Susceptibility of native plant species to *Phytophthora cinnamomi* and the spread of Phytophthora dieback in South Australia

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Thesis submitted for the degree of

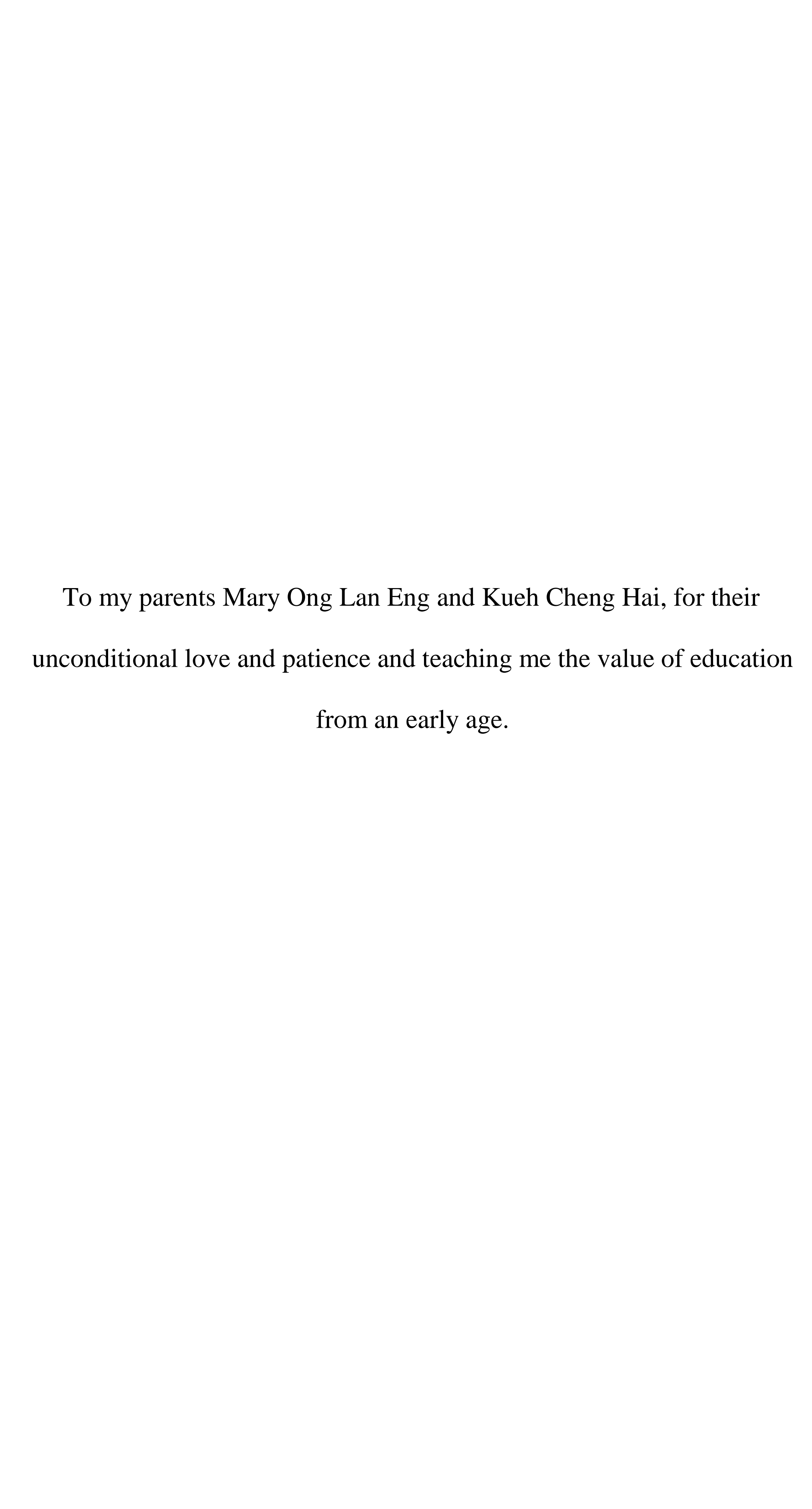
Doctor of Philosophy

at The University of Adelaide

School of Agriculture, Food and Wine

Faculty of Sciences

November 2011



To my parents Mary Ong Lan Eng and Kueh Cheng Hai, for their unconditional love and patience and teaching me the value of education from an early age.

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Abstract

Phytophthora dieback, caused by *Phytophthora cinnamomi* Rands, affects a wide range of Australian native plants. In South Australia, the pathogen has affected large areas of native vegetation to threaten plant biodiversity. Lack of information on the disease in the local environment hampers management. The main objectives of this project were to: a) determine the rate of pathogen and disease spread in naturally infested native vegetation, b) assess the susceptibility of plant species native to South Australia to the disease and c) assess ability of antagonistic soil actinomycetes to protect susceptible species from Phytophthora dieback.

A confirmed *P. cinnamomi*-infested site, with gentle slope, at Mount Bold Reservoir Catchment Reserve in the Mount Lofty Ranges, was selected to assess pathogen and disease spread in native vegetation. The soil was loamy sand. The vegetation was open woodland dominated by *Eucalyptus obliqua* L'Hérit with an understorey dominated by *Xanthorrhoea semiplana* F. Muell, a highly susceptible species which was used as an indicator to assess disease spread. An area of 70 m x 70 m, extending from two disease fronts into the adjoining healthy vegetation, was marked into 10 m x 10 m quadrats. The number of dead and dying *X. semiplana* was counted and soil samples from each quadrat, collected every spring and autumn from 2008 to 2010, were baited for *P. cinnamomi* using cotyledons of *E. sieberi* L.A.S. Johnson. *P. cinnamomi* was regularly detected along the disease front. However, the pathogen did not spread across the slope into the adjoining healthy vegetation despite annual rainfall of 626 to 900 mm for three consecutive years (2008 to 2010). The slow spread of the pathogen was reflected in the small numbers of dead and dying *X. semiplana* observed in each quadrat at each assessment time. The limited spread of the pathogen may be due to unfavourable weather conditions. In winter (June to August), when the

precipitation was high (*ca*. 50% of the annual rainfall), soil temperature was generally too low (average temperature 9.3°C) for formation of sporangia. On the contrary when the temperature was warm (≥ 15°C) during spring (September to November) and autumn (March to May), the average soil water potential, ≤ -200 kPa, may have been too low for movement of zoospores. Further, sporadic distribution of *P. cinnamomi* and the patchiness of disease spread might have reflected the efficiency of the baiting technique.

Thirty-seven South Australian native plant species, including 15 threatened or locally endangered species, were assessed for susceptibility to Phytophthora dieback in a greenhouse from October 2009 to July 2010. Seedlings or cuttings were raised in potting mix for native species then transplanted to 15 cm-diameter pots filled with limed University of California mix or Bio Gro® (Bio Gro, South Australia). Plants were inoculated with *P. cinnamomi* via pine wood-inoculum plugs when up to 6 months old, maintained in moist conditions and monitored for disease symptoms for 3 to 6 months. Twenty-four of the 37 species studied, including 8 threatened species, were susceptible to the disease. Nine of these 24 species were ranked as highly susceptible. Another nine species were assessed as resistant. All species classed as susceptible were trees or shrubs while herbs were unaffected. In South Australia, where native vegetation has been extensively cleared or degraded, Phytophthora dieback represents an additional threat to the remnant native flora that might cause the extinction of native plant species, particularly the rare and endangered species, if not brought under control.

Actinomycetes were isolated from soil collected from roots of *Acacia pycnantha* Benth and young, healthy *X. semiplana* growing close to dead *X. semiplana* at the field site. Of 127 actinomycetes isolates selected, 78% inhibited *P. cinnamomi* in dual culture. Eight *Streptomyces* spp. which exhibited strong to weak antagonism, were

compared in the greenhouse for ability to protect 2-month old *E. sieberi*. One isolate delayed infection of *E. sieberi* by *P. cinnamomi*, although none prevented disease. The high soil moisture ($\geq$ -10 kPa) required to induce disease was probably not conducive for the growth of the actinomycetes.

Knowledge generated in this project can be used in Phytophthora management to help prioritise threatened plant species in South Australia for protection, inform revegetation programs and to provide the basis for further research in the state.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Kueh Kiong Hook and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Acknowledgements

I am very grateful to the State Government of Sarawak for providing me with a scholarship to study at The University of Adelaide, South Australia.

I wish to thank my supervisors Professor Eileen Scott, Dr Amanda Able, Associate Professor Jose Facelli and Associate Professor Chris Franco for their supervision and support with this project over the course of my candidature. I appreciate the dedication of Eileen Scott and Amanda Able in supervising my research and their untiring effort in commenting on my drafts are invaluable. I have personally benefited immeasurably from their guidance. Thanks also to ARC Research Associates Drs Suzanne McKay and Evelina Facelli for their cooperation in the field study and the School of Agriculture, Food and Wine for support in my project.

Many thanks to Paul Ingram, Andreas Flench'e and Andrew McGregor, Plant Growth Services, SARDI, for their help on the use of the greenhouse facilities; Eerden Bert (now retired) and Ken Ruge, Mount Bold Reservoir Office, for the weather data and supply of pine wood; Dr Nari Williams, Centre for *Phytophthora* Science and Management, Murdoch University, Western Australia for confirming identification of isolates 71a and SC4; and Dr Phillip Ainsley, Seed Conservation Centre, Department of Environment and Natural Resources, for providing seeds and seedlings of threatened species; Dr. Cameron Grant for showing me how to determinate soil texture; Kaewkla Onuma, Flinders University, for kindly teaching me the use of PCR for identification of actinomycetes.

I am grateful to my fellow PhD students, Steve Coventry, Amanda Benger, Kieren Arthur, Carol Walker, Macklin Dayod and Margaret Abat for many fruitful

discussion and friendship. I also thank Tanya Kovalenko, SARDI tissue culture group, for her friendship and spending coffee breaks together.

Finally, I would especially like to thank my wife, Vu Thanh Tu Anh, who has supported and helped me in so many ways during the course of my study.

Publications and conference proceedings

The following publications and conference proceedings were produced during the PhD candidature:

Kueh KH, McKay SF, Facelli E, Facelli JM, Velzeboer RMA, Able AJ, Scott ES, 2012. Response of selected South Australian native plant species to *Phytophthora cinnamomi.* Manuscript published in Plant Pathology on 17 February 2012 (DOI: 10.1111/j.1365-3059.2012.02593.x) and presented in Chapter 4.

Kueh KH, Franco C, Able JA, Facelli J, Scott E, 2009. Screening for soil actinomycetes antagonistic to *Phytophthora cinnamomi* in a native ecosystem in South Australia. Abstract and oral presentation in Microbial Ecology Workshop: Concepts and techniques for disease control, a workshop of the 17th Australasian Plant Pathology Society Conference, held on the 27 September, at Newcastle Civic Centre, Newcastle, NSW, Australia.

McKay SF, Kueh KH, Able AJ, Velzeboer RMA, Facelli JM, Scott ES, 2009. Impact of *Phytophthora cinnamomi* on native vegetation in South Australia. Abstract in Proceedings of the 16th Australasian Plant Pathology Society Conference, held on 29 September to 1 October at New Civic Centre, Newcastle, NSW, Australia.

List of Abbreviations

ANOVA	analysis of variance
bp	base pair
CFU	colony forming unit
CGM	casein glycerol medium
CMA	cornmeal agar
CPSM	Centre for *Phytophthora* Science and Management
CTAB	hexadecyltrimethylammonium bromide
d	day
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleic acid
ETDA	ethylenediamine *tetra* acetic acid
h	hour
HA	humic acid-vitamin agar
ISP medium 2	International *Streptomyces* Project medium 2
LSD	least significant difference
min	minute
MS	mannitol-soy medium
OA	oatmeal agar
P_{10}ARPH	corn meal agar with antibiotics
PCNB	pentachloronitrobenzene
PCR	polymerase chain reaction
PDA	potato dextrose agar
rRNA	ribosomal ribonucleic acid

rDNA	ribosomal deoxyribonucleic acid
s	second
SARDI	South Australian Research and Development Institute
SDS	sodium dodecyl sulphate
UC	University of California
V8	V8 juice
WYE	water yeast extract medium
YCED	casamino-yeast extract-glucose agar

Chapter 1 Literature review

1.1 Introduction

Phytophthora cinnamomi, the causal agent of Phytophthora dieback, has caused considerable damage to the native vegetation in Australia for the last 90 years (Podger, 1972). The disease is present in all States of Australia but the native vegetation most seriously affected is in the south eastern and south western parts of the country, including the temperate forest in Tasmania, the woodland in Victoria and the jarrah forest and *Banksia* woodlands in Western Australia. Tens of thousands of hectares are estimated to be infested with the pathogen with potential to spread further (Bluett *et al.*, 2003). The origin of *P. cinnamomi* in Australia is not known with certainty but it is generally believed to have been brought into the country through contaminated planting material, claimed by Markus (2009) to be citrus seedlings imported from Asia. The pathogen is soil-borne and attacks roots, causing root rot which leads to dieback and eventually plant death. *P. cinnamomi* has a wide host range, estimated to be several thousand species, particularly native plant species of Australia (Shearer *et al.*, 2004a).

In South Australia, death of native plants associated with *P. cinnamomi* was first reported in 1973 in the Adelaide Hills (Wicks, 1973). Since then, the pathogen has been found widely in a mosaic of infected areas in the Mount Lofty Ranges, Fleurieu Peninsula and Kangaroo Island regions (Department of the Environment and Water Resources, 2001). These are regions of high conservation value (Holliday *et al.*, 1994; Pobke, 2007; Wilson & Bignall, 2009). The presence of the disease in these regions poses a serious threat to the continued survival of native plants, particularly threatened species (Peters & Weste, 1997; Barker & Wardlaw, 1995). Populations of threatened

plants are, by definition small and the presence of *P. cinnamomi* in their vicinity would make these plants even more vulnerable to extinction (Weste & Ruppin, 1975). It is therefore a matter of urgent concern that action be taken to protect these plants from Phytophthora dieback (Barker *et al.*, 1996; Reiter *et al.*, 2004; Shearer *et al.*, 2007).

Currently, eradication of *P. cinnamomi* from the soil is rarely feasible, particularly in natural ecosystems (Dunstan *et al.*, 2010). In South Australia, protection and conservation of native plant species and plant communities has relied on hygiene, such as restricted access to conservation parks during wet weather and provision of car wash facilities, to reduce the spread of *P. cinnamomi* to uninfested areas. The Department of Environment and Natural Resources (DENR), South Australia has been active in promoting hygiene to the general public, and has produced detailed guidelines for bushwalkers and horse riders giving details about responsible access and hygiene procedures (O'Gara *et al.*, 2005). However, in view of the fact that the disease is already present widely in the State, hygiene measure alone might not be adequate to protect native plant species *in situ* (Jilian *et al.*, 2006). A new Phytophthora management strategy is therefore needed. As such, the Department of Environment and Heritage, (DEH, now known as Department of Environment and Natural Resources), South Australia developed a risk assessment and priority setting strategy in 2005 for management of threatened plant species in the State (Velzeboer *et al.*, 2005). The goal of the species risk assessment was to prioritise threatened plant species for management and conservation (Velzeboer *et al.*, 2005). The potential risk that Phytophthora dieback posed to each threatened plant species was estimated based on the conservation status of the species and proximity to sites known or likely to be infested by *P. cinnamomi*. Areas deemed vulnerable to infestation by *P. cinnamomi* or which were already infested by *P. cinnamomi* were divided into high, moderate and

low risk zones depending on the distance from a *P. cinnamomi*-infested site (Velzeboer *et al.*, 2005). However, information on the response of South Australian native plant species to infection by *P. cinnamomi* and the rate of spread of disease and *P. cinnamomi* in native vegetation is lacking. This lack of information has hampered management of Phytophthora dieback in the State. The use of beneficial soil microflora, such as fungi, actinomycetes, and other bacteria which are antagonistic to *P. cinnamomi*, represents a potential method for protection of threatened native plant species from Phytophthora dieback in revegetation programmes (Broadbent *et al.*, 1971; Malajczuk *et al.*, 1977b; Walchhuetter, 2002).

1.2 History and impact of Phytophthora dieback on native vegetation

P. cinnamomi is an aggressive pathogen of global distribution, affecting many horticultural crops, like avocado (*Persea americana*), pineapple (*Ananas comosus*) and citrus (*Citrus* spp.) (Erwin & Ribeiro, 1996). *P. cinnamomi* also infects species of native vegetation (Erwin & Ribeiro, 1996). In Spain and Portugal, *P. cinnamomi* had been implicated in the decline of cork oak, *Quercus suber*, and holm oak, *Q. ilex*. In the United States of America *P. cinnamomi* decimated wild chestnut trees (*Castana dentata*) in the 1820s (Money, 2007). These reports contrast with the situation in Australia, where a broad spectrum of native plants is killed by the disease (Money, 2007).

The term Phytophthora dieback refers to the progressive deterioration of the crowns after plants have been infected by *P. cinnamomi*. The disease was first reported in 1921 in small areas near Karagullen in the northern jarrah (*Eucalyptus marginata*) forest in Western Australia (Batini & Hopkins, 1972), while in eastern Australia, the

disease was first associated with death of natural vegetation in coastal New South Wales in 1949 and 1956 (Fraser, 1956). However, with the increase of logging activities, particularly the use of machinery after World War II, the disease spread very rapidly and by the 1950s had became epidemic in the jarrah forest of Western Australia (McKinnell, 1981). The disease killed not only the dominant jarrah trees, but also many understorey species. Initially the death of the jarrah trees was attributed to several ecological factors such as drought, waterlogging, salt, toxic nutrients, fire and disturbance of native vegetation but these factors failed to account for all aspects of the disease (Batini & Hopkins, 1972). The possibility that a soil-borne pathogen was responsible was only explored when pine trees (*Pinus radiata*) at a number of places adjacent to the jarrah forest showed dieback symptoms similar to those observed in *Pinus radiata* trees in New Zealand after infection by *Phytophthora* spp. (Newhook, 1959). In 1964, Zentmyer isolated *P. cinnamomi* Rands from soil beneath dying jarrah for the first time in Western Australia (Podger, 1972). Later, pathogenicity tests of isolates on jarrah and indigenous plant species confirmed *P. cinnamomi* was the cause of the disease in 1967 (Podger, 1972).

Within three decades, from 1950 to 1980, severe diebacks had been reported in East Gippsland, the Grampians, Wilson's Promontory National Park and the Brisbane Ranges, Victoria, killing species like *Banksia serrata*, *Xanthorrhoea australis* and *Sprengelia incarnata* (Weste & Taylor, 1971; Marks *et al*., 1972; Weste, 1974), and in the wet sclerophyll forest in the north-east of Tasmania, killing *Eucalyptus delegatensis* (Bird *et al*., 1974). Phytophthora dieback also occurred, but to a lesser extent, in Queensland, New South Wales and Northern Territory, affecting native vegetation e.g. in patches of *Banksia integrifolia* at Coolum, Queensland (Pratt *et al*., 1972a), in dry sclerophyll forest affecting *Woollsia pungens*, *Epacris purpurascens* and *Xanthosia*

tridentata at Black Mountain, NSW (Fraser, 1956; Cahill *et al*., 1989), and in patches of *Eucalyptus tetradonta* in the Gove area, NT (Blowes & Pitkethley, 1981). In South Australia, native plants such as *Xanthorrhoea semiplana*, *Banksia marginata* and *Pultenaea involucrata* exhibiting symptoms of disease were first noted in the Cleland Conservation Park in 1973, from which *P. cinnamomi* was isolated (Wicks, 1973).

The most immediate danger posed by Phytophthora dieback is the extinction of rare and endangered plant species which are susceptible to the disease. Endangered plant species threatened with extinction by Phytophthora dieback include *Banksia brownii* (McCredie *et al.*, 1985), *Wollemia nobilis* (Bullock *et al.*, 2000), *Grevillea chrysophaea* and *Pultenaea graveolens* (Peters and Weste, 1997), to name only a few. Severe infection of native vegetation by *P. cinnamomi* can lead to changes in structure and composition of plant communities as susceptible plants die and are replaced by resistant species (Weste, 1994). Changes in the structural composition of native vegetation could have adverse impacts on fauna which depend on the susceptible species for food and habitat (Weste, 1994). *P. cinnamomi* can also exert a strong selection pressure on the composition of native vegetation. For example, Pratt & Heather (1973) noted that, in eastern Australia, eucalypts and understorey species in moist sites, which favour the growth of *P. cinnamomi*, tend to be resistant to *P. cinnamomi* while those on drier sites tend to be susceptible.

Phytophthora dieback is recognised as a “key threatening process” in Australia under the Environment Protection and Biodiversity Conservation Act 1991. Under the Act, Federal and State Governments have the obligation to put in place action plans to mitigate the impact of the disease based on scientific findings and knowledge.

1.3 Life cycle of *Phytophthora cinnamomi*

Phytophthora is currently classified among eukaryotes in the Kingdom Stramenopila; Class Oomycota; Sub-class Peronosporomycetidae; Order Peronosporales; Family Pythiaceae (Van West *et al.*, 2003). As such they are more closely related to brown algae and diatoms than the 'true' fungi (Judelson & Blanco, 2005), though their growth pattern is 'fungus-like' (Van West *et al.*, 2003). Molecular studies have confirmed that *P. cinnamomi* is more closely related to brown algae than to true fungi. In addition, the cell wall of *P. cinnamomi* consists mainly of cellulose and glucan and not chitin as in true fungi. The hyphae of *P. cinnamomi* are coenocytic (not partitioned by septa). The nuclei of mycelium are diploid. *P. cinnamomi* is heterothallic, which means it requires two mating types, designated as A1 and A2, for sexual reproduction. The pathogen also undergoes asexual reproduction.

1.3.1 Sexual life cycle of *Phytophthora cinnamomi*

Sexual reproduction is by gametangial contact between an oogonium (female reproductive cell) and antheridium (male reproductive cell). When the two mating types, A1 and A2, grow together in the same host or in the same culture, the antheridium grows towards the oogonium that contains an unfertilised egg. When the two come together, the male clamps onto the surface of the oogonium and fertilises the oogonium. The fertilised egg develops into an oospore. The formation of male and female reproductive cells involves meiosis and recombination of genetic material. Thus, new strains of *P. cinnamomi* are formed, with new characteristics that might include pathogenicity different from that of the parental strains. Oospores are survival structures. They are enclosed with a thick wall and can survive adverse conditions

(Shea, 1975). In favourable conditions, oospores can germinate either directly into mycelium or indirectly into sporangia (Zentmyer, 1981).

1.3.2 Asexual life cycle

The mycelium of *P. cinnamomi* can form either chlamydospores or sporangia depending on environmental conditions (Figure 1.1). When the environmental conditions are hot and dry, mycelium forms chlamydospores (Grant & Byrt, 1984). Like oospores, chlamydospores are survival structures which are enclosed within a thick cell wall. Chlamydospores not only allow *P. cinnamomi* to survive during unfavourable weather conditions but also enable the pathogen to be dispersed to new areas. When the environment becomes favourable for growth, chlamydospores germinate and produce mycelium again.

When the environmental conditions are warm, 18-22°C and wet such that water is available for metabolic processes, the mycelium forms sporangia. Sporangia are reproductive structures and are produced at the tip of sporangiophores. When a sporangium matures, it releases 20 to 30 motile zoospores through a pore at the tip of the sporangium (Hine *et al*., 1964). A zoospore possesses two flagella, a whiplash flagellum at the posterior end and a tinsel at the anterior end that enables it to move short distances (20-35 mm) in moist soil (Duniway, 1976). Zoospores encyst and then germinate to infect the root. As zoospores play an important role in the spread of *P. cinnamomi* from one plant to another, the infection process is the focus of the next section.

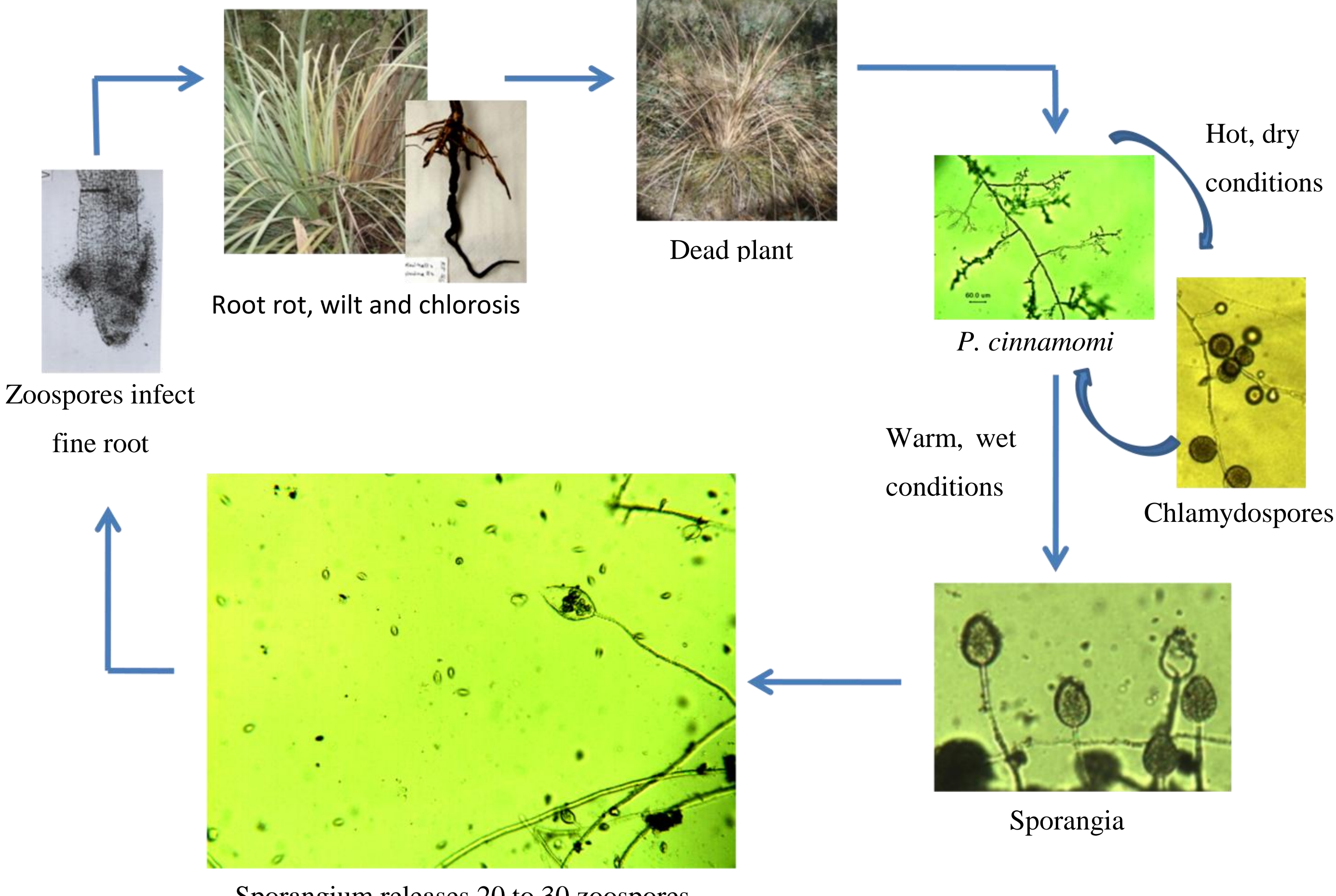

Figure 1.1 Asexual life cycle of *Phytophthora cinnamomi*. Disease symptoms in *X. semiplana* caused by *P. cinnamomi* are also shown.

1.4 Infection of plants by *Phytophthora cinnamomi*

Research carried out over the last 35 years has provided a clearer understanding of the process of infection of roots by *P. cinnamomi*. In natural ecosystems, infection of the plant is generally via root to root contact (Shea & Dillon, 1980; Hill *et al.*, 1994) and zoospores (Davison, 1994), although both mycelium and chlamydospores are also capable of infecting plants (Tippett *et al.*, 1976; Malajczuk *et al.*, 1977a; O'Gara *et al.*, 1996). Zoospores are motile and are chemotactically attracted to the sugar and amino acids present in the exudates of roots. Zoospores encyst close to the root surface at the zone of elongation, which coincides with the regions of maximum exudation (Hinch & Weste, 1979). The cysts then germinate by producing germ tubes. Penetration of root tissue by hyphae of *P. cinnamomi* involves formation of appressoria-like structures and is facilitated by secretion of a range of enzymes, which include polygalacturonases that breakdown cell walls in advance of the invading hyphae (Hardham & Blackman, 2010). After the hyphae have colonised plant tissue, the hyphae obtain nutrients from the plant. This allows *P. cinnamomi* to grow quickly and spread throughout the cortical tissue to the vascular tissue, mainly by growing intercellularly and less frequently intracellularly (Malajczuk *et al.*, 1977a). Infected cells became necrotic and die.

