle. For intact mycelium, the *Agrobacterium* suspension, un-diluted or diluted 1:100, was added to cover the mycelium so that isolates were covered with *Agrobacterium* containing the pBINHLGUS, pVBK1 or pBINHL1 plasmids. Co-transformation was tested by combining pVBK1 and pBINHL1. For damaged mycelium, the same transformed *Agrobacterium* cultures and combination cultures were used. The macerated mycelium was mixed separately with 10 ml of each *Agrobacteria* suspension. Mixtures were spread onto sterile cellophane that had been placed on induction medium. All plates from both intact or damaged mycelia were incubated for 1, 2 or 3 days at 28 ºC under cool white fluorescent light before the cellophane was transferred to a NARPH (Appendix 2) plate containing 400 µg hygromycin ml^{-1} (Roche Diagnostics, Germany) or cellophane cultures were covered by molten NARPH agar (400 µg hygromycin ml^{-1}). Plates were observed for the next month to observe if any mycelial growth occurred on the medium. Experiments were repeated on *P. drechsleri* (MU14, MU13), *P. cinnamomi* (MU33. MU35, MU31, MP97-16A), *P. cambivora* (MU136A), *P. citricola* (MU1A), *P. megasperma* (MP41B) and *P. cryptogea* (MU28, MU25) (collaboratively with E. Moek, an exchange student from Germany).

Non-sterile zoospores of *P. cinnamomi* were also used for transformation. To produce non-sterile zoospores, approximately ten cubes (5 mm^2) from a clarified V8 agar culture of *P. cinnamomi* (isolates MP94-15, MP127, MP128 and MP103) was placed in a 9 cm Petri dish and covered with clarified V8 broth. These plates were incubated at 25 ºC for two days in the dark. The clarified V8 broth was removed and the culture washed three times

with sterile water. Soil leachate (10 % (w/v) potting mix, incubated overnight and filtered through Whatman No. 1 filter paper), was added to the plates to cover the growing culture. Plates were incubated at 25 ºC for three days under cool white fluorescent light. Cultures were checked for the presence of sporangia and were placed at 4 ºC for 30 minutes. Plates were then stored at 25 ºC until zoospores were released. Water containing zoospores was collected in a 50 ml tube and adjusted to approximately $5x10^7$ zoospores ml^{-1} (Vijn and Govers 2003).

Transformation of non-sterile zoospores of *P. cinnamomi* was attempted using the method of Vijn and Govers (2003). To 50 ml tubes containing 1 ml of zoospores, 1 ml of either the non-diluted or 1:100 dilution of *Agrobacterium* was added. Co-transformation was tested with *Agrobacterium* containing either pBINHL1 or pVBK1. Tubes were left at room temperature for 30 minutes before zoospore encystment was induced by vigorous manual shaking for 2 minutes. Tubes were incubated at 25 ºC for 2 hours to allow germination and then centrifuged for 5 minutes at 260 *g*. The supernatant was discarded and the remaining pellet was resuspended in 50 µl sterile water and spread onto induction medium. After 1, 2 and 3 days incubation, plates were checked for growth and if growth was observed, this was subcultured to NARPH plates containing hygromycin (400 µg ml^{-1}).

Transformation of Phytophthora cinnamomi using the protoplast method

To produce sterile zoospores of *P. cinnamomi*, the method of O'Gara (1998) was followed, a modification of the method by Dolan and Coffey (1986). Mycelial plugs (5 mm^2) from clarified V8 agar plates (*P. cinnamomi* isolates MP94-15, MP127, and MP103) were transferred mycelial side down to 6 cm^2 sterile cheesecloth on clarified V8 agar plates. Cheesecloth had previously been prepared by boiling for two hours in water containing 0.02 % (w/v) $EDTANa_2$, washed with tap water and boiled for a further two hours in water. Cheesecloth was removed from the water, excess water squeezed out, placed in 250 ml wide-mouthed Erlenmeyer flasks and autoclaved at 121 ºC for 20 minutes on three consecutive days.

Plates with cheesecloth inoculated with *P. cinnamomi* were wrapped in aluminium foil and incubated at 25 ºC for six days. Agar plugs on the cheesecloth were removed and the cheesecloth was placed in sterile 250 ml wide-mouthed Erlenmeyer flasks to which 50 ml of clarified V8 broth was added. The flasks were incubated overnight at 25 ºC under cool white fluorescent light with shaking (100 rpm). The V8 broth was removed and the cheesecloth washed 5 times with sterile deionised water before adding 50 ml of a mineral salts solution (Appendix 2). Flasks were incubated overnight under cool white fluorescent light at 25 ºC with shaking (100 rpm). Cheesecloth was transferred to a sterile 9 cm Petri dish. Sterile, deionised water was added to cover the cheesecloth and plates were incubated at 4 ºC for 30 minutes. Plates were transferred to a light box and left until the sporangia began releasing zoospores. If zoospores were not released after 90 minutes, plates were incubated again at 4 ºC for 30 minutes and then returned to the light box until zoospore release. Zoospores were distributed between four 9 cm Petri dishes and 15 ml of clarified

V8 broth was added. Water was again added to cheesecloth and the cold shock treatment was repeated so more Petri dishes could be filled with zoospores until 16 Petri dishes of zoospores and clarified V8 broth was obtained. Plates were incubated at 25 ºC under cool white fluorescent light between 36 and 48 hours.

To prepare protoplasts, the 16 plates containing mycelium from zoospores was transferred to 50ml Falcon tubes (Sarstedt; Australia) and centrifuged for 5 minutes at 960 *g*. The liquid was discarded and the mycelium resuspended in 10 ml KC osmoticum (0.64 M KCl, 0.2 M $CaCl_2$). Mycelium was pelleted (150 *g,* 2 minutes) and the supernatant discarded. The mycelial pellet was resuspended in 10 ml of KC osmoticum. To this suspension, 5 mg ml^{-1} lysing enzymes from *Trichoderma harzianum* (Sigma; Germany) and 3 mg ml^{-1} Cellulase "Onozuka" R-10 (Yakult Pharmaceutical; Japan) was added. The suspension was incubated at room temperature for approximately 1 hour.

The solution was then filtered through sterilised cheesecloth into a fresh 50 ml Falcon tube. The protoplasts were pelleted (830 *g*, 4 minutes), the supernatant discarded and protoplasts resuspended in 10 ml KC buffer. This was centrifuged for a further 4 minutes at 830 *g*, the supernatant discarded and the pellet resuspended in 10 ml of a KC/ MT buffer solution (MT buffer – 1 M Mannitol, 10 mM Tris.HCl (pH 7.5), 20 mM $CaCl_2$). This was centrifuged (830 *g*, 4 minutes), the supernatant discarded and the pellet resuspended in 10 ml MT buffer and again centrifuged at 830 *g* for 4 minutes. The supernatant was discarded and the pellet completely resuspended in 1 ml MT buffer.

Between 20 – 30 μg of plasmid p34GFN was added to the 1 ml suspension of protoplasts (approximately 10^6 protoplasts) in MT buffer and mixed by gentle rolling of the tube before

incubating for 5 minutes at room temperature. Turning the tube slowly, 1 ml of freshly prepared 50 % (w/v) Polyethylene glycol 4000 (BDH; England) was added over a 30 second period. The suspension was allowed to sit for 2 minutes before inverting the tube once and allowing incubation for a further 3 minutes at room temperature. 2 ml of V8 broth with 1M mannitol was added to the tube, inverting gently to mix. After 2 minutes, a further 6 ml of V8 broth with 1M Mannitol was added and the tube again inverted once to mix. Three minutes later, the entire contents were added to approximately 10 ml V8 broth with 1 M mannitol on a 9 cm sterile Petri dish. This was incubated for 2 – 3 days at 25 ºC and the mixture centrifuged in a 50 ml Falcon tube for 5 minutes at 830 *g*. The supernatant was discarded and the pellet was resuspended in 200 µl of clarified V8 broth. This was spread over a 9 cm Petri dish containing clarified V8 agar (with 20 µg geneticin ml^{-1} [Sigma, Germany]).

Protoplast growth was followed under the microscope and any growth observed over a 4 week period was transferred to fresh selective plates and also checked under the microscope (Olympus BX51; Olympus, Australia) using blue excitation (wavelength 480 nm) for fluorescence.

6.2.3 Southern Blot Analysis

DNA extraction

Putative transformants were considered those cultures that continued to grow on selective medium after three subcultures (Judelson *et al*. 1993). DNA from all putative transformants (approximately 24 cultures from three parents) and their non-transformed parents was extracted using Graham's extraction method (Graham *et al.* 1994) with some modifications. Approximately 100 – 400 mg of mycelium was collected for each isolate to which 5 ml of extraction buffer (2 % (w/v) Hexadecyltrimethyl-ammonium bromide, 100 mM Tris.HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA) was added. The suspension was incubated at 65 ºC for 60 minutes. The suspension was separated through centrifugation at 15 000 *g* for 5 minutes, the supernatant being transferred to a fresh eppendorf tube and mixed with an equal volume of chloroform: isoamylalcohol (24:1). The mixture was centrifuged at 15 000 *g* for 5 minutes. The upper aqueous layer was collected to which 0.1 volumes of 7.5 M ammonium acetate and 2 volumes of absolute ethanol were added. To precipitate the DNA, samples were incubated at -20 ºC for 16 hours. Samples were then centrifuged at 1 500 *g* for 2 minutes, the supernatant discarded and the pellet washed once with 70 % ethanol. Pellets were dried in a vacuum before resuspending in 500 µl of sterile distilled water. DNA concentration was measured for each sample using the Nanodrop ND-1000 Spectrophotometer (Biolab, Australia).

Restriction Digestion of Phytophthora DNA

Digestion of *Phytophthora* DNA was carried out using 10 µg DNA, 5 µl of enzyme from *Xanthomonas badrii* (*Xba*1; Promega, Australia), 15 µl buffer (90 mM Tris-HCL (pH 7.9), 2.25 NaCl, 90 mM $MgCl_2$, 15 mM dithiothreitol) and sterile water to make the final volume to 100 µl. The reaction was incubated in a 37 ºC waterbath for 18 hours. The products were concentrated using ethanol precipitation as described earlier and resuspended in 10 µl sterile water. The samples were run alongside a 1 kb DNA ladder (Promega, Australia) and plasmid p34 GFN 1, 10, 100 and 1000 copy number controls on a 1 % electrophoresis gel for 18 hours at 25 V. This process was repeated using 50 µg DNA for the initial digestion.

Southern blot hybridisation

Gels were soaked in 0.25 M HCl for 5 minutes, followed by rinsing in fresh water for another 5 minutes. With gentle agitation, gels were soaked in 0.5 M NaOH and 1.5 M NaCl combined for 45 minutes. Gels were rinsed with fresh water before soaking in neutralisation buffer (0.5 M Tris-HCl (pH 7.2), 1.5 M NaCl, 0.001 M EDTA Na_2) for 30 minutes. The gels were washed in 2x SSC (0.3 M NaCl, 30 mM sodium citrate) for 5 minutes.

A capillary transfer system was set up (Figure 6.6). A dish was filled with 20x SSC (3 M NaCl, 300 mM sodium citrate), a long piece of chromatography paper was soaked in the solution and overlayed on a glass plate separated from 20x SSC by a plastic block. The gel with DNA to be transferred was placed on top, surrounded but not covered by parafilm and a Hybond-N^+ Nucleic acid transfer membrane (0.45 µm; Amersham, Australia) was placed on top. Chromatography paper was rested on the membrane and a wad of paper towels

separated the membrane from the top glass plate on which a weight was placed. This was left for 24 hours at room temperature to allow the DNA transfer to take place.

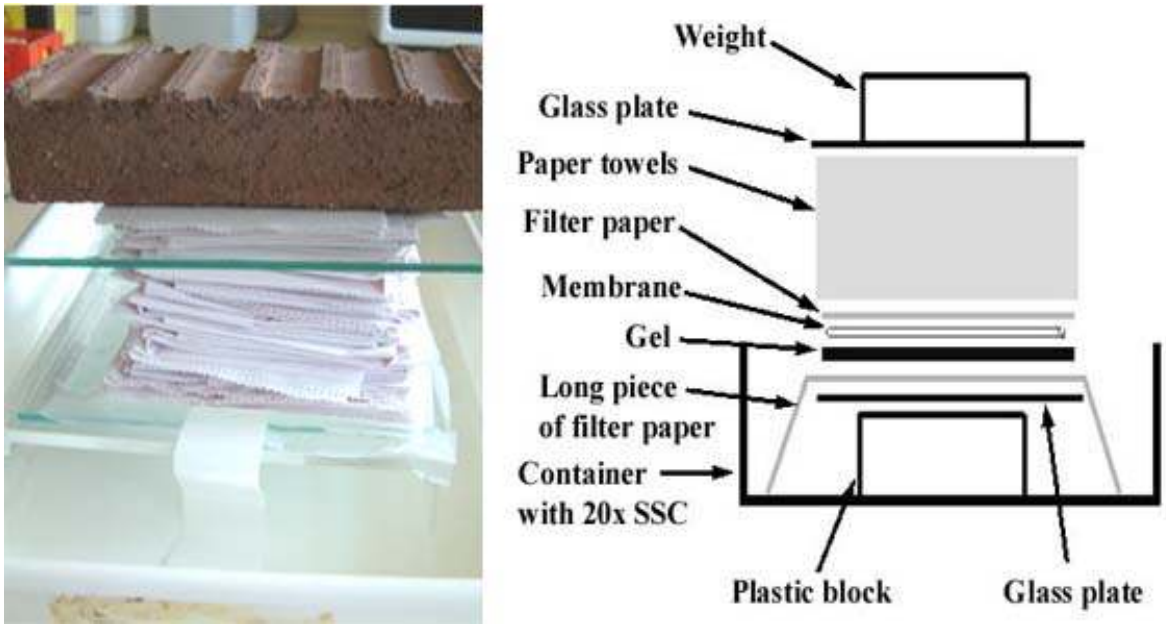

Figure 6.6: Capillary transfer system for transferring DNA from an agarose gel to a Hybond-N^+ Nucleic acid transfer membrane (0.45 µm; Amersham, Australia).

The capillary transfer system was dismantled, the slots of the gel marked with pencil onto the Hybond N^+ membrane. The gel was soaked in ethidium bromide (1 µg ml^{-1}) for 10 minutes and then viewed on an ultraviolet transilluminator (Gibco BRL TFX-35M Life Technologies) to determine the success of the DNA transfer. The membrane was soaked in 6x SSC (0.9 M NaCl, 90 mM sodium citrate) to remove remaining agar. The membrane was allowed to dry for 30 minutes and DNA was fixed to the membrane by exposure to UV light (150 m Joule) using the GS Gene Linker UV chamber (BioRad, Australia).

For DNA hybridization, each membrane was immersed in 6x SSC until it was thoroughly wet. The membrane containing 12 µg DNA was placed into a heat sealable bag to which 0.2 ml of pre-hybridisation buffer (1 M NaCl, 10 % sodium dodecyl sulfate (SDS), 50 %

formamide) was added for each square centimetre of the membrane. Most of the air was removed from the bag and the open end was sealed with a heat sealer. The bag containing the membrane was submerged in a 42 ºC waterbath for 2 hours. In repeat experiments, both the 12 µg DNA and 50 µg DNA membranes were pre-hybridised in Rapid-hyb buffer (Amersham Biosciences, UK) for 30 minutes in 0.2 ml for each square centimetre of the membrane.

After pre-hybridisation, 100 µg denatured calf thymus ml^{-1} was added to the 12 µg DNA membrane in pre-hybridisation buffer. Template DNA, plasmid p34GFN, was labelled using the Megaprime DNA Labelling Systems (Amersham Biosciences, Australia), according to the manufacturer instructions using Redivue [α-^{32}P]dCTP (Amersham Biosceinces, Australia). Redivue 5'-[α-^{32}P]dCTP pipette tips (3000 Ci $mmol^{-1}$) (Amersham Biosciences) were used for the 12 µg DNA membrane in pre-hybridisation buffer and in experiments with membranes hybridised in Rapid-hyb buffer, the template DNA was labelled in the same way but with Redivue 5-[α-^{32}P]dCTP stored in vials(Amersham Biosciences). 2 ng ml^{-1} labelled probe was added to the membranes soaking in buffers. Bags were resealed and the 12 µg DNA membrane in pre-hybridisation buffer was submerged in a 42 ºC waterbath for 18 hours, while the 50 µg DNA membrane and the 12 µg DNA membrane in the repeat experiment in Rapid-hyb buffer were incubated in a 65 ºC waterbath for 2 hours.

The 12 µg DNA membrane that had been in pre-hybridisation buffer was washed in 2x SSC and 0.1 % (w/v) SDS at room temperature for 10 minutes and then submerged in this solution for a further hour. This was followed by washing twice for 30 minutes in 0.1x

SSC and 0.1 % (w/v) SDS. The 50 µg and 12 µg DNA membranes that had been in Rapid-hyb buffer were washed twice for 10 minutes at room temperature in 2 x SSC and 0.1 % (w/v) SDS, followed by soaking in 1x SSC and 0.1 % (w/v) SDS for 15 minutes at 65 ºC. Finally, the membrane was washed in 0.1x SSC and 0.1 % SDS for 50 minutes at 65 ºC. Excess liquid was removed and the membrane wrapped in plastic (Gladwrap, Australia) and placed inside a X-ray film cassette with an X-ray film (Super HR-G 30, Fujifilm, Germany) on top. The X-ray film cassette was incubated at -80 ºC for 1 day and the X-ray film was developed using a film processor (FPM 3000, Fuji, Germany). The X-ray film was replaced and incubated for a further 7 days.