Within 2 to 3 days after infection, hyphae of *P. cinnamomi* emerge from the root surface and produce sporangia again. Formation of zoospores is a very rapid process and together with the short disease cycle of 2 to 3 days allows rapid build up of inoculum which can lead to an epidemic when environmental conditions are conducive for the growth of *P. cinnamomi*.

1.5 Disease symptoms

P. cinnamomi attacks the fine roots of plants and spreads to the vascular system. The primary symptoms of infection are root lesions resulting in stunted roots, discolouration of the root and eventually root rot (Halsall, 1978). Necrosis of the lateral and major roots reduces water and nutrient uptake from the soil, which causes the infected plant to show secondary symptoms similar to those produced by water stress, such as wilting and loss of turgor pressure. This is followed by leaf chlorosis, twig dieback, occasional leaf fall and eventual plant death. Depending on the environmental conditions, the infected plant may die within a few months or years. Occasionally, woody plants may recover and produce epicormic shoots (Podger, 1972).

Plants of highly susceptible species, such as *Xanthorrhoea australis*, *Isopogon ceratophyllus* and *Banksia grandis*, die rather quickly, generally within a few months after infection. These species express disease symptoms readily. Plants of *Eucalyptus calophylla*, now known as *Corymbia calophylla*, and *E. wandoo* are more tolerant of the disease, express disease symptoms gradually and can remain alive for many years (Malajczuk *et al.*, 1977a).

1.6 Distribution and variation of *Phytophthora cinnamomi*

P. cinnamomi is present in a wide range of climatic, topographical and floristic environments throughout Australia (Pratt & Heather, 1973). Both mating type A1 and A2 are found in Australia, however, *P. cinnamomi* in Australia is predominantly of the A2 mating type. Of the 372 Australian *P. cinnamomi* isolates assessed for mating type, only five isolates were found to be of A1, 366 were A2 and one was undetermined (Pratt *et al.*, 1972b). Records from the literature show that A1 has only been isolated

from the following places in Australia: Beerburrum, Cooloola, Bribie Island and Coolum in Queensland; Kioloa, Ourimbah, Murwillumbah in New South Wales; Stirling Ranges, Kelmscott and the Fitzgerald River National Park (GEStJ Hardy, pers. com., 2012), in Western Australia; Nhulunbuy in Northern Territory; Canberra in the Australian Capital Territory, and Southern Tasmania (Podger, 1989; Dudzinski *et al.*, 1993). No isolate of *P. cinnamomi* mating type A1 has been reported in South Australia. Since the mating type A1 is rarely found in soil, it is likely that few oospores are formed in forest soil; instead *P. cinnamomi* reproduces asexually through the formation of sporangia and spreads in the form of genetically distinct lineages in different parts of Australia (Money, 2007).

There have been few studies of variability among isolates of *P. cinnamomi* in Australia. Pratt & Shepherd (1972) compared the morphological features of 344 isolates of *P. cinnamomi* and found significant variation among the isolates collected from within Australia in terms of optimum growth temperature, colony growth rate on agar and ability to produce sporangia and oospores. Podger (1989) found no significant differences in pathogenicity when comparing 14 isolates of *P. cinnamomi* collected from Queensland, Western Australia, Tasmania and New South Wales. The pathogenicity was assessed based on its ability to cause mortality in five highly susceptible species: *Sprengelia incarnata*, *Epacris corymbiflora*, *Melaleuca squamea*, *Baeckia leptocaulis* and *Boronia parviflora*. However, this finding was in contrast to the results of Dudzinski *et al.* (1993) who reported significant differences in the pathogenicity of 42 isolates of *P. cinnamomi*, consisting of both mating type A1 and A2, collected from Queensland, Tasmania, New South Wales, Victoria, Australian Capital Territory, Northern Territory and Western Australia. The pathogenicity of the 42 isolates was assessed based on their ability to induce disease symptoms on a single

clone of *Eucalyptus marginata* and the authors concluded that pathogenicity was a stable characteristic of the isolate and was unrelated to mating type, isozyme properties or the source of the isolate. These different findings between these two studies might reflect the larger number of isolates of *P. cinnamomi* collected from a broader area used in the study by Dudzinski (1993). Due to the variation in pathogenicity among isolates of *P. cinnamomi*, Shearer *et al*. (2007) recommended the use of more than one virulent isolate for testing of susceptibility to *P. cinnamomi*.

1.7 Host range

Australia has an estimated 21,000 to 23,000 native vascular plant species belonging to 240 families (2700 genera) of which about 85% are endemic (Thiele & Adams, 2001). Australian native flora are particularly susceptible to Phytophthora dieback. In the South-West Botanical Province of Western Australia, about 2284 of the 5700 described plant species are estimated to be susceptible to the disease (Shearer *et al.*, 2004a) and, in Tasmania, 136 species from 31 families were listed as hosts of *P. cinnamomi*, many of which were highly susceptible (Podger *et al.*, 1990). An additional 36 of 47 native threatened plant species in Tasmania were later assessed by Barker & Wardlaw (1995) as susceptible. In a *P. cinnamomi*-infested site in the Grampians National Park in Victoria, Kennedy and Weste (1986) reported that 54% of the 108 species present were susceptible.

Most species in the Protecaceae, Epacridaceae and Papilionaceae tend to be susceptible to Phytophthora dieback. Asteraceae and Mimosaceae contain few susceptible species (Cahill *et al.*, 2008). Species in the Myrtaceae are biased towards resistant groups (Shearer *et al.*, 2004a). Species of Cyperaceae (sedges) are mainly

resistant (Shearer *et al.*, 2004a). There is considerable interspecific and intraspecific variation in susceptibility within families (Shearer *et al.*, 2004a). Podger & Batini (1971) assessed the susceptibility of 36 species of *Eucalyptus* and found 11 species were highly susceptible while another six species were resistant. Within a species, individual plants showed wide variation in susceptibility to Phytophthora dieback. There is also considerable variation in the susceptibility of species within the same genus from different geographical ranges or provenances. For example, McCredie *et al*. (1985) found that *Banksia* species from the east coast of Australia were less susceptible to Phytophthora dieback than *Banksia* species from the west coast (Cahill *et al.*, 2008). As such, species from a particular provenance should be assessed by experimentation for susceptibility and information generated cannot be extrapolated to similar species from another provenance.

Due to its wide host range, *P. cinnamomi* poses a threat to the long-term survival of rare and threatened plant species in Australia (Barker & Wardlaw, 1995; Peters & Weste, 1997; Reiter *et al.*, 2004; Shearer *et al.*, 2004a). Threatened plant species should be tested for susceptibility to Phytophthora in order to protect wild populations from the disease. In South Australia, few native species have been tested for susceptibility to Phytophthora dieback (O'Gara *et al.*, 2005). Most of the information on susceptibility of the South Australian native species has been based on field observation and anecdotal evidence. As there is variation in susceptibility of species from different areas and States, threatened species in South Australia should therefore be tested for susceptibility.

1.8 Response of plants to infection by *Phytophthora cinnamomi*

As noted in section 1.7, Australian native plants are divided into susceptible, tolerant (sometimes also referred to as resistant but a host) and resistant (non-host) to Phytophthora dieback based on the response to infection by *P. cinnamomi*. Plant species are categorised as susceptible if *P. cinnamomi* successfully infects and colonise host tissue, resulting in expression of disease symptoms including death. Species highly susceptible to Phytophthora dieback include *Xanthorrhoea australis* and *Banksia grandis*. However, if *P. cinnamomi* successfully penetrates and establishes itself inside the host tissue but causes no disease symptoms, the species may be regarded as tolerant (British Mycological Society, 1950). On the contrary, if the pathogen fails to establish itself in the plant tissue even if it initially gains entry into the host, due to some physiological or anatomical character, the plant species is categorised as resistant (American Phytopathological Society, 1940). These terms had been elaborated by White (1950).

As zoospores are responsible for causing infection, attraction of zoospores to root exudates may determine the susceptibility of a species, in that a susceptible species may attract more zoospores than a resistant species (Hinch & Weste, 1979). This was found to be the case when two species of blueberry, *Vaccinium corymbosum* (susceptible) and *V. ashei* (resistant) (Milholland, 1975) were compared and, likewise *Eucalyptus st johnii* (resistant) and *E. obliqua* (susceptible). The roots of the susceptible species were found to attract more zoospores than the roots of the resistant species (Tippett *et al.*, 1976) (Table 1.1). However, evidence linking chemotaxis of zoospores to susceptibility is not unequivocal. Malajczuk & McComb (1977) (Table 1.1) reported that chemotaxis of zoospores in response to exudates from roots was not

species-specific in the case of *E. marginata* and *C. calophylla* but emphasised that microflora present in the rhizosphere might influence the number of zoospores that were able to cyst and germinate on the surface of roots. This suggested that the resistance of *C. calophylla* to *P. cinnamomi* was influenced by the presence of microflora in the rhizosphere (Malajczuk & McComb, 1979).

Halsall (1978), Hinch & Weste (1979) and Cahill *et al*. (1989) have also found that chemotaxis of zoospores to roots was non-host specific and that zoospores were abundantly attracted to roots of a range of Australian native plants regardless of whether the species was susceptible, tolerant or resistant (Table 1.1). Zoospores successfully infected plants of all species. These observations led to the conclusion that differential susceptibility between species was not due to chemotaxis of zoospores to roots but rather was determined by the response of the species post-infection.

In susceptible species, such as *Eucalyptus sieberi* and *Xanthorrhoea australis*, hyphae of *P. cinnamomi* spread rapidly throughout the host tissue after infection, causing necrosis of the root system. New roots grew but eventually succumbed to infection by *P. cinnamomi* (Malajczuk *et al.*, 1977a). This resulted in expression of severe symptoms of the disease. On the contrary, in tolerant species such as *Corymbia maculata* and *Gahnia radula*, plants responded to infection by *P. cinnamomi* by forming necrophylactic periderm, callose, tylose or accumulation of phenolic compounds which stops the spread of *P. cinnamomi* within the tissue (Tippett *et al.*, 1976; Phillips *et al.*, 1987) (Table 1.1). Although *P. cinnamomi* was prevented from spreading in the tissue, the pathogen remained viable and broke out of the periderm and invaded new cells (Cahill *et al.*, 1989). The viability of *P. cinnamomi* in the tissue suggested that tolerant plants could be a threat to native vegetation because they can harbour *P. cinnamomi* without showing any disease symptoms.

In resistant species (non-host), *P. cinnamomi* failed to persist in the plant tissue due to hypersensitive reaction by the plant (Tippett & Malajczuk, 1979). Initial infection of plants by *P. cinnamomi* triggered a reaction in which apposition of phenolic compound and tylose occurred close to invading hyphae, causing hyphal lysis *P. cinnamomi* (Tippett & Malajczuk, 1979) (Table 1.1). A state of incompatibility appeared to exist between the host and the pathogen. An example of a species which exhibited a hypersensitive reaction is *Acacia pulchella* (Tippett & Malajczuk, 1979).

Work on pathogenicity conducted by Huberli *et al.* (2002) showed that jarrah clonal planting materials varied in response to infection by *P. cinnamomi* and that severity of disease expression was influenced by temperature (section 1.9) and method of inoculation. However, when assessing susceptibility or resistance to infection by *P. cinnamomi*, Hüberli *et al*. (2002) recommended inoculation using zoospores and measurement of the length of colonisation by *P. cinnamomi*, rather than lesion length, for more consistent results.

1.9 Factors affecting survival and pathogenicity of *Phytophthora cinnamomi*

In forest soils, population density of *P. cinnamomi* is primarily influenced by three factors; soil temperature, soil moisture and microbial activity (Weste & Ruppin, 1975). These factors act in concert in determining the population densities and, potentially, the severity of disease and its rate of spread (Weste & Ruppin, 1977).

Table 1.1 Resistance attributes that have been identified as components of the reaction of plants to *Phytophthora cinnamomi*.

Reference	**Species examined**	**Methods**	**Resistance component**	**Major finding/conclusion or inference**
Milholland (1975)	*Vaccinium corymbosum* (susceptible) *V. ashei* (resistant)	- Inoculated with mycelium and zoospores - Histopathology	- Variability of pathogenicity of *P. cinnamomi* isolates - Resistant species attracted fewer zoospores than susceptible species	- Differences in susceptibility were due to different numbers of zoospores attracted to roots
Tippett *et al*. (1976)	*Eucalyptus st johnii* (tolerant) *E. obliqua* (susceptible)	- Inoculated with zoospores and mycelium - Light and electron microscopy and staining	- Fewer zoospores encysted on roots of resistant species - Formation of callose sealed off *P. cinnamomi*	- Infection process of root by zoospores is the same for susceptible and resistant species
Tippett *et al*. (1977)	*Eucalyptus st johnii* (tolerant) *E. obliqua* (susceptible) *E. sieberi* (susceptible)	- Inoculated with zoospores - Light and electron microscopy and staining	- Tolerant of water logging - Regeneration of new roots	- Plant response to infection by *P. cinnamomi* was the same in all species
Malajczuk *et al*. (1977a)	*Corymbia calophylla* *E. marginata*	- Inoculated with mycelium - Light and electron microscopy and staining	- Rhizosphere microflora reduced zoospore penetration of *C. calophylla*	- Rhizosphere microflora contributed to resistance of *C. calophylla*
Malajczuk & McComb (1977)	*Corymbia calophylla* *E. marginata*	- Light microscopy - Chromatography	- Rhizosphere microflora affected the concentration of exudates which in turn affected the number of zoospores attracted to roots	- Differences in plant susceptibility were not due to differences in root exudates but to rhizosphere microflora

Cont…

Table 1.1 Resistance attributes that have been identified as components of the reaction of plants to *Phytophthora cinnamomi*.

Reference	Species examined	Method	Resistance component	Major finding/conclusion or inference
Halsall (1978)	*Eucalyptus sieberi* *E. maculata*	- Inoculated with zoospores - Photography	- Regeneration of new roots in resistant species - Cell-free extract of *P. cinnamomi* induced dieback symptoms in both species but was more severe in *Eucalyptus sieberi*	- Zoospores attracted in equal numbers to both susceptible and resistant species
Tippett & Malajczuk (1979)	*Acacia pulchella* (resistant)	- Inoculated with zoospores - Light and electron microscopy	- Formation of callose and hypersensitive reaction causing death of hyphae	- First report of hypersensitive reaction
Hinch & Weste (1979)	23 Australian native species from different families of different susceptibility to *P. cinnamomi*	- Inoculated with zoospores - Light microscopy	- Chemotaxis of zoospore was non-specific and not-host oriented	- Pattern of zoospore accumulation on roots
Tippett *et al*. (1983)	*Eucalyptus marginata* (susceptible)	- Inoculated with mycelium - Interference contrast microscopy	- Formation of phenolic, necrophylactic periderm and kino vein	- Environment factors and plant physiology influenced resistance of plant
Phillips & Weste (1984)	*Lepidosperma laterale* *Gahnia radula* *Poa sieberana* (tolerant)	- Inoculated with zoospores - Histopathology	- Formation of necrophylatic periderm - Rapid regeneration of new roots	- *P. cinnamomi* remained viable in tolerant plants

Cont…

Table 1.1 Resistance attributes that have been identified as components of the reaction of plants to *Phytophthora cinnamomi*.

Reference	Species examined	Method	Resistance component	Major finding/conclusion or inference
Tippett *et al*. (1985)	21 *Eucalyptus* spp.	- Inoculated with zoospores - Microscopy	- Basic incompatibility - Formation of kino vein and wound periderm	- Seasonal influence on host-pathogen interaction
Phillips *et al*. (1987)	*Persea americana* (moderately resistant)	- Inoculated with zoospores - Histopathology	- Formation of necrophylactic periderm	- Resistance may be contributed by biochemical Factors
Cahill *et al*. (1989)	*Xanthorrhoea australis* *X. resinosa* *Themeda australis* *Eucalyptus marginata* *E. sieberi* *Acacia melanoxylon* **(susceptible species)** *Acacia pulchella* *Corymbia calophylla* *E. maculata* *Gahnia radula* *Juncus bufonius* *Zea mays* *Triticum aestivum* **(resistant species)**	- Inoculated with zoospores - Histopathology	- Formation of callose, tylose, lignification of cell walls, deposition of phenolic materials - Regeneration of new roots in resistant species - Hypersensitive reaction in *Acacia pulchella*	- Resistance was determined by the plant's physiology
Hüberli *et al*. (2002)	*Eucalyptus marginata* clonal material	- Wounded underbark inoculation and zoospore - mortality, lesion length and length of colonisation	- bark cortical cell and cell layers around phloem	- extent of tissue colonisation by *P. cinnamomi* needed to be assessed for screening resistance

1.9.1 Temperature

Temperature is one of the most significant environmental factors that influence the growth and pathogenesis of fungi in relation to their host plants (Zentmyer *et al.*, 1976). The cardinal temperatures for vegetative growth of *P. cinnamomi*, based on results of 50 Australian isolates on corn meal agar, are: minimum, 5-10°C; optimum, 20.5-30°C; and maximum, 31-35°C (Shepherd & Pratt, 1974). Maximal sporangial production occurred at 24°C, and no sporangia were produced at 15°C or below, or at 36°C or above (Nesbitt *et al.*, 1979b). Reflecting the temperature-growth relationship of *P. cinnamomi*, infection and development of Phytophthora dieback can only occur at temperatures which are suitable for the growth of the pathogen. Severe heart rot and root rot of pineapple, caused by *P. cinnamomi*, occurred only at a temperature of between 19 and 25°C (Hine *et al.*, 1964). At 30°C, the disease progressed slowly and at 36°C, no disease occurred. Severe root rot of avocado occurred at 15 to 27°C (Zentmyer, 1981). This is because the rate of lesion development in plant tissue was strongly dependent on temperature (Grant & Byrt, 1984; Shearer *et al.*, 1987b). Within a range of 10 to 30°C, the relationship between rate of lesion extension in *Banksia grandis* and *Eucayptus marginata* and temperature was shown to be linear (Shearer *et al.*, 1987b).

Soil temperature and moisture have also been shown to affect the seasonal pathogenic activities of *P. cinnamomi* in soil (Shea, 1975; Weste & Ruppin, 1977). When adequate soil moisture from autumn to late spring (April to October) was available, the population of *P. cinnamomi* in soil was low primarily due to low temperature (Weste & Ruppin, 1977). This resulted in less disease activity in winter because of soil temperatures below 10°C. However, *P. cinnamomi* may survive over

winter inside root tissue or may exist as resistant spores such as chlamydospores or oospores in plant tissue. Oospores of *Phytophthora* are more tolerant of low temperatures than is mycelium (Drenth *et al.*, 1995).

P. cinnamomi has not generally been regarded as a significant pathogen in cold climates. However, the observation that *P. cinnamomi* caused death of native plants such as *Oxylobium arborescens*, *Tasmannia purpurascens* and *Lycopodium deuterodensum* in subalpine vegetation in New South Wales, where temperature fluctuated between 16 and 3°C, suggests otherwise (McDougall *et al.*, 2003).

Investigation carried out by Halsall & Williams (1984) showed that zoospores of *P. cinnamomi* were indeed produced and infection could occur at temperature as low as 6°C, although disease symptoms were not expressed until the temperature became warmer.

1.9.2 Soil moisture

As the name “water mould” implies, *P. cinnamomi* thrives in wet conditions. The asexual life cycle of *Phytophthora* species, involving production and release of motile zoospores, is very closely tied to the aquatic environment (Irwin *et al.*, 1995). While mycelium of *P. cinnamomi* may grow at water potentials as low as -1,000 kPa (Sommers *et al.*, 1970), water potentials for sporangium production are in the range -10 to -30 kPa, with the maximum production at -16 kPa (Gisi *et al.*, 1980). Soil water potential strongly influences the release and dispersal of zoospores. Motile zoospores of *Phytophthora* can readily swam through a film of water in coarse textured soil at -0.1 kPa to reach seedlings at a distance of 20-35 mm (Duniway, 1976). However, the active movement of zoospores was much reduced at -1 kPa and was not observed at -5kPa indicating that high water potentials are needed for movement of zoospores.

In waterlogged condition, infection of roots increased due to increased mobility of zoospores in flooded soil and increased attraction of zoospores to ethanol, a product of anaerobically respiring roots (Davison & Tay, 1987). Waterlogging also causes vessels in the stem and tap root to become blocked with tyloses and they eventually die (Davison & Tay, 1985). Thus infected plants in waterlogged conditions develop more severe disease symptoms faster than infected plants in non-waterlogged conditions (Davison & Tay, 1987). The water potential of plant tissue can greatly influence disease development (Tippett *et al.*, 1987; Smith & Marks, 1986). Growth of *P. cinnamomi* in stems of *E. marginata* was slower in plants experiencing water stress than in unstressed plants. When water deficit in plant tissue reached -1,500 kPa, growth of *P. cinnamomi* stopped (Tippett *et al.*, 1987). These results suggest that in susceptibility experiments, soil moisture must be maintained at sufficient levels for infection and development of disease. When temperature and soil moisture are favourable, soil type may influence the rate of disease development, with more serious disease occurring in heavier-textured silt than in sandy soil (Batini & Cameron, 1975; Shea, 1975).

1.9.3 Interaction of *Phytophthora cinnamomi* with soil microorganisms

The critical role of the soil microflora in influencing disease development is borne out by observations that outbreaks of Phytophthora dieback rarely occur in certain areas even though both susceptible plant species and *P. cinnamomi* are present (Podger, 1972). Several researchers investigating the biological properties of this “suppressive” soil discovered that this soil contained populations of soil microorganisms, including bacteria, fungi and, in particular, actinomycetes, which antagonise *P. cinnamomi* (Broadbent *et al.*, 1971; Halsall, 1982; Keast & Tonkin, 1983). These microorganisms

were found to affect the survival of *P. cinnamomi* by parasitism (Nesbitt *et al.*, 1979a), suppression of sporulation and antibiosis (Broadbent *et al.*, 1971; Keast & Tonkin, 1983). Microorganisms which strongly inhibit mycelial growth, zoospore discharge and spore germination formed a greater proportion of the rhizosphere of tolerant species such as *Acacia pulchella* than in the rhizosphere of highly susceptible species such as *Banksia grandis* (Murray, 1987).

Actinomycetes are quantitatively and qualitatively important in the rhizosphere of native plants (Broadbent *et al.*, 1971; Murray, 1987; Keast & Tonkin, 1983) and can be antagonistic to *P. cinnamomi* (Broadbent *et al.*, 1971). Actinomycetes synthesise antibiotics and metabolites active against a broad spectrum of soil-borne pathogens, including *P. cinnamomi* (Strap, 2006). *Streptomyces griseoloalgus*, isolated from Snowbrush Ceanothus, (*Ceanothis velutinus*), showed strong antibiosis against *P. cinnamomi* (Rose *et al.*, 1980). Actinomycetes are usually abundant in the rhizosphere of plants because they are attracted to the exudates from roots (Strap, 2006). Similarly, zoospores of *P. cinnamomi* are also attracted to the roots and depend on root exudates for encystment and germination. Actinomycetes in the rhizosphere therefore compete and antagonise the zoospores on the root surface making them ideally suited for use as biocontrol agents (Raaijmakers *et al.*, 2009). Control of Phytophthora dieback using biological agents which produce natural antibiotics within the microhabitat of the rhizosphere is less polluting than the use of chemical fungicides (Strap, 2006). Thus, the potential of using antagonistic soil microorganisms for control of Phytophthora dieback in forest situation merits more intensive research.

1.10 Spread of *Phytophthora cinnamomi*

P. cinnamomi can spread through active and passive dispersal. In active dispersal, zoospores move by themselves. Zoospores of *P. cinnamomi* are motile and can swim at *ca*. 200 µm s^{-1} in saturated soil toward roots (Hardham, 2005). *P. cinnamomi* can also spread through growth of mycelium in root to root contact and this process may account for the upslope movement of a disease front. Spread through root to root contact was shown to be responsible for the slow expansion of a disease front by *ca*. 1 m per year on undulating sandy soils in *Banksia* woodland (Hill *et al.*, 1994) and upslope extension in the jarrah forest in Western Australia (Shea & Dillon, 1980). In passive dispersal, zoospores or other forms of inoculum are carried in subsurface or surface water flow and are responsible for the rapid spread of disease downslope. In the Brisbane Ranges, for example, the disease front extended at an estimated rate of 4 m per month in moderately drained soil and 7 m per month in waterlogged soils (Weste & Taylor, 1971). Lateral dispersal of zoospores carried in water flowing through soil overlying a concrete duricrust horizon 1 m below the soil surface has been responsible for the extensive outbreak of Phytophthora dieback in the jarrah forests of Western Australia (Shea *et al.*, 1983; Kinal, 1993). Detection of *P. cinnamomi* in soil represents an important aspect of field studies of the dynamics of the disease.

1.11 Methods for detecting *Phytophthora cinnamomi* in soil

A reliable technique for the detection and identification of *P. cinnamomi* in soil and *in planta* is a prerequisite for monitoring the spread of *P. cinnamomi* in soil and development of an effective management of the disease in the State.

1.11.1 Soil baiting

The standard method for isolation and identification of *P. cinnamomi* involves baiting wood debris and soil slurry with susceptible tissues such as cotyledons of *Eucalyptus sieberi*, roots of *Lupinus angustifolius* and pear fruit (Greenhalgh, 1978; Eden *et al.*, 2000; Davison & Tay, 2005), and subsequent plating of infected bait on selective media and examination of morphology of the pathogen. The technique requires considerable expertise in recognising the morphology of *P. cinnamomi* and other species of *Phytophthora*. Although, baiting is generally considered as an effective technique to detect the presence of *P. cinnamomi* in a soil sample, it can produce false negative result especially when the population of the pathogen in the soil sample is small. Nonetheless, baiting is widely used in detection of *P. cinnamomi* in Australia because the technique is easy to use and cheap (O'Gara *et al.*, 2005).

1.11.2 Monoclonal antibodies

Enzyme immunoassays are an alternative method that have been researched in a bid to improve sensitivity of detection. This method uses monoclonal antibodies that are specific for zoospores or cysts of *P. cinnamomi* and is used together with soil baiting (Gabor *et al.*, 1993). Zoospores that are produced from soil samples are trapped on a membrane and then incubated with the antibodies. The resultant conjugated antibody-antigen can be visualised, after treatment with appropriate chemicals, either as fluorescence using immuno-fluorescence microscopy (Gabor *et al.*, 1993) or change of colour (Cahill & Hardham, 1994). The technique can be modified into a dipstick assay suitable for use in the field. This technique is about 10 times more sensitive than soil baiting. However, one deficiency of the monoclonal antibody technique is that antibodies sometimes cross-reacts with antigens of other pathogens, such as *Pythium*

aphanidermatum (Drenth *et al.*, 2006). This lack of species-specificity explains in part why monoclonal antibody techniques are not widely adopted for detection of *P. cinnamomi* in soil.

1.11.3 DNA-based assay

A number of DNA-based methods has been developed for the detection of *P. cinnamomi* in soil (Kong *et al.*, 2003; Drenth *et al.*, 2006; O'Brien, 2008; Williams *et al.*, 2009). Although DNA-based assays improved the sensitivity of detecting *P. cinnamomi* compared with the baiting assay, cross-reaction can occur with other pathogens such as *Pythium* or other species within the genus *Phytophthora* (Kong *et al.*, 2003). The use of DNA-based assays also encountered problems with inhibition due to presence of “soil DNA” and other organic molecules which could interfere with amplification of DNA. Due to the high cost and the technical problems discussed above, DNA-based assays for detection of *P. cinnamomi* in soil have not been widely adopted (Drenth *et al.*, 2006). However, application of minor groove blinder (MGB)-DNA probes may offer hope of improving the species-specificity for the detection of *P. cinnamomi* in soil in the near future (Applied Biosystems, 2010).

1.12 Current management of Phytophthora dieback in South Australia

Currently, eradication of *P. cinnamomi* from a site once it is infested is not feasible, except on a small scale (Dunstan *et al.*, 2010). In this situation, prevention is more effective than cure. As human activities such as logging, earthworks, nursery activities, revegetation and recreational activities have the potential to spread *P. cinnamomi* far and wide, existing on-ground management focuses primarily on controlling and

modifying human behaviour and implementation of hygiene procedures to limit the spread of *P. cinnamomi* and to ensure that existing infested areas remain localised. Containment methods include use of different sets of machinery for logging operations in infested and uninfested areas, ensuring soil and gravel used in road construction is free of *Phytophthora*, postponement of activities to dry conditions and restriction of access to uninfested areas. In South Australia, restriction of access to known *Phytophthora*-infested sites is a common practice. Spraying of footwear with disinfectant is a requirement after passing through a *P. cinnamomi*-infested area (Department of Environment and Natural Resources South Australia, 2002).

Methods for mitigating the impact of *P. cinnamomi* at infested sites include the use of phosphite and *ex situ* conservation. Hardy *et al*. (2001) have written an informative and comprehensive overview on the use of phosphite to control *P. cinnamomi* in natural ecosystems in Australia. Phosphite, the anionic form of phosphonic acid (HPO_3^{2-}), acts directly on the pathogen, including *P. cinnamomi*, and indirectly by boosting the plant resistance to the pathogen and, ultimately, inhibits pathogen growth *in planta* (Suddaby *et al.*, 2008). Phosphite is currently used as an aerial spray (at 12 to 24 g L^{-1}) in Western Australia and Victoria, specifically to protect populations of critically endangered but susceptible plant species, such as *Banksia brownii*, and to slow down the spread of *P. cinnamomi* in a landscape (Hardy *et al.*, 2001). Experiments conducted by Shearer *et al*. (2004b) indicated that phosphite, when taken up by a plant, can reduce disease extension through root to root contact by 30 to 50%.

However, the effectiveness of phosphite to control Phytophthora dieback varies from species to species. Phosphite is also known to cause phytotoxicity, growth abnormalities, and reduced reproductive capacity in some plant species (Aberton *et al.*,

1999; Barrett *et al.*, 2004). Thus a judicious approach is needed when using phosphite for the management of *P. cinnamomi* in natural ecosystems. More research is required to determine the optimal use of the chemical.

Ex situ conservation involves the transfer of plants or regenerative plant material from one place to another. In certain conditions, where there is an imminent threat to the survival of a population, *in situ* conservation, defined as preservation of biological diversity in the wild (Primack, 2006), alone cannot guarantee the survival of the population or species. In that situation, *ex situ* conservation should be implemented at the same time, to store a stock of viable propagules that can be accessed just in case a population is lost or a species becomes extinct within its environment due to disease.

1.13 Risk assessment priority setting strategy

Phytophthora dieback has occurred widely in Australia and demands substantial resources to contain it. As resources are limited, management of Phytophthora dieback should be evaluated and priorities set so that resources can be invested in areas where benefits can be maximised (O'Gara *et al.*, 2005). In this context, risk assessment was introduced in Australia to identify where the impact of the pathogen is likely to be greatest and to determine risk management priorities. Risk assessment for Phytophthora dieback is still at a development stage and most states in Australia have identified broad zones where biodiversity is vulnerable to the threat of *P. cinnamomi* due to the coincidence of susceptible vegetation and environmental conditions that are conducive to the establishment of *P. cinnamomi* (O'Gara *et al.*, 2005). In Victoria, a risk map was developed showing areas in which topographical and climatic parameters are suitable for the pathogen, the known distribution of the pathogen and the

distribution of susceptible plant species (O'Gara *et al.*, 2005). In Tasmania, the presence of susceptible and threatened plant species (based on nationally- and State-listed plant species) was a criterion for the identification of areas to be prioritised for protection from Phytophthora dieback (Barker *et al.*, 1996).

However, in South Australia, risk areas have been identified based on presence of threatened plant species [listed in the Environment Protection and Biodiversity Conservation (EPBC) Act 1999 and National Parks and Wildlife (NP&W) Act 1972] and areas deemed vulnerable for *P. cinnamomi* to become established (Velzeboer *et al.*, 2005). Areas with more than 400 mm average annual rainfall and that have acidic to neutral soil with poor drainage are regarded as potentially vulnerable to infestation by *P. cinnamomi* (Velzeboer *et al.*, 2005). Species were prioritised for protection and management based on the risk faced by each species.

1.14 Significant gaps in knowledge

In South Australia, there has been no long-term study of the impact of *P. cinnamomi* on native vegetation. As such, empirical data are lacking on the current distribution and rate of spread of *P. cinnamomi*, its impact and the extent of susceptibility in native vegetation. The susceptibility of threatened plant species to *P. cinnamomi* is simply not known in South Australia (O'Gara *et al.*, 2005). The lack of empirical data on the impact of *P. cinnamomi* on plant species and communities represents a distinct impediment to the risk assessment and management of native vegetation in South Australia. Data and information used in evaluating the risk of Phytophthora dieback to threatened species are based on field observations and on information which has been generated in other States, which may not be applicable to South Australia because of

the different plant communities and environmental conditions. Furthermore, rhizosphere microflora, particularly the actinomycetes, which represent a potential for biological control of Phytophthora dieback in native ecosystem, have not been studied in South Australia.

1.15 Conclusion

This review has highlighted the significant threat posed by Phytophthora dieback to native vegetation in Australia in general and to South Australia, in particular, and the need to protect rare and threatened native plant species from the disease. The disease has been destroying native vegetation in South Australia for at least the last 40 years and threatens endangered plant species with extinction. The existing hygiene measures are deemed inadequate to protect threatened plant species from the disease. A more targeted Phytophthora management strategy based on assessment of risk to threatened plant species offers hope of protecting threatened plant species from extinction due to disease. Major gaps in knowledge needed for the effective implementation of this strategy have been identified.

Therefore, to support the development of a new Phytophthora management strategy in South Australia, the aims of this project were as follows:

(1) To assess the response of selected South Australian native plant species to Phytophthora dieback

(2) To determine the rate of spread of disease and *P. cinnamomi* in a typical native vegetation in South Australia and

(3) To isolate soil antagonists, particularly actinomycetes, from rhizosphere soil and test for ability to protect susceptible native plants from Phytophthora dieback.

Chapter 2 General materials and methods

This chapter describes the materials and methods that are common to several studies carried out in this project. Materials and methods specific to particular experiments will be presented in the relevant chapter.

2.1 Preparation of *Phytophthora*-selective medium P_{10}ARPH

The *Phytophthora*-selective medium P_{10}ARPH was prepared based on the formulation first developed by Jeffers and Martin (1986). However, the concentration of pimaricin in the medium was 10 µg mL^{-1} instead of 5 µg mL^{-1} as proposed by Tsao and Ocana (1969). Cornmeal agar (CMA, Sigma-Aldrich), 8.5 g, was added to 500 mL of distilled water and autoclaved at 121°C for 20 min. After cooling to between 45 and 50°C, the medium was supplemented with five antibiotics, *viz*. pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB) and hymexazol, thus called P_{10}ARPH. Pimaricin used was a 2.5% aqueous suspension of the sodium salt of the parent compound. Concentrated stock solutions of the other four antibiotics were first prepared, then appropriate amounts were added to give final concentrations in the medium as follows: ampicillin 250 µg mL^{-1}, rifampicin 10 µg mL^{-1}, PCNB 25 µg mL^{-1} and hymexazol 25 µg mL^{-1}. Pimaricin, ampicillin and rifampicin were pipetted into the medium but PCNB and hymexazol were filter-sterilised (using a Millipore disposable filter, 0.45 µm pore size) into the medium. The antibiotics and the medium were mixed thoroughly before dispensing 18 to 20 mL aliquots aseptically into 9 cm-diameter Petri dishes.

2.2 *Phytophthora cinnamomi*

2.2.1 Sources of isolates

P. cinnamomi isolates 71a and SC4 were obtained in South Australia. Isolate 71a was obtained from a composite soil sample collected from around roots of a dead plant of each of *X. semiplana* ssp. *tataena* and *Isopogon ceratophyllum* on Kangaroo Island, South Australia (35°47.558'S, 137°27.333'E, elevation 62 m) on 5 November 2007 (Williams *et al.*, 2007). Isolate SC4 was obtained from a dead *X. semiplana* in Scott Creek Conservation Park, South Australia (35°5.102'S, 138°41.530'E, elevation 380 m) on 3 June 2008. Both isolates were collected by Dr S. McKay, the University of Adelaide, Waite Campus, as follows. Soil samples were baited for *P. cinnamomi* using cotyledons of *E. sieberi* (Marks & Kassaby, 1974). Infected cotyledons were blotted dry with a sterile paper towel and then plated onto P_{10}ARPH medium. Pure cultures of *P. cinnamomi* were established by excising single hyphal tips, with the help of a dissecting microscope, and culturing these on CMA. Pure cultures of both isolates were sent to the Centre for *Phytophthora* Science and Management, Murdoch University, Western Australia and confirmed as *P. cinnamomi* using polymerase chain reaction in June 2008 (S. McKay, pers. com., 2009).

2.2.2 Maintenance of isolates

Pure *P. cinnamomi* was grown on CMA plates at 24°C in the dark. After incubating the culture in the dark for 6 days, 5 mm-diameter mycelial discs were taken from the edge of the colony and transferred to McCartney bottles containing 10 mL of sterile distilled water. These bottles were stored in the dark at 24°C and discs retrieved and grown on

fresh CMA when required. Mycelial discs were grown up on fresh CMA and placed in storage every 6 months.

2.3 Inoculation method

Before the susceptibility experiments could be carried out, the inoculation method was refined to ensure that the inoculation would cause infection and disease. The inoculation method was based on that reported by Shearer *et al*. (2004a). Two preliminary experiments were conducted in September and October 2008 for the purpose of selecting an appropriate soil type and inoculum dosage to optimise methods for susceptibility experiments later (Chapter 4).

2.3.1 Inoculum dosage experiment

2.3.1.1 Preparation of pine (*Pinus radiata*) wood-inoculum plugs

The method of preparing pine wood inoculum plugs was adapted from that used by Butcher *et al.* (1984). Live branches, about 1-1.5 cm diameter, were obtained from mature *Pinus radiata* trees located near Mount Bold Reservoir Office (35º07'16.44" S, 138º40'37.64" E, elevation 254 m). The branches were debarked, cut into plugs of 2 cm long and soaked in distilled water overnight. The plugs were rinsed thrice in distilled water, placed in 250 mL tissue culture tubs (50 plugs each). A small amount of distilled water was added to the tubs before autoclaving at 121°C for 20 min. The tubs were autoclaved for a second time at least 24 h later.

Mycelial mats of *P. cinnamomi* used to inoculate the pine plugs were prepared following the method of Butcher *et al.* (1984). V8 juice broth was prepared by adding V8 juice (Campbell's Soup, Campbell Australia Pty Ltd) to distilled water in the ratio

of 1:4 in a glass beaker. For 354 mL of 20% V8 juice, 5 g of calcium carbonate (AnalaR) was added, stirred for 20 min and then centrifuged at 4000 rpm for 20 min. The supernatant was then dispensed into McCartney bottles, 10 mL each, and autoclaved at 121°C for 20 min. Ten discs of CMA, 5 mm diameter each, taken from the edges of a 6 day-old culture were added to the sterile V8 juice in each McCartney bottle. The bottles were incubated at 24°C in the dark for six days, then the resulting dense mycelial mats transferred to the tissue culture tubs using aseptic technique. The tubs were gently shaken to disperse the mycelium among the pine wood plugs and shaken again a few times during the incubation period. The plugs were incubated at 24°C in the dark for about 3 weeks before use. Pine plugs infested with isolates 71a and SC4 were prepared separately.

2.3.1.2 Growing *Eucalyptus sieberi* and *Xanthorrhoea semiplana* in greenhouse

Seedlings of *E. sieberi* used for the preliminary experiments were raised in greenhouse located at Waite Campus (34°58.153'S, 138°38.258'E, elevation 140 m). The temperature in the greenhouse fluctuated between 17 and 29°C depending on the season.

Seeds of *E. sieberi* and *X. semiplana* were purchased from Blackwood Nursery, South Australia and germinated in Jiffy-7 pots (Smoults, South Australia). When the seedlings were at the two-leaf stage, about 6 to 8 weeks after sowing, they were transplanted to 15 cm-diameter plastic pots filled with Bio gro® (Bio Gro, South Australia), one plant per pot. A hole was made in the centre of the potting mix with a spoon, then the Jiffy-7 pot containing the most vigorous seedling was placed inside the hole and gently pressed in. Two plastic centrifuge tubes, 10 cm long and 1 cm diameter (Sarstedt, Australia), with lids were inserted into the potting mix close to the roots on

opposite sides of the plant for placement of inoculum plugs later in the experiment. At the time of transplanting, 3 g of Osmocote pellets, a slow-release fertiliser with formulation 17N:1.6P:8.7K+ Trace elements (Scotts Australia Pty Ltd), were scattered onto the surface of each pot. No further fertiliser was applied. All the plants were maintained in the greenhouse and watered daily. The plants were inoculated when about 4-month-old. Inoculum dosage used was as follows:

(i) sham-inoculated with blank pine plugs

(ii) two pine-plugs inserted into one hole

(iii) two pine-plugs inserted into two holes

(iv) four pine-plugs inserted into two holes and

(v) six pine-plugs inserted into two holes

Test plants were 4-month-old *E. sieberi* and 5- to 6-month old *X. semiplana* grown in 15 cm-diameter pots filled with Bio Gro® potting mix.

At the time of soil inoculation, the plastic tubes were removed from the pots. For the pathogen-free control, the plants were sham-inoculated with blank pine plugs. For treatment (ii), one pine plug infested with isolate 71a and another pine plug infested with SC4 were inserted into the same hole. For treatments (iii), (iv) and (v), one, two or three pine plugs infested with isolate 71a were inserted into one hole and another one, two or three pine plugs infested with isolate SC4 were inserted into the other hole. There were eight replicates, consisting of four plants each of *E. sieberi* and *Xanthorrhoea semiplana*, for each treatment.

The dates on which plants developed disease symptoms and died were recorded and plants were harvested. Root tissue samples from the dead plants were plated onto

P_{10}ARPH to reisolate *P. cinnamomi*. Kaplan-Meier survival curves were plotted for each treatment and the survival curves were compared using The Log-rank (Mantel-Cox) test at $P \leq 0.05$. Eighty two days after inoculation, no plant had died in the control treatment (Appendix A1). The maximum number of plant deaths due to Phytophthora dieback was four each in treatments (iv) and (v). There were no significant differences among the five survival curves at $P \leq 0.05$ suggesting that soil inoculation with four pine plugs per pot was as effective as six pine plugs in causing infection and disease.

2.3.2 Effect of soil type on infection and disease

The experiment was conducted on 3 October 2008. The four soil types used were as follows; Bio gro®, pasteurised field soil from Mount Bold Catchment Reserve, limed University of California (UM) mix (Baker, 1957) and UC mix without lime. Initial pH value of the four soil types were 5.8, 5.4, 6.5 and 3.5 respectively. The test plants were *E. sieberi* and *X. semiplana*. The number of replicates was ten, consisting of five plants each of *E. sieberi* and *X. semiplana*. Each plant was inoculated with two pine plugs infested with isolate 71a and another two pine plugs infested with SC4. Another set of plants for each treatment was sham-inoculated with sterilised pine plugs as the pathogen-free control. Eighty two days after inoculation, no control plants died (Appendix A2). The largest number of plant deaths was five in the limed UC mix followed by four in Bio Gro®, three in pasteurised field soil and one in UC mix without lime. The Log-rank test showed there were significant differences in the survival curves among the four soil types at $P < 0.05$ suggesting that limed UC mix was the most conducive for infection and disease development.

2.3.3 Final conditions for inoculation

Based on the results of these preliminary experiments, plants for susceptibility testing were grown in limed UC mix, except where otherwise stated, and soil inoculated with four pine plugs in greenhouse conditions. The preparation of UC mix is presented in Appendix B. *E. sieberi* was used as positive control because disease symptoms were easier to observe on *E. sieberi* than *X. semiplana*.

In the greenhouse, a shade cloth was attached to the ceiling, which could be rolled down to reduce sunlight by 50% when the sun was strong. Two sodium vapour lights bulbs (Philips, supplied by Smoults, South Australia), each 400 watts, were installed at one side of the greenhouse to balance the shading effect of the adjacent atrium. The bulbs were on from 06:00 to 18:00 everyday throughout the year.

2.4 Baiting of soil samples for *Phytophthora cinnamomi*

Soil samples collected in the field study or potting mix from greenhouse experiments were baited for *P. cinnamomi* following the technique first developed by Marks and Kassaby (1974). Distilled water, 150 mL, was added to 20 g of soil or potting mix in a 260 mL plastic denture cup (Advanced Australian Packaging, South Australia) and stirred thoroughly. Floating organic matter was removed using a metal sieve.

Seeds of *E. sieberi* were germinated in 260-mL-denture cups filled with vermiculite (Smoults, South Australia) then placed in a tray filled with tap water to a depth of 1 cm. Cotyledons from 3 week-old seedlings were used for baiting for *P. cinnamomi*. Pairs of cotyledons were removed from five or six seedlings (by pinching the stem with tweezers) and floated adaxial surface up on the soil suspension in each cup. The denture cups were then covered with lids and incubated at ambient

temperature, approximately 20-24°C, for 4 to 8 days. The reddish adaxial surface of the cotyledons slowly turned green after being colonised by *P. cinnamomi*. When observed under power magnification of 100x, hyphae and sporangia of *P. cinnamomi* could be seen growing out from the edges of cotyledons (Marks & Kassaby, 1974).

In greenhouse experiments, when a plant died it was removed from its pot and washed under running tap water. The collar region, lateral roots and fine roots were carefully checked for presence of rotting, lesions or discolouration of tissue. For plants with obvious lesions, sections of the collar region and root around the lesions were excised, surface sterilised and plated onto P_{10}ARPH to reisolate *P. cinnamomi*. If no lesion was observed, roots were selected at random. Sections of collar region and root were first sterilised in 1% sodium hypochlorite for 1 min, rinsed three times with sterile distilled water and allowed to dry on a piece of sterile paper towel. The epidermis of the roots was removed with a sterile scalpel and both ends of each section, which might have absorbed sodium hypochlorite, were discarded. The root section was cut longitudinally and the cut surface gently pressed onto the P_{10}ARPH medium to secure good contact with the medium. The collar region was similarly processed before plating onto the medium. If *P. cinnamomi* was present, coenocytic hyphae could be observed to emerge from the tissue within 24 h of incubation at 24°C in the dark.

2.5 Statistical analysis

Australian native flora are mainly open-pollinated plants and, therefore susceptibility to a particular disease is likely to differ from one individual to another. Intra-specific variation in susceptibility among individuals of the same species can vary from resistant to highly susceptible. In an inoculation experiment involving an open-

pollinated plant species, it is likely that individual plants will remain alive and healthy at the termination of the trial. In such a situation, it is considered appropriate to analysis the data using survival curve analyse (Machin *et al.*, 2006). Other statistical analyses, such as analysis of variance (ANOVA) using the statistical software GenStat version 11.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK) and G-test are described in the individual chapters.

Chapter 3 Spread of *Phytophthora cinnamomi* and Phytophthora dieback in native vegetation in South Australia

3.1 Introduction

Phytophthora cinnamomi spreads through soil and water (Ristaino & Gumpertz, 2000). Although movement of contaminated soil and planting materials has been implicated in the dispersal of *P. cinnamomi* over large distances, rapid reproduction and hyphal growth in roots are the primary mechanisms for local spread of the pathogen and disease when favourable environmental conditions are present. Zoospores dispersed in water moving downslope as surface and overland flow are responsible for the rapid spread of the disease along drainage lines and in areas with impeded drainage (Podger, 1972). Even when soil water is not sufficient for zoospore production, *P. cinnamomi* spreads when mycelia in roots spread to neighbouring roots (Marx & Bryan, 1969; Hill *et al.*, 1994), leading to the slow uphill or lateral spread of disease along slopes on apparently free-draining soil.

Favourable soil conditions for development of *P. cinnamomi* appear to include a water matric potential of between -8 and -16 kPa for sporulation (Gisi *et al.*, 1980) and ≥ 0.1 kPa for release of zoospores (Duniway, 1976), temperatures between 18 and 22°C (Halsall & Williams, 1984), and a soil pH between 4.5 and 7.2 (Blaker, 1983). The waterlogged conditions observed during spring and autumn in some Australian environments may therefore provide a favourable environment for sporulation by *P. cinnamomi*. Soil texture and soil water repellency also affect the spread of *P. cinnamomi*. Large pores in coarse textured soils favour movement of zoospores while

fine pores in fine textured soils impede movement of zoospores (Shearer & Tippett, 1989). Water infiltration and preferential flow path of surface water flow is affected by soil water repellency (Dekker & Ritsema, 1994) thereby affecting the pattern of spread of the pathogen.

In areas where environmental conditions favour the formation of zoospores, such as in an open sclerophyll forest in the Brisbane Ranges National Park (Victoria), rate of disease extension downhill through zoospores carried in surface runoff was estimated at 400 m per year (Weste & Law, 1973). In the *Banksia* woodland of Bassendean Dune and the Jarrah forest (*Eucalyptus marginata* Donn ex Smith) in Western Australia, where *P. cinnamomi* was found to spread through mycelium via root to root contact, disease extension uphill occurred at a slow rate of only 0.77 to 1.10 m per year despite receiving a high annual rainfall of 780 to 1,200 mm (Shea & Dillon, 1980; Hill *et al.*, 1994).

The standard method used to estimate the rate of disease spread in native vegetation is to monitor changes in the health status of highly susceptible species, such as *Xanthorrhoea australis* and *Banksia attenuata* (Hill *et al.*, 1994), along a transect at regular intervals followed by confirmation of presence of *P. cinnamomi* by isolation from soil and roots. Investigation of the rate of spread of *P. cinnamomi* involves seasonal collection of soil and fine root samples at regular points along that transect from a disease front into an adjacent area of healthy vegetation. The soil samples are then baited for the presence of *P. cinnamomi* with tissue from susceptible hosts, such as roots of *Lupinus angustifolius* (Eden *et al.*, 2000) or cotyledons of *Eucalyptus sieberi* (Marks & Kassaby, 1974).

P. cinnamomi is found extensively in South Australia and information on the rates of spread of disease and pathogen is needed to guide management plans to protect populations of threatened plant communities from advancing disease fronts, defined as the boundary between healthy and diseased areas (Phytophthora Technical Group, 2003). Given that the composition of native plant species, annual rainfall, and soil moisture and temperature regimes are different in South Australia, rates of pathogen and disease spread are likely to differ from those in Victoria and Western Australia, where most research has been conducted. This field study was undertaken to estimate the rates of spread of the pathogen and disease in a typical woodland in South Australia.

3.2 Materials and methods

3.2.1 Study site

The site selected to monitor the spread of the pathogen and the disease was a confirmed *P. cinnamomi*-infested site (R. Velzeboer, pers. com., 2007) located in the Mount Bold Catchment Reserve (35°05.061'S, 138°43.108'E, elevation 270 m) about 60 km south east of Adelaide (Figure 3.1). The topography of the site ranges from flat to a steep slope. The vegetation at the site is regarded as open woodland. Dominant tree species are *Eucalyptus obliqua* and *E. cosmophylla.* Shrubs such as *Acacia pycnantha* and *A. myrtifolia* occur as scattered understorey species. *Xanthorrhoea semiplana* (yacca) is the dominant understorey species. Other associated understorey species include *Pultenaea daphnoides*, *Leptospermum* spp., *Platylobium obtusangulum*, *Isopogon ceratophyllus*, *Phyllota pleurandroides* and *Correa decumbens*, growing interspersed with *X. semiplana*. Altogether, at least 34 native plant species were identified at the site

(Appendix C). The area has a Mediterranean-type climate with a wet and cool winter and a warm and dry summer. Based on the past 55 years of local records (*http://www.bom.gov.au*), the area received an average annual rainfall of 784 mm (range 462 to 1180 mm) (Table 3.1). Winter (June, July and August) was the wettest season, receiving 322 mm of rain on average while summer (December, January and February) was the driest season with only 82 mm of rain. Autumn (March, April and May) and spring (September, October and November) received a similar amount of rainfall, at 188 to 192 mm.

Preliminary analysis carried out by Soil and Plant Analysis (CSBP Limited, Perth) showed that the soil at the site was low in fertility with an average nitrate content of 2.0 mg kg^{-1} soil, ammonium nitrogen 10.3 mg kg^{-1}, phosphorus 2.5 mg kg^{-1}, potassium 90 mg kg^{-1}, sulphur 3.6 mg kg^{-1} soil and organic carbon 2.1%. Conductivity of the soil was 0.04 dS m^{-1}. The pH_{water} of the soil was determined using 10 g of air-dried soil (2 mm sieved) added into 50 mL of distilled water. The suspension was shaken for 1 h at room temperature and allowed to settle before taking the pH using a pH meter (Oakton®, Extech Equipment Pty Ltd, Australia). The soil at the site was slightly acidic (pH 5.2).

3.2.2 Counting of dead and dying *Xanthorrhoea semiplana* indicator plants

A patch, about 1 ha, of dense and continuous healthy *X. semiplana* understorey contiguous with a patch of diseased *X. semiplana* was selected to monitor spread of disease and pathogen in the field. *X. semiplana* was chosen as the sensitive indicator plant to monitor changes in disease at the site as it is highly susceptible to infection by *P. cinnamomi*, has wide distribution and the persistent remains enable recognition of

old, diseased plants (Aberton *et al.*, 2001). Two disease fronts were observed (Figure 3.2). The first disease front was represented by a few dead *X. semiplana* which lay on

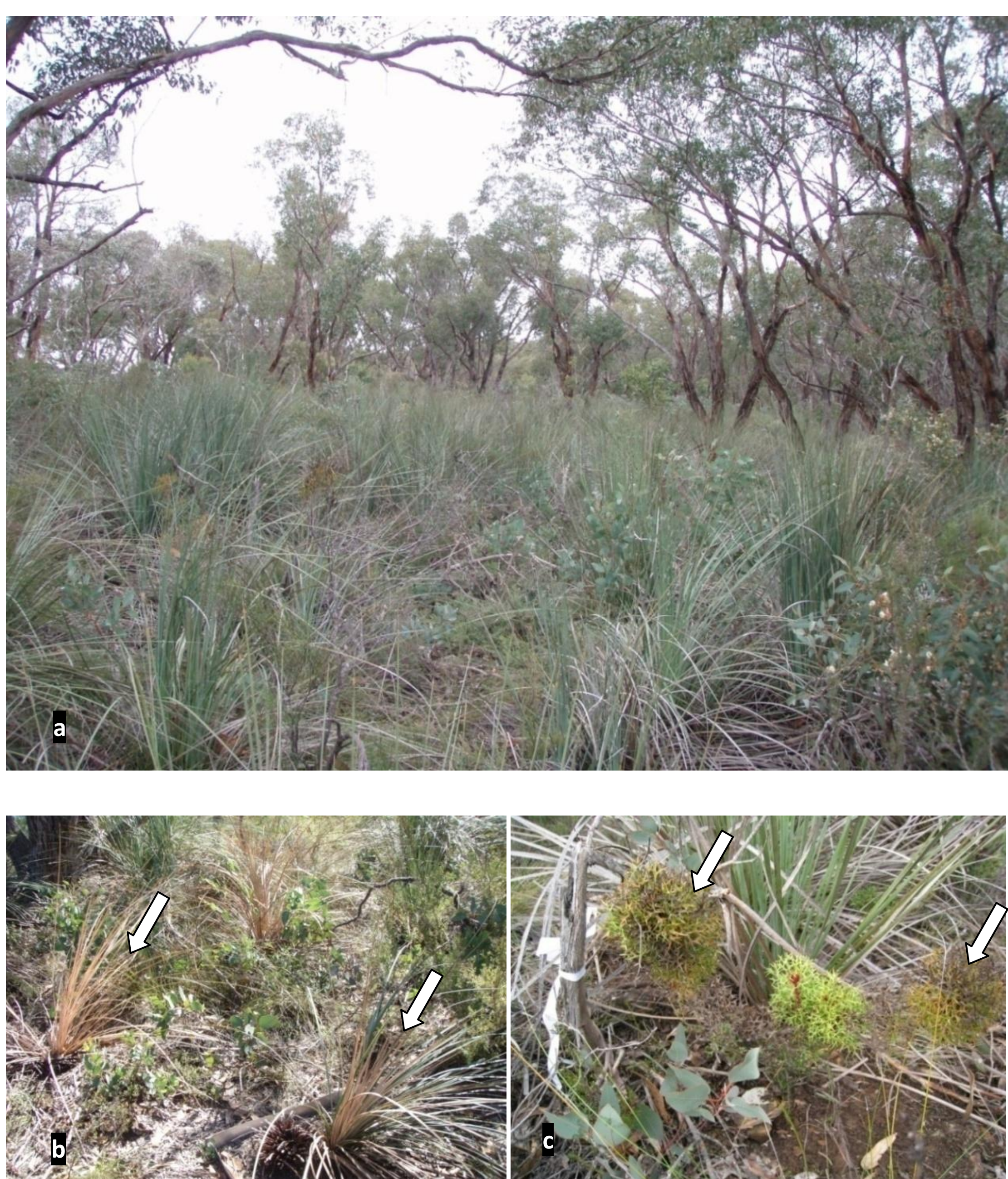

Figure 3.1 The open woodland vegetation at the experimental site at Mount Bold Catchment Reserve, South Australia: (a) species of *Eucalyptus* and *Acacia* are the main tree species while *Xanthorrhoea semiplana* forms a dense and continuous understorey vegetation; (b) dead *X. semiplana* plants indicated by arrows; and (c) dead *Isopogon ceratophyllus* indicated by arrows, both due to Phytophthora dieback.