6.3 Results

Plasmid preparation

Plasmids pBINHL1, pHAMT35G, pVW2 and p34GFN were successfully extracted (Figure 6.7). Of the four different enzymes (*Kpn*1, *Bam*H1, *Sph*1, *Sst*1) used to digest pBINHL1, only enzymes *Kpn*1 and *Sst*1 were found to cut plasmid pBINHL1 once (Figure 6.8). This was important for future experiments for insertion of single genes into the plasmid and *Kpn*1 was subsequently chosen for digestion reactions.

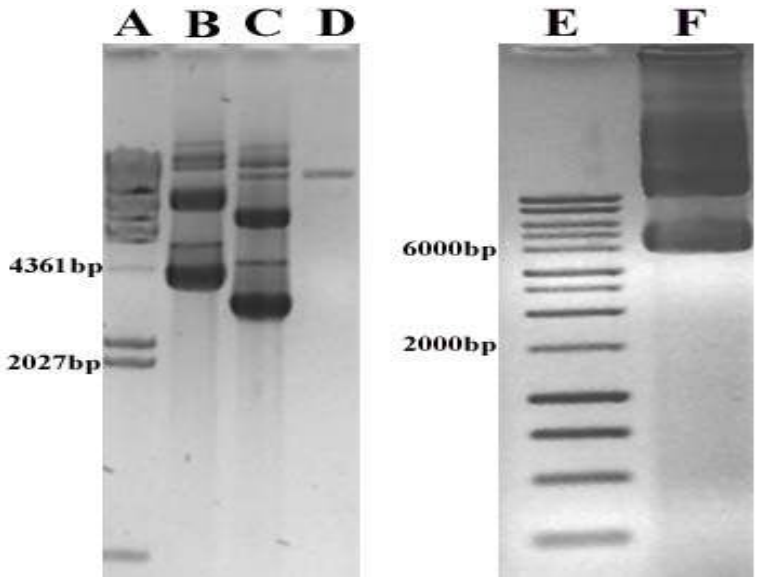

Figure 6.7: Products of plasmid extraction. **A.** Lamba *Hin*dIII Marker (Biotech); **B.** pHAMT35G; **C.** pVW2; **D.** pBINHL1; **E.** 1kb Marker (Promega); **F.** p34GFN.

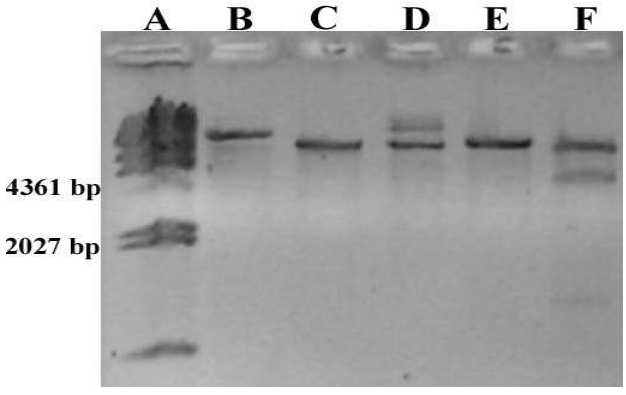

Figure 6.8: Digestion of pBINHL1 with enzymes *kpn*1 (**C**), *Bam*H1 (**D**), *Sst*1 (**E**) and *Sph*1 (**F**). **A.** Lambda *Hin*dIII Marker (Biotech). **B.** pBINHL1 undigested control.

Following the PCR amplification of the GUS and GFP genes from pVW2 and pHAMT35G and the incorporation of these into the binary vector pBINHL1, banding patterns were only observed in some of the cultures screened for 40 ng μl^{-1} pHAMT35G (Figure 6.9). No banding patterns were observed in cultures screened for pVW2. The concentration of pHAMT35G at 40 ng μl^{-1} was found to be the concentration at which recombinants formed with the vector pBINHL1 at the highest frequency.

The *Hae*III marker was unsuitable for the gel electrophoresis in Figure 6.8 as its banding pattern ranged between 72 – 1353 bp but bands were observed in the 40 ng μl^{-1} pHAMT35G lanes indicating a product larger than this range (Figure 6.9). However, the exact size could not be determined as no marker was available for comparison, therefore, positive bands were extracted from the gel in Figure 6.9 (F) and digested with *Nco*1 to confirm banding patterns typical of the GUS gene (Figure 6.10 D & E). Therefore, the integration of the GUS gene into pBINHL1 and subsequent transformation into *E. coli* was deemed successful. These products were named pBINHLGUS.

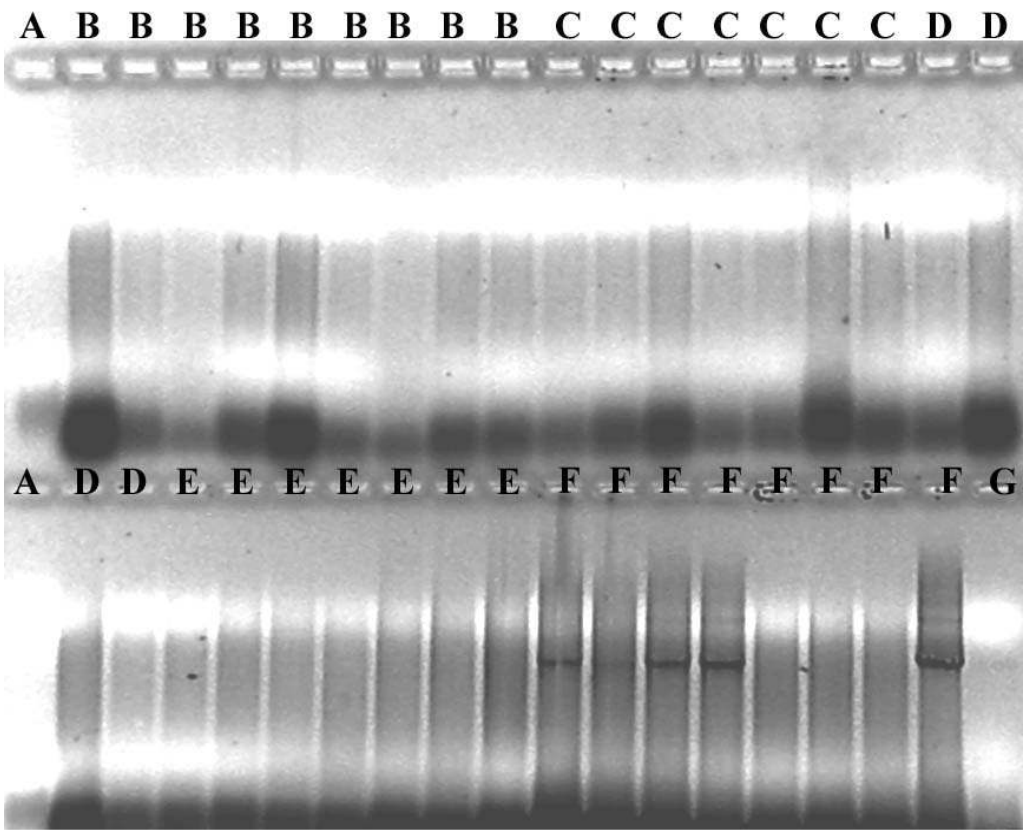

Figure 6.9: PCR reactions to screen the success of the integration of the GFP (from pVW2) gene and the GUS (from pHAMT35G) gene into the pBINHL1 plasmid and subsequent incorporation into *Esherichia coli*. Observable bands were only present in lanes **F. A.** *Hae*III Marker (Biotech); **B.** Transformation using 20 ng μl^{-1} pVW2 DNA; **C.** Transformation using 40 ng μl^{-1} pVW2 DNA; **D.** Transformation using 10 ng μl^{-1} pHAMT35G DNA; **E.** Transformation using 20 ng μl^{-1} pHAMT35G DNA; **F.** Transformation using 40 ng μl^{-1} pHAMT35G; **G.** Blank control. NB – Multiple lanes with same letter indicate replicate *E. coli* plasmids.

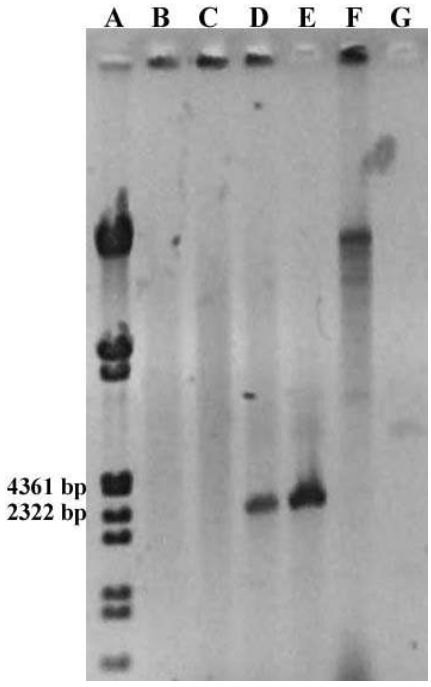

Figure 6.10: Banding patterns resulting from the digestion of the PCR product from pBINHL1 with *Nco*1 to confirm the success of integration of the GUS gene into plasmid pBINHL1. **A.** Lambda *Hin*dIII Marker (Biotech); **B.** Positive control – original GUS plasmid; **C - F.** Digestion of positive PCR bands in Figure 6.9. Lanes **D & E** showed a product in the range 2322 bp to 4361 bp, indicative for the length of the desired fragment; **G.** Blank control.

Cultures of *A. tumefaciens* growing on selective agar plates after conjugal transfer were confirmed by PCR as successfully transformed with pBINHLGUS, pBINHL1 and pVBK1 (Figure 6.11). The primers used for the PCR of pBINHLGUS amplified the desired fragment of 1200 bp, the PCR of pBINHL1 amplified the desired fragment of 367 bp and the PCR of pVBK1 amplified the desired fragment of 524 bp (Figure 6.11).

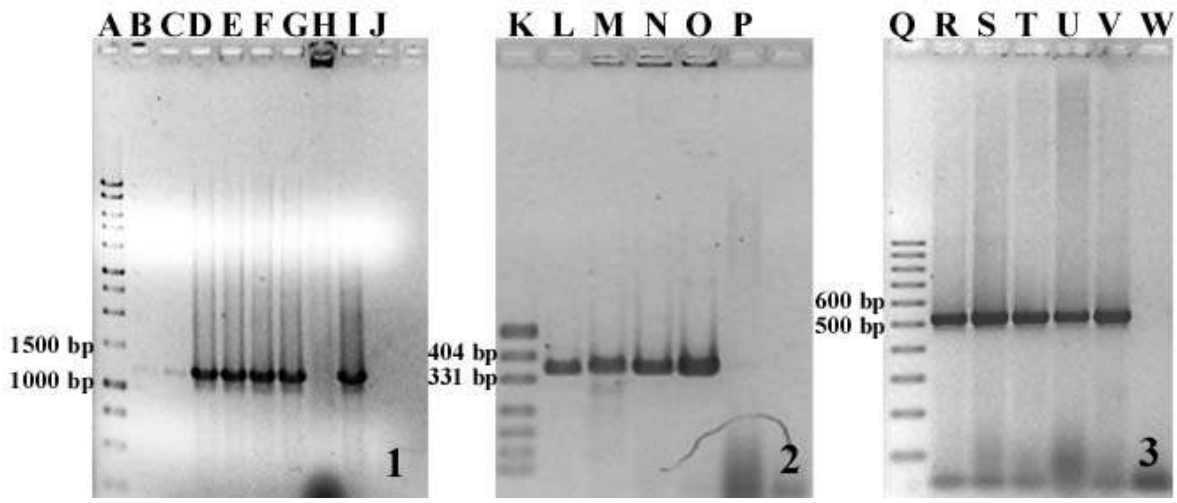

Figure 6.11: PCR screening for successful transformation of pBINHLGUS (**1**), pBINHL1 (**2**) and pVBK1 (**3**) plasmids into *Agrobacterium tumefaciens* isolates LB4044 and AGLO.
A. 1kb Marker (Promega); **B & E** Positive control (*Escherichia coli* + pBINHLGUS); **C & F.** AGLO + pBINHLGUS; **D & G.** LB4044 & pBINHLGUS; **H.** Negative control (AGLO + PCR mix); **I.** Positive control (pHAMT35G); **J.** Blank control.
K. pUC19DNA/ *Hpa*II marker (Biotech); **L.** Positive control (*E. coli* + pBINHL1); **M.** AGLO + pBINHL1; **N & O.** LB4044 + pBINHL1; **P.** Blank control.
Q. 100 bp Marker (Promega); **R.** Positive control (*E. coli* + pVBK1); **S.** Positive control (*E. coli* + pVW2); **T.** AGLO + pVBK1; **U & V.** LB4044 + pVBK1. **W.** Blank control.

Transformation of Phytophthora using Agrobacterium tumefaciens

No method tested for transformation of *Phytophthora* using the transformed *A. tumefaciens* was successful as there was no survival or growth on selective medium. Neither intact or damaged mycelium of *P. cinnamomi*, *P. drechsleri*, *P. cambivora*, *P. citricola*, *P. megasperma* or *P. cryptogea* was transformed at either diluted or un-diluted concentrations of *A. tumefaciens* containing pBINHL1, pVBK1 or pBINHLGUS genes. Co-transformation using pVBK1 and pBINHL1 was also unsuccessful. Zoospores produced under non-sterile conditions were only used for this method and also showed no transformation.

Protoplast Transformation

Treatment of *P. cinnamomi* mycelium with lysing enzymes from *Trichoderma harzianum* and cellulase weakened the cell walls which allowed protoplasts to be released into KC osmoticum (Figure 6.12).

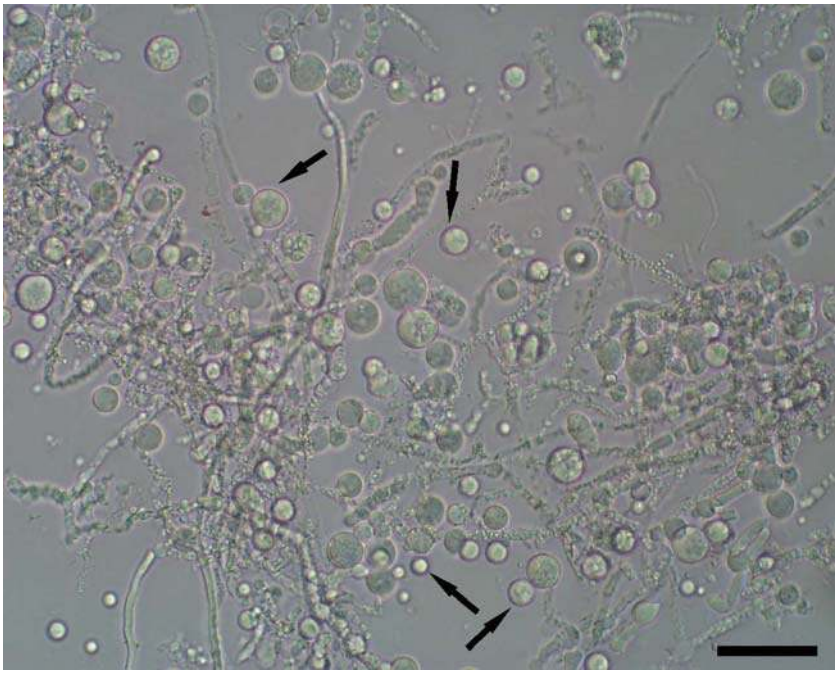

Figure 6.12: Mycelium of *Phytophthora cinnamomi* releasing protoplasts into KC osmoticum. (Arrows indicate some protoplasts). Bar = 50 µm.

After transformation of the protoplasts with the plasmid p34GFN and their regeneration on V8 broth for 2 -3 days, it was found that many of the growing protoplasts clumped together when transferred to selective medium and were difficult to spread evenly across the plate. This meant that after 10 days, colonies of many regenerating protoplasts (Figure 6.13) were difficult to distinguish individually and it was possible that more that one protoplast contributed to the single colonies that were subcultured to a fresh plate. For this reason, tips of hyphae were then subcultured to ensure single colonies but as a result, transformation efficiency could not be determined.

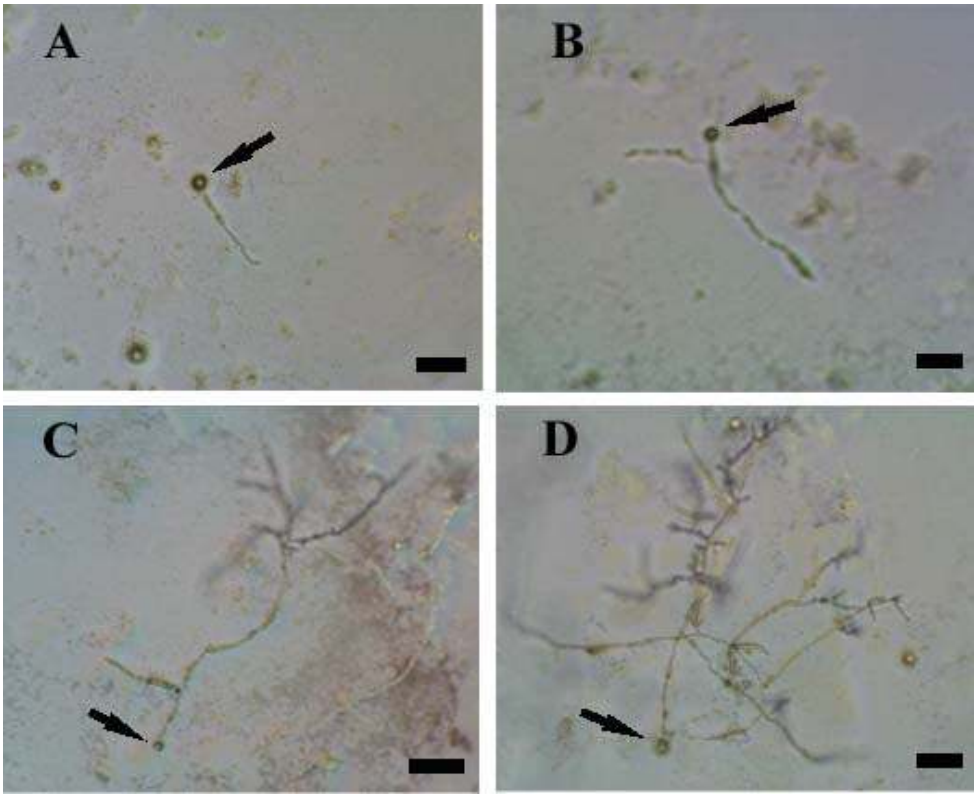

Figure 6.13: Different stages of growth of regenerating protoplasts of *Phytophthora cinnamomi* on media containing geneticin for selection. **A.** Initial germination of protoplast (1 germination tube); **B.** Germ tube branching; **C & D.** Further branching of germ tube. Arrows point to original protoplast. Bar = 50 µm.

Protoplasts from *P. cinnamomi* isolates MP127, MP103 and MP94-15 were successfully transformed as determined by fluorescence (Figure 6.14). Fluorescence was observed in the cytoplasm of the mycelium and chlamydospores. Isolates did not fluoresce until after 9 days in culture and as cultures aged, fluorescence intensity increased. Hyphae of *P. cinnamomi* that had not been transformed did not fluoresce under blue excitation to give green fluorescence.

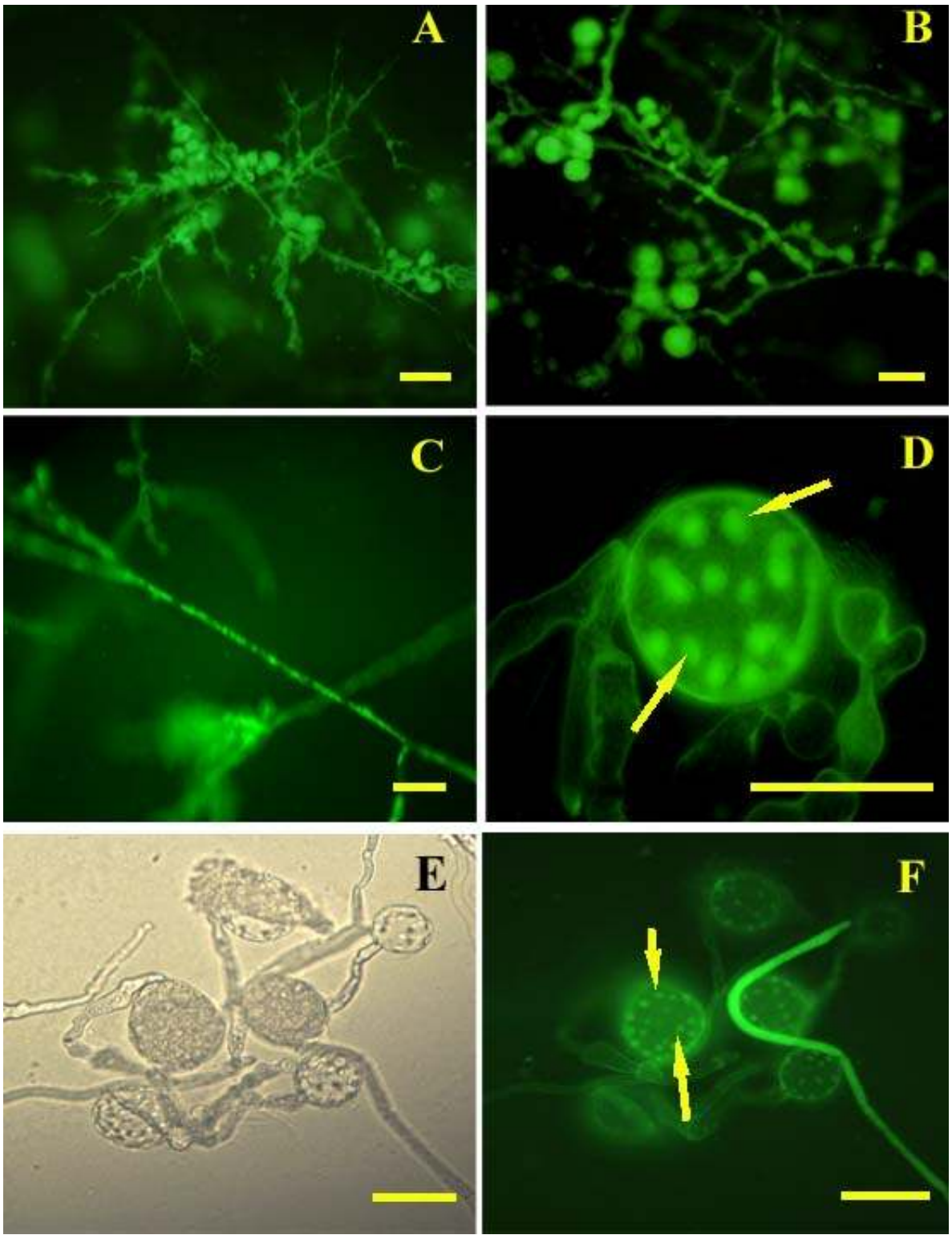

Figure 6.14: Isolates of *Phytophthora cinnamomi* fluorescing after transformation under light at 480 nm. **A, B & C.** Fluorescent mycelium; **D.** Fluorescent patches in chlamydospores; **E.** Chlamydospores under bright light; **F.** Same as E but under light at 480 nm. Arrows point to some fluorescence. Bar = 50 µm.

Southern blot analysis

The Southern blot analysis was only able to detect DNA as low as 100 copy numbers of the gene and was not sensitive enough to detect the 10 and 1 copy number dilutions (Figure 6.15). Variations in initial DNA concentration, incubation time of the labelled membrane with the X-ray film, hybridisation buffers, radioisotope storage, washing buffers and times did not affect the sensitivity of the probe. Background was reduced when the membrane was washed in 2x SSC and 0.1 % (w/v) SDS for 10 minutes and then submerged in this solution for an hour, followed by washing twice for 30 minutes in 0.1x SSC and 0.1 % (w/v) SDS (Figure 6.15 A & B).

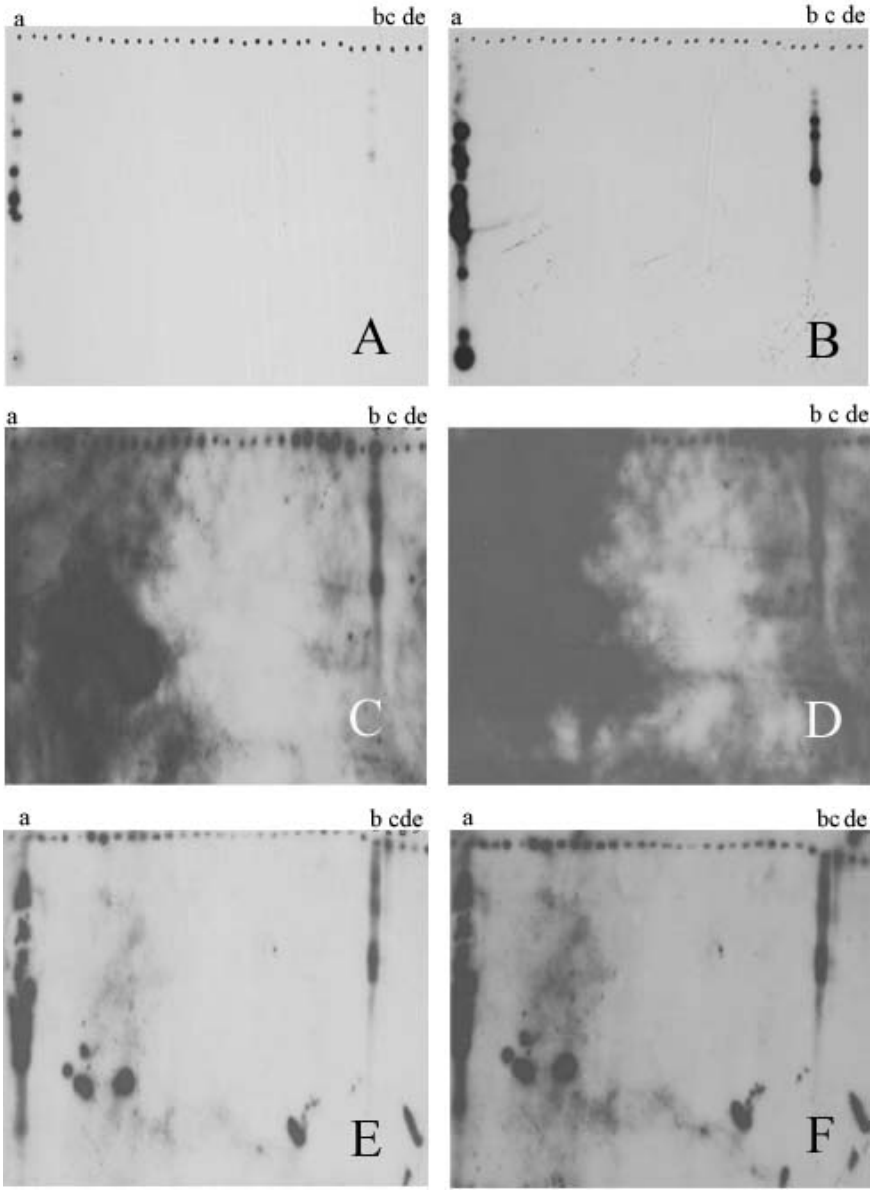

Figure 6.15: Southern blot analysis of putative transformants to assess the success of integration of the Green Fluorescent Protein and geneticin resistance genes. Membranes were blotted from gels containing 12 µg DNA (**A, B, C & D**) and 50 µg DNA (**E & F**). Hybridisation buffer, source of radioisotope for labelling, incubation and membrane washing times for (**A & B**) differed to that used for (**C, D, E & F**). **A, C & E.** 1 day incubation period at -80 ˚C after labelling. **B, D & F.** 7 day incubation period at -80 ˚C after labelling. **a.** Molecular marker, **b.** Control 1000 copy number, **c.** Control 100 copy number, **d.** Control 10 copy number, **e.** Control 1 copy number. Background interference from the probe was high for **C, D, E & F**.

6.4 Discussion

Phytophthora cinnamomi was successfully transformed to express GFP and geneticin resistance using mycelial protoplasts treated with polyethylene glycol. Transformation using protoplasts and polyethylene glycol has been used quite extensively for *Phytophthora* species (Judelson *et al*. 1991, 1993; Kamoun *et al.* 1998; van West *et al*. 1999) but *P. cinnamomi* had not previously been transformed using this method.

Protoplast transformation using polyethylene glycol was found to be successful for each of the three *P. cinnamomi* isolates tested. Improving transformation efficiency was beyond the scope of this experiment although previous researchers have found transformation efficiency of *Phytophthora* spp. using the protoplast transformation method to be around 0.1 – 2 transformants per μg DNA or per 10^8 protoplasts (Judelson and Michelmore 1991; van West *et al.* 1999) but variations within species have been observed (Si-Ammour *et al.* 2003). It was found that transformed cultures needed to be at least 9-days-old before their fluorescence was easily visible which is consistent with previous research that has found transformed cultures may take between 9 – 20 days before fluorescing under blue excitation (Champouret and Kamoun 2004).

Fluorescence was confined to the cytoplasmic material within the mycelium and developing chlamydospores. In fully formed, aging chlamydospores, fluorescence was seen in small unidentified vesicles. This has not been clearly described in previous research and thin-sectioning is required to determine where the green fluorescent protein is accumulating in the spore. Hyphal walls of the transformants produced a dull green colour similar to that seen in non-transformed controls. This autofluorescence was easily recognisable from the vibrant green colour produced in cytoplasm of transformants.

Time constraints meant the Southern blot was unable to be optimised to confirm the stability of the integration or determine the copy number of p34GFN DNA integration into the DNA of *P. cinnamomi.* Previous reports have shown that stable integration of p34GFN into *P. brassicae* and *P. infestans* occurred in 85 % of transformants (Si-Ammour *et al.* 2003), showing the necessity to optimise the Southern blot to determine transient and stable transformants in the current experiment. Future optimisation strategies for Southern blot analysis could involve using more ^{32}P-labelled probe, a combination of radio-labelled dNTPs and the use of a shorter probe to increase specificity and sensitivity.

In contrast to protoplast transformation, transformation attempts using *A. tumefaciens* were unsuccessful. The development of a plasmid containing the hygromycin resistance gene and the gene for the GUS protein was successful. The GFP gene however was not successfully incorporated into a separate plasmid containing the hygromycin gene but this may have occurred if experiments were repeated.

Transformation of pBINHL1, pVBK1 and the newly developed pBINHLGUS into separate strains of *A. tumefaciens* was successful. However, using the *Agrobacterium*-mediated transformation system to subsequently transform *Phytophthora* species with these plasmids was unsuccessful as no cultures of *Phytophthora* species grew on selective media containing hygromycin. This showed either that the antibiotic resistant gene had not been incorporated into the genome of the organism or it was not being expressed by the pathogen.

Previously, the GUS and hygromycin resistance gene with a *Ustilago maydis* hsp 70 promoter (pCM54) had been successfully incorporated into *P. cinnamomi* using the biolistics approach to transformation (Bailey *et al.* 1993). However, Judelson *et al.* (1993) compared the vector used by Bailey *et al.* (1993) to vectors with promoters from an oomycete organism (ie. *Bremia lactucae*). They could not obtain transformation of *P. megasperma* unless the promoters were from an oomycete organism and suggested that pCM54 was unstable in *P. megasperma* (Judelson *et al.* 1993). This had previously been reported by Bailey *et al.* (1993) as they had found pCM54 to be extrachromosomally inherited and unstable in *P. capsici* and *P. parasitica*. Another possibility is that vectors used for transformation of filamentous fungi were unsuitable for *Phytophthora* due to the different sequence requirements for transcription (Judelson and Michelmore 1991; Judelson *et al*. 1993).

The lack of success in *Agrobacterium*-transformation of *Phytophthora* spp. using pHAMT35GUS or the co-transformation of pVBK1 and pBINHL1 provides further evidence of the unsuitability of non-oomycete promoters for *P. cinnamomi* transformation. The inserted hygromycin resistance gene being flanked by a promoter and terminator from the yeast fungus, *Ustilago maydis,* appears not to have been expressed in *P. cinnamomi* as the organism is unable to recognise the promoter and terminator sequences flanking the hygromycin resistance gene with the result that the organism was unable to grow on medium containing hygromycin.

The transformation of *P. cinnamomi* to incorporate a GFP gene provides an important molecular tool for the study of the biology of the pathogen, in particular, the way it survives and grows within plant and soil material.

CHAPTER 7

The Effect of GFP Transformation on Fitness and Pathogenicity of *Phytophthora cinnamomi*

7.1 Introduction

Transformants of *P. brassicae* and *P. infestans* with p34GFN are known to have reduced fitness in *in vitro* growth compared to non-transformed controls (Si-Ammour *et al.* 2003). Similarly, Bae and Knudsen (2000), studying GFP transformed *Trichoderma harzianum* found transformants grew significantly slower than the wild type on Potato Dextrose Agar, so that by day 3, there was an 18 % reduction in growth. The stability of GFP expression has been found to vary (Bottin *et al.* 1999; Cvitanich and Judelson 2003; Si-Ammour *et al.* 2003; Vijn and Govers 2003) and there are reports of many subcultures of putative GFP transformed *P. megasperma* f.sp. *glycinea* failing to grow on selective media (Judelson *et al*. 1993; Cvitanich and Judelson 2003).

Tests were designed to examine the stability of GFP expression and the effect of the transformation process on culture fitness in *P. cinnamomi* by assessing their root colonisation ability and colony growth rates compared to their non-transformed parents. Due to time constraints of the project, the Southern blot analysis was not optimised and was unable to determine success of the integration. Therefore only these preliminary tests could be performed on two putative transformants.

7.2 Methods and Materials

Experimental Design

Putative transformed cultures of *P. cinnamomi*, MP103 (1/44) and MP127 (17/27), were selected based on their ability to grow on selective media after three subcultures (Judelson *et al.* 1993) and the intensity of their fluorescence after this time. Controls were the parent non-transformed *P. cinnamomi* isolates MP103 and MP127. All isolates were passaged through aseptically grown lupins (*L. augustifolius*) prior to experimentation. Lupin seedlings were prepared by surface sterilising the seeds in 6 % solution of sodium hypochlorite for 30 minutes before washing three times in sterile water and placing them on filter paper moistened with sterile water in a 90 mm Petri dish 7 days at 25 ºC. A 5 mm^2 cube taken from the outer growing edge of a one-week-old culture of *P. cinnamomi* growing on V8 agar (Appendix 2) was inoculated onto the tips of the lupin roots and seedlings were incubated for a further 7 days before the roots were excised and plated onto NARPH (Appendix 2). Mycelium growth from these roots was then plated onto V8 agar and incubated for 1 week before a 5 mm^2 colonised agar cube from the outer colony edge was used, unless otherwise stated.