Table 3.1 Historical weather data associated with the study site. Average monthly rainfall based on records from 1952 to 1986 and 1988 to 2007 at Mount Bold Reservoir Office. The average minimum and maximum air temperatures were based on records from 1998 to 2011 at Kuitpo Forest Reserve (35.23ºS, 138.69ºE), which is located about 4.2 km from Mount Bold Reservoir Office, South Australia.

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Rainfall (mm)	24	23	32	62	95	108	113	101	83	66	43	36	786
Min. Temp. (ºC)	22.0	22.7	19.3	15.9	13.8	11.3	11.2	11.6	13.1	15.4	19.1	19.4	-
Max. Temp. (ºC)	28.9	29.1	27.7	22.8	17.5	14.5	13.3	15.3	17.5	28.0	26.7	25.5	-

Source: *http://www.bom.gov.au*

the northern side of the site (shown by yellow triangles in Figure 3.2). This disease front, which ran parallel to the gravel road, was considered as the original disease front, as *P. cinnamomi* is often introduced into an area through movement of soil for construction of gravel roads (Bumbieris & Boardman, 1979). A second, probably more recent, disease front was represented by a patch of dead *X. semiplana* on the western side of the field (shown by yellow region in Figure 3.2). This disease front was considered as the western disease front.

In August 2008, a plot 60 m x 70 m was marked out at the site, extending from the boundaries of the two disease fronts to the adjoining healthy *X. semiplana* vegetation. The plot was divided into a grid of regular quadrats, each 10 m x 10 m. Each quadrat was labelled according to the number of the column (T1 to T6) and row (Q1 to Q7) in which it was located. In spring 2009, another column of quadrats, TA was added to the western side of the plot. If TA represented the pathogen front, then soil samples collected from quadrats along TA would be expected to yield *P. cinnamomi*. Altogether, the total number of quadrats in the experimental plot was 41. The boundary of each quadrat was marked out with a flagging tape. To determine the rate of disease spread from the disease front into the healthy area, numbers of healthy, chlorotic and dead *X. semiplana* plants were counted in each quadrat every autumn and spring for two and half years from August 2008 to November 2010. A plant with green foliage was categorised as healthy, while a plant with partial yellowing of the foliage was regarded as chlorotic (Figure 3.3). If the foliage of a plant had completely turned yellow and the crown had collapsed, the plant was considered dead. The proportion of symptomatic plants (both dead and chlorotic plants) and of healthy *X. semiplana* between seasons was tested for homogeneity using a G-test at $P \leq 0.05$ (Sokal & Rohlf, 1981) .

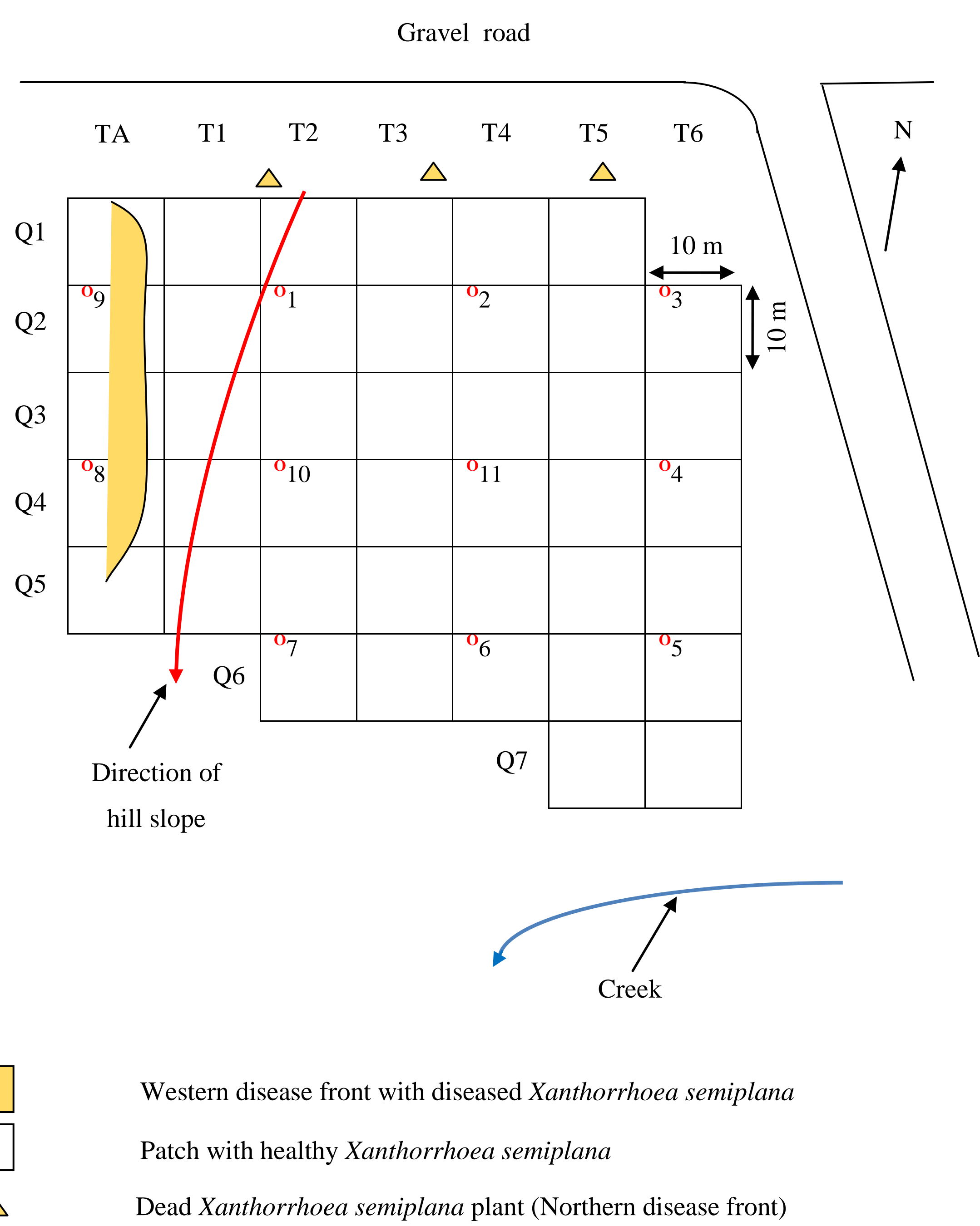

Figure 3.2 Grid layout of the field plot at Mount Bold Catchment Reserve, South Australia. The total number of quadrats within the experimental plot was 41. The red circles indicate the position where soil samples were collected for determination of soil texture and the number near the red circle indicates the number of the pit. Column (T) and Row (Q) and the adjacent letter and numbers refer to the positions of the quadrats in the column and row.

Figure 3.3 Classification of *Xanthorrhoea semiplana* plants into healthy and symptomatic based on the following symptoms: (a) healthy plant with green foliage; (b) chlorotic plant with yellowing of leaves; and (c) dead plant with brown and collapsed foliage.

3.2.3 Seasonal soil sampling for detection of *Phytophthora cinnamomi*

To estimate the rate of pathogen spread from the disease fronts into the plot, soil from each quadrat was sampled and tested for the presence of *P. cinnamomi* each time *X. semiplana* was counted. After removing the surface litter, soil samples together with root material, were collected from around the roots of *X. semiplana* to a depth of about 15 cm using a trowel. To maximise the chance of recovering *P. cinnamomi*, dead and dying *X. semiplana* plants in each quadrat were prioritised for soil sampling. Within each quadrat, soil was collected from around three to five symptomatic plants, placed in a ziplock bag to make a composite soil sample of about 400 g and sealed to prevent loss of moisture. If no dying plants were available in a quadrat, then the soil sample was collected from at least four healthy *X. semiplana* plants, one from each corner of the quadrat. After each quadrat was sampled, the trowel was surface sterilised with

70% ethanol before sampling the next quadrat to avoid cross contamination. To prevent inadvertent spread of the pathogen between quadrats, soil samples were always collected first from the quadrats furthest from the disease fronts and progressing towards the disease fronts. Soil samples were taken to the laboratory immediately and tested for the presence of *P. cinnamomi* on the same or the next day. Altogether, five rounds of soil sampling were carried out, each on the same day as the counting of *X. semiplana* (Section 3.2.2).

3.2.4 Baiting of soil samples with cotyledons of *Eucalyptus sieberi*

Soil samples were baited for *P. cinnamomi* using cotyledons of *E. sieberi* as described by Marks and Kassaby (1974) in section 2.4. Each soil sample was baited in duplicate. Infected cotyledons were plated onto *Phytophthora*-selective medium, P_{10}ARPH (Jeffers & Martin, 1986), for identification to species. Morphological features of the sporangia, such as shape and size, were used to identify the *Phytophthora* isolates to species (Stamps *et al.*, 1990).

3.2.5 Random soil and water sampling

In addition to systematic soil sampling, soil and water samples were collected at random within and outside the experimental plot to test for the presence of *P. cinnamomi*. Soil samples were collected from around roots of known susceptible species, such as *Isopogon ceratophyllus* R. Br., *Pultenaea daphnoides* J.C.Wendl. and *Platylobium obstusangulum* Hook., growing within and outside the plot. Water samples from the nearby reservoir and creek were also collected. A total of 47 random soil samples and five water samples were collected in 2009 and 2010 and baited for the presence of *P. cinnamomi* as per section 2.4.

3.2.6 Measure of steepness and direction of slope

Within each quadrat, the direction and steepness of the slope were measured and recorded using a compass and a clinometer. The clinometer was placed on the ground in the direction of the steepest slope in the quadrat to measure the steepness of the slope.

3.2.7 Determination of soil texture

In December 2009, topsoil and subsoil samples were collected from 11 pits in the experimental plot (marked with red circles in Figure 3.2) to determine the texture of the soil. At each plot, a small pit, 30 cm wide by 30 cm long was dug into the soil with a shovel to the subsoil or until there was a change in the colour of the soil, which was then regarded as subsoil. At each pit, about 500 g of topsoil (0-25 cm from soil surface) and 500 g of subsoil (25-36 cm below the soil surface) were collected, and placed in separate zip-lock polyethylene bags and labelled accordingly. The soil samples were taken to the laboratory and spread out on trays to dry at ambient temperature for 4-5 day after which the soil was sieved with a 2-mm mesh.

Soil texture was determined using the method for rapid determination of clay content (< 2 mm) developed by Smith and Tiller (1977). Between 10 and 20 g of < 2 mm soil, referred to as M_s, from each sample was weighed into a 250 mL plastic bottle. Ten mL of 10% calgon (sodium hexametaphosphate) solution, 0.5 mL of 0.6 M NaOH and 150 mL distilled water were added to the soil to disperse the soil particles. The bottle was sealed with a cap and agitated on a rotating wheel for up to 24 h. The soil suspension was then decanted quantitatively into a tall-form beaker and diluted to 300 mL with additional distilled water (V_t). The soil suspension was left undisturbed to

allow sedimentation to occur. After settling for 3 h, 25 mL of soil suspension (V_s) was taken at a sampling depth of 3 cm with a pipette and deposited in a pre-weighed plastic container with a lid. This was placed in an oven at 105°C for 24 h, cooled in a vacuum desiccator and re-weighed to obtain an apparent weight of clay. The apparent weight of clay was corrected for the mass of calgon, sodium hydroxide and soil water content. The corrected mass of clay (M_{clay}) was used to calculate % of clay content in the soil using the equation below;

$$\% \text{ clay} = \frac{\frac{M_{clay}}{V_s} \times V_t}{M_{OD\ soil}} \times 100$$

where M_{clay} = corrected mass of clay

V_s = sample volume

V_t = total volume

$M_{OD\ soil}$ = total mass of oven dry soil sample

Total content of sand (% sand) was determined using the suspension remaining after the clay sample had been taken. The suspension containing clay and silt was carefully poured off, leaving the sand at the bottom of the cylinder. The cylinder was refilled with tap water to a height of 15 cm, the contents stirred and allowed to sediment for 5 min before pouring off the suspension. This process was repeated several times until only sand was left in the cylinder. The sand was then oven-dried to obtain the mass of sand (M_{sand}). The sand content (%) in the soil was calculated using the equation below:

$$\% \text{ sand} = \frac{M_{sand}}{M_{OD\ soil}} \times 100$$

where $M_{OD\ soil}$ is the mass of oven dried soil.

The sand was finally sieved with a 53 μm mesh to determine the percentage of coarse sand. The percentage of silt was calculated by subtraction of the percentages of clay and silt.

The gravimetric water content of a soil, θ_m, is the water associated with 1 kg of a dry soil (Brady & Weil, 2002). To determine the gravimetric water content of the soil, about 10 g of each soil sample (<2 mm fraction), in duplicate, was weighed and then dried in an oven at 105°C for at least 24 h and finally weighed again. The gravimetric water content of soil was calculated from the equation below:

$$\theta_m = \frac{\chi_w}{\chi_s}$$

where χ_w is the weight of water loss upon drying and χ_s is the weight of dry soil. The gravimetric water content of the soil was used to calculate the oven dried weight of the soil samples.

3.2.8 Determination of soil surface water repellency

In the laboratory, 10 g of < 2 mm air-dried soil sample (prepared as per section 3.2.7), from each quadrat was placed in Petri dishes and dried at 40°C for 48 h. To determine the water repellency of the soil, a drop (*ca.* 40 μL) of distilled water was placed on the smoothed soil surface and the time taken for the water drop to infiltrate completely into the soil was recorded as water drop penetration time (WDPT). For each soil sample, the WDPT for five drops was recorded. The soil surface water repellency obtained was classified as wettable (< 5 s), slight (5 to < 60 s) or strong (≥ 60s) according to Dekker and Ritsema (1994).

3.2.9 Environmental parameters

Automatic meters were installed to assess soil temperature and moisture regimes, two main factors that affect variation in disease and pathogen spread (Shea, 1975). A Tinytag Plus 2 Logger (Gemini Hastings Data Logger, Australia) was installed at the centre of the plot, in quadrat T2Q3 (refer to Figure 3.2) with open canopy in May 2009 to record the soil temperature. The probe was buried 15 cm deep in the soil and was set to record temperature at hourly intervals.

Soil water tension was measured using GBUG (Measurement Engineering Australia 2.21) data loggers. Two data loggers were used: one at quadrat T2Q2 and another at T4Q4 (Figure 3.2). The soil moisture probes were buried 15 cm below the soil surface. The GBUG was set to record soil water tension at 2-hourly intervals for 18 months, from May 2009 to October 2010. Daily rainfall was recorded from a rain gauge installed at the Mount Bold Reservoir Office, about 10 km from the field site.

3.2.10 Statistical analysis

The seasonal count of symptomatic dead and chlorotic *X. semiplana* plants in the healthy area was tested for independence of proportionality using the G-test at $P \leq 0.05$ (Sokal and Rohlf, 1981). The means of the WDPTs from the 41 quadrats were subjected to ANOVA using GenStat (section 2.5). Multiple comparison of means of WDPT were carried out using the Duncan's multiple range test and the least significant difference (LSD) method at $P \leq 0.05$.

3.3 Results

3.3.1 Slope of quadrats

The slope in each quadrat ranged from flat to a steep slope of 24 degrees (Figure 3.4). The quadrats closer to the top of the hill were generally gentler, with slopes of less than 9 degrees. The quadrats became steeper from north to south. The quadrat with the steepest slope was T3Q6 with 24 degrees. The results of the slope measurement suggested that the patch with healthy *X. semiplana* was located higher than the western disease front but lower than the northern disease front.

3.3.2 Texture of topsoil and subsoil at Mount Bold experimental site

The texture of topsoil taken from within each quadrat ranged from loamy sand to sandy loam (Table 3.2). The textural distribution of the topsoil (0-25 cm) was on average 76.4% sand (mainly coarse sand > 53 μm), 6.3% clay and 15.5% silt (Table 3.2). In the subsoil, the texture became more clayey, with an average of 67.8% sand, 15.7% clay and 16.5% silt. Subsoil of Pit 4 from quadrat T6Q4 was the most clayey, with about 37.8% clay.

3.3.3 Soil surface water repellency

The spatial variability of water repellency was very high, with WDPT ranging from 12 to more than 300 s (Table 3.3). Nearly 78% of the 41 quadrats showed strong water repellency, with WDPT of 60 s or above. Of these, 17% showed extreme water repellency, with water drops remaining for more than 180 s. Quadrats in column T1, which flanked the western disease front, had WDPT ranging from 60 to 142 s,

indicating that rain water could remain on the soil surface for 1 to 2 min before infiltrating into the soil.

3.3.4 Environmental parameters

3.3.4.1 Seasonal rainfall

The total annual rainfall received at the field site for the years 2008, 2009 and 2010 was 626, 802 and 900 mm, respectively (Figure 3.5). However, the seasonal rainfalls were very variable and erratic, particularly in summer. Most of the rain fell in winter, with 329 to 395 mm (*ca.* 65 rain days), which represents 41 to 45% of the annual rainfall, followed by autumn and spring with 177–203 mm and 67–242 mm, respectively. Summer was the driest season, with only 39 and 45 mm of rain in 2008 and 2009, respectively. There were 63 to 75 days without rain. An unusual wet spell occurred in summer 2010/11 when 192 mm of rain fell in February 2011 (data not shown).

Most of the rain events that occurred were of very low intensity, 5 mm or below (Table 3.4). Rain events of between 10 and 20 mm were few, occurring on average about 20 times per year, and mostly in winter. Rain events of more than 30 mm were very rare.

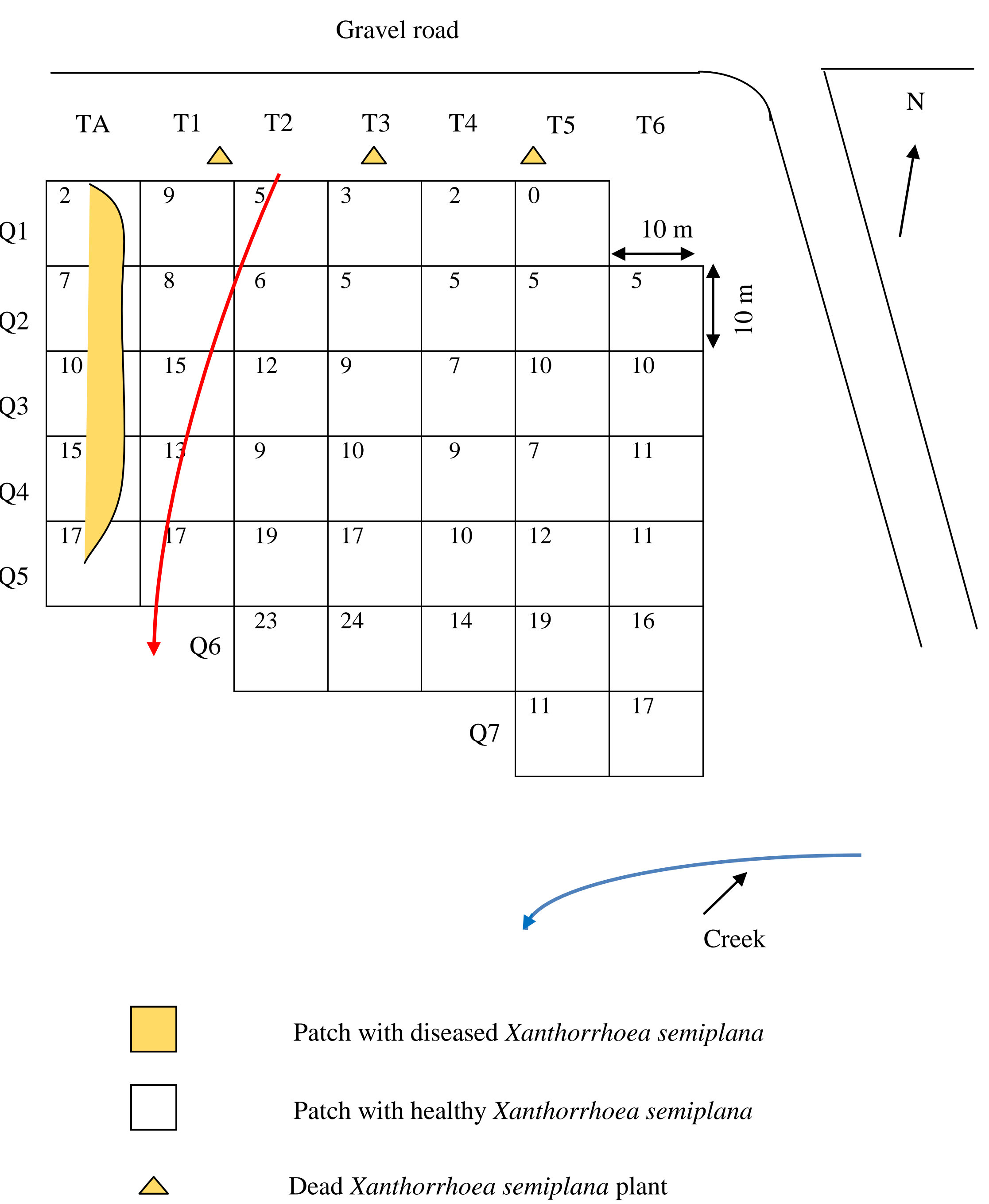

Figure 3.4 Direction and steepness of slope in each quadrat at the experimental site at Mount Bold Catchment Reserve. The red arrow indicates the direction of the slope while the number in each quadrat indicates the steepness (in degrees) of slope. The quadrats near the road were more flat while the quadrats in the south were steeper. Column (T) and Row (Q) and the adjacent letter and numbers refer to the positions of the quadrats in the columns and rows.

Table 3.2 Percentage (%) of sand, clay and silt of topsoil and subsoil samples from the experimental site at Mount Bold Catchment Reserve. The pits were numbered as per Figure 3.2.

Topsoil (0-25 cm)					Subsoil (25-36 cm)				
Pit No	Soil texture	% sand	% clay	% silt	Pit No	Soil texture	% sand	% clay	% silt
1	Loamy sand	81.2	9.1	9.7	1	Sandy loam	71.9	13.5	14.7
2	Loamy sand	75.8	5.1	19.1	2	Sandy clay loam	58.9	24.3	16.8
3	Loamy sand	86.3	6.4	7.3	3	Sandy loam	67.7	15.5	16.8
4	Loamy sand	79.7	6.0	14.2	4	Clay loam	43.0	37.8	19.2
5	Loamy sand	76.2	7.0	16.8	5	Sandy loam	69.4	15.2	15.4
6	Loamy sand	79.3	8.0	12.8	6	Sandy loam	72.8	11.8	15.4
7	Sandy loam	70.1	4.1	25.8	7	Sandy loam	76.7	7.2	16.1
8	Sandy loam	74.9	6.9	18.2	8	Sandy loam	71.8	9.3	18.9
9	Sandy loam	68.7	5.0	26.2	9	Loamy sand	79.3	7.6	13.1
10	Loamy sand	75.0	4.9	20.1	10	na	na	na	na
11	Sandy loam	72.7	6.8	20.5	11	Sandy loam	66.2	14.9	18.9
Average		76.4	6.3	15.5			67.8	15.7	16.5

na = not analysed

Table 3.3 Water droplet penetration time (s) of soil collected from the 41 quadrats. (T) Column; (Q) Row; Adjacent letters and numbers refer to the positions of the quadrats in the column and row; (R) replicate.

Quadrat No	Replicate						
	R1	R2	R3	R4	R5	Mean* (s)	
TAQ1	144	62	18	47	110	76	abcdefg
TAQ2	300	53	113	122	32	124	bcdefghijk
TAQ3	85	102	145	104	154	118	bcdefghijk
TAQ4	51	35	110	42	69	61	abcdf
TAQ5	58	39	231	300	199	165	bcdefghijklmn
T1Q1	274	285	16	104	29	142	bcdefghijkl
T1Q2	176	39	206	64	37	104	bcdefghi
T1Q3	2	131	33	54	101	64	abcdef
T1Q4	259	17	2	229	132	128	bcdefhijk
T1Q5	61	52	36	61	89	60	abcdf
T2Q1	300	300	300	300	300	300	efghijklmno
T2Q2	300	205	124	300	300	246	defghijklmno
T2Q3	40	282	93	290	300	201	defghijklmn
T2Q4	59	204	199	294	260	203	defghijklmn
T2Q5	283	41	300	252	300	235	defghijklmno
T2Q6	110	178	45	8	3	69	abcdef
T3Q1	300	286	213	136	26	192	bcdefghijklmn
T3Q2	289	96	300	300	169	231	defghijklmno
T3Q3	136	112	32	18	72	74	abcdef
T3Q4	3	139	58	70	41	62	abcdef
T3Q5	125	26	154	216	8	106	bcdefghi
T3Q6	12	10	5	19	12	12	a
T4Q1	300	58	89	156	62	133	bcdefghijk
T4Q2	139	28	56	34	156	83	abcdefgh
T4Q3	145	26	32	33	41	55	abcdef
T4Q4	37	32	20	154	124	73	abcd
T4Q5	300	300	300	300	300	300	efghijklmno
T4Q6	78	48	21	17	15	36	abc
T5Q1	300	58	109	76	300	169	bcdefghijklmn
T5Q2	66	41	85	17	63	54	abcdef
T5Q3	44	174	300	67	15	120	bcdefghijk
T5Q4	4	62	22	45	140	55	abcdef
T5Q5	13	8	23	65	70	36	adc
T5Q6	27	63	21	18	78	41	abcd
T5Q7	2	2	3	38	98	29	ab
T6Q1	67	144	40	10	10	54	abcdef
T6Q2	74	126	124	121	124	114	bcdefghijk
T6Q3	28	69	68	71	16	50	abcde
T6Q4	19	12	26	51	36	29	ab
T6Q5	30	19	41	63	62	43	abcd
T6Q6	34	26	31	26	65	36	abc

*Multiple comparisons of means were carried out using Duncan's multiple range test. Means with different letters are significantly different at $P \leq 0.05$. LSD = 89.80.

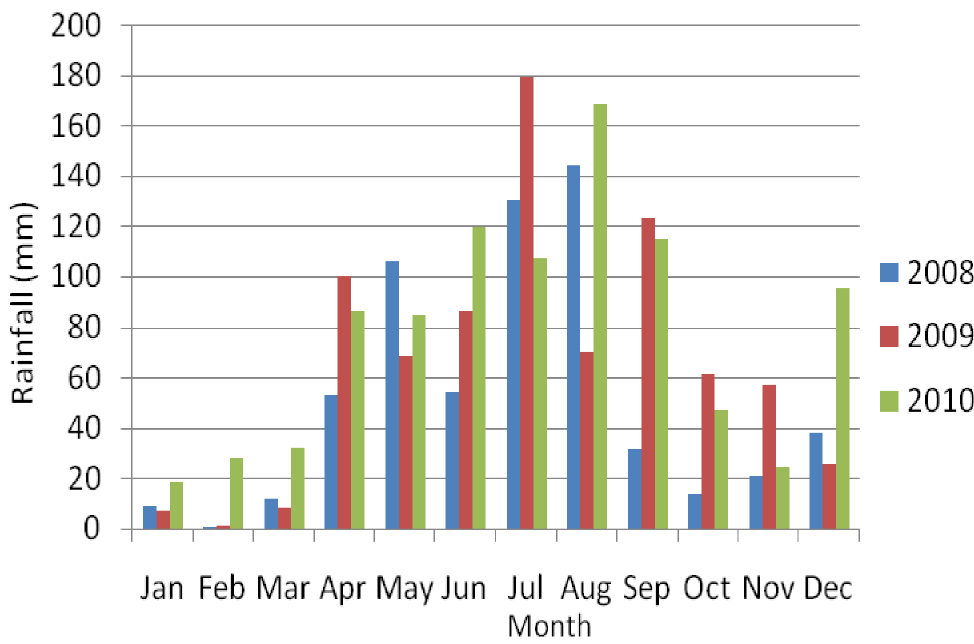

Figure 3.5 Monthly rainfall at Mount Bold Reservoir Office for 2008, 2009 and 2010.

Table 3.4 Daily rainfall (mm) recorded in spring, summer, autumn and winter in 2008 to 2010.