Experiments

Comparisons between growth of transformed and non-transformed isolates

Five replicates of each transformed and non-transformed isolate of *P. cinnamomi* were grown on V8 agar and V8 agar containing 20 μg geneticin ml^{-1} at 25 ºC until cultures reached the edge of a 90 mm Petri dish. Colony diameter was measured daily. The experiment was repeated twice on media without geneticin and once for media with geneticin.

The effect of repeated subculturing on the fluorescence emitted by the GFP gene and growth on geneticin medium

Five replicates of each transformed and non-transformed isolate of *P. cinnamomi* were grown on V8 agar, with or without geneticin (20 μg ml^{-1}) for 1 week at 25 ºC. After 7 days, colonies were subcultured to fresh V8 agar and V8 agar containing 20 μg geneticin ml^{-1}. After 4 weeks, a 5 mm^2 agar plug from the mid-radial section of the colony was squashed underneath a coverslip on a microscope slide and observed under blue excitation (480 nm) at 400 – 1000x magnification (Olympus BX51; Olympus, Australia) for fluorescence. This was repeated for 10 subcultures, each subculture being one week apart.

Comparisons between aggressiveness of colonisation of lupin roots for transformed and non-transformed isolates

Lupin seeds were grown aseptically for one week as described above. Four lupin seedlings were then evenly placed around the edges of a 140 mm Petri dish with roots resting towards the centre on filter paper moistened with sterile water. 15 plates were set up in this way for each parent or transformant. A 2 mm^2 colonised agar cube taken from the outer growing edge of a one week culture of a transformed or non-transformed culture of *P. cinnamomi* was placed on each root tip so that in each plate, one lupin seedling was inoculated with each isolate (MP103 (1/44), MP127 (17/27), MP103 or MP127). Plates were sealed with clingfilm (Glad wrap, Australia) to reduce evaporation. After 4 days incubation at 25 ºC, the extent of colonisation in each root was determined. The agar cube was removed and roots of each lupin were cross-sectioned into 5 mm lengths so that the first segment contained the initial inoculation site. Segments were plated onto NARPH media. Plates

were incubated for 3 days after which segments were scored as positive or negative for the presence of *P. cinnamomi*. The experiment was repeated twice.

Statistical Analysis

Statistical analysis was performed using Minitab's General Linear Model for each experiment. Pairwise t-tests were conducted where appropriate.

7.3 Results

Comparisons between growth of transformed and non-transformed isolates

The difference between the behaviour of the isolates in repeat experiments was insignificant ($P > 0.05$) so data were bulked from all experiments. The transformed isolates grew significantly ($P < 0.05$) slower on V8 agar than the non-transformed isolates so that by day number 5, when MP103 reached the edge of the agar plate, the percentage reduction in growth for MP103 (1/44) was 18.8 % and 12.3 % for MP127 (17/27) compared to their non-transformed parents (Figure 7.1).

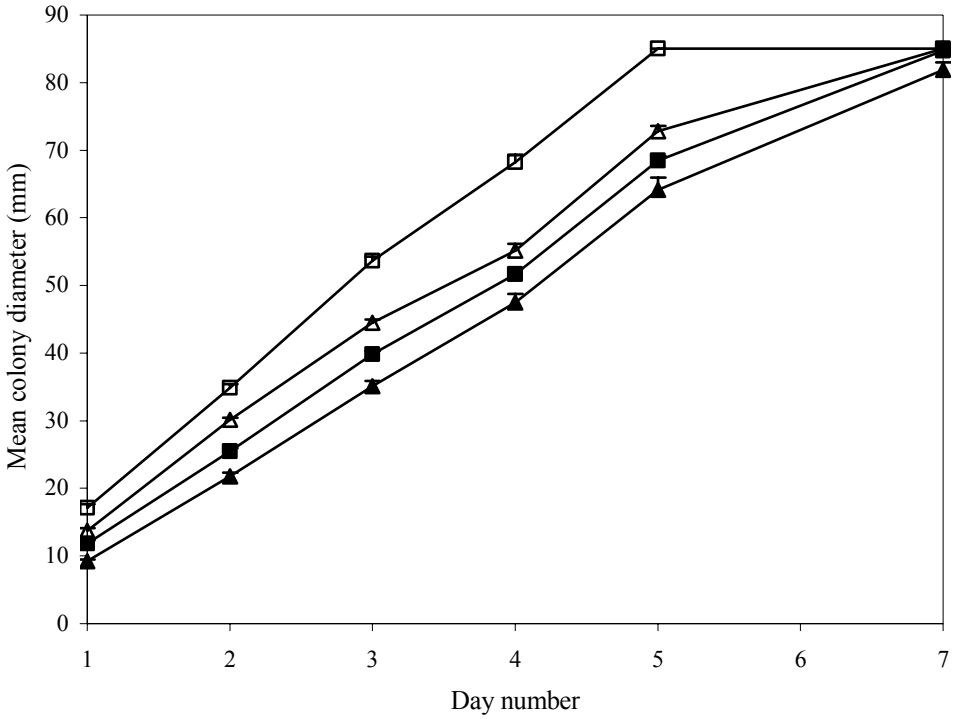

Figure 7.1: Mean colony diameter for non-transformed isolates of *Phytophthora cinnamomi* compared to transformed isolates on V8 agar. (Δ) MP127 control; (▲) MP127 (17/27) transformed; (□) MP103 control; (■) MP103(1/44) transformed. Bars represent positive standard error of the mean.

The diameter of non-transformed isolates on selective V8 agar (20 µg geneticin ml^{-1}) was never greater than 10 mm whereas transformed isolates grew to a colony diameter of approximately 70 mm (MP127 (17/27)) and 40 mm (MP103 (1/44)) (Figure 7.2). However, once these transformed organisms reached this diameter, their radial growth stopped. When subcultured to fresh V8 agar containing 20 µg geneticin ml^{-1}, the mycelium began to grow again. No significant ($P > 0.05$) difference was found between the two experiments run for either transformed isolate MP127 (17/27) or MP103 (1/44) so the data were bulked.

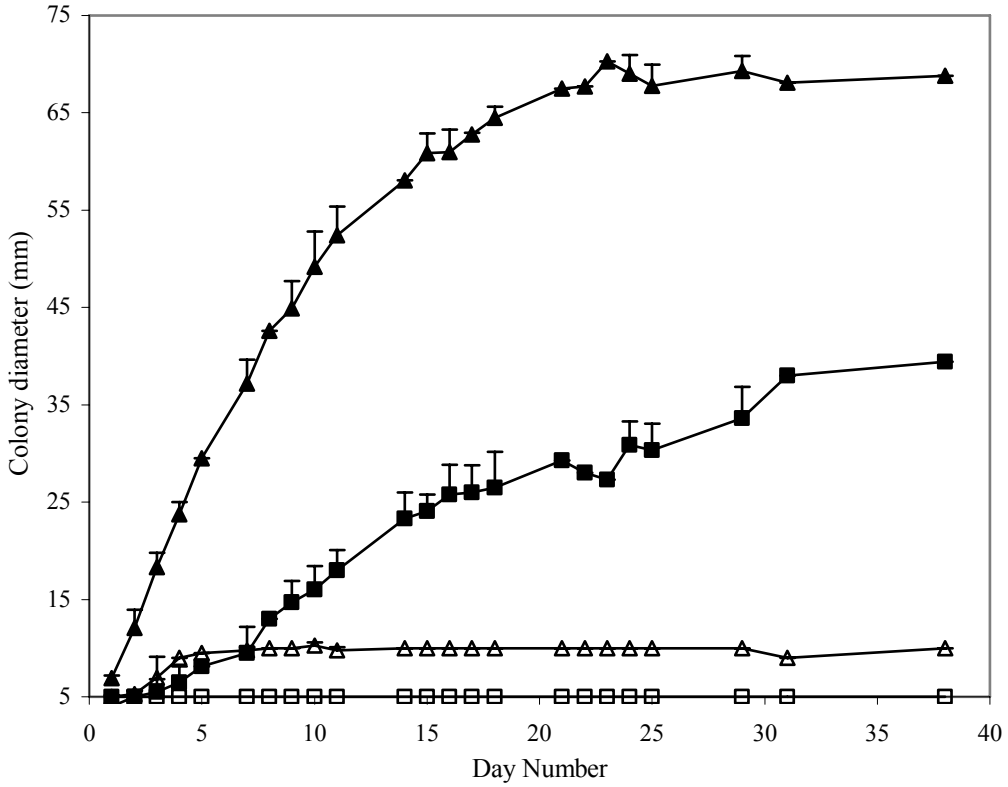

Figure 7.2: Mean colony diameter for non-transformed isolates of *Phytophthora cinnamomi* compared to transformed isolates on selective V8 agar (+ 20 µg geneticin ml^{-1}). (Δ) MP127 control; (▲) MP127 (17/27) transformed; (□) MP103 control; (■) MP103 (1/44) transformed. Bars represent positive standard error of the mean.

The effect of repeated subculture on the fluorescence emitted by the GFP gene and growth on geneticin medium

Transformed cultures of *P. cinnamomi* were found to fluoresce for a total of five subcultures but fluorescence emitted at each subculture appeared to fade over this time. By the sixth subculture, no fluorescence could be observed in transformed isolates MP127 (17/27) and MP103 (1/44). Both MP127 (17/27) and MP103 (1/44) were capable of growing on V8 agar containing geneticin (20 µg ml^{-1}) for the full 10 subcultures. Non-transformed isolates, MP127 and MP103, did not fluoresce nor could they grow on selective medium.

The effect of transformation on the pathogenicity of the organism

There was no significant ($P > 0.05$) difference between the three repeat experiments on growth of transformed and non-transformed *P. cinnamomi* in lupin roots so data were bulked. There was a significant ($P < 0.05$) difference in pathogenicities of the transformed and non-transformed organisms as assessed by the length of the root colonised by the pathogen using a pairwise t-test (Figure 7.3). The percentage reduction in root length colonised was 19.3 % for MP127 (17/27) and 14.1 % for MP103 (1/44) as compared to their parent lines for the same period of time.

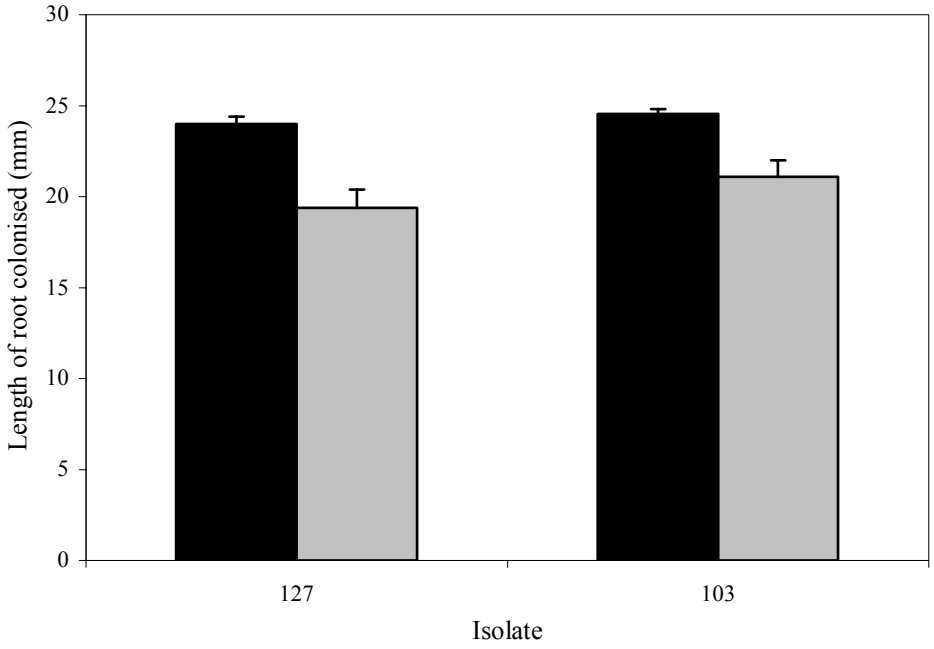

Figure 7.3: Length of aseptic lupin root colonisation with non-transformed (black bars) and transformed (grey bars) *Phytophthora cinnamomi* isolates, MP127, MP127 (17/27), MP103, MP103 (1/44). Roots were incubated with the isolates for 4 days. Bars represent positive standard error of the mean.

7.4 Discussion

Transformants were found to be pathogenic as they colonised sterile lupin roots but they were less aggressive than their non-transformed parents. Radial growth rate of transformants on V8 agar was slower than their non-transformed parents. Interestingly, the larger reduction in growth observed for MP103 (1/44) than MP127 (17/27) on V8 agar did not correspond to a larger reduction in aggressiveness in pathogenicity testing where it was found that MP127 (17/27) was the less aggressive of the two transformants by colonising lupin roots slower than MP103 (1/44).

The reduction in growth rate of transformants compared to non-transformants is similar to previously that reported (Bae and Knudsen 2000; Si-Ammour *et al.* 2003). It is possible that the differences noted in growth of transformants as opposed to non-transformants may be in part due to the metabolites utilised in the production of an extra protein. However, Lee *et al.* (2002) and Si-Ammour *et al.* (2003) have found isolates with similar fitness of growth to their wild type parents in transformants of *Ophiostoma piceae* and *P. infestans.* Therefore, screening of more transformants of *P. cinnamomi* may find transformants with similar fitness characteristics to their wild type parents.

The stability of the transgene in *P. cinnamomi* isolates MP127 (17/27) and MP103 (1/44) is uncertain as the two transformants studied showed reduction in the intensity of the GFP fluorescence over five subcultures until by subculture 6, no fluorescence could be observed. Cultures continued to grow on the selective medium on which their wild type parents could not. This is similar to studies by Bottin *et al.* (1999) where only 13 % of transformants of *P. parasitica* var. *nicotianae* fluoresced and Vijn and Govers (2003) who found that only 60 % of zoospore cultures arising from transformants, showed GUS expression, yet all

transformants were capable of growing on selective medium which non-transformants could not. A Southern blot analysis showed that the GUS gene was present but they could not explain why GUS expression was lost but thought it may be due to transcriptional gene silencing (Judelson *et al.* 1993; Bottin *et al.* 1999; Cvitanich and Judelson 2003; Vijn and Govers 2003). Loss of DNA expression in transgenic organisms including fungi is a well documented phenomenon, especially for DNA sequences that do not confer a selective advantage (Monke and Schaefer 1993). However, GFP expression in *P. brassicae* and *P. infestans* with p34GFN has been stable for at least two years with fortnightly subculturing on selective medium, quantified by GFP intensity (Si-Ammour *et al.* 2003). This indicates that further screening of transformants is required.

There has been an observed link between the integration of multiple copies of donor DNA and methylation in *Schizophyllum commune* transformed with hygromycin B (*hph*) (Mooibroek *et al.* 1990). It was proposed that after transformation, multiple copies of integrated DNA are methylated to prevent over-expression (Mooibroek *et al.* 1990). Organisms transformed using the PEG/ protoplast system have been shown to often carry more than one transgene copy (de Groot *et al.* 1998; Covert *et al.* 2001; Vijn and Govers 2003). Time constraints limited optimisation of the Southern blot to determine the number of transgene copies (Chapter 6) but future studies in number of donor DNA inserted into *P. cinnamomi* could provide a possible explanation why the intensity of fluorescence declined over 10 subcultures in two transformants.

Although transformed isolates grew successfully on V8 agar with geneticin, colonies never reached the edge of the plate and ceased to grow at approximately 45 mm and 65 mm (MP127 (17/27) and MP103 (1/44), respectively). Robinson and Deacon (2001) described

a similar occurrence in transformed *Rhizoctonia solani.* They observed transformants stopping growth at 35 or 65 mm colony diameters, depending on the gene insert. In these cases, the explanation was probably non-integrative transformation as 99 % of these isolates fail to grow upon subculture (Monke and Schafer 1993; Robinson and Deacon 2001). However, in the case of *P. cinnamomi*, upon subculturing from the outer edge of MP127 (17/27) and MP103 (1/44), to fresh selective media, cultures continued to grow but again did not exceed the 45 or 65 mm colony diameter. This would suggest that the gene had been successfully integrated into the genome and the cessation of growth might be due to the build-up of staling products in the media (Park 1961; Griffin 1994). A Southern blot analysis would be required to determine if the gene had been integrated but due to time restraints of the project, this could not be achieved.

More work is required to analyse other transformants to find those isolates that behave more closely in growth rates and aggressiveness to their non-transformed parent as it has previously been found that fitness can vary across transformants (Si-Ammour *et al.* 2003). Due to time constraints of this project, this was not possible but results obtained here highlight the necessity that future experiments always use non-transformed controls to ensure all behavioural characteristics observed were real and not a result of the reduced fitness of the pathogen.