Range of daily rainfall (mm)	2008	2009	2010	2008	2009	2010
	Spring			Summer		
	Number of days					
0	60	51	44	75	71	63
> 0 to ≤ 5	26	28	38	12	15	19
> 5 to ≤ 10	4	4	3	1	3	1
> 10 to ≤ 20	0	7	3	0	0	2
> 20 to ≤ 30	0	1	0	1	0	1
> 30 to ≤ 40	0	1	1	0	0	0
> 40 to ≤ 50	0	0	1	0	0	2
> 50 mm	0	0	0	0	0	0
	Autumn			Winter		
	Number of days					
0	57	51	57	27	28	27
> 0 to ≤ 5	25	32	20	44	43	42
> 5 to ≤ 10	5	4	9	8	12	10
> 10 to ≤ 20	6	5	4	11	6	8
> 20 to ≤ 30	0	2	1	0	2	3
> 30 to ≤ 40	0	1	0	0	1	0
> 40 to ≤ 50	0	0	0	0	0	0
> 50 mm	1	0	0	0	0	1

3.3.4.2 Soil water potential in autumn and spring 2010

Due to technical problems, soil water potential was recorded for only a short period during autumn (2 March to 15 April 2010) and spring 2010 (2 September to 3 November 2010) (Figure 3.6 a and b). Soil water potential in autumn was lower and more variable than during spring. Soil water potential in autumn ranged from -26.1 to -163.4 kPa with an average of -86.2 kPa, while soil water potential during spring ranged from -12.6 to -42.1 kPa with an average of -26.2 kPa, indicating that soil water potential during spring was conducive for formation of sporangia by *P. cinnamomi*.

3.3.4.3 Soil temperature

Soil temperature at the experimental site was recorded from May 2009 to November 2010 (Figure 3.7). Total monthly rainfall for each month of the corresponding period has been included in Figure 3.7 to show availability of moisture with temperature. Soil temperature in winter was cool, from 5.4 to 13.0°C with the coolest time around mid-July. Summer was warm with soil temperatures in the range of 18 to 22°C. Mid-January was the warmest time of the year. Temperatures in autumn and spring fluctuated more widely, ranging from 12 to 20°C. In early autumn (April), the soil was warm, 17-20°C and this dropped to <10°C in the second half of autumn. Conversely, early spring was cool, with soil temperature in the range of 9-12°C (September) before rising to 12-20°C at the end of spring (November). Based on the regimes of soil temperature and moisture, late spring (October - November) appeared most conducive for the spread of *P. cinnamomi* at the site.

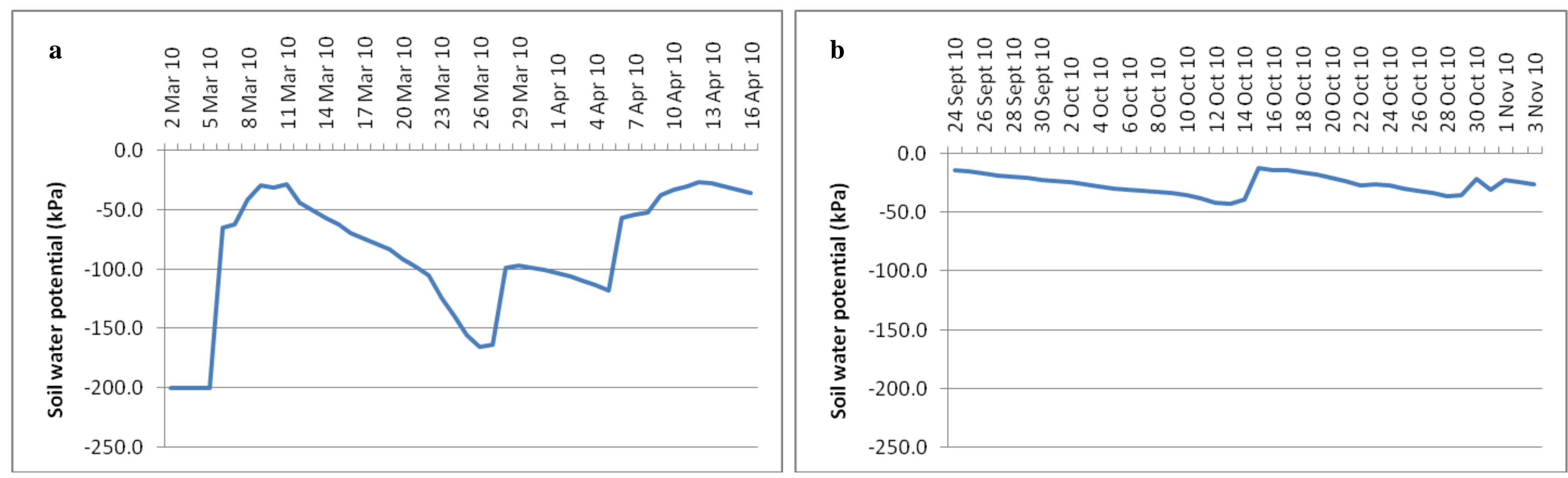

Figure 3.6 Soil water potential (kPa) 15 cm below the surface at the Mount Bold Catchment Reserve experimental site for (a) autumn and (b) spring 2010. The soil moisture was recorded using MEA (Measurement Engineering Australia 2.21) moisture probes which were set to record moisture every 2 h.

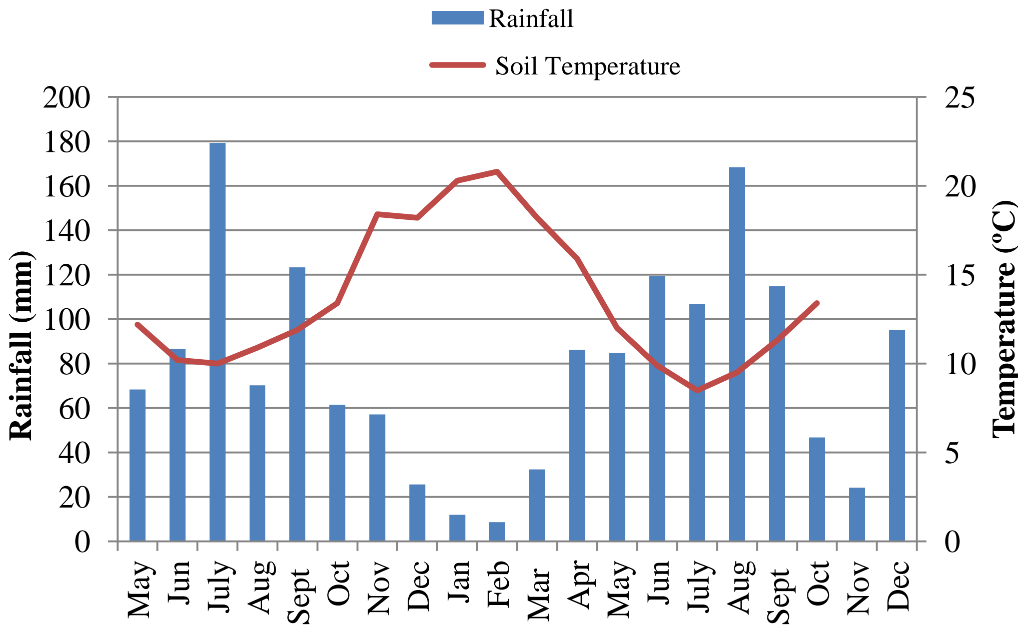

Figure 3.7 Average monthly soil temperature 15 cm below soil surface with open canopy at Mount Bold Catchment Reserve from May 2009 to October 2010. The temperature was recorded with a Tinytag Plus 2 which was set to record temperature at hourly intervals. The monthly rainfall data were collected from Mount Bold Reservoir Office, located about 10 km from the experimental site.

3.3.5 Population of *Xanthorrhoea semiplana* within the field plot

The total number of *X. semiplana* within the healthy area was estimated at 3,044 plants, with an average population density of 83 plants/quadrat (100 m^2). However, the distribution of *X. semiplana* within each plot was very uneven, ranging from 11 plants in quadrat TAQ5 to 160 plants in quadrat T6Q6 (Figure 3.8).

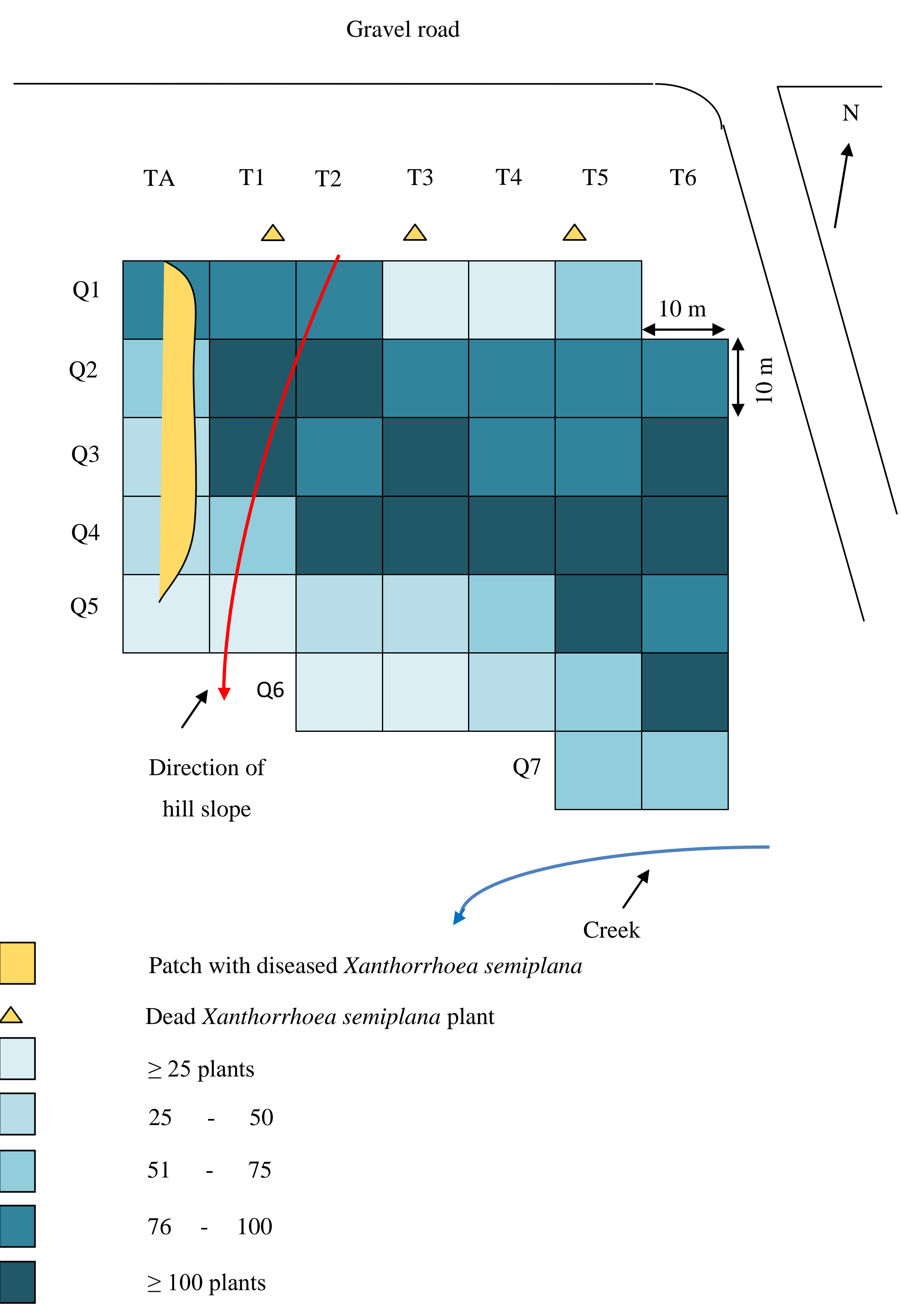

Figure 3.8 Numbers of *Xanthorrhoea semiplana* plants in each quadrat in August 2008. Column (T) Row (Q) and adjacent letter and numbers refer to the positions of quadrats in the columns and rows.

3.3.5.1 Number of dead and dying *Xanthorrhoea semiplana*

The number of dead and dying *X. semiplana* plants in all quadrats in column TA was high, with 59, 50, 44 and 41 plants recorded in autumn 2009, spring 2009, autumn 2010 and spring 2010, respectively (Figure 3.9 and Table 3.5). In autumn 2009, quadrats TAQ1 and TAQ2 had 21 and 16 dead *X. semiplana,* respectively, by far the most dead *X. semiplana* recorded per quadrat. The number of dying *X. semiplana* in quadrat TAQ1 was also high, at seven plants.

The total numbers of dead *X. semiplana* recorded in all quadrats combined in the healthy area were 20, 14, 23, 15 and 17 for the five seasonal counts (Table 3.5). The highest number of the dead *X. semiplana* in the healthy area were found in T1Q2 and T1Q3 with eight and five dead *X. semiplana* in spring 2010 and spring 2008, respectively. The number of dying *X. semiplana* recorded in the healthy area remained more or less constant over the study period, at 48, 47, 48, 43 and 59 for the five seasons counted. The majority of the quadrats in the healthy area contained one to three dying *X. semiplana*, depending on the season. Attempts were made to pull the chlorotic leaves from the dying plants. Leaves could be easily pulled out from a few chlorotic plants. The ends of the leaves appeared cut off due to insects as beetles could be seen crawling out of the caudex of the plants.

In the G-test, seasonal counts of symptomatic plants for all quadrats in the healthy area were combined to prevent errors in the analysis caused by low numbers of symptomatic plants in each quadrat. The test showed that there was no significant difference in the proportion of symptomatic *X. semiplana* plants from one season to the next at $P \leq 0.05$, indicating that there was no significant increase in the number of symptomatic *X. semiplana* plants in the 3 years (Table 3.6).

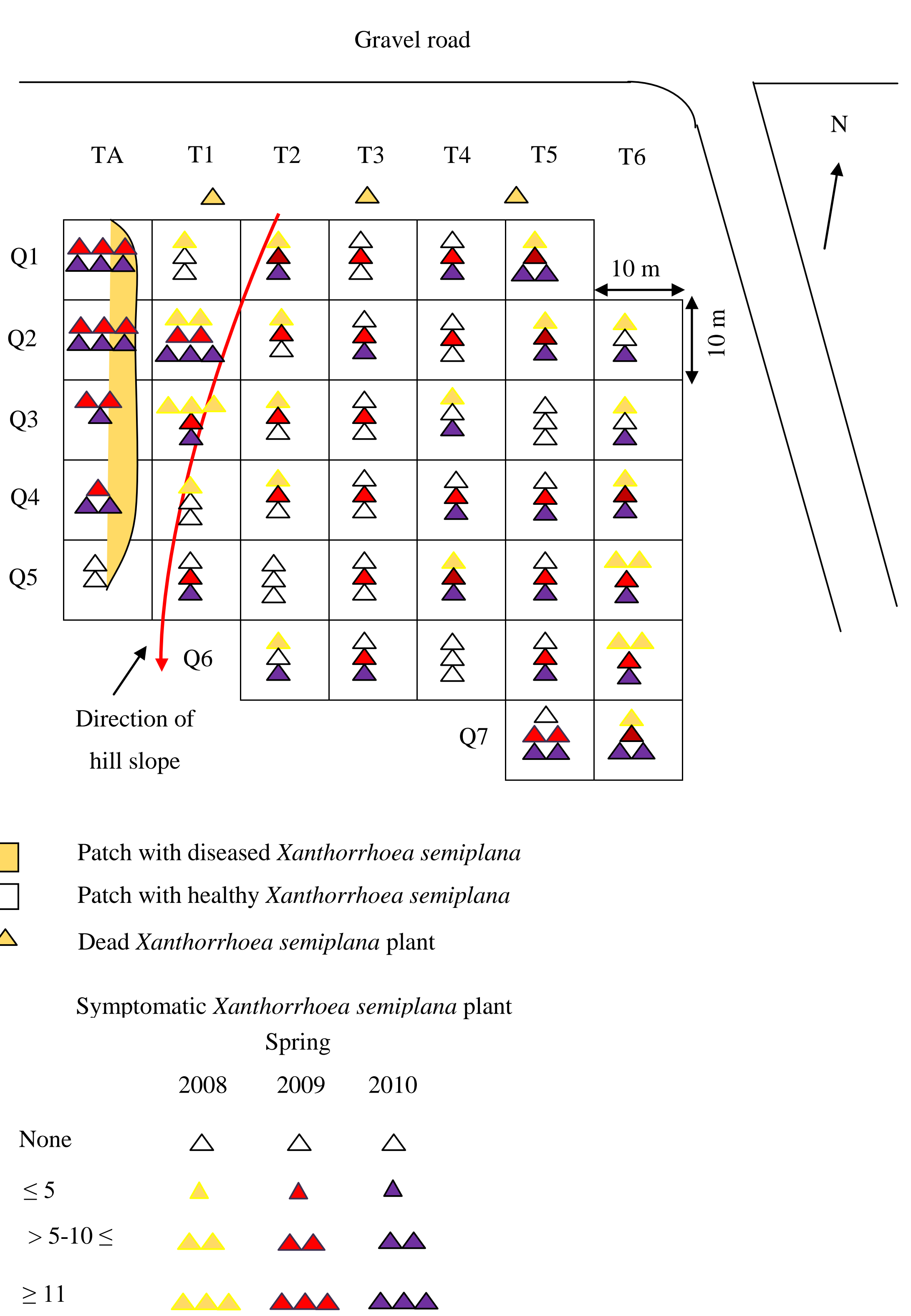

Figure 3.9 Numbers of symptomatic *Xanthorrhoea semiplana* (dead and chlorotic) in each quadrat counted in spring 2008-2010. Column (T) and Row (Q) and the adjacent letter and numbers refer to the positions of quadrats in the column and row.

Table 3.5 Numbers of symptomatic (dead, dying) and healthy *Xanthorrhoea semiplana* recorded in each quadrat at Mount Bold Catchment Reserve for the five seasonal counts starting from spring 2008 to spring 2010.

Quadrat	*Xanthorrhoea semiplana* counting season														
	Spring 2008			Autumn 2009			Spring 2009			Autumn 2010			Spring 2010		
	Dead	Dying	Healthy	Dead	Dying	Healthy	Dead	Dying	Healthy	Dead	Dying	Healthy	Dead	Dying	Healthy
TAQ1	nc	nc	nc	21	7	51	14	5	53	15	3	52	19	0	51
TAQ2	nc	nc	nc	16	2	46	16	4	44	10	4	44	8	3	45
TAQ3	nc	nc	nc	4	1	41	4	2	40	1	2	40	3	1	39
TAQ4	nc	nc	nc	5	1	26	5	0	27	8	1	22	6	1	22
TAQ5	nc	nc	nc	1	1	9	0	0	10	0	0	10	0	0	10
T1Q1	0	2	75	0	0	77	0	0	75	0	0	75	0	0	75
T1Q2	7	0	97	5	1	96	7	1	94	6	2	93	8	3	92
T1Q3	5	5	94	2	2	97	4	0	97	2	1	96	1	1	96
T1Q4	1	3	55	1	2	56	0	0	58	0	0	58	0	0	58
T1Q5	0	0	15	0	0	15	2	2	11	0	0	12	1	0	11
T2Q1	0	5	79	0	1	83	1	0	82	0	0	82	0	1	81
T2Q2	0	1	110	0	0	110	1	0	109	0	2	107	0	0	107
T2Q3	0	2	92	0	0	94	0	2	92	0	0	94	0	0	94
T2Q4	0	2	135	0	2	135	0	1	136	0	1	136	0	0	137
T2Q5	0	0	31	0	2	29	0	0	29	0	0	29	0	0	29
T2Q6	0	1	14	0	3	12	0	0	14	0	1	13	0	1	13
T3Q1	0	0	44	0	2	42	0	5	39	0	4	40	0	0	40
T3Q2	0	0	81	0	0	81	0	1	80	0	1	80	0	1	80
T3Q3	0	0	117	0	0	117	0	2	115	0	2	115	0	0	117
T3Q4	0	0	107	0	2	105	0	3	104	0	0	107	0	0	107
T3Q5	0	0	48	0	0	48	0	2	46	0	1	47	0	0	48
T3Q6	0	0	24	0	1	23	0	2	22	0	1	22	0	1	23
T4Q1	0	0	47	0	0	47	0	1	46	1	0	46	1	4	41
T4Q2	0	0	86	0	0	86	0	1	85	0	0	86	0	0	86
T4Q3	0	3	96	0	3	96	0	0	99	0	2	97	0	2	95
T4Q4	0	0	118	0	0	118	0	2	116	0	1	117	0	2	116
T4Q5	1	2	69	1	2	69	0	2	69	0	1	70	0	2	69
T4Q6	0	0	40	0	0	40	0	0	40	0	0	40	0	0	40

Cont...

Table 3.5 Numbers of symptomatic (dead, dying) and healthy *Xanthorrhoea semiplana* recorded in each quadrat at Mount Bold Catchment Reserve for the five seasonal counts starting from spring 2008 to spring 2010.

Quadrat	*Xanthorrhoea semiplana* counting season														
	Spring 2008			Autumn 2009			Spring 2009			Autumn 2010			Spring 2010		
	Dead	Dying	Healthy	Dead	Dying	Healthy	Dead	Dying	Healthy	Dead	Dying	Healthy	Dead	Dying	Healthy
T5Q1	2	1	52	3	1	51	2	2	50	2	4	48	2	4	48
T5Q2	0	3	73	0	3	73	0	1	75	0	1	75	0	1	75
T5Q3	0	0	92	0	5	87	0	0	92	0	0	92	0	0	92
T5Q4	0	0	123	1	2	120	1	2	120	0	1	121	0	2	120
T5Q5	0	0	140	0	0	140	1	0	139	0	1	138	0	2	137
T5Q6	0	0	75	0	0	75	0	1	74	1	0	74	0	2	72
T5Q7	0	0	55	0	1	54	1	7	47	0	3	51	1	7	56
T6Q1	0	2	97	0	2	97	0	0	99	0	2	97	0	3	96
T6Q2	0	2	143	0	2	143	0	1	144	0	3	142	0	5	140
T6Q3	0	1	115	0	1	115	1	0	115	1	0	114	0	3	111
T6Q4	1	5	88	0	0	88	0	4	84	0	3	85	0	3	85
T6Q5	2	5	112	1	3	114	1	2	115	1	2	115	1	3	114
T6Q6	1	3	157	0	4	156	1	1	158	1	3	156	2	6	152
Total	20	48	2996	61	59	3162	62	59	3144	49	53	3138	53	64	3120

A *Xanthorrhoea semiplana* plant with green foliage was classified as healthy, partial yellowing of foliage as chlorotic and plant which had completely turned brown either with upright or collapsed crown was classified as dead.

T = column; Q = row; Letter and number adjacent to T and Q refer to the positions of quadrats in the column and row (see Fig 3.2).

nc = not counted.

Table 3.6 The proportion of symptomatic (inclusive of dead and dying plants) and healthy *Xanthorrhoea semiplana* within the "healthy" area for all five seasonal counts tested for independence of proportionality using the G-test at $P \leq 0.05$ (Sokal and Rohlf, 1981).

Season	Symptomatic and healthy *X. semiplana* in healthy area			% of symptomatic *X. semiplana**
	Symptomatic (dead and dying) *X. semiplana*	Healthy *X. semiplana*	Total	
Spring 2008	68	2996	3064	2.2
Autumn 2009	61	2989	3050	2.0
Spring 2009	71	2970	3041	2.3
Autumn 2010	58	2970	3028	1.9
Autumn 2010	76	2953	3029	2.5
Total	334	14878	15212	10.7

*There was no significant difference in the proportion of symptomatic *X. semiplana* among the five seasonal counts at $P \leq 0.05$

3.3.5.2 Detection of *Phytophthora cinnamomi* and other *Phytophthora* species

Between 2008 and 2010, *P. cinnamomi* was detected in soil from all quadrats in column TA, except TAQ3 (Figure 3.10). *P. cinnamomi* was detected in quadrat TAQ4 at three consecutive soil samplings, in spring 2009, autumn 2010 and spring 2010, and in quadrat TAQ2 it was detected in spring 2009 and 2010 but not in autumn 2010. *P. cinnamomi* was not detected in quadrat TAQ1 in spring 2009 but was detected in the subsequent two seasons, in autumn and spring 2010. For quadrat TAQ5, *P. cinnamomi* was detected only in autumn 2010. None of the samples from the quadrats near to the road, except for TAQ1, yielded *P. cinnamomi*.

When soil samples from these quadrats were baited with cotyledons of *E. sieberi*, non-caducous and non-papillate obpyriform sporangia, borne at the end of long sporangiophores, were produced by hyphae colonising the cotyledons (Figure 3.11). Some soil samples produced prolific sporangia. While being observed under a low power microscope, a few sporangia matured and 20 to 30 zoospores were seen swimming out from each sporangium. Based on measurements of 20 mature sporangia, the mean length x breadth of sporangia was 72.3 x 46.7 µm, with length/breadth ratio of 1.50 (Table 3.7). The shape and size of sporangia indicated that these were likely to be *P. cinnamomi*. By plating the infected cotyledons onto P_{10}ARPH medium, pure cultures of *P. cinnamomi* were obtained from quadrats TAQ1, TAQ2, TAQ4 and TAQ5, comprising coenocytic hyphae with prominent coralloid structure.

In quadrat T1Q3, *P. cinnamomi* was detected in spring 2008 and autumn 2009 but not in subsequent soil samples. A pure culture of *P. cinnamomi* from T1Q3 was obtained in autumn 2009. Although sporangia were observed from soil samples collected from T5Q7 in spring 2008 and again in quadrats T2Q6, T3Q6 and T5Q7 in

autumn 2009, the organism could not be positively identified as *P. cinnamomi*, as the sporangia produced from these quadrats were more spherical (globose) and smaller, with average length of 43.9 µm and breadth of 35.8 µm, than expected for *P. cinnamomi*. The length/breadth ratio of these sporangia was about 1.2, which resembles sporangia of *Phytophthora cryptogea* or *Phytophthora citricola*. A few of these sporangia were observed to release zoospores when viewed with a microscope (magnification 100x). Several attempts to produce hyphae from these sporangia failed because of the presence of other soil microorganisms which contaminated the plates. Neither *P. cinnamomi* nor sporangia were detected in soil samples collected from other quadrats throughout the study period.

3.3.5.3 Detection of *Phytophthora* from random soil and water samples

P. cinnamomi was detected from soil samples collected from around roots of *X. semiplana* from quadrat TAQ4 on various occasions (Table 3.7). It was detected from *Platylobium obstusangulum* located in quadrat TAQ2 on 10 April 2010 and from an old dead *X. semiplana* located at about 12 m down the slope near the reservoir on 16 June and again on 3 November 2010 (Figure 3.10). However, *P. cinnamomi* was not detected from soil samples collected from *P. daphnoides*, which is a known susceptible species. *P. cinnamomi* and *P. megasperma* were suspected to be present in water in the reservoir and in a creek located within the Mount Bold Catchment Reserve (Figure 3.11). Appendix D shows the baiting results of random soil samples collected from within and outside the experimental plot.

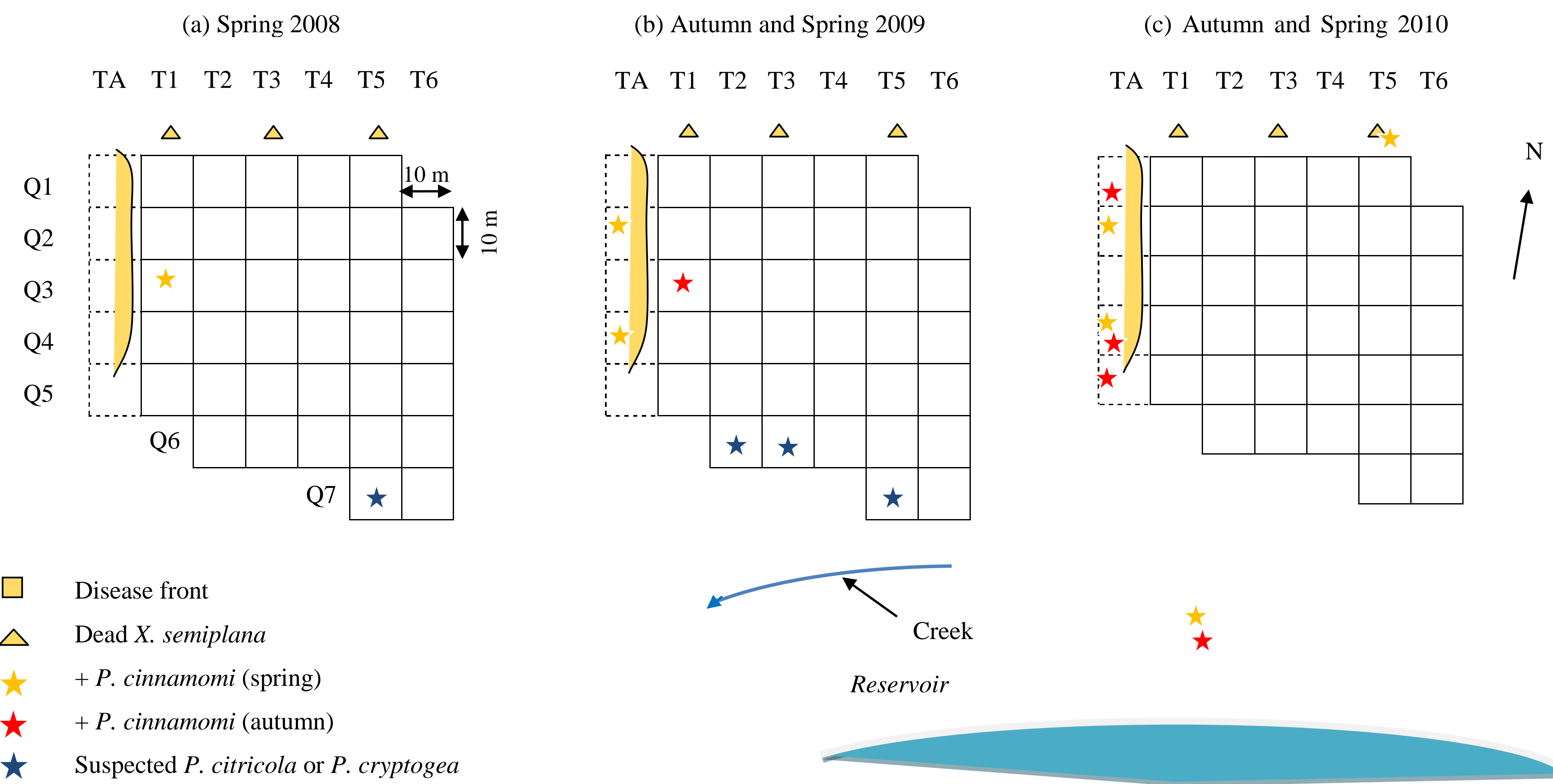

Figure 3.10 Quadrats in which *Phytophthora cinnamoni* was detected in autumn (red star) and spring (yellow star) in (a) 2008, (b) 2009 and (c) 2010. *P. cinnamomi* was detected from two dead *Xanthorrhoea semiplana*, one located near to the shore of the reservoir and one located outside quadrat T5Q1 in spring 2010. Column (T); Row (Q).

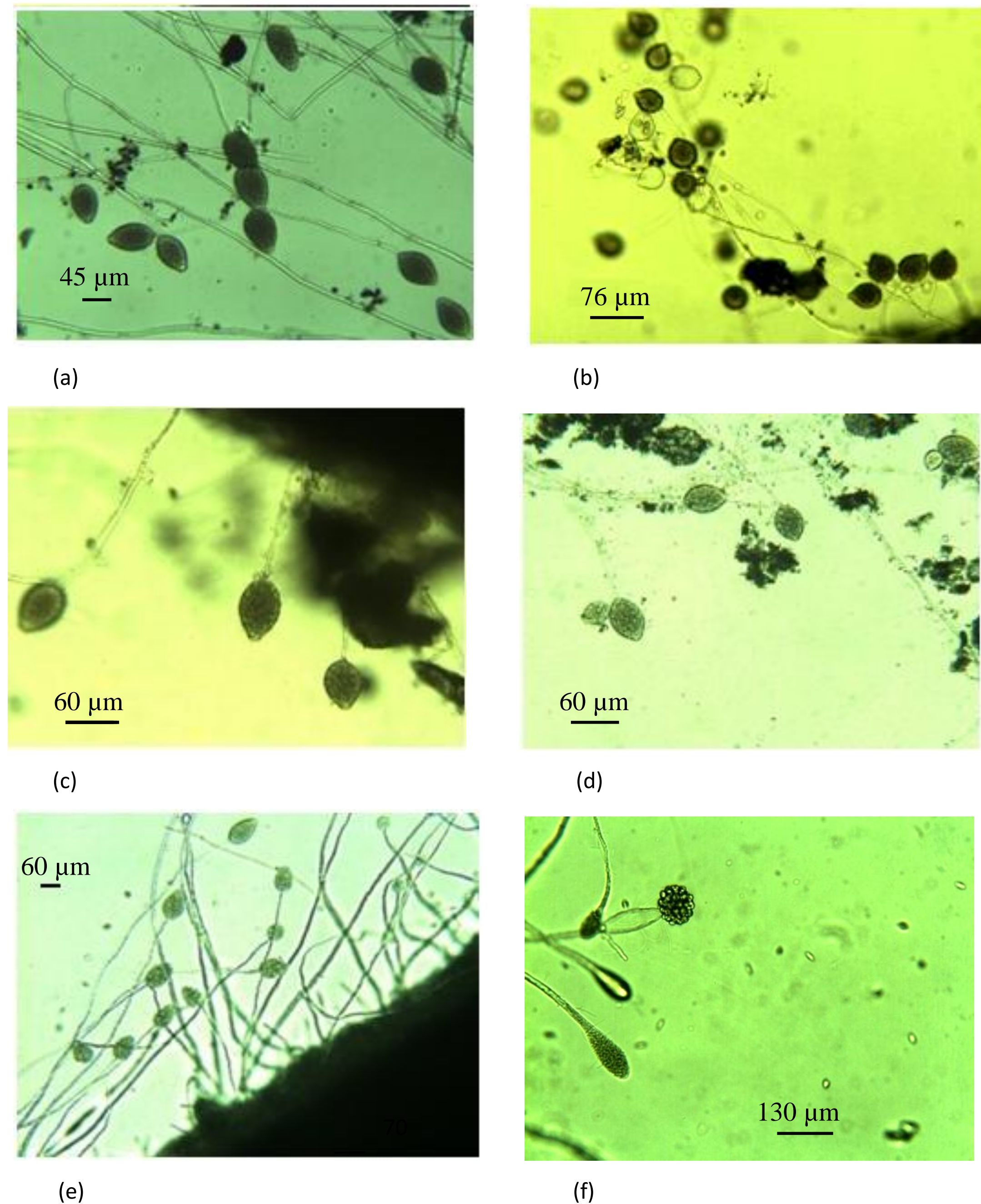

Figure 3.11 Sporangia of various shapes and sizes obtained from different sources at Mount Bold Catchment Reserve, South Australia: (a) Obpyriform sporangia from quadrat TAQ4 identified as *Phytophthora cinnamomi*; (b) Globose sporangia from quadrat T2Q6 suspected to be *P. cryptogea* or *P. citricola*; (c) Ovoid sporangia from an old dead *Xanthorrhoea semiplana* near reservoir suspected to be *P. cinnamomi*; (d) sporangia from soil collected near roots of symptomatic *Platylobium obtusangulum* identified as *P. cinnamomi*; (e) Ovoid and obpyriform sporangia from creek water collected on 13 August 2009, suspected to be *P. cinnamomi*; (f) Limoniform sporangia from reservoir water suspected to be *P. megasperma*.

Table 3.7 Shape and size of sporangia from various sources at the Mount Bold Catchment Reserve experimental site.

Date of sampling Source	8 May 2009 T2Q6	8 May 2009 T3Q6	8 May 2009 T5Q7	12 August 2009 Creek	30 October 2009 TAQ4	16 December 2009 TAQ4
Soil/water	Soil (Symptomatic *Xanthorrhoea semiplana*)	Soil (Symptomatic *X. semiplana*)	Soil (Symptomatic *X. semiplana*)	Water	Soil (Healthy *X. semiplana*)	Soil (Healthy *X. semiplana*)
n= Sample size	16	12	9	6	6	20
Average (µm) (Length x Breadth)	46.6 x 36.7	43.9 x 35.8	42.8 x 35.1	55.4 x 43.7	58.2 x 43.0	64.6 x 44.8
Range (µm): Length	37.2 – 55.3	40.0 – 47.2	40.0 – 47.0	38.9 – 68.6	49.7 – 65.6	47.7 – 81.9
Range (µm): Breadth	33.0 – 40.8	30.7 – 38.0	28.7 – 38.7	35.8 – 58.2	31.1 – 53.5	39.2 – 50.0
Ratio: Length/Breadth	1.20	1.27	1.22	1.27	1.35	1.44
Phytophthora species identified	*P. citricola* or *P. cryptogea*	*P. citricola* or *P. cryptogea*	*P. citricola* or *P. cryptogea*	(?)	*P. cinnamomi*	*P. cinnamomi*

Cont...

Table 3.7 Shape and size of sporangia from various sources at the Mount Bold Catchment Reserve experimental site.

Date of sampling Source	22 March 2010 TAQ4	22 March 2010 TAQ4	22 March 2010 TAQ5	16 April 2010 TAQ5	16 April 2010 TAQ1	16 April 2010 TAQ2
Soil/water	Soil (Healthy *X. semiplana*)	Soil (Healthy *X. semiplana*)	Soil (Healthy *X. semiplana*)	Soil (Healthy *X. semiplana*)	Soil (Symptomatic *X. semiplana*)	Soil (Symptomatic *Platylobium obtusangulum*)
n= Sample size	20	20	20	20	20	20
Average (µm) (Length x Breadth)	62.6 x 40.4	72.3 x 46.7	68.7 x 47.6	63.9 x 42.5	67.3 x 43.4	67.3 x 44.3
Range (µm): Length	42.6 – 87.3	54.6 – 85.8	60.2 – 81.4	54.5 – 73.1	51.9 – 84.9	55.0 – 88.5
Range (µm): Breadth	29.5 – 49.4	39.5 – 53.6	40.2 – 55.3	34.6 – 53.9	31.0 – 55.4	32.7 – 51.1
Ratio: Length/Breadth	1.55	1.55	1.44	1.50	1.55	1.52
Phytophthora species suspected	*P. cinnamomi*	*P. cinnamomi*	*P. cinnamomi*	*P. cinnamomi*	*P. cinnamomi*	*P. cinnamomi*

Cont...

Table 3.7 Shape and size of sporangia from various sources at the Mount Bold Catchment Reserve experimental site.

Date of sampling Source	10 June l 2010 Creek	10 June 2010 Creek	16 July 2010
Soil/water	Water	Water (2nd baiting)	Dead *X. semiplana* plant near to reservoir
n= Sample size	3	6	20
Average (µm) (Length x Breadth)	60.0 x 36.2	128.3 x 36.7	53.0 x 41.0
Range (µm): Length	60.0	125.0 - 130	49.6 – 65.9
Range (µm): Breadth	35.0 – 40.0	30.0 – 40.0	39.5 – 50.6
Ratio: Length/Breadth	1.64	3.5	1.29
Phytophthora species identified	*P. cinnamomi* (suspected)	*P. megasperma* (suspected)	*P. cinnamomi*

3.4 Discussion

P. cinnamomi was found at the experimental field site, however, seasonal counting of symptomatic *X. semiplana* and soil samplings showed no detectable or noticeable spread of the disease or pathogen from the disease fronts to the adjacent healthy vegetation over a 2-year period.

Surveys of the symptomatic *X. semiplana* plants showed that most of them were located in quadrats lying along the western disease front (Figure 3.2). *P. cinnamomi* was detected in these quadrats frequently enough to conclude that it was the cause of death of *X. semiplana*. *P. cinnamomi* was also isolated from a dead *X. semiplana* in the northern disease front (Figure 3.10), from a dead *X. semiplana* located near to quadrat T5Q1 in spring 2010, although *P. cinnamomi* was likely to have been present there since the beginning of the study. The size and shape of the sporangia recorded strongly indicated that these were sporangia of *P. cinnamomi*. In the healthy area, quadrat T1Q3 was the only quadrat from which *P. cinnamomi* was isolated in the first two rounds of soil samplings. Subsequently, soil sampling did not detect *P. cinnamomi* in this quadrat or any quadrat in the healthy area.

Symptomatic *X. semiplana* plants were also observed in some quadrats in the healthy area, but *P. cinnamomi* was not detected from these plants. Some plants may have become chlorotic because of water stress. It was also possible that another pest or pathogen could have caused the death or chlorosis of *X. semiplana* in these areas (Sivansesan & Sutton, 1985; Borsboom, 2005). Insects such as weevils and beetles were seen crawling out of plants when the leaves of some of these dying plants were pulled out from the plants. A wide range of microfungi (Sivanesan & Sutton, 1985) and

insects (Borsboom, 2005) have been reported to infest *Xanthorrhoea* plants. *Armillaria luteobubalina* Watling and Kile, which also causes root rot, has been reported to cause scattered deaths of many understorey species including *Xanthorrhoea* species in native vegetation (Kile *et al.*, 1983) and black beetle (*Hololepta lissopyga* Marseul) has been found infesting the caudex (leaf base) of *Xanthorrhoea* (Hawkeswood, 2006). Infestation of *Xanthorrhoea* plants by such pests and pathogens could have produced symptoms resembling those of Phytophthora dieback. Some plants which turned chlorotic could have recovered, particularly if the insect did not cause serious injury to the plants or when sufficient water was available. This could explain why the number of dead and dying *X. semiplana* decreased over time in some quadrats, such as T1Q4 and T2Q1.

Based on the baiting results obtained in this study, there appeared to be no noticeable or detectable spread of *P. cinnamomi* at the study site. None of the quadrats in the healthy area, in particular those closest to both the western and northern disease fronts, was diagnosed with *P. cinnamomi*. Subsequent soil sampling in spring 2009 to spring 2010 did not detect *P. cinnamomi* from any quadrat in the healthy area nor was there any dramatic increase in the number of dead and dying *X. semiplana*. This suggested that *P. cinnamomi* did not spread from the disease front to the neighbouring quadrats during the study period.

The putative *P. citricola* or *P. cryptogea* in quadrats T2Q6 or T3Q6 and *P. megasperma* were identified based on morphology. However, identification of *Phytophthora* species based solely on morphological characters is not reliable. Recent re-evaluation based on sequencing of internal transcribed spacer (ITS) region of the rDNA carried out in WA has revealed that many isolates thought to be *P. citricola*, *P.cryptogea* or *P. megasperma* based on morphology actually represent new species of

Phytophthora (Burgess *et al.*, 2009). Thus there is a need to sequence the putative *P. cryptogea*, *P. citricola* and *P. megasperma* to assign them to species.

The apparent lack of spread of *P. cinnamomi* at the site could be attributed to several factors as discussed below.

Phytophthora cinnamomi is not uniformly distributed throughout soil in natural ecosystems, rather, it is likely to be present in woody material in moist micro-environments. As such the 20 g soil samples with two replications used for baiting were too small for reliable detection of the presence of *P. cinnamomi*. This was borne out by the fact that on many occasions *P. cinnamomi* was detected in one of the two replicates. To increase the chance of detecting P. cinnamomi, one possible alternative is to increase the number of replicates or bait larger quantities of soil using bigger containers. Furthermore, the containers should be left uncovered as *P. cinnamomi* forms sporangia only in aerobic condition (Davison & Tay, 1987).

For *P. cinnamomi* to spread through zoospores, free water must be present, at high water potential of higher than -16 kPa (MacDonald & Duniway, 1978; Gisi *et al.*, 1980) and temperature warm, between 18 to 22°C (Halsall & Williams, 1984), for a sustained period of time. These conditions are necessary to stimulate the formation and release of zoospores and to provide sufficient water flow to disperse zoospores, either through surface runoff or soil infiltration (Kinal, 1993). Soil baiting conducted in the laboratory indicated that mycelium of *P. cinnamomi* formed sporangia and released zoospores after incubation for 3-4 days in water. However, judging by the rainfall pattern over 2009 and 2010, long periods of continuous wet conditions were unlikely to have occurred at the field site.

Although relatively high annual rainfall was recorded from 2008 to 2010, most of the rain fell in winter when the temperature was too cold (below 10ºC) for *P. cinnamomi* to sporulate (Halsall & Williams, 1984). Conversely, when the temperature was suitable for the formation of zoospores in summer, soil moisture might be too low (generally less than 50 mm of rain in June, July and August) for *P. cinnamomi* to thrive in soil, except for the *P. cinnamomi* already inside the tissue of plants (Shearer *et al.*, 1987b; Shearer & Tippett, 1989). Therefore, the temperature and rainfall required to produce an environment conducive for the growth and sporulation of *P. cinnamomi* did not coincide.

Spring and autumn were the times when moisture and temperature were most likely to coincide to provide an environment more conducive for the spread of *P. cinnamomi*, but even then these conditions were sub-optimal and occurred only intermittently. From the data collected, spring and autumn received a seasonal rainfall of 66 to 242 mm, which, though moderate, was only able to raise the soil water potential intermittently to an average of -94.4 kPa (range -26.1 to -200 kPa) in autumn and -26 kPa (range -12.6 to -42.9 kPa) in spring. These conditions were sub-optimal for the sporulation and spread of zoospores (Duniway, 1976; Gisi *et al.*, 1980).

At the northern disease front, the flat topography, the porous sandy loam soil and the generally low rain (mainly less than 10 mm) suggested rain water would infiltrate directly into subsoil and the topsoil would dry up rather quickly. These conditions would not favour the spread of disease from the northern front. However, further study to test the presence of *P. cinnamomi* in the subsoil needs to be carried out. Even in the two quadrats, T4Q2 and T6Q3, where the clay content in the subsoil was relatively high, the numbers of symptomatic *X. semiplana* were low, while in TAQ2 and TAQ4, where the clay content of subsoil was low (9.3%), the numbers of dead and

dying *X. semiplana* were high, suggesting that the distribution of dead and dying *X. semiplana* at the site was not influenced by the clay content of the subsoil but rather could be influenced by the infiltration of water on soil surface.

The soil in all quadrats which flanked the western and northern disease fronts had strong surface water repellency and therefore rain water, particularly rain of low intensity, was unlikely to infiltrate the soil but would instead flow along a preferential path. The preferential path of water flow could have influenced the disease spread in the western disease front. These quadrats with high water repellency represent a barrier for the spread of the pathogen to the healthy area. Furthermore, the western disease front was located at a lower elevation than the healthy area, thus surface water was more likely to infiltrate from the healthy area to the disease front rather than from the disease front to the healthy area.

The topography of the disease front ranged from gentle slope in the north to steep slope in the south, particularly in TAQ4 and TAQ5. Quadrats TAQ4 and TAQ5, which had yielded *P. cinnamomi*, would disperse *P. cinnamomi* by zoospores down the hillslope if continuous rain occurred during the summer season. The pathogen detected from a dead *X. semiplana* located 12 m downhill near the shore of the reservoir could have originated from propagules washed downhill from the western disease front, although dispersal of propagules of *P. cinnamomi* by wild animals could not be discounted as footprints were often observed in the area. The presence of suspected *P. cinnamomi* in the reservoir and creek water was a cause of concern as it means that *P. cinnamomi* could have, or potentially had, been dispersed far and wide through water flow from the reservoir. An effective *Phytophthora* management plan is urgently needed to contain the spread of *P. cinnamomi* at the site.

Rock structure was present at the soil surface in quadrats T3Q3 and T3Q4 which could have caused water logged conditions. However, the rocky structures were located 20-30 m away from the disease front and so would be unlikely to influence the spread of *P. cinnamomi* in the site itself.

Even though environmental conditions were not conducive for the spread of *P. cinnamomi* through zoospores, *P. cinnamomi* may have spread as mycelium via root to root contact, but such a mechanism of spread would be too slow to be detected in two and half years of study. According to Shearer *et al.* (1987b) the rate of invasion by *P. cinnamomi* along root tissue of *Banksia grandis* and *Eucalyptus marginata* in optimal conditions was about 1-2 m per year following the temperature-growth relationship as follows:

$$y = (-2.610 \pm 0.616) + (0.454 \pm 0.030)T \ (\pm s.e)$$

where y is the growth rate in mm per day and T is the temperature between 10 and 30°C.

Assuming that the equation is applicable to *X. semiplana* plants and using the monthly soil temperature recorded at Mount Bold Catchment Reserve, the model predicts disease extension of about 1.5 m per year, which arguably could be much slower if the environmental conditions were only intermittently conducive or if the plants experienced water stress (Smith & Marks, 1986). A smaller quadrat size of about 1 m x 1 m might provide greate r 'resolution' to detect this slow spread of *P. cinnamomi*. Alternatively, a longer term monitoring would be required to assess the spread of *P. cinnamomi* in native vegetation.

In this study, detection of *P. cinnamomi* was carried out following the standard method, i.e. by baiting soil and fine root samples with cotyledons of *Eucalyptus*

sieberi. Although considered to be an effective method to test for the presence of *P. cinnamomi* (Williams *et al.*, 2007), soil baiting can sometimes produce false negative results, particularly when the soil inoculum is small (Davison & Tay, 2005). According to Davison and Tay (2005), the proportion of soil samples from which *P. cinnamomi* is not detected can be used to estimate the minimum number of soil samples to show that a site is not infested by *P. cinnamomi* using the equation given as follows:

$$P = (1 - r)^n$$

where *P* is the probability of all samples being negative, r is the proportion of samples from which *P. cinnamomi* was isolated and n is the sample size. From the 31 soil samples collected from the healthy area in each season, only one sample from quadrat T1Q3 yielded *P. cinnamomi*. Thus the proportion of soil samples from which *P. cinnamomi* was not detected was 0.9677. Using this value, the minimum number of soil samples (at a 95% confidence level) that would have to be tested to indicate that the area was free of *P. cinnamomi* (if all tested negative for *P. cinnamomi*) was 107 samples. From 2008 to 2010, more than 150 soil samples collected from the “healthy” area had tested negative for *P. cinamomi*, thus it could reasonably be concluded that the “healthy” area was free of *P. cinamomi* at present rather than reflecting false negative baiting results.

In this survey, other species of *Phytophthora* were also detected. Sporangia of *P. citricola* or *P. cryptogea* were suspected to be present at the site. Detection of *P. cryptogea*, *P. megasperma* var. *sojae* and *P. drechsleri* at Mount Bold Catchment Reserve area had been reported by Davison and Bumbieris (1973) and Pratt and Heather (1973). These species have been reported to be capable of infecting native plants such as species of *Banksia* and *Eucalyptus* in Australia (Weste, 1975; Shearer *et*

al., 1987a; Tynan *et al.*, 1998). A survey conducted by Pratt and Heather (1973) showed that *P. cryptogea* was the main pathogen associated with death of pine trees in the Adelaide Hills. In this field study, species of *Phytophthora* detected in association with dying *X. semiplana* in quadrats T2Q6, T3Q6 and T5Q7 was suspected to be *P. cryptogea*. This strongly indicated a need to assess the threat these species of *Phytophthora* may pose to native flora in South Australia.

The spread of *P. cinnamomi* at the site was found to be negligible, probably due to the topography, soil texture and environmental conditions. However, further monitoring and expansion of that monitoring to cover a wider area is recommended in view of the fact that *P. cinnamomi* had spread to the shore of the reservoir. With the expected increase in temperature and higher intensity rains under a climate change scenario (Brasier, 1996), the activity and rate of spread of *P. cinnamomi* at existing locations might increase in the future.

STATEMENT OF THE CONTRIBUTIONS TO JOINTLY AUTHORED PAPERS

Chapter 4 comprises a manuscript submitted to Plant Pathology on 29 August 2011. This manuscript is presented according to the instructions to authors for the journal available on the following website: http://www.wiley.com/bw/submit.asp?ref=0032-0862&site=1. Following revision, the manuscript was published in Plant Pathology on 17 February 2012 (DOI: 10.1111/j.1365-3059.2012.02593.x).

K. H. Kueh, S. F. McKay, E. Facelli, J. M. Facelli, R. M. A. Velzeboer, A. J. Able, E. S. Scott, 2012. Response of selected South Australian native plant species to *Phytophthora cinnamomi*.

Author contributions: KHK and SFM designed and individually conducted all research experiments and analysed the data. Later EF helped in the research. ESS, AJA, JMF and RMAV contributed to planning the research. KHK took the lead in writing the manuscript. All co-authors contributed to the final version of the manuscript.

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Short title: *Phytophthora*-susceptibility of South Australian plants

Chapter 4 Response of selected South Australian native plant species to *Phytophthora cinnamomi*

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Keywords: *P*hytophthora dieback, native vegetation, susceptibility, survival, threatened species

Abstract

Thirty-seven South Australian native plant species from 11 families, including 15 threatened species in the state (of which six are listed as threatened under the federal Environment Protection and Biodiversity Conservation Act 1999) were assessed for response to infection by *Phytophthora cinnamomi*. Seedlings, 3-6 months old and grown in a greenhouse, were inoculated by placing infested pine wood plugs in the potting mix, maintained in moist conditions and assessed for mortality and disease symptoms for between 3 and 10 months. Thirty species were found to be susceptible, of which nine were highly susceptible, 15 moderately susceptible and six slightly susceptible. Three species were found to be resistant and results for four species were inconclusive. Six of the 15 threatened, rare or locally endangered species tested (*Eucalyptus viminalis* var. *viminalis*, *Correa aemula*, *C. calycina*, *Olearia pannosa* ssp. *pannosa*, *Pomaderris halmaturina* ssp. *halmaturina* and *Prostanthera eurybioides*) were moderately susceptible, while two (*Allocasuarina robusta* and *Pultenaea graveolens*) were highly susceptible. Significant populations of at least five of the threatened species susceptible to the disease are located close to confirmed or suspected *Phytophthora*-infested areas or growing in areas conducive for *P. cinnamomi*. An effective management strategy is therefore required to avoid extinction of such species due to infection by the Phytophthora dieback pathogen.

Introduction

Phytophthora dieback is a soil-borne disease which causes damage to many horticultural crops worldwide (Erwin & Ribeiro, 1996). In Australia, the disease also causes damage of epidemic proportions to native vegetation in many regions, particularly in the south eastern and south western regions of the country. The disease was first associated with the death of a large number of native plants in the jarrah forests in Western Australia in 1921 (Podger, 1972) and in 1952 in Gippsland in the south-east of Victoria (Marks *et al.*, 1972). By the 1990s, the disease had destroyed large areas of native vegetation in Western Australia, Tasmania, Queensland and Victoria, causing local susceptible species to disappear (Weste, 1986). Phytophthora dieback continues to threaten the survival of susceptible native plant species, particularly endangered species such as *Banksia brownii* in Western Australia and *Wollemia nobilis* in New South Wales (O'Gara *et al.*, 2005; Reiter *et al.*, 2004). As such, Phytophthora dieback is recognised as a key threatening process to the natural ecosystem under the Environment Protection and Biodiversity Conservation (EPBC) Act 1999 of Australia.

The disease is caused by *Phytophthora cinnamomi* Rands, which attacks the roots causing root and crown rots, resulting in shoot dieback, chlorotic foliage and defoliation. When rot has girdled the collar region, plants invariably wilt and die. Young eucalypt seedlings tend to show greater sensitivity to Phytophthora dieback than older plants (Peace, 1962). The spread and severity of disease depends very much on prevailing weather conditions (Tregonning & Fagg, 1984). Being a water mould, *P. cinnamomi* thrives in warm and wet conditions, when the pathogen sporulates and releases motile zoospores which are attracted to and infect the roots. Infected plants may die during hot, dry spells following wet weather, as plants with rotted roots are

unable to take up sufficient water to compensate for increased water loss. This pattern is common in the Mediterranean-type environments of the south western and south eastern parts of Australia where a warm and wet spring is followed by a hot and dry summer.

P. cinnamomi affects a large number of species in Australia's diverse and unique flora, from large trees to prostrate shrubs (Barker & Wardlaw, 1995; Shearer *et al.*, 2004). Barker & Wardlaw (1995) found that 36 of 47 species native to Tasmania tested were susceptible. Assessment of the susceptibility of 749 native plant species from 253 genera in Western Australia led Shearer *at al*. (2004) to estimate 40% of the 5,710 described plant species in the South-West Botanical Province to be susceptible to this pathogen. Of these, about 800 species were ranked as highly susceptible. A field evaluation of susceptibility by Weste (1986) showed that up to 75% of the native flora in an infested native vegetation site in Brisbane Ranges National Park, Victoria, were susceptible. The disease caused not only local extinction of susceptible species and endangered the continued survival of rare species but also changed the species composition of the plant communities (Weste, 1986).