CHAPTER 8

General Discussion

This thesis has investigated the short-falls in our knowledge on the saprophytic ability of *Phytophthora cinnamomi.* The research provided evidence to support the accepted knowledge on the existence of thick-walled chlamydospores and challenged the assumption that oospores play no role in the survival of the pathogen. The major findings and the further work that is required included:

- ***P. cinnamomi* is not a saprophyte** except under conditions of low microbial competition (Chapter 2). While the pathogen was not considered a saprophyte of ecological importance, it displayed a competitive ability in the colonisation of added organic matter that had been sterilised by autoclaving. This fits with the ability of *P. cinnamomi* to colonise healthy plant tissue such as the roots and collars of healthy plants in the bush (Shearer *et al.* 1981). Such tissue would not contain microbial competitors.
- **Solid evidence for the existence of thick-walled chlamydospores of *P. cinnamomi*** *in vitro* under sterile and non-sterile conditions (Chapter 3). Production of thick-walled spores was however sporadic and investigations into the stimulus (environmental, chemical or biological) for their production is required.
- **Evidence that the ontogeny of thick-walled chlamydospores might be different from previous reports** (Chapter 3). Thick-walled chlamydospores formed from hyphae with similar wall thickness. This is in contrast to previous research by Hemmes and Wong (1975) who found that chlamydospores walls were originally thin, thickening after the basal septum was formed. The preliminary research of this thesis indicates that

chlamydospore formation and how their walls thicken may need to be re-assessed and further work is required.

- **An *in vitro* technique to produce selfed oospores of *P. cinnamomi*** (Chapter 5). In Australia, the role of oospores in survival of *P. cinnamomi* is believed to be of little importance as only the A2 mating type is abundant and widespread (Weste and Vithanage 1979; Zentmyer 1980; Weste 1983a). In areas where A1 and A2 isolates occur in the same area, no evidence for sexual reproduction has been found (Dobrowolski *et al.* 2002). However, the current research has shown oospores, always with amphigynous antheridia, forming in four A2 isolates of *P. cinnamomi in vitro* therefore behaving as a homothallic species in the absence of stress factors (eg. presence of volatiles or *Trichoderma* spp.). As selfed oospores on modified Ribeiro's medium has previously not been reported, a PCR using specific primers confirmed their identity. Prior to all experiments, isolates were passaged through sterile lupin roots to reduce any stress associated with long-term storage and constant subculturing on agar. Large numbers of oospores only occurred in one isolate tested and testing of more isolates is required. A constraint of the project was time to investigate whether these isolates of *P. cinnamomi* are capable of producing selfed oospores in the natural environment and what triggers their germination.

- **The stimulation of chlamydospore production *in vitro* by phosphite, while oospore production was inhibited** (Chapter 4 and 5). The inhibition of selfed oospores by phosphite was similar to that observed in oospores produced by mating of *P. cinnamomi* (Coffey and Joseph 1985). The initial inhibition of chlamydospore production during *in vitro* growth was similar to research of Coffey and Joseph (1985). However, chlamydospore production was then stimulated *in vitro* in the presence of phosphite, which has not been previously reported and is of concern for the management of the pathogen.

- **The induction of dormancy of chlamydospores by the presence of phosphite** during their formation (Chapter 4). Chlamydospores produced in the presence of phosphite *in vitro* displayed higher levels of dormancy than controls. This, combined with an increase in chlamydospore numbers as a result of phosphite, has implications for survival of the pathogen during phosphite treatments in the natural environment. Conditions that may break dormancy of chlamydospores and whether chlamydospore production is being stimulated by phosphite *in planta* in the natural environment needs to be assessed.
- **The development of a transformed isolate of *P. cinnamomi* containing the Green Fluorescent Protein** for future survival studies in non-sterile systems (Chapter 6). Transformed isolates of *P. cinnamomi* contained the Green Fluorescent Protein gene and the *npt*II gene for geneticin resistance. Time constraints limited the chance to test the stability of the transgene but early indications suggested successful stable transformation due to the pathogen still being capable of growing on media containing geneticin which parental cultures could not after 10 subcultures (Chapter 7). However, the intensity of the fluorescence produced by the GFP gene declined until, by the sixth subculture, fluorescence could not be observed in the two putative transformants tested (Chapter 7). Further screening of transformants is required.

Future research and management directions

Difficulties in the visualisation of mycelium growing through soil for saprophytic studies and distinguishing propagules such as chlamydospores and oospores of *P. cinnamomi* from spores of other fungi, led to the development of transgenic cultures of *P. cinnamomi*. In the past, it has been difficult to find hyphae and other structures of *P. cinnamomi* in plant tissues especially woody plant tissues, even using staining and other techniques (Shea *et al.* 1980; Old *et al.* 1984; Schild 1995). Transgenic isolates should facilitate the location of the

pathogen in plant tissues in the future and help in studies on survival, infection, colonisation and defence. If transgenic cultures are used in the future, it will not be necessary to germinate propagules found in roots or soil as the presence of green fluorescence will be sufficient to identify them as *P. cinnamomi*.

Rehabilitation of bauxite mine sites, forestry and road building operations may involve the movement of large volumes of soil (Colquhoun and Hardy 2000; Hardy *et al.* 2001). While mapping of disease sites and soil baiting can be used to determine the disease status of a site (Colquhoun and Hardy 2000; Hardy *et al.* 2001), it does not allow for the possibility of pathogen dormancy. Chlamydospore dormancy, although widely stated to exist (Blackwell 1949; Zentmyer and Erwin 1970; Erwin and Ribeiro 1996), has had no definitive evidence. In the current thesis, dormant thin-walled chlamydospores were produced in the presence of phosphite, however, the dormancy status of thick-walled chlamydospores was not determined. The dormancy of selfed oospores *in vitro* was shown and it is possible that the same may occur in the natural environment. This aspect of the biology of *P. cinnamomi* needs further study. The suggestion that false-negative results often observed during soil baiting experiments are due to propagule dormancy (Hüberli *et al.* 2000) may be confirmed using a transgenic (eg. transformed with GFP) *P. cinnamomi* strain by examining soil and plant material for dormant oospores and chlamydospores in glasshouse and laboratory experiments. Propagule dormancy is an important issue to resolve for quarantine and hygiene reasons. Better detection of the dormant pathogen could reduce the accidental movement of infested soils, believed to be free of *Phytophthora* as determined by baiting and disease mapping. Additional research is required to understand the mechanisms of dormancy and to develop quick and reliable ways to break dormancy to ensure 100 % reliability.

Phosphite is an effective control for *Phytophthora* diseases in Western Australia (Hardy *et al.* 2001). However, from results obtained in this thesis the effectiveness may be limited. Currently, foliar applications of phosphite to run-off in Western Australia are applied at 5 g L^{-1} (Hardy *et al.* 2001). Seven days after spraying *Corymbia calophylla* to run-off at 5 g L^{-1}, 1094 µg and 2205 µg phosphite g^{-1} tissue accumulated in mature roots and root tips, respectively (Fairbanks *et al.* 2000). As phosphite at 40 and 100 µg ml^{-1} (approximately 40 and 100 µg g^{-1} medium) stimulated chlamydospore production *in vitro* in this thesis, it is possible that the same may occur in root tissue of phosphite treated plants in the natural environment where phosphite is accumulating and *P. cinnamomi* is present. Further investigations are required for management to determine whether phosphite treatment increases the inoculum level of *P. cinnamomi in planta* in the natural environment. It is also important to determine which propagules, chlamydospores or oospores, contribute more to survival of *P. cinnamomi* as phosphite stimulates chlamydospore production while inhibiting oospore production *in vitro.*

Oospores and thick-walled chlamydospores are thought to survive long periods due to the protection from their thick outer wall from desiccation and microbial antagonists (Weste and Vithanage 1979; Zentmyer 1980; Weste 1983). The development of thick-walled chlamydospores from hyphae of a similar thickness could indicate that production of thick-walled hyphae are also a survival mechanism for the pathogen. Mycelium of *P. cinnamomi* is known to lay down septa with age (Waterhouse 1970) and this could be an alternative mode of survival for the pathogen, taking advantage of its thick-walled hyphae. This needs further investigation.

The reliance on phosphite to induce host defence responses to protect against *Phytophthora* invasion (Colquhoun and Hardy 2000; Hardy *et al.* 2001) needs further investigation as its benefits may only be short-term. If the *in vitro* studies of the current thesis are shown to produce similar results *in planta* under more natural conditions, it is possible that high inoculum levels of *P. cinnamomi* may be accumulating in plants in the presence of phosphite. It is important to understand exactly how the pathogen survives and what triggers dormant spores to germinate before management mechanisms to eliminate *P. cinnamomi* might be developed.

APPENDIX 1

Soil characteristics

Table A1: Soil characteristics of Jarrah Forest soil, Bassendean sand, Potting mix and red loam Havel classified 'Q' jarrah vegetation type soils as analysed by CSBP Soil and Plant Analysis Service (Bibra Lake, Western Australia).

	Jarrah Forest Soil	**Bassendean Sand**	**Potting Mix**	**Red loam Havel classified 'Q' jarrah vegetation type**
Texture	1.5	1.5	1.5	2.0
Gravel (%)	40 - 45	-	5	15 - 20
Colour	Brown/ Grey	Grey	Dark Brown	Dark brown
Nitrate Nitrogen (mg kg^{-1})	3	1	5	9
Ammonium Nitrogen (mg kg^{-1})	2	3	1	12
Phosphorus Colwell (mg kg^{-1})	1	2	5	2
Potassium Colwell (mg kg^{-1})	25	15	95	105
Sulfur (mg kg^{-1})	4.0	3.4	14.5	7.0
Organic Carbon (%)	1.65	0.93	3.59	4.38
Reactive Iron (mg kg^{-1})	633	207	233	1321
Conductivity (dS m^{-1})	0.034	0.035	0.119	0.103
pH level ($CaCl_2$)	5.0	4.3	4.7	5.2
pH level (H_2O)	6.2	5.7	5.8	6.1
Copper (mg kg^{-1})	0.40	0.34	0.81	0.98
Zinc (mg kg^{-1})	0.75	0.37	1.69	0.60
Manganese (mg kg^{-1})	2.99	1.02	5.00	26.73
Iron (mg kg^{-1})	21.41	32.99	29.21	53.91

APPENDIX 2

Agar and Broth Recipes

Phytophthora-selective agar medium (NARPH)

Recipe published by Shearer and Dillon (1995) and modified by Hüberli (2001).

Ingredient	Product Name	Quantity	Manufacturer
Nystatin	Nilstat	1 ml	Wyeth-Ayerst, Australia
Sodium Ampicillin		100 mg	Fisons, Australia
Rifampicin	Rifadin	500 µl	Hoechst Marion Roussel, Australia
Pentachloronitrobenzene (PCNB)	Terrachlor	100 mg	Uniroyal, Australia
Hymexazol	Tachigaren	50 mg	Sankyo Company, Japan
Corn meal agar (CMA)	Oxoid	17 g	Unipath, UK
Deionised water		1 L	

CMA was autoclaved in the deionised water for 20 minutes at 121 ºC. The other ingredients were dissolved in 10 ml of sterile, deionised water at room temperature before being added to agar when the agar had cooled to approximately 50 ºC.

Pea agar

Based on recipe published by Erwin and Ribeiro (1996).

Ingredient	Quantity	Manufacturer
Blended peas	160 g	Heinz, Australia
Sucrose	5 g	
Bacto Agar	15 g	Difco Laboratories, USA
β-sitosterol	0.02 g	Sigma, USA
Water	1 L	

Blended peas were soaked in the water for 30 minutes with stirring before filtering through cheesecloth. β-sitosterol was dissolved in 5 ml 100 % ethanol and added along with the other ingredients to the pea water. The media was made up to 1 L with water. The medium was autoclaved at 121 ºC for 20 minutes.

Modified Ribeiro's minimal medium

Based on recipe published by Ribeiro *et al.* (1975).

Microelement stock solution:

Ingredient	Quantity
H_3BO_3	4.32 g
$MnCl_2.4H_2O$	2.77 g
$CuSO_4$	80 mg
$ZnSO_4.7H_2O$	288 mg
$Na_2MoO_4.2H_2O$	48 mg
NaCl	580 mg
$CoCl_2.6H_2O$	2.38 mg
Deionised water	100 ml

Ferric stock solution:

Ingredient	Quantity
$FeCl_3.6H_2O$	50 mg
EDTA	2.6 g
KOH	1.5 g
Deionised water	100 ml

EDTA and KOH were dissolved in deionised water and made up volume to 100 ml. Add $FeCl_3.6H_2O$.

Thiamine stock solution:

Ingredient	Quantity	Manufacturer
Thiamine.HCl	0.1 mg	Sigma-Aldrich, Germany
Deionised water	100 ml	

Basal medium:

Ingredient	Quantity	Manufacturer
Glucose	4.5 g	
L-asparagine	0.1 g	Sigma-Aldrich, Germany
KNO_3	0.15 g	
KH_2PO_4	1.0 g	
$MgSO_4.7H_2O$	0.5 g	
$CaCl_2$	0.1 g	
Bacto agar	17 g	Difco Laboratories, USA
Deionised water	1 L	
Microelement stock solution	1 ml	
Ferric stock solution	1 ml	
β-sitosterol	0.02 g	

β-sitosterol was dissolved in 5 ml 100 % ethanol before addition. PH of the basal medium was adjusted with 6 M KOH to 6.2. The medium was autoclaved at 121 ºC for 20 minutes. After the medium had cooled to approximately 50 ºC, 1ml of thiamine stock solution was added through a 0.22 µm Millipore filter (Schleicher and Schuell, Australia).

Clarified V8 agar and broth

Based on the recipe published by Hardham *et al.* (1991).

Ingredient	Quantity	Manufacturer
Cleared Vegetable-8 Juice *	100 ml	Campbells, Australia
$CaCO_3$	0.1 g	
β-sitosterol	0.02 g	Sigma, USA
Bacto agar	15 g	Difco Laboratories, USA
Water	900 ml	

* Vegetable-8 Juice was cleared by centrifugation at 10 600 *g* for 10 minutes before filtering the supernatant through Whatman No. 1 filter paper (Springfield Mill, UK).

β-sitosterol was dissolved in 5 ml 100 % ethanol before addition. The pH of the medium was adjusted to 6.2 with KOH before autoclaving at 121 ºC for 20 minutes. To make V8 broth, the Bacto agar was omitted.

S + L medium

Based on recipe published by Ann and Ko (1988).

Basal salt solution:

Ingredient	Quantity
$(NH_4)_2SO_4$	100 mg
$MgSO_4.7H_2O$	100 mg
$CaCl_2.2H_2O$	30 mg
$ZnSO_4.7H_2O$	3 mg
KH_2PO_4	30 mg
K_2HPO_4	60 mg
Distilled water	100 ml

The solution was filter sterilised through a 0.22 µm Millipore filter (Schleicher and Schuell, Australia).

Basal medium:

Ingredient	Quantity	Manufacturer
Lecithin (98 % Lectihin granules)	100 mg	Lowan, Australia
Glucose	20 mg	
Bacto agar	15 g	Difco Laboratories, USA
Deionised water	1 L	
*Nystatin	1 ml	Wyeth-Ayerst, Australia
*Sodium Ampicillin	100 mg	Fisons, Australia
*Pentachloronitrobenzene (PCNB)	100 mg	Uniroyal, Australia
* Basal salt solution	1 ml	

* These were added to the basal medium after the basal medium had been autoclaved and cooled to approximately 50 ºC.

The basal medium was brought to pH 7 with 1 M KOH before autoclaving at 121 ºC for 20 minutes.

Hooykaas minimal medium

Based on recipe published by Hookaas (1988).

K-salt solution:

Ingredient	Quantity
K_2HPO_4	20.5 g
KH_2PO_4	14.5 g
Distilled water	1 L

The solution was autoclaved at 121 ºC for 20 minutes.

M-salt solution:

Ingredient	Quantity
$MgSO_4.7H_2O$	3 g
$(NH_4)_2SO_4$	2.5 g
NaCl	1.5 g
Distilled water	1 L

The solution was autoclaved at 121 ºC for 20 minutes.

Basal medium:

Ingredient	Quantity
$CaCl_2$	1 mg
Glucose	200 mg
Bacto agar	1.8 g
Distilled water	965 ml
* K-salt solution	10 ml
* M-salt solution	25 ml

* These were added to the basal medium after the basal medium had been autoclaved at 121 ºC for 20 minutes and allowed to cool to approximately 50 ºC.

Induction medium

Based on recipe published by Erwin and Ribeiro (1996).

Z-salt solution:

Ingredient	Quantity
$ZnSO_4.H_2O$	0.01 g
$CuSO_4.5H_2O$	0.01 g
H_3BO_3	0.01 g
$MnSO_4.H_2O$	0.01 g
Distilled water	1 L

The pH of the solution was adjusted to 7.0 and the solution was autoclaved at 121 ºC for 20 minutes.

Basal medium:

Ingredient	Quantity
NH_4NO_3	50 mg
$CaCl_2$	1 mg
Glucose	400 mg
0.1 M Acetosyringone	1 ml
Distilled water	909 ml
* K-salt solution	10 ml
* M-salt solution	20 ml
* Z-salt solution	5 ml
** 2-[N-Morpholino]ethanesulfonic acid (MES)	40 ml
*** 0.5 % (w/v) solution of Glycerol	5 ml
*** 0.01 % (w/v) $FeSO_4$	10 ml

* This was added to the cooled autoclaved basal medium.
** A 1M solution of MES was adjusted to pH 6.3 before the desired aliquot was added to the cooled, autoclaved basal medium.
*** This was autoclaved prior to addition to the cooled, autoclaved basal medium.