In comparison, very little is known about the susceptibility of the native flora of South Australia to this pathogen. *P. cinnamomi* was first isolated in South Australia in 1969 (Davison, 1970) and, since then, has been found extensively in many areas of native vegetation with high conservation value, such as the Mount Lofty Ranges, Fleurieu Peninsula (Lee & Wicks, 1977) and Kangaroo Island (Williams *et al.*, 2007). While *P. citricola, P. cryptogea*, *P. drechsleri* and *P. megasperma* have been isolated from forest nurseries and pine plantations (Davison & Bumbieris, 1973; Pratt & Heather, 1973) and have the potential to cause dieback of native plants such as *Banksia*

and *Eucalyptus* spp. (Weste, 1975; Shearer *et al*., 1987) *P. cinnamomi* is considered to cause most damage to native vegetation in South Australia (Velzeboer *et al*., 2005).

Protection of native flora from Phytophthora diseases has relied on hygiene measures, such as provision of car wash-down facilities and restriction of access to conservation areas during wet periods, to prevent or reduce the spread of the pathogen to non-infested areas. However, in areas where the pathogen has already spread widely, hygiene measures alone are no longer effective to protect the vegetation from the disease. More effective and targeted strategies require evaluation of the susceptibility of native plant species to Phytophthora dieback and prioritisation for protection accordingly. Velzeboer *et al*. (2005) reported the potential risk posed by Phytophthora dieback to 145 threatened South Australian native plant species according to their proximity to an infestation and vulnerability to infection by species of *Phytophthora*. However, reliable information about the effect of *P. cinnamomi* on South Australian native plant species, and threatened plant species in particular, is very limited. Most of the species in South Australia assumed to be susceptible have been designated as such based on observation of disease symptoms in the field or isolation of *P. cinnamomi* from root and soil samples. In many cases, *P. cinnamomi* was not confirmed as the cause of disease. This lack of knowledge has hampered the management of Phytophthora dieback in South Australia (Velzeboer *et al.*, 2005). Therefore, the aim of this study was to assess the susceptibility of selected South Australian native plant species to *P. cinnamomi* in a greenhouse, where conditions were conducive for disease.

Materials and methods

Experiments were carried out to assess the susceptibility of South Australian native plant species to *P. cinnamomi* and to assess the effect of seedling age on susceptibility to infection by *P. cinnamomi*. The method of inoculation was adapted from Shearer *et al*. (2004). Based on the results of preliminary experiments, seedlings were planted in limed University of California (UC) mix, unless otherwise stated, and the soil inoculated with infested pine wood plugs.

Testing the susceptibility of South Australian native plant species

Preparation of planting material

Plant species native to South Australia were selected for testing on the basis of a list of threatened species compiled by Velzeboer *et al*. (2005), their relative importance in the communities of the Mount Lofty Ranges and availability of seeds. Seeds and seedlings for the experiments were obtained from the Seed Conservation Centre, Adelaide Botanic Gardens and Blackwood Nursery, South Australia. Although seeds of 45 species were sown in potting mix, only 37 species, including 15 threatened species, were successfully raised in numbers sufficient for testing. Two species, *Prostanthera eurybioides* and *Correa decumbens* were prepared through cuttings. Cuttings of *Pr. eurybioides* were obtained from five plants raised from seeds obtained from the Seed Conservation Centre, while *C. decumbens* was propagated through cuttings obtained from a small population at Mount Bold Conservation Park (35º05’06.11”S, 138º43’10.83”E, elevation 270 m a.s.l). As the numbers of seedlings were limited and obtained at different times, susceptibility testing was carried out in batches and with different numbers of replicates. In all, 37 species from 11 families were assessed,

representing 22 common, six endangered, four vulnerable, four rare and one uncommon species (Velzeboer *et al.*, 2005) (Table 1). Seeds of *Eucalyptus sieberi*, a known susceptible species, were purchased from Forest Products Commission, Western Australia and included as a susceptible control, where disease was expected to develop after inoculation.

All experimental plants were maintained in a greenhouse at the Waite Campus, South Australia (34°58'15.13"S, 138°38'25.18"E, elevation 140 m a.s.l). Seedlings were raised in potting mix for native species and later transplanted to 15-cm diameter pots, free-draining pots filled with limed UC mix with a pH of about 6.5. UC mix was prepared as described by Naseri *et al*. (2008). At the time of transplanting, 3 g of Osmocote (Scotts Australia Pty Ltd), a slow release fertiliser for Australian native plants with formulation 17 N: 1.6 P: 8.7 K + trace elements, were applied to each pot. No fertiliser was applied thereafter. Six species (*Pr. eurybioides*, *Pultenaea graveolens*, *Pomaderris halmaturina* ssp. *halmaturina*, *Olearia pannosa* ssp. *pannosa*, *Oreomyrrhis eriopoda* and *Glycine tabacina*) failed to thrive in UC mix. Freshly prepared seedlings or cuttings were transplanted into Bio Gro® native potting mix (Bio Gro, South Adelaide). *Allocasuarina robusta* was tested twice in UC mix at different times and *Pr. eurybioides* was tested twice in UC mix and once in Bio Gro® to determine if soil type was likely to have a major effect on response to inoculation with *P. cinnamomi*.

At transplanting, two centrifuge tubes (1 x 10 cm) were inserted close to the roots on opposite sides of the plant to allow for subsequent placement of inoculum without disturbing the substrate or the roots. The pots were watered daily to saturation and left to drain. Temperature in the greenhouse fluctuated between 17 and 29°C depending on the season. During summer when sunlight was strong, shade cloth was

used to reduce sunlight by 50%. The plants were maintained in this way until inoculation.

Phytophthora cinnamomi isolates

Phytophthora cinnamomi isolates 71a and SC4, both from South Australia, were used for susceptibility testing. Isolate 71a was obtained from a soil sample collected from around the roots of a dead *Xanthorrhoea semiplana* ssp. *tateana* on Kangaroo Island (35°47' 55.78"S, 137°27' 33.27"E, 62 m a.s.l.) on 5 October 2007 (Williams *et al.*, 2007) and SC4 was isolated from soil collected around a dead *X. semiplana* in Scott Creek Conservation Park (35°05' 10.24" S, 138°41' 52.98" E, 380 m a.s.l.) on 3 June 2008. Both isolates were confirmed as *P. cinnamomi* in June 2008 using a polymerase chain reaction assay at the Centre for Phytophthora Science and Management, Murdoch University, Western Australia, and diagnostic sequences of the internal transcribed spacer regions submitted to Genbank (accession numbers JQ306322 and JQ306323).

Preparation of infested pine wood plugs

Live branches, about 1.0-1.5 cm in diameter, were obtained from mature *Pinus radiata* trees, processed and inoculated with *P. cinnamomi* as described by Butcher *et al.* (1984). Infested pine plugs for isolates 71a and SC4 were prepared separately.

Inoculation of pots

When seedlings were 3-6 months old, the centrifuge tubes were removed from the pots and two pine plugs infested with *P. cinnamomi* isolate 71a were inserted into one hole and two infested with isolate SC4 were inserted into the other hole. Between four and

15 plants of each species were inoculated. *E. sieberi* was included as a susceptible control. Up to five plants of each species, including *E. sieberi*, were mock-inoculated with sterilised blank pine plugs as pathogen-free controls. After inoculation, the pots containing the plants were placed in 2-L plastic containers and flooded with tap water for 48 h to stimulate production of zoospores (Davison & Tay, 1987). The water was then decanted such that up to 4 cm depth was maintained in the container to keep the potting mix moist. A disinfected probe (G Bug, Measurement Engineering Australia) was later inserted into one pot to measure the water potential of the potting mix for the duration of the experiment. Water potential was always in the range of -1 to -9 kPa.

Plants were arranged in a randomised complete block design and watered daily. All plants were observed daily for dieback symptoms and date of plant death was recorded. Altogether, the 37 plant species were tested in seven batches and all plants tested were inoculated in winter and spring 2010 except for batch 3 which was inoculated in summer (January).

Effect of seedling age on susceptibility to Phytophthora dieback

Six species that differed in susceptibility, as determined in this study, were selected to determine the effect of seedling age on the response to infection by *P. cinnamomi*. Seeds of *Eucalyptus goniocalyx*, *E. viminalis* var. *viminalis*, *E. cladocalyx*, *Al. robusta*, *Kunzea pomifera* and *Acacia paradoxa* were procured from Blackwood Nursery (South Australia), sown in potting mix in March and June 2010 and seedlings maintained as described above. Seedlings 100 and 180 days from the date of sowing were inoculated with infested pine plugs on 6 October 2010, 15 replicates of each species. Another five plants of each age and species were inoculated with blank plugs as pathogen-free controls. *Eucalyptus sieberi* seedlings, 70 days old, were inoculated as susceptible

controls. All plants were arranged in a randomised complete block and monitored for disease symptoms and mortality for 12 weeks.

Data collection

Plants (whether inoculated or control) were harvested at death and the date recorded. At the time of harvest, soil was baited to assess presence of *P. cinnamomi*. About 30 g of the potting mix together with root pieces from each pot were collected in a plastic cup containing 150 mL of reverse osmosis (RO) water. Five to six pairs of cotyledons of 3-week-old *E. sieberi* were floated on the resulting soil suspension as bait for *P. cinnamomi* (Marks & Kassaby, 1974). *Phytophthora cinnamomi* was assumed to be present if diagnostic sporangia could be seen growing from the edges of cotyledons under x100 magnification after incubation in the dark at ambient temperature for 4 to 8 days. The harvested plants were washed under running tap water and the roots were examined for lesions and rot. Sections of fine and lateral roots and the collar region with lesions were excised, surface sterilised in 1% sodium hypochlorite for 1 min, rinsed three times in sterile distilled water and allowed to dry on sterile paper towel. If no lesion was observed, root sections were selected at random. The epidermis of the roots was removed and both ends of each section, which might have absorbed sodium hypochlorite, were cut and discarded. The root sections were cut in half and the cut surface gently pressed onto *Phytophthora*-selective medium, P_{10}ARPH (O'Gara *et al.*, 1996), to ensure good contact with the medium. The collar region was prepared in a similar manner. Generally about 10 to 12 pieces of root tissue from each plant were plated. If coenocytic hyphae with numerous coralloid structures and spherical swellings could be observed to emerge from the root tissue within 1-2 days of incubation in the dark at 25°C, *P. cinnamomi* was assumed to be present. If tissue samples, particularly

from the collar region, collected at the time of plant death yielded *P. cinnamomi*, then Phytophthora root rot was considered to be the cause of death; otherwise, the plant was considered as censored in the calculation of mortality (see below). Reisolation of *P. cinnamomi* from root tissue was considered part of the definition of mortality, to reduce the risk of misclassifying a response as susceptible when the cause of death was, for example, excessively moist soil (Davison & Tay, 1987).

At the end of experiments, all surviving plants were harvested (and treated as censored in the analyses), washed and roots examined for symptoms, such as lesions. Root samples were collected from each plant to test for the presence of *P. cinnamomi* and all potting mix from each pot was baited as described above.

Statistical analysis

Percentage plant mortality due to Phytophthora dieback was calculated based on the number of dead plants from which *P. cinnamomi* was reisolated from root samples and the total number of plants inoculated. Kaplan-Meier survival curves of the inoculated and the non-inoculated plants within the same species were compared using a Log-Rank (Mantel-Cox) test performed using GraphPad Prism (version 5.04 for Windows, GraphPad Software) at $P \leq 0.05$. Kaplan-Meier survival curves were prepared for all species in batches 1, 2 and 3, except where control plants were not included, viz. *E. odorata* and *C. aemula*, for which only four plants were available. Percentage plant mortality and severity of disease symptoms observed were used to categorise species as highly susceptible, moderately susceptible, slightly susceptible, resistant but host and resistant and non-host, as described by Barker & Wardlaw (1995).

Results

Disease symptoms on positive control, *Eucalyptus sieberi*

The high mortality in the positive control indicated that the *P. cinnamomi* isolates used were pathogenic (Table 2). Inoculated plants of *E. sieberi* started to wilt and die 2 weeks after inoculation and mortality increased steeply in the following 6 weeks (Figures 1-3). By the 17th week after inoculation, over 80% of the inoculated *E. sieberi* had died in all three batches. Plants wilted suddenly, followed by rapid death. Roots of all dead plants had rotted completely and turned dark brown. Neither leaf chlorosis nor defoliation was observed prior to plant death. *Phytophthora cinnamomi* was readily and consistently reisolated from this material (Table 2). Soil samples collected when each plant died consistently showed the presence of *P. cinnamomi* (Table 3). One non-inoculated *E. sieberi* plant in Batch 1 and two in Batch 2 died but *P. cinnamomi* was not reisolated from root samples. Comparison of the survival curves showed that mortality of inoculated *E. sieberi* seedlings was significantly greater ($P < 0.01$) than that of non-inoculated seedlings (Table 2). The survival curves of inoculated *E. sieberi* in the three batches were similar ($P > 0.05$). Between 71.4 and 100% of all potting mix samples from pots that contained inoculated *E. sieberi* yielded *P. cinnamomi* (Table 3). The pathogen was not isolated from the non-inoculated controls.

Susceptibility of 37 South Australian native plant species to infection by *P. cinnamomi*

The species tested varied in response to inoculation by *P. cinnamomi*. The majority of the inoculated plants of *Eucalyptus baxteri*, *Pultenaea daphnoides*, *Pu. largiflorens*, *Pu. graveolens*, *Platylobium obstusangulum*, *Al. robusta*, *Al. muelleriana*, *Banksia*

marginata and *K. pomifera* died quickly after inoculation. By the end of the 16th week after inoculation, over 80% of the inoculated plants of these species had died, suggesting they were highly susceptible (Table 2; Figures 1-3). Diseased plants of all these species exhibited sudden and severe wilt, followed by rapid plant death. At the time of death they showed severe root necrosis with almost complete loss of fine roots and in many cases rot had girdled the collar region. Root samples from fine and lateral roots and the collar region consistently yielded *P. cinnamomi* (Table 2). A few control plants of these species also died during the period of observation. One plant each of *E. baxteri*, *Pu. daphnoides*, *Pu. graveolens*, two each of *Pl. obtusangulum*, *B. marginata* and *K. pomifera* and one *A. robusta* in batch 3 died. Root samples from these dead plants did not yield *P. cinnamomi*. Mortality of the inoculated plants was significantly greater than that of the corresponding non-inoculated controls ($P < 0.05$) for all species except for *Pl. obtusangulum* and *Pu. daphnoides* (Table 2).

Individual plants of 15 species, namely *Eucalyptus cladocalyx*, *E. viminalis* var. *viminalis*, *E. odorata*, *Leptospermum continentale*, *L. myrsinoides*, *L. juniperium*, *Acacia verniciflua*, *Allocasuarina verticillata*, *O. pannosa* ssp. *pannosa*, *Hakea rostrata*, *C. decumbens*, *C. aemula*, *C. calycina*, *Prostanthera eurybioides* and *Po. halmaturina* ssp. *halmaturina*, varied in susceptibility to *P. cinnamomi*. A variable number of inoculated plants, ranging from two to 11 from each of these species, died of Phytophthora dieback during the period of observation (Table 2). Responses varied from sudden death to slow decline. In the case of slow decline, leaves initially turned pale green and wilted and, in the case of *Eucalyptus* species, turned brown and abscised. Diseased plants were weak with flaccid and pale green leaves. Death occurred weeks or months after the initial appearance of symptoms. However, a small number of plants of *E. viminalis* var. *viminalis*, *C. aemula* and *H. rostrata* showed

sudden wilting and rapid death. Plants of these species showed varying severity of root rot, from lesions confined to a few spots along fine roots to extensive rot, suggesting they were moderately susceptible (Table 4). *P. cinnamomi* was regularly isolated from fine and lateral root samples that showed spot lesions and occasionally from the collar. In one plant of *E. viminalis* var. *viminalis*, *P. cinnamomi* was observed to have spread from one lateral root to a branch along one side of the stem, causing the infected branch to wilt and die. Some plants of each of these species remained alive and healthy until the end of experiment, although root samples and potting mix consistently yielded *P. cinnamomi* (Tables 2 and 3). A variable number of the non-inoculated control plants also died during the observation period, ranging from one plant each of *E. viminalis* var. *viminalis*, *L. myrsinoides*, *Po. halmaturina* ssp. *halmaturina* and *O. pannosa* ssp. *pannosa* to four plants of *H. rostrata*. Except for *Correa decumbens*, there was no significant difference between mortality of the inoculated and non-inoculated plants within each species ($P > 0.05$).

Mortality of inoculated *Pr. eurybioides,* which was tested twice in UC mix, ranged from 46.7 (batch 1, inoculated in spring) to 73.3% (batch 6, winter). Plant mortality was 46.7% when tested in Bio Gro® in batch 7 (inoculated in winter). *Allocasuarina. robusta* was tested twice in UC mix, with 80% mortality in the first test, inoculated in summer, and 20% in the second, in winter.

For six species, namely *Acacia paradoxa*, *A. melanoxylon*, *A. spooneri*, *A. leiophylla*, *Eucalyptus microcarpa* and *E. dalrympleana*, mortality of inoculated plants was very low (Table 2). Only one plant each of *A. paradoxa*, *A. melanoxylon*, *A. spooneri* and *E. microcarpa,* and two plants each of *A. leiophylla* and *E. dalrympleana* died and reisolation yielded *P. cinnamomi*. Root lesions, if any, did not develop into rot but remained confined to small localised lesions along fine roots from which *P.*

cinnamomi was reisolated. The majority of plants of these species remained healthy for at least 11 weeks after inoculation suggesting they were only slightly susceptible (Figures 1-3). Among the non-inoculated plants, only one plant of *E. dalrympleana* died. There was no significant difference in mortality between the inoculated and the non-inoculated plants for any of these species.

All inoculated plants of *Acacia enterocarpa* and *A. pinguifolia* remained healthy and continued to grow vigorously, reaching a height of 78.7 cm, an increase of 65 cm by the end of experiment. No chlorosis appeared in the phyllodes of either the inoculated or non-inoculated plants. Roots remained light brown and healthy. However, root tissue from 50% and 40% of inoculated seedlings of *A. enterocarpa* and *A. pinguifolia*, respectively, yielded *P. cinnamomi* at the end of experiment, suggesting that these plants were resistant to Phytophthora dieback. None of the control plants of these species died or yielded *P. cinnamomi*.

Brachyscome diversifolia was the only species from which *P. cinnamomi* was never reisolated from root tissue, suggesting that this species might not be a host of the pathogen (Table 4). Of the nine inoculated plants, two *Br. diversifolia* plants died during the study period, but *P. cinnamomi* was not reisolated from the roots. The remaining seven plants continued to grow without showing any noticeable dieback symptoms at the end of the experiment.

Results for *Austrodanthonia carphoides*, *Glycine tabacina*, *Hakea rugosa* and *Oreomyrrhis eriopoda* were inconclusive because all of the inoculated and non-inoculated plants died during the experiment.

Effect of age of seedlings on plant susceptibility to Phytophthora dieback

Mortality of the susceptible control plants, *E. sieberi,* reached 66.7% 12 weeks after inoculation, which was significantly more than for the non-inoculated control (Table 4), indicating that the experimental conditions were conducive for *P. cinnamomi* to infect and kill plants.

Of the six species tested, seedling age influenced the response only of *E. viminalis* var. *viminalis* to infection by *P. cinnamomi*. Eight seedlings of *Eucalyptus viminalis* var. *viminalis* inoculated when 100 days old died during the period of observation (12 weeks) whereas none inoculated when 180 days old died (significant difference, $P < 0.01$). The response of *Eucalyptus goniocalyx*, *E. cladocalyx*, *Ac. paradoxa*, *Al. robusta* and *K. pomifera* to inoculation with *P. cinnamomi* at 100 and 180 days did not differ. At 12 weeks after inoculation, mortality of *Al. robusta* was 40% and *K. pomifera* 26.7% to 33.3% while that for both *E. goniocalyx* and *E. cladocalyx* was 6.7%. Three control plants, one from *E. goniocalyx* at 180 days old and two from *E. viminalis* var. *viminalis*, one each at 100 and 180 days old, died during the experiment, but *P. cinnamomi* was not reisolated from the roots.

Discussion

The 37 species of South Australian native plants studied showed various degrees of susceptibility to Phytophthora dieback. Of the 15 threatened or uncommon species tested, two were highly susceptible, six moderately susceptible, two slightly susceptible, three resistant and results for two species were inconclusive. *B. diversifolia*, an endangered perennial herb, was the only species not infected by *P. cinnamomi*. Of these 15 species, only *Pu. graveolens*, from the provenance of Brisbane

Ranges, Victoria, had been tested for susceptibility before and, likewise, found to be highly susceptible (Peters & Weste, 1997).

Of the 22 common species tested, seven were highly susceptible to the disease, nine moderately susceptible, four slightly susceptible and results from two species were inconclusive. The high susceptibility of *E. baxteri* to Phytophthora dieback, which is consistent with a report by Podger & Batini (1971), may be a cause for concern because this species is abundant in the high rainfall areas of South Australia, namely southern parts of the Mount Lofty Ranges and Kangaroo Island, where *P. cinnamomi* is widespread (Williams *et al.*, 2007).

Although *Pl. obstusangulum* and *Pu. daphnoides* were highly susceptible to Phytophthora dieback, dieback of these species in their natural habitats in South Australia has not been reported. At Mount Bold Reservoir, Mount Lofty Ranges, where both species are common and *P. cinnamomi* has been isolated, mortality of these species was not evident and most of the dead plants were *X. semiplana* and *Isopogon ceratophyllus* (data not shown). The apparent differences in plant susceptibility between greenhouse and field may reflect differences in environmental and soil conditions. In the greenhouse, the plants were exposed to *P. cinnamomi* in conditions of temperature, moisture and medium conducive for infection and disease, while in the field environment, the conditions fluctuate widely.

Considerable variation in susceptibility among plants from different genera and among species (inter-specific) from the same family was observed among the plants tested. Within the Fabaceae, species of *Acacia* tended to be resistant to the disease while all three *Pultenaea* species tested were highly susceptible. Also reported to be highly susceptible in greenhouse experiments were *Pultenaea hibbertioides*, *Pu. paleacea*, *Pu. prostrata* in Tasmania (Barker & Wardlaw, 1995) and *Pu. subalpina* in

Victoria (Reiter *et al.*, 2004). Within the Myrtaceae, responses among species of *Eucalyptus* to inoculation with *P. cinnamomi* varied widely, in that *E. microcarpa* and *E. dalrympleana* were resistant, *E. viminalis* var. *viminalis* and *E. cladocalyx* were moderately susceptible and *E. baxteri* was highly susceptible. This may reflect the genetic variability of *Eucalyptus* species that occupy different ecosystems. Con-generic and inter-specific variation in susceptibility to Phytophthora dieback among Australian native plant species is commonly reported in literature (Wills, 1993; Barker & Wardlaw, 1995; Shearer *et al.*, 2004).

P. cinnamomi is generally considered to be a pathogen of woody perennials, with herbs, grasses and geophytes not affected (Wills, 1993; Shearer *et al.*, 2004). In the present study, none of the herbaceous species tested; *viz. Br. diversifolia*, *G. tabacina*, *Or. eriopoda* and *Au. carphoides* were affected by the pathogen. Although most plants of *G. tabacina*, *O. eriopoda* and *Au. carphoides*, both inoculated and non-inoculated, died, *P. cinnamomi* was isolated only from one inoculated plant of *O. eriopoda*. Therefore, it cannot be concluded that Phytophthora dieback was the cause of death. Barker & Wardlaw (1995) reported high mortality of the herbs *Mitrasacme distylis*, *Stylidium despectum* and *S. perpusillum* in the greenhouse; death may be due to the short-lived nature of these plants and, perhaps, the moist potting mix rather than *P. cinnamomi*.

Although herbs and orchids are unlikely to be affected, there is a need to assess the indirect impact of the disease on these species (Shearer *et al.*, 2007). Of the 145 threatened South Australian native plant species listed by Velzeboer *et al.* (2005) as potentially vulnerable to infection by *P. cinnamomi*, 38% are trees or woody shrubs, 30% are herbs and 32% are orchids. Several populations of the woody species, such as *Euphrasia collina* ssp. *osbonii*, *Sprengelia incarnata*, *Melaleuca squamea* and

Grevillea aquifolium, are located in *P. cinnamomi*-infested areas. Common overstorey species such as eucalypts and acacias were included in the study because death due to Phytophthora dieback may affect understorey plants and hence alter the community structure and function (Weste 1986). However, the impact on South Australian native herbs, grasses and geophytes remains to be determined.

The response of plant species to infection by *P. cinnamomi* is sometimes inconsistent even in the greenhouse. For example, *Al. robusta* was highly susceptible in the first test in UC mix, inoculated in summer, but moderately susceptible in the second. Whereas *Pr. eurybioides* was consistently deemed susceptible when tested twice in UC mix and once in Bio Gro®, mortality rates were 46.7% in Bio Gro® (batch 7), and 46.7 and 73.3% in UC mix (batch 1 and batch 6, respectively). Unfortunately, *E. sieberi* was not included as a susceptible control in batches 4-7. Differences between batches may arise from variation in experimental conditions, possibly seasonal, between tests. Slight variation in the time of inoculation (seasonal susceptibility), pH of the potting mix, temperature and watering regime can affect the growth and pathogenicity of *P. cinnamomi* (You *et al.*, 1996; King & Dale, 2002). All these factors could cause variation in response to inoculation between tests. The variation in susceptibility between tests suggests that assigning a level of susceptibility to a species should be based on the results of multiple tests and not on a single experiment (Shearer *et al.*, 2007). The greater consistency of response observed for *E. sieberi* may reflect the use of younger seedlings, which are highly susceptible (Smith & Marks, 1982).

Of the six species selected to assess the effect of seedling age on susceptibility to *P. cinnamomi,* only *E. viminalis* var. *viminalis* showed decreased susceptibility with increase in age. All species tested were fast-growing plants, and seedlings at 100 and 180 days old had mature root systems with dense lateral and feeder roots. The

susceptibility to *P. cinnamomi* of plants with mature root systems and of younger seedlings having only seminal roots should be compared.

At the end of the experiment, root samples from 70% of all inoculated plants yielded *P. cinnamomi*. Of particular concern, two endangered plant species which are included in revegetation projects, *Ac. enterocarpa* and *Ac. pinguifolia* (Obst, 2005), were hosts of the pathogen. If asymptomatic plants with cryptic infection were planted in the natural ecosystem, they would provide a long-term source of inoculum for susceptible native plants in the vicinity. Therefore, ensuring that plant materials intended for revegetation are free from *P. cinnamomi* before being planted in the field is of the utmost importance. Furthermore, the use of *Acacia* spp. as companion plants to protect susceptible native species from infection by *P. cinnamomi* (D'Souza *et al.*, 2004) should be carried out with caution.

Susceptible and endangered species such as *Al. robusta* and *E. viminalis* var. *viminalis*, which occur in small populations with restricted geographical distribution, are at risk from *P. cinnamomi*. For example, the total population of *Al. robusta* in South Australia is estimated to be 200 plants, all of which are confined to one location on the lower Fleurieu Peninsula (Obst, 2005). Similarly, *E. viminalis* var. *viminalis*, a rare species in South Australia, is confined to higher parts of the central Mount Lofty Ranges (Nicolle, 1997). About half of the populations of these species are located near to areas known to be infested with *P. cinnamomi*, putting them at a high risk of extinction if a chance infection occurs (Velzeboer *et al.*, 2005).

This is the first detailed study of the susceptibility of South Australian native flora to Phytophthora dieback and findings contribute to knowledge of the threat posed by the disease to South Australia. The information generated here may help in choosing species for conservation purposes. Natural populations of susceptible species identified

in this study, particularly the endangered and rare species such as *Al. robusta* and *C. aemula*, should be located and tested for *P. cinnamomi* and, if at risk, appropriate measures, including *ex-situ* conservation, should then be taken to protect them. For the common and susceptible species such as *E. baxteri*, research on selection of resistant plants and use of *Acacia* spp. with ability to protect native species from *P. cinnamomi* should be investigated to facilitate conservation and revegetation.

Acknowledgements

The authors thank the Australian Research Council for funding this research and the following for financial and logistical support; South Australian Department of Environment and Natural Resources, SA Water, Adelaide Hills Council, Adelaide and Mount Lofty Natural Resource Management Board, City of Tea Tree Gully Council, Department of Transport, Energy and Infrastructure, Forestry SA, Primary Industries and Resources South Australia Forestry, South Australian Murray Darling Basin Natural Resource Management Board and the University of Adelaide. We also thank the Sarawak State Government, Malaysia for sponsoring the PhD research of Mr KHK; Dr N. M. Williams, D. White and Dr T. Burgess, Centre for Phytophthora Science and Management, Murdoch University, Western Australia for confirming identification of isolates 71a and SC4 and ITS sequencing; and Phillip Ainsley, Seed Conservation Centre, Department of Environment and Natural Resources for providing seeds and seedlings of threatened species.