The basal medium was autoclaved at 121 ºC for 20 minutes.

Mineral salts solution

Based on the recipe of Chen and Zentmyer (1970).

Ingredient	Quantity
$Ca(NO_3)_2.4H_2O$	2.36 g
KNO_3	0.51 g
$MgSO_4.7H_2O$	0.70 g
* 0.1 M FeEDTA	1 ml
Distilled water	1 L

* This was filter sterilised through a 0.22 µm Millipore filter. The desired aliquot was added to the remaining medium after the medium had been autoclaved at 121 ºC for 20 minutes and allowed to cool to room temperature.

REFERENCES

Aberton, M., Wilson, B. A., and Cahill, D. (1999). The use of potassium phosphonate to control *Phytophthora cinnamomi* in native vegetation at Anglesea, Victoria. *Australasian Plant Pathology* **28**, 225 - 234.

Afek, U., and Sztejnberg, A. (1989). Effects of Fosetyl-Al and phosphorus acid on scoparone, a phytoalexin associated with resistance of citrus to *Phytophthora citrophthora*. *Phytopathology* **79**, 736 - 739.

Ann, P. J., and Ko, W. H. (1988). Induction of oospore germination of *Phytophthora parasitica*. *Phytopathology* **78**, 335 - 337.

Bae, Y., and Knudsen, G. R. (2000). Cotransformation of *Trichoderma harzianum* with β-Glucuronidase and Green Fluorescent Protein genes provides a useful tool for monitoring fungal growth and activity in natural soils. *Applied and Environmental Microbiology* **66**, 810 - 815.

Bailey, A. M., Mena, G. L., and Herrera-Estrella, L. (1991). Genetic transformation of the plant pathogens *Phytophthora capsici* and *Phytophthora parasitica*. *Nucleic Acids Research* **19**, 4273 - 4278.

Bailey, A. M., Mena, G. L., and Herrera-Estrella, L. (1993). Transformation of four pathogenic *Phytophthora spp.* by microprojectile bombardment on intact mycelia. *Current Genetics* **23**, 42 - 46.

Barchietto, T., Saindrenan, P., and Bompeix, G. (1989). Characterisation of phosphonate uptake in two *Phytophthora* spp. and its inhibition by phosphate. *Archives of Microbiology* **151**.

Barchietto, T., Saindrenan, P., and Bompeix, G. (1992). Physiological responses of *Phytophthora citrophthora* to a subinhibitory concentration of phosphonate. *Pesticide Biochemistry and Physiology* **42**, 151 - 166.

Bartnicki-Garcia, S., and Wang, M. (1983). Biochemical aspects of morphogenesis in *Phytophthora*. *In* "*Phytophthora:* Its biology, taxonomy, ecology and pathology" (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.), pp. 392. APS Press, St Paul, Minnesota.

Birnboim, H., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513.

Blackwell, E. (1949). Terminology in *Phytophthora*. *Mycological Papers* **30**.

Blowes, W. M., Heather, W. A., Malajczuk, N., and Shea, S. R. (1982). The distribution of *Phytophthora cinnamomi* Rands at two sites in southern Western Australia and at Durras in south-eastern New South Wales. *Australian Journal of Botany* **30**, 139 - 145.

Bottin, A., Larche, L., Villalba, F., Gaulin, E., Esquerre-Tugaye, M., and Rickauer, M. (1999). Green fluorescent protein (GFP) as gene expression reporter and vital marker for studying development and microbe-plant interaction in the tobacco pathogen *Phytophthora parasitica* var. *nicotianae*. *FEMS Microbiology Letters* **176**, 51 - 56.

Bower, L. A., and Coffey, M. D. (1985). Development of laboratory tolerance to phosphorus acid, fosetyl-Al and metalaxyl in *Phytophthora capsici*. *Canadian Journal of Plant Pathology* **7**, 1 - 6.

Brasier, C. M. (1971). Induction of sexual reproduction in single A2 isolates of *Phytophthora* species by *Trichoderma viride*. *Nature* **231**, 283.

Brasier, C. M. (1972). Observations on the sexual mechanism in *Phytophthora palmivora* and related species. *Transactions of the British Mycological Society* **58**, 237 - 251.

Brasier, C. M. (1978). Stimulation of oospore formation in *Phytophthora* by antagonistic species of *Trichoderma* and its ecological implications. *Annuals of Applied Biology* **89**, 135 -139.

Brasier, C. M., and Sansome, E. (1975). Diploidy and gametangial meiosis in *Phytophthora cinnamomi, P. infestans* and *P. drechsleri*. *Transactions of the British Mycological Society* **65**, 49 - 65.

Broadbent, P., and Baker, K. (1974). Association of bacteria with sporangium formation and breakdown of sporangia in *Phytophthora* species. *Australian Journal of Agricultural Research* **25**, 139 - 145.

Bruzzese, E., and Hasan, S. (1983). A whole leaf clearing and staining technique for host specificity studies of rust fungi. *Plant Pathology* **32**, 335 - 338.

Bundock, P., den Dulk-Ras, A., Beijersbergen, A., and Hooykaas, P. J. J. (1995). Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *The EMBO Journal* **14**, 3206 - 3214.

Bunny, F. J. (1996). The biology, ecology and taxonomy of *Phytophthora citricola* in native plant communities in Western Australia. PhD thesis, Murdoch University, Perth, Western Australia.

Cahill, D., Legge, B., Grant, B., and Weste, G. (1989). Cellular and histological changes induced by *Phytophthora cinnamomi* in a group of plant species ranging from fully susceptible to fully resistant. *Phytopathology* **79**, 417 - 424.

CALM, (Department of Conservation and Land Management) (1990). Lane Poole Reserve Management Plan 1990 - 2000. *In* "Management Plan", pp. 160, Perth, Western Australia.

Carswell, C., Grant, B. R., Theodorou, M. E., Harris, J., Niere, J. O., and Plaxton, W. C. (1996). The fungicide phosphonate disrupts the phosphate-starvation response in *Brassica nigra* seedlings. *Plant Physiology* **110**, 105 - 110.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802 - 805.

Champouret, N., and Kamoun, S. (2004). An improved protocol for the protoplast method of stable DNA transformation of *Phytophthora infestans*. http://www.KamounLab.net.

Chee, K. H., and Newhook, F. J. (1965a). Improved methods for use in studies on *Phytophthora cinnamomi* and other *Phytophthora* species. *New Zealand Journal of Agricultural Research* **8**, 88 - 95.

Chee, K. H., and Newhook, F. J. (1965b). Variability of *Phytophthora cinnamomi* Rands. *New Zealand Journal of Agricultural Research* **8**, 96 - 103.

Chen, D., and Zentmyer, G. A. (1970). Production of sporangia by *Phytophthora cinnamomi* in axenic culture. *Mycologia* **62**, 397 - 402.

Chen, W., Hoitink, H. A. J., Schmitthenner, A. F., and Tuovinen, O. H. (1988). The role of microbial activity in suppression of damping-off caused by *Pythium ultimum*. *Phytopathology* **78**, 314 - 322.

Chen, X., Stone, M., Schlagnhaufer, C., and Romaine, C. P. (2000). A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. *Applied and Environmental Microbiology* **66**, 4510 - 4513.

Coffey, M. D., and Bower, L. A. (1984). *In vitro* variability among isolates of eight *Phytophthora* species in response to phosphorus acid. *Phytopathology* **74**, 738 - 742.

Coffey, M. D., and Joseph, M. C. (1985). Effects of phosphorus acid and fosetyl-Al on the life cycle of *Phytophthora cinnamomi* and *P. citricola*. *Phytopathology* **75**, 1042 - 1046.

Cohen, S. D. (1984). Detection of mycelium and oospores of *Phytophthora megasperma* f. sp. *glycinea* by vital stains in soil. *Mycologia* **76**, 34 - 39.

Colquhoun, I. J., and Hardy, G. E. S. (2000). Managing the risks of Phytophthora root and collar rot during bauxite mining in the *Eucalyptus marginata* (jarrah) forest of Western Australia. *Plant Disease* **84**, 116 - 127.

Cother, E. J., and Griffin, D. M. (1973). Formation of chlamdospores by *Phytophthora drechsleri. Transactions of the British Mycological Society* **61**, 379 - 402.

Covert, S. F., Kapoor, P., Lee, M., Briley, A., and Nairn, C. J. (2001). *Agrobacterium tumefaciens*-mediated transformation of *Fusarium circinatum*. *Mycological Research* **105**, 259 - 264.

Cvitanich, C., and Judelson, H. S. (2003). Stable transformation of the oomycete, *Phytophthora infestans,* using microprojectile bombardment. *Current Genetics* **42**, 228 - 235.

Daniel, R., Wilson, B. A., and Cahill, D. M. (2003). *Phytophthora cinnamomi* in native vegetation communities of southern Victoria - morphological variation and paragyny among isolates. *Australasian Plant Pathology* **32**, 403 - 409.

Daniel, R., Wilson, B. A., and Cahill, D. (2005). Potassium phosphonate alters the defence response of *Xanthorrhoea australis* following infection by *Phytophthora cinnamomi*. *Australasian Plant Pathology* **34**, 541 - 548.

Darakis, G., Bourbos, V. A., and Skoudridakis, M. T. (1997). Phosphonate transport in *Phytophthora capsici*. *Plant Pathology* **46**, 762 - 772.

Davis, R. M. (1989). Effectiveness of fosetyl-Al against *Phytophthora parasitica* on tomato. *Plant Disease* **73**, 215 - 217.

Darmono, T. W., and Parke, J. L. (1990). Chlamydospores of *Phytophthora cactorum*: their production, structure and infectivity. *Canadian Journal of Botany* **68**, 640 - 645.

Davison, E. M., and Shearer, B. L. (1989). *Phytophthora* spp. in indigenous forests in Australia. *New Zealand Journal of Forestry Science* **19**, 277 - 289.

DeBruyn, H. L. G. (1922). The saprophytic life of *Phytophthora* in soil. *Meded. Landbouwhoogeschool* **24** (4), 1 - 38.

de Groot, M. J. A., Bundock, P., Hooykaas, P. J. J., and Beijersbergen, A. (1998). *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology* **16**, 839 - 842.

Dobrowolski, M. P., and O'Brien, P. A. (1993). Use of RAPD-PCR to isolate a species specific DNA probe for *Phytophthora cinnamomi*. *FEMS Microbiology Letters* **113**, 43 - 48.

Dobrowolski, M. P., Tommerup, I. C., and Blakeman, H. D. (2002). Non-mendelian inheritance revealed in a genetic analysis of sexual progeny of *Phytophthora cinnamomi* with microsatellite markers. *Fungal Genetics and Biology* **35**, 197 - 212.

Dobrowolski, M. P., Tommerup, I. C., Shearer, B. L., and O'Brien, P. A. (2003). Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. *Phytopathology* **93**, 695 - 704.

Dolan, T. E., and Coffey, M. D. (1986). Laboratory screening technique for assessing resistance of four avocado root stocks of *Phytophthora cinnamomi*. *Plant Disease* **70**, 115 - 118.

Drenth, A., Janssen, E. M., and Govers, F. (1995). Formation and survival of oospores of *Phytophthora infestans* under natural conditions. *Plant Pathology* **44**, 86 - 94.

Duncan, J. M. (1985a). Effect of temperature and other factors on *in vitro* germination of *Phytophthora fragariae* oospores. *Transactions of the British Mycological Society* **85**, 455 - 462.

Duncan, J. M. (1985b). Effect of fungicides on survival, infectivity and germination of *Phytophthora fragariae* oospores. *Transactions of the British Mycological Society* **85**, 585 - 593.

Duncan, M. J., and Keane, P. J. (1996). Vegetation changes associated with *Phytophthora cinnamomi* and its decline under *Xanthorrhoea australis* in Kinglake National Park, Victoria. *Australian Journal of Botany* **44**, 355 - 369.

Duniway, J. M. (1975). Limiting influence of low water potential on the formation of sporangia by *Phytophthora drechsleri* in soil. *Phytopathology* **65**, 1089 - 1093.

Duvenhage, J. A. (1994). Monitoring the resistance of *Phytophthora cinnamomi* to fosetyl-Al and H_3PO_3. *South African Avocado Growers' Association Yearbook* **17**, 35 - 37.

Eren, J., and Pramer, D. (1966). Application of immunofluorescent staining to studies of the ecology of soil microorganisms. *Soil Science* **101**, 39 - 45.

Erwin, D. C., and Ribeiro, O. K. (1996). "Phytophthora Diseases Worldwide.," APS Press, St Paul, Minnesota.

Fabritius, A., Cvitanich, C., and Judelson, H. S. (2002). Stage-specific gene expression during sexual development in *Phytophthora infestans*. *Molecular Microbiology* **45**, 1057 - 1066.

Fairbanks, M. M., Hardy, G. E. S., and McComb, J. A. (2000). Comparisons of phosphite concentrations in *Corymbia (Eucalyptus) calophylla* tissues after spray, mist or soil drench applications with the fungicide phosphite. *Australasian Plant Pathology* **29**, 96 - 101.

Fairbanks, M. M., Hardy, G. E. S., and McComb, J. A. (2002). Effect of the fungicide phosphite on pollen fertility of perennial species of the *Eucalyptus marginata* forest and northern sandplains of Western Australia. *Australian Journal of Botany* **50**, 769 - 779.

Fawcett, R. G., and Collis-George, N. (1967). A filter paper method for determining the moisture characteristic of soil. *Australian Journal of Experimental Agriculture and Animal Husbandry* **7**, 162 - 167.

Fenn, M. E., and Coffey, M. D. (1984). Studies on the *in vitro* and *in vivo* antifungal activity of fosetyl-Al and phosphorus acid. *Phytopathology* **74**, 606 - 611.

Fenn, M. E., and Coffey, M. D. (1985). Further evidence for the direct mode of action of fosetyl-Al and phosphorus acid. *Phytopathology* **75**, 1064 - 1067.

Fenn, M. E., and Coffey, M. D. (1989). Quantification of phosphonate and ethyl phosphonate in tobacco and tomato tissues and significance for the mode of action of two phosphonate fungicides. *Phytopathology* **79**, 76 - 82.

Fermentas (Date accessed: 12/07/2005). www.fermentas.com.

Ferraris, L., Cardinale, F., Valentino, D., Roggero, P., and Tamietti, G. (2004). Immunological discrimination of *Phytophthora cinnamomi* from other *Phytophthorae* pathogenic on chestnut. *Phytopathology* **152**, 193 - 199.

Fincham, J. R. S. (1989). Transformation in fungi. *Microbiological Reviews* **53**, 148 - 170.

Fraser, L. R. (1956). *Phytophthora cinnamomi* attacking native plants. *Australian Plant Disease Recorder* **8**, 30.

Galindo, J., and Zentmyer, G. A. (1964). Mating types in *Phytophthora cinnamomi*. *Phytopathology* **54**, 238 - 239.

Gao, Z. M., Zheng, X. B., Lu, J. Y., and Ko, W. H. (1998). Effect of culture media on antheridial configuration in *Phytophthora boehmeriae*. *Canadian Journal of Botany* **76**, 2177 - 2179.

Garbelotto, M. (2001). Composting as a control for sudden oak death disease. *Journal of Composting and Organics Recycling*, 53 - 56.

Garrett, S. D. (1956). "Biology of the root-infecting fungi," Cambridge University Press, Cambridge.

Garrett, S. D. (1970). "Pathogenic root-infecting fungi.," Cambridge University Press, Cambridge.

Gerrettson-Cornell, L. (1983). A compendium of the morphology of *Phytophthora cinnamomi* Rands from Australia. *Acta Botanica Hungarica* **29**, 91-105.

Gilpatrick, J. (1969). Effect of soil amendments upon inoculum survival and function in Phytophthora root rot of avocado. *Phytopathology* **59**, 979 - 985.

Glenn, O. F., Parker, C. A., and Sivasithamparam, K. (1988). Use of 14C-labelled wheat tissue to demonstrate saprophytic growth of *Gaeumannomyces graminis var.tritici* in soil. *Transactions of the British Mycological Society* **90**, 545 - 550.

Graham, G. C., Meyers, P., and Henry, R. J. (1994). A simplified method for preparation of fungal DNA for PCR and RAPD analysis. *Biotechniques* **16**, 48 - 50.

Griffin, D. M. (1972). "Ecology of Soil Fungi," Chapman and Hall, London.

Griffin, D. H. (1994). "Fungal Physiology," 2/Ed. Wiley-Liss, New York.

Griffith, J. M., Smillie, R. H., Niere, J. O., and Grant, B. (1989). Effect of phosphate on the toxicity of phosphite in *Phytophthora palmivora*. *Archives of Microbiology* **152**, 425 - 429.

Groves, C. T., and Ristaino, J. B. (2000). Commercial fungicide formulations induce *in vitro* oospore formation and phenotypic change in mating type in *Phytophthora infestans*. *Phytopathology* **90**, 1201 - 1208.

Guest, D. I. (1984). Modification of defence responses in tobacco and capsicum following treatment with Fosetyl-Al (Aluminium tris (o-ethyl phosphonate)). *Physiological Plant Pathology* **25**, 125 – 134.

Guest, D. I., and Grant, B. (1991). The complex action of phosphonates as antifungal agents. *Biological Reviews* **66**, 159 - 187.

Halsall, D. (1978). A comparison of *Phytophthora infestans* infection in *Eucalyptus sieberi*, a susceptible species, and *E. marginata*, a field resistant species. *Australian Journal of Botany* **26**, 643 - 655.

Hamblin, A. P. (1981). Filter-paper method for routine measurement of field water potential. *Journal of Hydrology* **53**, 355 - 360.

Hardham, A. R. (1986). Monoclonal antibodies to isolate-, species-, and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. *Canadian Journal of Botany* **64**, 311 - 321.

Hardham, A. R. (1987). Ultrastructure and serial section reconstruction of zoospores of the fungus *Phytophthora cinnamomi*. *Experimental Mycology* **11**, 297 - 306.

Hardham, A. R. (1989). Lectin and antibody labelling of surface components of spores of *Phytophthora cinnamomi*. *Australian Journal of Plant Physiology* **16**, 19 - 32.

Hardham, A. R. (2005). Pathogen profile *Phytophthora cinnamomi*. *Molecular Plant Pathology* **6**, 289 - 604.

Hardham, A. R., Gubler, F., Duniec, J., and Elliot, J. (1991). A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens. *Journal of Microscopy* **162**, 305 - 318.

Hardy, G. E. S., Barrett, S., and Shearer, B. L. (2001). The future of phosphite as a fungicide to control the soilborne plant pathogen *Phytophthora cinnamomi* in natural ecosystems. *Australasian Plant Pathology* **30**, 133 - 139.

Havel, J. J. (1975). Site-vegetation mapping in the northern jarrah forest (Darling Range). 1. Definition of site-vegetation types. *Forests Department*, Perth, Western Australia Bulletin **86**.

Hegnauer, H., and Hohl, H. R. (1978). Cell wall architecture of sporangia, chlamydospores, oogonia and oospores in *Phytophthora*. *Experimental Mycology* **2**, 216 - 233.

Hemmes, D. E. (1983). Cytology of *Phytophthora*. *In* "*Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology." (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.). APS Press, USA.

Hemmes, D. E., and Wong, L. D. S. (1975). Ultrastructure of chlamydospores of *Phytophthora cinnamomi* during development and germination. *Canadian Journal of Botany* **53**, 2945 - 2957.

Hendrix, J. W. (1967). Light-cholesterol relationships in morphogenesis of *Phytophthora palmivora* and *P. capsici* sporangia. *Mycologia* **59**, 1107 - 1111.

Hepting, G. H. (1963). Climate and Forest Diseases. *Annual Review of Phytopathology* **1:** 31 - 50.

Ho, H. H., and Zentmyer, G. A. (1977). Morphology of *Phytophthora cinnamomi*. *Mycologia* **69**, 701 - 713.

Ho, H. H., Zhuang, W. Y., Liang, Z. R., and Yu, Y. N. (1983). *Phytophthora cinnamomi* on black locust (*Robinia pseudoacacia*) in Jiangsu province of China. *Mycologia* **75**, 881 - 886.

Hoitink, F. A. J., and Fahy, P. C. (1986). Basis for the control of soil spore plant pathogens with composts. *Annual Review of Phytopathology* **24**, 93 - 114.

Hood, M. T., and Stachow, C. (1992). Influence of polyethylene glycol on the size of *Schizosaccharomyces pombe* electropores. *Applied and Environmental Microbiology* **58**, 1201 - 1206.

Hooykaas, P. J. J. (1988). *Agrobacterium* molecular genetics. *In* "Plant Molecular Biology Manual" (S. B. Gelvin, R. A. Schilperoort and D. P. S. Verma, eds.). Kluwer Academic Publishers, Belgium.

Hüberli, D. (2001). Phenotypic variation of two localised populations of *Phytophthora cinnamomi* from Western Australia and how they impact on *Eucalyptus marginata* resistance. PhD thesis, Murdoch University, Perth.

Hüberli, D., Tommerup, I. C., and Hardy, G. E. S. (1997). The role of paragynous and amphigynous antheridia in sexual reproduction of *Phytophthora cinnamomi*. *Mycological Research* **101**, 1383 - 1388.

Hüberli, D., Tommerup, I. C., and Hardy, G. E. S. (2000). False-negative isolations or absence of lesions may cause mis-diagnosis of diseased plants infected with *Phytophthora cinnamomi*. *Australasian Plant Pathology* **29**, 164 - 169.

Hunsley, D. (1973). Apical wall structure in hyphae of *Phytophthora parasitica*. *New Phytologist* **72**, 985 - 990.

Hwang, S. C., and Ko, W. M. (1978). Biology of chlamydospores, sporangia and zoospores of *Phytophthora cinnamomi* in soil. *Phytopathology* **68**, 726 - 731.

Irwin, J. A. G., Cahill, D., and Drenth, A. (1995). *Phytophthora* in Australia. *Australian Journal of Agricultural Research* **46**, 1311 - 1137.

Irwin, J. A. G., and Mackie, J. M. (2000). Sexual reproduction by *Phytophthora macrochlamydospora*: a group VI species. *Australian Systematic Botany* **13**, 817 - 821.

Jackson, T. J., Burgess, T., Colquhoun, I., and Hardy, G. E. S. (2000). Action of the fungicide phosphite on *Eucalyptus marginata* inoculated with *Phytophthora cinnamomi*. *Plant Pathology* **49**, 147 - 154.

Jefferson, R. A., Burgess, S. M., and Hirsh, D. (1986). Beta-glucuronidase (uidA) from *E. coli* as a gene fusion marker. *Biochemistry* **83**, 8447 - 8451.

Jiang, J., and Erwin, D. C. (1990). Morphology, plasmolysis and tetrazolium bromide stain as criteria for determining viability of *Phytophthora* oospores. *Mycologia* **82**, 107 - 113.

Jiang, J., Stephenson, L. W., Erwin, D. C., and Leary, J. V. (1989). Nuclear changes in *Phytophthora* during oospores maturation and germination. *Mycological Research* **92**, 463 - 469.

Judelson, H. S., Coffey, M. D., Arredondo, F. R., and Tyler, B. M. (1993). Transformation of the oomycete pathogen *Phytophthora megasperma* F.sp. *glycinea* occurs by DNA integration into single or multiple chromosomes. *Current Genetics* **23**, 211 - 218.

Judelson, H. S., and Michelmore, R. W. (1991). Transient expression of genes in the oomycete *Phytophthora infestans* using *Bremia lactucae* regulatory sequences. *Current Genetics* **19**, 453 - 459.

Judelson, H. S., Tyler, B. M., and Michelmore, R. W. (1991). Transformation of the oomycete pathogen *Phytophthora infestans*. *Molecular Plant-Microbe Interactions* **4**, 602 - 607.

Judelson, H. S., Tyler, B. M., and Michelmore, R. W. (1992). Regulatory sequences for expressing genes in oomycete fungi. *Molecular and General Genetics* **234**, 138 - 146.

Kadooka, J. Y., and Ko, W. H. (1973). Production of chlamydospores by *Phytophthora palmivora* in culture media. *Phytopathology* **63**, 559 - 562.

Kamoun, S. (2001). Nonhost resistance to *Phytophthora*: novel prospects for a classical problem. *Current Opinion in Plant Biology* **4**, 295 - 300.

Kamoun, S., van West, P., and Govers, F. (1998). Quantification of late blight resistance of potato using transgenic *Phytophthora infestans* expressing β-glucuronidase. *European Journal of Plant Pathology* **104**, 521 - 525.

Keane, P. J., Kile, G. A., Podger, F. D., and Brown, B. N. (2000). "Diseases and Pathogens of Eucalypts.," CSIRO Publishing, Victoria.

Khan, A. J., Vernenghi, A., and Ravise, A. (1986). Incidence of fosetyl Al and elicitors on the defence reactions of citrus attacked by *Phytophthora* spp. *Fruits* **41**, 587 - 595.

Ko, W. H. (1978). Heterothallic *Phytophthora*: Evidence for hormonal regulation of sexual reproduction. *Journal of General Microbiology* **107**, 15 - 18.

Ko, W. H. (1980). Sexuality, evolution and origin of *Phytophthora*. *Plant Protection Bulletin* **22**, 141 - 151.

Komorek, B. M., Shearer, B. L., Blumberg, M. V., and Fairman, R. G. (1997). Potassium phosphite - effective chemical tool in the protection of native flora threatened by *Phytophthora*. *In* "Australasian Plant Pathology Society 11th Biennial Conference", Perth, Western Australia.

Kuhlman, E. G. (1964). Survival and pathogenicity of *Phytophthora cinnamomi* in several south western Oregon soils. *Forest Science* **10**, 151 - 158.

Kuhn, D. N. (1991). Parasexuality in *Phytophthora*? *In* "*Phytophthora*" (J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke, eds.), pp. 242 - 255. Cambridge University Press, Cambridge, UK.

Lacey, J. (1965). The infectivity of soils containing *Phytophthora infestans*. *Annals of Applied Biology* **56**, 363 - 380.

Latijnhouwers, M., and Govers, F. (2003). A *Phytophthora infestans* G-Protein β subunit is involved in sporangium formation. *Eukaryotic Cell* **2**, 971 - 977.

Lee, S., Kim, S. H., and Breuil, C. (2002). The use of the green fluorescent protein as a biomarker for sapstain fungi. *Forest Pathology* **32**, 153 - 161.

Linde, C., Soo, S. H., and Drenth, A. (2001). Sexual recombination in *Phytophthora cinnamomi in vitro* and aggressiveness of single-oospore progeny to *Eucalyptus*. *Plant Pathology* **50**, 97 - 102.

Lockwood, J. L. (1977). Fungistasis in soils. *Biological Review,* Cambridge Philosophical Society **52**, 1 - 43.

MacDonald, J. D., and Duniway, J. M. (1979). Use of fluorescent antibodies to study the survival of *Phytophthora megasperma* and *Phytophthora cinnamomi* zoospores in soil. *Phytopathology* **69**, 436 - 441.

Mackay, A., Weste, G., and Sharpe, K. (1985). Survival of *Phytophthora cinnamomi* in buried Eucalypt roots. *Phytopathologische zeitschrift* **114**, 214 - 223.

Malajczuk, N. (1983). Microbial Antagonism to *Phytophthora. In* "*Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology" (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.). APS Press, USA.

Malajczuk, N., Bowen, G. D., and Greenhalgh, F. C. (1978). A combined fluorescent antibody and soil sieving technique to count chlamydospores of *Phytophthora cinnamomi* in soil. *Soil Biology and Biochemistry* **10**, 437 - 438.

Malajczuk, N., and McComb, A. J. (1977). Root exudates from *Eucalyptus calophylla* R.Br. and *Eucalyptus marginata* Donn ex Sm. Seedlings and their effects on *Phytophthora cinnamomi* Rands. *Australian Journal of Botany* **25**, 501 - 514.

Malajczuk, N., McComb, A. J., and Parker, C. A. (1975). An immunofluorescence technique for detecting *Phytophthora cinnamomi* Rands. *Australian Journal of Botany* **23**, 289 - 309.

Malajczuk, N., McComb, A. J., and Parker, C. A. (1977a). Infection by *Phytophthora cinnamomi* Rands of roots of *Eucalyptus calophylla* R.Br. And *Eucalyptus marginata* Donn. Ex Sm. *Australian Journal of Botany* **25**, 483 - 500.

Malajczuk, N., Nesbitt, H., and Glenn, A. R. (1977b). A light and electron microscope study of the interaction of soil bacteria with *Phytophthora cinnamomi* Rands. *Canadian Journal of Microbiology* **23**, 1518 - 1525.

Malajczuk, N., Sanfelieu, C. L., and Hossen, S. (1983). Production and survival of *Phytophthora cinnamomi* zoospores in suppressive and conducive soils. *Transactions of the British Mycological Society* **80**, 305 – 312.

Malajczuk, N., and Theodorou, C. (1979). Influence of water potential on growth and cultural characteristics of *Phytophthora cinnamomi*. *Transactions of the British Mycological Society* **72**, 15 - 18.

Marks, G. C., Kassaby, F. Y., and Fagg, P. C. (1975). Variation in population levels of *Phytophthora cinnamomi* in Eucalyptus forest soils of eastern Victoria. *Australian Journal of Botany* **23**, 435 - 449.

May, K. J., Drenth, A., and Irwin, J. A. G. (2003). Interspecific hybrids between the homothallic *Phytophthora sojae* and *Phytophthora vignae*. *Australasian Plant Pathology* **32**, 353 - 359.

McDonald, A. E., Niere, J. O., and Plaxton, W. C. (2001). Phosphite disrupts the acclimation of *Saccharomyces cerevisiae* to phosphite starvation. *Canadian Journal of Microbiology* **47**, 969 - 978.

Mircetich, S. M., and Zentmyer, G. A. (1966). Production of oospores and chlamydospores of *Phytophthora cinnamomi* in roots and soil. *Phytopathology* **56**, 1076 - 1078.

Mircetich, S. M., and Zentmyer, G. A. (1967). Existence of *Phytophthora cinnamomi* as chlamydospores and oospores in roots and soil. *California Avocado Society Yearbook* **51**.

Mircetich, S. M., and Zentmyer, G. A. (1969). Effect of carbon and nitrogen compounds on germination of chlamydospores of *Phytophthora cinnamomi* in soil. *Phytopathology* **59**, 1732 - 1735.

Mircetich, S. M., Zentmyer, G. A., and Kendrick, J. B. J. (1968). Physiology of germination of chlamydospores of *Phytophthora cinnamomi*. *Phytopathology* **58**, 666 - 671.

Mitchell, D. J., and Zentmyer, G. A. (1971). Effects of oxygen and carbon dioxide tensions on sporangium and oospore formation by *Phytophthora* species. *Phytopathology* **61**, 807 - 811.

Monke, E., and Schafer, W. (1993). Transient and stable expression in the fungal maize pathogen *Cochliobolus heterostrophus* after transformation with the β-glucuronidase (GUS) gene. *Molecular and General Genetics* **241**, 73 - 80.

Mooibroek, H., Kuipers, A. G. J., Sietsma, J. H., Punt, P. J., and Wessels, J. G. H. (1990). Introduction of hygromycin B resistance into *Schizophyllum commune*: Preferential methylation of donor DNA. *Molecular and General Genetics* **222**, 41 - 48.

Murray, D. I. L., Darling, D. D., and McGann, L. R. (1985). Indirect effect of floristic composition on production of sporangia by *Phytophthora cinnamomi* in jarrah forest soils. *Australian Journal of Botany* **33**, 109 - 113.

Nemestothy, G. S., and Guest, D. I. (1990). Phytoalexin accumulation, phenylalanine ammonia lyase activity and ethylene biosynthesis in fosetyl-Al treated resistant and susceptible tobacco cultivars infected with *Phytophthora nicotianae* var. *nicotianae*. *Physiological and Molecular Plant Pathology* **37**, 207 - 219.

Nesbitt, H., Malajczuk, N., and Glenn, A. R. (1979). Effect of organic matter on the survival of *Phytophthora cinnamomi* Rands in soil. *Soil Biology and Biochemistry* **11**, 133 - 136.

Newall, S. Y., Miller, J. D., and Fallon, R. D. (1987). Ergosterol content of salt-marsh fungi: Effect of growth conditions and mycelial age. *Mycologia* **79**, 688 - 695.

Niere, J. O., DeAngelis, G., and Grant, B. (1994). The effect of phosphonate on the acid-soluble phosphorus components in the genus *Phytophthora*. *Microbiology* **140**, 1661 - 1670.

Niere, J. O., Griffith, J. M., and Grant, B. R. (1990). ^{31}P NMR studies on the effect of phosphite on *Phytophthora palmivora*. *Journal of General Microbiology* **136**, 147 - 156.

O'Gara, E. T. (1998). Infection and disease of *Eucalyptus marginata* (Jarrah), caused by *Phytophthora cinnamomi* in rehabilitated bauxite mines in the south-west of Western Australia. PhD thesis, Murdoch University, Perth.

O'Gara, E., Hardy, G. E. S., and McComb, J. A. (1996). The ability of *Phytophthora cinnamomi* to infect through unwounded and wounded periderm tissue of *Eucalyptus marginata. Plant Pathology* **45**, 955 - 963.

O'Brien, I. G. (1991). Induced homothallism in *Phytophthora cinnamomi*: A bioassay for volatile bioactive compounds. *Phytochemistry* **30**, 3545 - 3548.

Old, K. M., Oros, J. M., and Malafant, K. W. (1984). Survival of *Phytophthora cinnamomi* in root fragments in Australian forest soils. *Transactions of the British Mycological Society* **82**, 605 - 613.

Oliver, R. P., Farman, M. L., Jones, J. D. G., and Hammond-Kosack, K. E. (1993). Use of fungal transformants expressing □-Glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. *Molecular Plant-Microbe Interactions* **6**, 521 - 525.

Ouimette, D. G., and Coffey, M. D. (1990). Symplastic entry and phloem translocation of phosphonate. *Pesticide Biochemistry and Physiology* **38**, 18 - 25.

Park, D. (1961). Morphogenesis, fungistasis and cultural staling in *Fusarium oxysporum* Snyder and Hansen. *Transactions of the British Mycological Society* **44**, 377 - 390.

Peberdy, J. (1991). Fungal protoplasts. *In* "More gene manipulations in fungi" (J. Bennett and L. Lasure, eds.), pp. 307 - 318. Academic Press, San Diego.

Perez, V., Mamdouh, A. M., Huet, J. C., Pernollet, J. C., and Bompeix, G. (1995). Enhanced secretion of elicitins by *Phytophthora* fungi exposed to phosphonate. *Cryptogamie, Mycology* **16**, 191 - 194.

Pittis, J. E., and Shattock, R. C. (1994). Viability, germination and infection potential of oospores of *Phytophthora infestans. Plant Pathology* **43**, 387 - 396.

Podger, F. D. (1972). *Phytophthora cinnamomi*: A cause of lethal disease in indigenous plant communities in Western Australia. *Phytopathology* **62**, 972 - 981.

Podger, F. D., Doepel, R. F., and Zentmyer, G. A. (1965). Association of *Phytophthora cinnamomi* with a disease of *Eucalyptus marginata* forest in Western Australia. *Plant Disease Reporter* **49**, 943 - 947.

Ponchet, M., Panabieres, F., Milat, M. L., Mikes, M., Montillet, J. L., Suty, L., Triantaphylides, C., Tirilly, Y., and Blein, J. P. (1999). Are elicitins cryptograms in plant-Oomycete communications? *Cellular and Molecular Life Sciences* **56**, 1020 - 1047.

Prell, H. H., Karlovsky, P., and Bahnweg, G. (1991). Towards transformation in *Phytophthora nicotianae*. *In* "*Phytophthora*" (J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke, eds.), pp. 312 - 325. Cambridge University Press, Cambridge, UK.

Rands, R. (1922). Streepkanker van Kaneel, veroorzaakt door *Phytophthora cinnamomi* n. sp. (Stripe canker of cinnamon caused by *Phytophthora cinnamomi* n. sp.). *Meded. Inst. Plantenziekten* **54**, 41pp (In Dutch).

Reeves, R. J. (1975). Behaviour of *Phytophthora cinnamomi* Rands in different soils and water regimes. *Soil Biology and Biochemistry* **7**, 19 - 24.

Reeves, R. J., and Jackson, R. M. (1972). Induction of *Phytophthora cinnamomi* oospores in soil by *Trichoderma viride*. *Transactions of the British Mycological Society* **59**, 156 - 159.

Reeves, R. J., and Jackson, R. M. (1974). Stimulation of sexual reproduction in *Phytophthora* by damage. *Transactions of the British Mycological Society* **84**, 303 - 310.

Reynolds, K. M., Benson, D. M., and Bruck, R. I. (1985). Epidemiology of Phytophthora root rot of Fraser fir: Root colonisation and inoculum potential. *Phytopathology* **75**, 1004 - 1009.

Ribeiro, O. K. (1983). Physiology of Asexual Sporulation and Spore Germination in *Phytophthora*. *In* "*Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology" (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.). APS Press, USA.

Ribeiro, O. K., Erwin, D. C., and Zentmyer, G. A. (1975). An improved synthetic medium for oospore production and germination of several *Phytophthora* species. *Mycologia* **67**, 1012 - 1019.

Robinson, L. H., and Deacon, J. W. (2001). Protoplast preparation and transient transformation of *Rhizoctonia solani*. *Mycological Research* **105**, 1295 - 1303.

Robinson, L. H., and Cahill, D. (2003). Ecotypic variation in the response of *Arabidopsis thaliana* to *Phytophthora cinnamomi*. *Australasian Plant Pathology* **32**, 53 - 64.

Roos, G. H. P., Loane, C., Dell, B., and Hardy, G. E. S. (1999). Facile high performance ion chromatographic analysis of phosphite and phosphate in plant samples. *Communications in Soil Science and Plant Annals* **17 & 18**, 2323 - 2329.

Royle, D., and Hickman, C. (1964). Observations on *Phytophthora cinnamomi*. *Canadian Journal of Botany* **42**, 311 - 318.

Ruben, D. M., Frank, Z. R., and Chet, I. (1980). Factors affecting behaviour and developmental synchrony of germinating oospores of *Pythium aphanidermatum*. *Phytopathology* **70**, 54 - 59.

Saindrenan, P., Barchietto, T., Avelino, J., and Bompeix, G. (1988). Effects of phosphite on phytoalexin accumulation in leaves of cowpea infected with *Phytophthora crytogea*. *Biological and Molecular Plant Pathology* **32**, 425 - 435.

Savage, E. J., Clayton, C. W., Hunter, J. H., Brennema, J. A., Laviola, C., and Gallegly, M. E. (1968). Homothallism, heterothallism and interspecific hybridisation in the genus *Phytophthora*. *Phytopathology* **58**, 1004 - 1021.

Schild, D. M. (1995). The survival of *Phytophthora cinnamomi* Rands in the northern jarrah (*Eucalyptus marginata* Donn Ex Sm.) forest of Western Australia. PhD Thesis, Murdoch University, Western Australia.

Schmitthenner, A. F., and Canaday, C. H. (1983). Role of chemical factors in development of *Phytophthora* diseases. *In* "*Phytophthora*: Its bioogy, taxonomy, ecology and pathology" (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.), pp. 189 - 196. American Phytopathological Society, St Paul, Minnesota, USA.

Schnurer, J., and Rosswall, T. (1982). Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. *Applied and Environmental Microbiology* **43**, 1256 - 1261.

Seymour, N. P. (1994). Phytoxicity of fosetyl Al and phosphonic acid to maize during production of vesicular-arbuscular mycorrhizal inoculum. *Plant Disease* **78**, 441 - 446.

Shea, S. R. (1977). Environmental factors of the northern jarrah forest in relation to pathogenicity and survival of *Phytophthora cinnamomi. Forests Department, Perth, Western Australia Bulletin* **85**.

Shea, S. R. (1979). *Phytophthora cinnamomi* Rands – a collar rot pathogen of *Banksia grandis* Willd. *Australasian Plant Pathology* **8**, 32 - 34.

Shea, S. R., Gillen, K. J., and Leppard, W. I. (1980). Seasonal variation in population levels of *Phytophthora cinnamomi* Rands in soil in diseased, freely-drained *Eucalyptus marginata* Sm sites in the northern jarrah forests of south-western Australia. *Protection Ecology* **2**, 135 - 156.

Shearer, B. L. (1992). Dieback of native plant communities caused by *Phytophthora* species - a major factor affecting land use in south-western Australia. *Land and Water Research News*, 15 - 26.

Shearer, B. L. (1994). The major plant pathogens occurring in native ecosystems in south-western Australia. *Journal of the Royal Society of Western Australia* **77**, 113 - 122.

Shearer, B. L., and Dillon, M. (1995). Susceptibility of plant species in *Eucalyptus marginata* forest to infection by *Phytophthora cinnamomi*. *Australian Journal of Botany* **43**, 113 - 134.

Shearer, B. L., and Fairman, R. G. (1997). Phosphite inhibits lesion development of *Phytophthora cinnamomi* for at least four years following trunk injection of *Banksia* species and *Eucalyptus marginata*. *In* "Australasian Plant Pathology Society 11th biennial conference", Perth, Western Australia.

Shearer, B. L., and Hill, T. C. (1989). Diseases of *Banksia* woodlands on the Bassendean and Spearwood dune systems. *Journal of the Royal Society of Western Australia* **71**, 113 - 114.

Shearer, B. L., and Shea, S. R. (1987). Variation in seasonal population fluctuations of *Phytophthora cinnamomi* within and between infected *Eucalyptus marginata* sites of south-western Australia. *Forest Ecology and Management* **21**, 209 - 230.

Shearer, B. L., Shea, S. R., and Deegan, P. M. (1987). Temperature-growth relationships of *Phytophthora cinnamomi* in the secondary phloem of roots of *Banksia grandis* and *Eucalyptus marginata*. *Phytopathology* **77**, 661 - 665.

Shearer, B. L., Shea, S. R., and Fairman, R. G. (1981). Infection of the stem and large roots of *Eucalyptus marginata* by *Phytophthora cinnamomi*. *Australasian Plant Pathology* **10**, 2 - 3.

Shearer, B. L., and Smith, I. W. (2000). Diseases of Eucalypts caused by soil-borne species of *Phytophthora* and *Pythium*. *In* "Diseases and pathogens of Eucalypts." (P. J. Keane, G. A. Kile, F. D. Podger and B. N. Brown, eds.). CSIRO Publishing, Australia.

Shearer, B. L., and Tippett, J. T. (1989). "Jarrah dieback: the dynamics and management of *Phytophthora cinnamomi* in the jarrah (*Eucalyptus. marginata*) forest of south-western Australia," Department of Conservation and Land Management, Como, Western Australia.

Shepherd, C. J. (1978). Mating behaviour of Australian isolates of *Phytophthora* species. I Inter- and intraspecific mating. *Australian Journal of Botany* **26**, 123 - 138.

Shew, H. D., and Benson, D. M. (1982). Qualitative and Quantitative Soil Assays for *Phytophthora cinnamomi*. *Phytopathology* **72**, 1029 - 1032.

Si-Ammour, A., Mauch-Mani, B., and Mauch, F. (2003). Quantification of induced resistance against *Phytophthora* species expressing GFP as a vital marker: β-aminobutyric acid but not BTH protects potato and *Arabidopsis* from infection. *Molecular Plant Pathology* **4**, 237 - 248.

Sneh, B., and McIntosh, D. L. (1974). Studies on the behaviour and survival of *Phytophthora cactorum* in soil. *Canadian Journal of Botany* **52**, 795 - 802.

Stamps, D., Waterhouse, G., Newhook, F., and Hall, G. (1990). Revised tabular key to the species of *Phytophthora*. *Mycological Papers* **162**, 28pp.

Stamps, D. J. (1953). Oospore production in paired cultures of *Phytophthora* species. *Transactions of the British Mycological Society* **36**, 255 - 259.

Sterne, R. E., Zentmyer, G. E., and Kaufmann, M. R. (1977). The influence of matric potential, soil texture and soil amendment on root disease caused by *Phytophthora cinnamomi*. *Phytopathology* **67**, 1495 - 1500.

Tippett, J. T., Shea, S. R., Hill, T. C., and Shearer, B. L. (1983). Development of lesions caused by *Phytophthora cinnamomi* in the secondary phloem of *Eucalyptus marginata*. *Australian Journal of Botany* **31**, 197 - 210.

Titze, J. F., and Palzer, C. R. (1969). Host list of *Phytophthora cinnamomi* with special reference to Western Australia. Forest and Timber Bureau, Canberra.

Tsao, P. H. (1970). Applications of the vital fluorescent labelling technique with brighteners to studies of saprophytic behaviour of *Phytophthora* in soil. *Soil Biology and Biochemistry* **2**, 247 - 256.

Tsao, P. H., and Oster, J. J. (1981). Relation of ammonia and nitrous acid to suppression of *Phytophthora* in soils amended with nitrogenous organic substances. *Phytopathology* **71**, 53 - 59.

Tsao, P. H., and Zentmyer, G. A. (1979). Suppression of *Phytophthora cinnamomi* and *Phytophthora parasitica* in urea amended soils. *In* "Soil-Borne Plant Pathogens" (B. Schippers and W. Gams, eds.). 686pp. Academic Press, London.

van West, P., de Jong, A. J., Judelson, H. S., Emons, A. M. C., and Govers, F. (1998). The *ipi*O gene of *Phytophthora infestans* is highly expressed in invading hyphae during infection. *Fungal Genetics and Biology* **23**, 126 - 138.

van West, P., Reid, B., Campbell, T. A., Sandrock, R. W., Fry, W. E., Kamoun, S., and Gow, N. A. R. (1999). Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*. *FEMS Microbiology Letters* **178**, 71 - 80.

Vijn, I., and Govers, F. (2003). *Agrobacterium tumefaciens* mediated transformation of the oomycete plant pathogen *Phytophthora infestans*. *Molecular Plant Pathology* **4**, 459 - 467.

Vujicic, R., and Park, D. (1964). Behaviour of *Phytophthora erthroseptica* in soil. *Transactions of the British Mycological Society* **47**, 455 - 458.

Waterhouse, G. M. (1931). The production of conidia in the genus *Phytophthora*. *Transactions of the British Mycological Society* **15**, 311 - 322.

Waterhouse, G. M. (1970). The genus *Phytophthora* de Bary. *Mycological Papers* **122**, 1 – 59.

Waterhouse, G. M., Newhook, F. J., and Stamps, D. J. (1983). Present criteria for classification of *Phytophthora*. *In* "*Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology" (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.). APS Press, USA.

Weaver, J. C. (1995). Electroporation theory. *In* "Electroporation Protocols for Microorganisms" (J. A. Nickoloff, ed.), pp. 1 - 26. Humana Press, Totowa, NJ.

Weiland, J. J. (2003). Transformation of *Pythium aphanidermatum* to geneticin resistance. *Current Genetics* **42**, 344 - 352.

Weste, G. (1979). The plant communities - susceptible - vulnerable - resistant. *In* "*Phytophthora* and forest management in Australia" (K. M. Old, ed.), pp. 7 - 13. CSIRO, Melbourne, Australia.

Weste, G. (1983a). Population dynamics and survival of *Phytophthora*. *In* "*Phytophthora*: Its biology, taxonomy, ecology and pathology" (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.). APS Press, St Paul, Minnesota.

Weste, G. (1983b). *Phytophthora cinnamomi*: the dynamics of chlamydospore formation and survival. *Phytopathologische zeitschrift* **106**, 163 - 176.

Weste, G., and Marks, G. C. (1987). The biology of *Phytophthora cinnamomi* in Australasian forests. *Annual Review of Phytopathology* **25**, 207 - 229.

Weste, G., and Ruppin, P. (1977). *Phytophthora cinnamomi*: Population densities in forest soils. *Australian Journal of Botany* **25**, 461 - 475.

Weste, G., and Vithanage, K. (1978a). Effect of *Phytophthora cinnamomi* on microbial populations associated with the roots of forest flora. *Australian Journal of Botany* **26**, 153 - 167.

Weste, G., and Vithanage, K. (1978b). Seasonal variation in numbers of chlamydospores in Victorian forest soils infested with *Phytophthora cinnamomi. Australian Journal of Botany* **26**, 657 - 662.

Weste, G., and Vithanage, K. (1979). Survival of chlamydospores of *Phytophthora cinnamomi* in several non-sterile, host-free forest soils and gravels at different soil water potentials. *Australian Journal of Botany* **27**, 1 - 9.

Wilkinson, C., Holmes, J., Tynan, K., Colquhoun, I., McComb, J., Hardy, G. E. S., and Dell, B. (2001a). Ability of phosphite applied in a glasshouse trial to control *Phytophthora cinnamomi* in five plant species native to Western Australia. *Australasian Plant Pathology* **30**, 343 - 351.

Wilkinson, C., Shearer, B. L., Jackson, T. J., and Hardy, G. E. S. (2001b). Variation in sensitivity of Western Australian isolates of *Phytophthora cinnamomi* to phosphite *in vitro*. *Plant Pathology* **50**, 83 - 89.

Wilkinson, C., Holmes, J. M., Dell, B., Tynan, K. M., McComb, J. A., Shearer, B. L., Colquhoun, I., and Hardy, G. E. S. (2001c). Effect of phosphite on *in planta* zoospore production of *Phytophthora cinnamomi*. *Plant Pathology* **50**.

Wongwathanarat, P., and Sivasithamparam, K. (1991). Effect of phosphonate on the rhizosphere microflora and the development of root rot (*Phytophthora cinnamomi*) in avocado (*Persea americana*) and pepper-corn (*Schinus molle*) tree seedlings. *Biology and Fertility of Soils* **11**, 13 - 17.

Wu, J. (2004). Transformation of *Rhizoctonia solani*. PhD thesis, Murdoch University.

You, M. P., and Sivasithamparam, K. (1994). Hydrolysis of flourescein diacetate in an avocado plantation mulch suppressive to *Phytophthora cinnamomi* and its relationship with certain biotic and abiotic factors. *Soil Biology and Biochemistry* **26**, 1355 - 1361.

Zambryski, P. (1992). Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annual Review of Plant Physiology and Plant Molecular Biology.* **43**, 465 - 490.

Zentmyer, G. A. (1952). A substance stimulating sexual reproduction in *Phytophthora cinnamomi*. *Phytopathology* **42**, 24.

Zentmyer, G. A. (1963). Biological control of *Phytophthora* root rot of avocado with alfalfa meal. *Phytopathology* **53**, 1383 - 1387.

Zentmyer, G. A. (1979). Stimulation of sexual reproduction in the A2 mating type of *Phytophthora cinnamomi* by a substance in avocado roots. *Phytopathology* **69**, 1129 - 1131.

Zentmyer, G. A. (1980). "*Phytophthora cinnamomi* and the diseases it causes." American Phytopathological Society, St Paul, Minnesota.

Zentmyer, G. A. (1983). The world of *Phytophthora*. *In* "*Phytophthora*: Its Biology, Taxonomy, Ecology and Pathology" (D. C. Erwin, S. Bartnicki-Garcia and P. H. Tsao, eds.). APS Press, USA.

Zentmyer, G. A., and Bingham, F. T. (1956). The influence of nitrate on the development of *Phytophthora* root rot of avocado. *Phytopathology* **46**, 121 - 124.

Zentmyer, G. A., and Erwin, D. C. (1970). Development and reproduction of *Phytophthora*. *Phytopathology* **60**, 1120 - 1127.

Zentmyer, G. A., and Mircetich, S. M. (1966). Saprophytism and persistence in soil by *Phytophthora cinnamomi. Phytopathology* **56**, 710 - 712.

Zheng, X. B., and Ko, W. H. (1996). Continuing variation in successive asexual generations of *Phytophthora cinnamomi* following sexual reproduction. *Canadian Journal of Botany* **74**, 1181 - 1185.