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Figure 1 Response of the eight plant species in Batch 1 to infection by *Phytophthora cinnamomi*. The plants were inoculated by inserting pine wood plugs infested with *P. cinnamomi* into the potting mix on 3 October 2010 and assessed 21 weeks later. Each figure shows the Kaplan-Meier survival estimates for the inoculated and non-inoculated plants within the same species. The numbers of replicates of the inoculated and non-inoculated plants within each species and the significance of the Log-Rank (Mantel-Cox) test are given in Table 2. The species, in order of increasing susceptibility to Phytophthora dieback, are: (a) *Acacia melanoxylon*; (b) *Acacia paradoxa*; (c) *Leptospermum continentale*; (d) *Leptospermum juniperinum*; (e) *Prostanthera eurybioides*; (f) *Acacia verniciflua*; (g) *Correa decumbens*; (h) *Platylobium obtusangulum*; (i) *Eucalyptus sieberi* as susceptible control. (—) control plants; (- - -) inoculated plants.

Figure 2 Response of the 10 plant species in Batch 2 to infection by *Phytophthora cinnamomi*. The plants were inoculated by inserting pine wood plugs infested with *P. cinnamomi* into the potting mix on 16 November 2010 and assessed 18 weeks later. Each figure shows the Kaplan-Meier survival estimates for the inoculated and non-inoculated plants within the same species. The numbers of replicates of the inoculated and non-inoculated plants within each species and the significance of the Log-rank (Mantel-Cox) test are given in Table 2. The species, in order of increasing susceptibility to Phytophthora dieback, are (a) *Acacia leiophylla*; (b) *Leptospermum myrsinoides*; (c) *Eucalyptus microcarpa*; (d) *Eucalyptus dalrympleana*; (e) *Eucalyptus cladocalyx*; (f) *Eucalyptus viminalis* var. *viminalis*; (g) *Eucalyptus baxteri*; (h) *Pultenaea largiflorens*; (i) *Pultenaea graveolens*; (j) *Pultenaea daphnoides*; (k) *Eucalyptus sieberi* as susceptible control; (—) control plants; (- - -) inoculated plants.

Figure 3 Response of the seven plant species in Batch 3 to infection by *Phytophthora cinnamomi*. The plants were inoculated by inserting pine wood plugs infested with *P. cinnamomi* into the potting mix on 6 January 2011 and assessed 12 weeks later. Each figure shows the Kaplan-Meier survival estimates for the inoculated and non-inoculated plants within the same species. The numbers of replicates of the inoculated and non-inoculated plants within each species and the significance of the Log-rank (Mantel-Cox) test are given in Table 2. The species, in order of increasing susceptibility to Phytophthora dieback, are (a) *Acacia enterocarpa*; (b) *Acacia pinguifolia:* (c) *Acacia spooneri;* (d) *Brachyscome diversifolia*; (e) *Allocasuarina robusta*; (f) *Banksia marginata*; (g) *Kunzea pomifera*; (h) *Eucalyptus sieberi* as susceptible control; (—) control plants: (- - -) inoculated plants.

Table 1 List of South Australian native plant species, arranged by conservation status, tested for susceptibility to Phytophthora dieback

No.	Species	Common name	Conservation Status[a] EPBC Act 1999	NPW Act 1972	Other	Family
Threatened species						
1	*Acacia enterocarpa*[b]	Jumping-jack wattle	E	E		Fabaceae
2	*A. pinguifolia*[b]	Fat-leaf wattle	E	E		Fabaceae
3	*Allocasuarina robusta*[b]	Mount Compass oak-bush		E		Casuarinaceae
4	*Brachyscome diversifolia*[b]	Tall daisy		E		Asteraceae
5	*Oreomyrrhis eriopoda*[b]	Australian carraway		E		Umbelliferae
6	*Prostanthera eurybioides*[b]	Monarto mintbush	E	E		Lamiaceae
7	*Correa calycina* var. *calycina*[b]	Hindmarsh correa	V	V		Rutaceae
8	*Glycine tabacina*[b]	Variable glycine		V		Fabaceae
9	*Olearia pannosa* ssp. *pannosa*[b]	Silver daisy-bush	V	V		Compositae
10	*Pomaderris halmaturina* ssp. *halmaturina*[b]	Kangaroo Island pomaderris	V	V		Rhamnaceae
11	*Acacia spooneri*[b]	Nectar brook wattle		R		Fabaceae
12	*Correa aemula*[b]	Hairy correa		R		Rutaceae
13	*Eucalyptus dalrympleana* ssp. d*alrympleana*[b]	Mountain gum		R		Myrtaceae
14	*Eucalyptus viminalis* var. *viminalis*[b]	Manna gum		R		Myrtaceae
15	*Pultenaea graveolens*[b,c]	Scented bushpea			U	Fabaceae
Common species						
16	*Acacia leiophylla*[b]	Golden coastal wattle				Fabaceae
17	*Acacia melanoxylon*[b]	Black wattle				Fabaceae
18	*Acacia paradoxa*[b]	Prickly wattle				Fabaceae
19	*Acacia verniciflua*[b]	Varnish wattle				Fabaceae
20	*Allocasuarina verticillata*[d]	Drooping sheoak				Casuarinaceae
21	*Allocasuarina muelleriana*[d]	Slaty sheoak				Casuarinaceae
22	*Austrodanthonia carphoides*[d]	Wallaby grass				Gramineae
23	*Banksia marginata*[d]	Silver banksia				Proteaceae
24	*Correa decumbens*	Spreading correa				Rutaceae
25	*Hakea rostrata*[d]	Beaked hakea				Proteaceae
26	*Hakea rugosa*[d]	Dwarf hakea				Proteaceae
27	*Kunzea pomifera*[b]	Muntries				Myrtaceae
28	*Eucalyptus baxteri*[d]	Brown stringybark				Myrtaceae
29	*Eucalyptus cladocalyx*[b]	Sugar gum				Myrtaceae
30	*Eucalyptus microcarpa*[b]	Grey box				Myrtaceae
31	*Eucalyptus odorata*[b]	Peppermint box				Myrtaceae
32	*Leptospermum continentale*[d]	Prickly tea tree				Myrtaceae
33	*Leptospermum juniperinum*[d]	Prickly tea tree				Myrtaceae
34	*Leptospermum myrsinoides*[d]	Heath tea-tree				Myrtaceae
35	*Platylobium obtusangulum*[d]	Common flatpea				Fabaceae
36	*Pultenaea daphnoides*[b]	Large-leaf bush pea				Fabaceae
37	*Pultenaea largiflorens*[b]	Twiggy bush-pea				Fabaceae

[a]Species listed as rare (R), vulnerable (V) or endangered (E) under the National Parks and Wildwife (NPW) Act 1972, South Australia (Velzeboer et al., 2005) and Environment Protection and Biodiversity Conservation (EPBC) Act 1999.

[b]Seeds or seedlings of the species obtained from Seed Conservation Centre, Adelaide.

Cont...

[c]Species has no listing under legislation but is of conservation concern in the Mount Lofty Ranges; listed as uncommon (U) by Ainsley & Guerin (2009).
[d]Seeds obtained from Blackwood Nursery, South Australia.

Table 2 Susceptibility of selected South Australian native plants to Phytophthora dieback

No.	Species[a]	Mortality (%)[b]	No. inoculated	No. dead	No. dead plants yielding *P. cinnamomi*	No. Surviving plants yielding *P. cinnamomi*	Average survival time and range (weeks)	Log-rank (Mantel-Cox) test[c]	No. Control plants	No. control plants dead[d]	Category of susceptibility[e]
Batch 1 (inoculated in spring)											
1	*Leptospermum continentale*	26.7	15	4	4	10	16.0 (7-21)	NS	5	0	MS
2	*Leptospermum juniperinum*	33.3	15	5	5	2	11.4 (7-21)	NS	5	0	MS
3	*Acacia paradoxa*	6.7	15	1	1	12	5 (5-21)	NS	5	0	SS
4	*Acacia melanoxylon*	6.7	15	1	1	11	21	NS	5	0	SS
5	*Acacia verniciflua*	46.7	15	8	7	6	9.6 (4-15)	NS	5	0	MS
6	*Correa decumbens*	66.7	15	13	10	0	9.3 (5-14)	S	5	0	MS
7	*Prostanthera eurybioides*	46.7	15	7	7	6	14.1 (4-21)	NS	3	0	MS
8	*Platylobium obstusangulum*	83.3	6	6	5	0	7 (3-16)	NS	2	2	HS
	Eucalyptus sieberi	86.7	15	15	13	0	4.8(2-5)	S	5	1	
Batch 2 (inoculated in spring)											
9	*Eucalyptus microcarpa*	14.3	7	1	1	6	18	NS	3	0	SS
10	*Eucalyptus dalrympleana*	13.3	15	3	2	11	6 (5-18)	NS	5	1	SS
11	*Eucalyptus baxteri*	86.7	15	15	13	0	5.3 (3-9)	S	3	1	HS
12	*Eucalyptus cladocalyx*	20.0	15	4	3	10	10.5 (5-18)	NS	4	0	MS
13	*Eucalyptus viminalis* var. *viminalis*	20.0	10	2	2	7	16.5 (16-17)	NS	3	1	MS
14	*Eucalyptus odorata*	25.0	4	1	1	1	5	-	0	0	MS
15	*Acacia leiophylla*	13.3	15	4	2	5	12.8 (8-18)	NS	5	0	SS
16	*Leptospermum myrsinoides*	26.7	15	5	5	9	14.4 (11-18)	NS	4	1	MS
17	*Pultenaea daphnoides*	100	5	5	5	0	6.8 (2-10)	NS	3	1	HS
18	*Pultenaea largiflorens*	80.0	5	5	4	0	3	S	3	0	HS
19	*Pultenaea graveolens*[f]	87.5	8	8	7	0	6.3 (4-8)	S	4	1	HS
20	*Correa aemula*	50.0	4	4	2	0	2.7 (2-4)	-	0	0	MS
	Eucalyptus sieberi	80.0	15	14	12	1	12.5 (8-17)	S	5	2	

Cont...

Batch 3 (inoculated in summer)											
21	*Acacia enterocarpa*	0	4	0	0	2	12	NS	3	0	RH
22	*Acacia pinguifolia*	0	5	0	0	2	12	NS	3	0	RH
23	*Acacia spooneri*	6.7	15	1	1	11	12	NS	5	0	SS
24	*Allocasuarina robusta*	80.0	15	14	12	0	5.5 (3-12)	S	5	1	HS
25	*Brachyscome diversifolia*	0	9	2	0	0	12	NS	3	0	R
26	*Banksia marginata*	100	15	15	15	0	6.3 (3-12)	S	5	2	HS
27	*Kunzea pomifera*	93.3	15	15	14	0	6 (3-12)	S	5	2	HS
	Eucalyptus sieberi	100	15	15	15	0	6.4 (3-12)	S	5	0	
Batch 4 (inoculated in winter)											
28	*Allocasuarina robusta*	20	10	3	2	7	11.7 (7-20)	NS	10	3	MS
29	*Austrodanthonia carphoides*	0	10	10	0	0	31.0 (20-36)	-	10	10	IC
Batch 5 (inoculated in winter)											
30	*Allocasuarina verticillata*	33.3	15	6	5	5	29.7 (27-36)	NS	5	0	MS
31	*Allocasuarina muelleriana*	100	15	15	15	0	7 (4-12)	S	5	0	HS
Batch 6 (inoculated in winter)											
32	*Prostanthera eurybioides*	73.3	15	14	11	0	8.6 (3-29)	NS	5	2	MS
33	*Correa calycina*	20.0	15	7	3	0	8.6 (3-29)	NS	5	2	MS
34	*Hakea rostrata*	30.8	13	7	4	2	15.1(3-44)	NS	5	4	MS
35	*Hakea rugosa*	11.1	9	6	1	0	27.6(3-44)	S	3	3	IC
Batch 7 (inoculated in winter)											
36	*Pomaderris halmaturina* spp. *halmaturina*[f]	40.0	10	6	4	4	19.7 (4-29)	NS	5	1	MS
37	*Olearia pannosa* ssp. *pannosa*[f]	45.5	11	8	5	3	21.3 (3-41)	NS	3	1	MS
38	*Oreomyrrhis eriopoda*[f]	10.0	10	10	1	0	24.4 (4-34)	NS	4	3	IC
39	*Prostanthera eurybioides*[f]	46.7	15	15	7	0	25.3 (3-35)	NS	5	4	MS
40	*Glycine tabacina*[f]	0	15	15	0	0	25.3 (3-35)	NS	4	3	IC

[a] The 37 plant species were tested in batches and *Eucalyptus sieberi* was included within batches 1-3 as a susceptible control. Plants were grown in UC potting mix unless shown otherwise.

[b] Mortality (%) calculated based on number of plants dead after first inoculation and which yielded *P. cinnamomi.*

Cont...

[c] Log-rank (Mantel-Cox) was used to test difference between the survival curves of the inoculated and non-inoculated plants within the same species at $P \leq 0.05$. (S, significant difference; NS, no significant difference).

[d] *P. cinnamomi* was not isolated from roots or potting mix from any of the non-inoculated controls.

[e] HS: highly susceptible; mortality due to *P. cinnamomi* was ≥80%. Disease symptoms included sudden wilt, extensive root and collar rot, rapid death; *P. cinnamomi* reisolated from root tissue. MS: moderately susceptible; mortality due to *P. cinnamomi* ranged from 20 to <80%; disease symptoms included slow dieback, variable root and collar rot; *P. cinnamomi* reisolated from root tissue. SS: slightly susceptible; mortality due to *P. cinnamomi* was <20%; disease symptoms included localised lesions on fine roots; *P. cinnamomi* reisolated from root tissue. RH: resistant but host; mortality due to *P. cinnamomi* was zero; inoculated plants remained healthy but *P. cinnamomi* reisolated from root tissue. R: resistant and non-host; mortality due to *P. cinnamomi* was zero; inoculated plants remained healthy and *P. cinnamomi* was not reisolated from root tissue. IC: inconclusive result due to death of both inoculated and non-inoculated plants.

[f] Plants were grown in Bio Gro® potting mix for Australian native plants (Bio Gro).

Table 3 Reisolation of *Phytophthora cinnamomi* from potting mix collected at the time of death of seedlings of South Australian native species and at end of experiments

Species	Number of pots sampled	Number of samples yielding positive result[a]	Samples yielding positive result (%)
Batch 1			
Leptospermum continentale	15	13	86.7
Leptospermum juniperinum	15	9	60.0
Acacia paradoxa	15	11	73.3
Acacia melanoxylon	15	13	86.7
Acacia verniciflua	15	15	100
Correa decumbens	15	15	100
Prostanthera eurybioides	14	10	71.4
Platylobium obstusangulum	5	5	100
Eucalyptus sieberi	13	13	100
Batch 2			
Eucalyptus macrocarpa	7	5	71.4
Eucalyptus dalrympleana	15	15	100
Eucalyptus viminalis var. *viminalis*	9	9	100
Eucalyptus odorata	4	3	75.0
Acacia leiophylla	14	14	100
Leptospermum myrsinoides	15	12	80.0
Pultenaea daphnoides	5	5	100
Pultenaea largiflorens	5	5	100
Pultenaea graveolens	8	8	100
Correa aemula	3	3	100
Eucalyptus sieberi	7	5	71.4
Batch 3			
Acacia enterocarpa	4	2	50.0
Acacia pinguifolia	5	5	100
Acacia spooneri	13	13	100
Allocasuarina robusta	13	13	100
Brachyscome diversifolia	9	5	55.6
Banksia marginata	15	15	100
Kunzea pomifera	14	14	100
Eucalyptus sieberi	15	15	100

[a] Potting mix suspension was baited with cotyledons of 3-week-old *E. sieberi*. If sporangia were produced, then *P. cinnamomi* was assumed to be present.

Note: *P. cinnamomi* was not isolated from roots or potting mix from any of the non-inoculated controls.

Table 4 Response of 100- and 180-day-old seedlings of six South Australian native plants species 12 weeks after inoculation with *Phytophthora cinnamomi.*

No	Species	Age of seedling (days)	Mortality (%)[a]	No. inoculated	No. dead	No. yielding *P. cinnamomi*	Mean weeks until death (range)	[b]Log-rank (Mantel-Cox) test[b]	Number control	[c]Number of control dead
1	*Eucalyptus viminalis* var. *viminalis*	180	0	15	0	0	-	S	5	1
		100	53.3	15	8	8	5 (3-7)		5	1
2	*Eucalyptus goniocalyx*	180	6.7	15	1	1	8	NS	5	1
		100	0	15	1	0	0		5	0
3	*Eucalyptus cladocalyx*	180	6.7	15	1	1	11	NS	5	0
		100	6.7	15	1	1	11		5	0
4	*Allocasuarina robusta*	180	40.0	15	8	6	5.7 (5-7)	NS	5	0
		100	40.0	15	9	6	6.2 (4-7)		5	0
5	*Kunzea pomifera*	180	33.3	15	6	5	6.8 (2-11)	NS	5	0
		100	26.7	15	4	4	5.5 (3-12)		5	0
6	*Acacia paradoxa*	180	0	15	0	0	0	NS	5	0
		100	6.7	15	2	1	11		5	0
	Eucalyptus sieberi[d]	100	66.7	15	11	10	5.7 (3-11)	S	5	0

[a] Percent mortality based on number of dead plants which yielded *P. cinnamomi.*
[b] Log-rank (Mantel-Cox) test was used to compare the survival curves of the inoculated and non-inoculated plants within the same species at P ≤ 0.05. (S = significant; NS = not significant).
[c] *P. cinnamomi* was not isolated from roots or potting mix from any of the non-inoculated controls.
[d] *Eucalyptus sieberi* was included as a susceptible control.

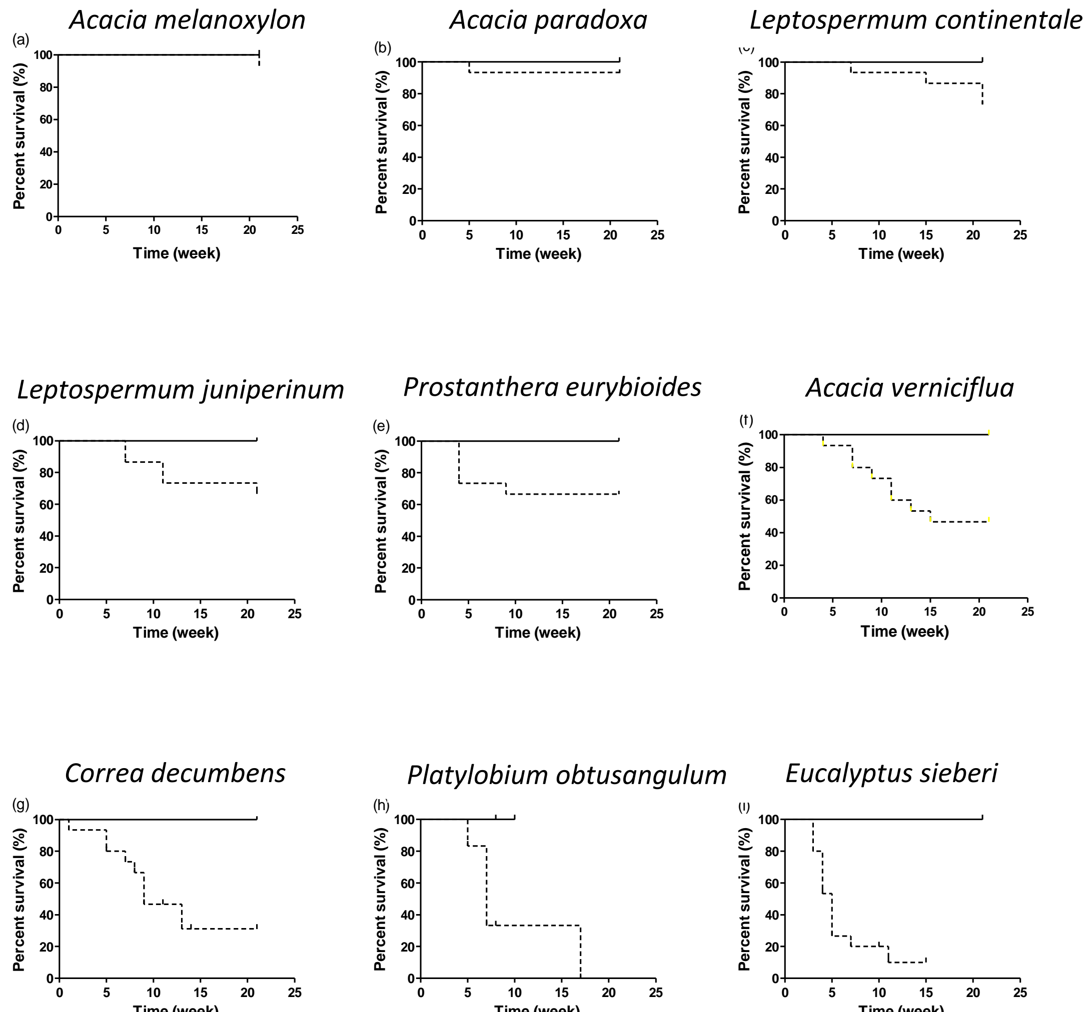

Figure 1

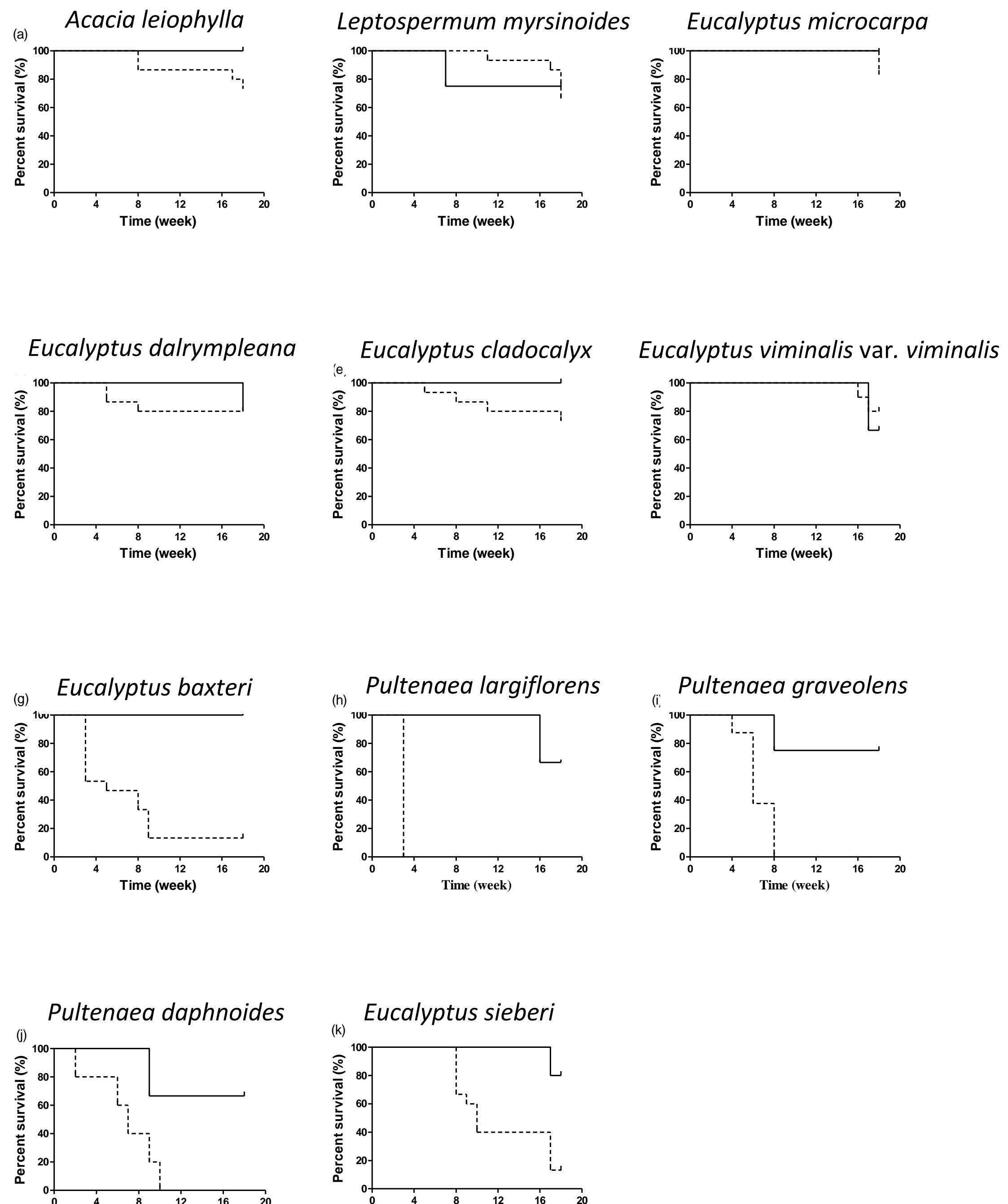

Figure 2

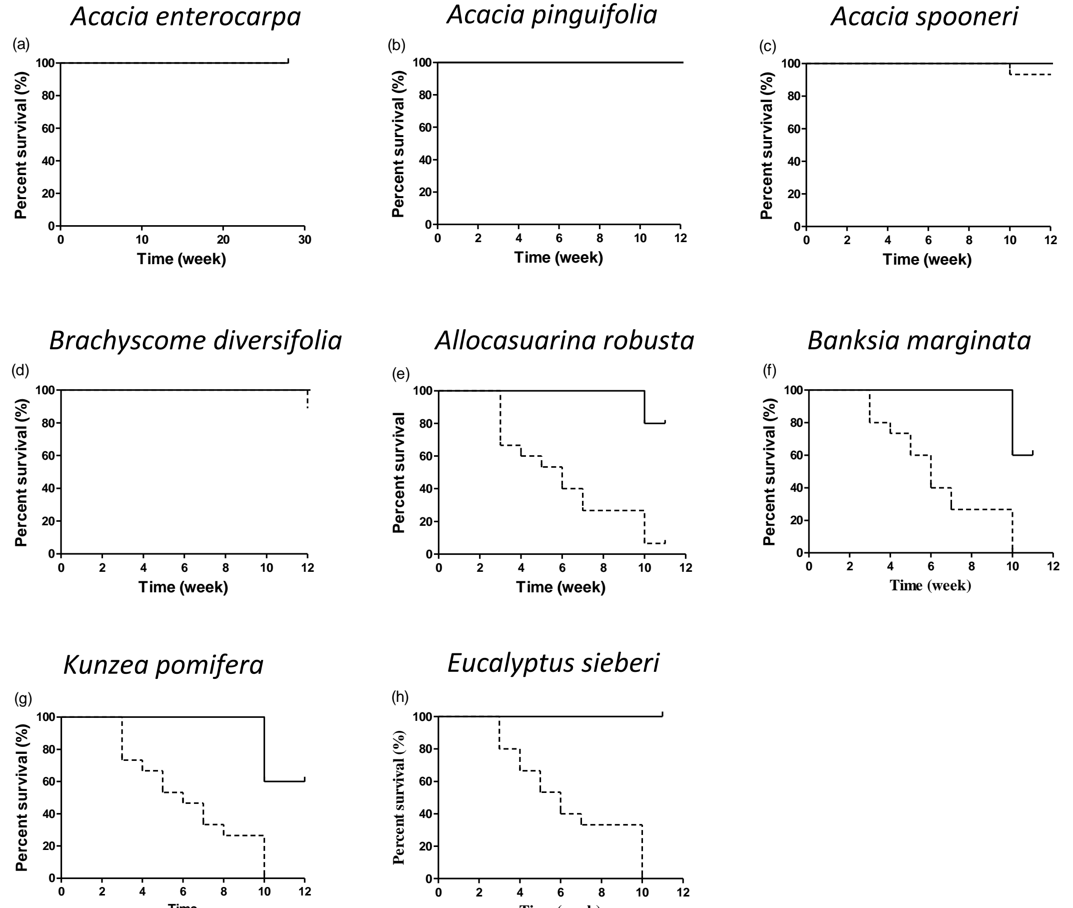

Figure 3

Chapter 5 Isolation of actinomycetes for control of Phytophthora dieback

5.1 Introduction

One of the major gaps in on-ground management of Phytophthora dieback in natural ecosystems is the lack of an effective method to control and eradicate *P. cinnamomi*. Since the 1990s, phosphite has been the main chemical used in Western Australia and Victoria, mainly to protect critically endangered but susceptible native plants in their natural habitats (Hardy *et al.*, 2001). Phosphite, however, does not kill *P. cinnamomi* and only provides temporary protection to plants by inducing defence mechanisms (Hardy *et al.*, 2001). No other chemical has been recommended specifically for the control of *P. cinnamomi* in native ecosystems. Due to the difficulty of controlling the disease in forest situations by chemical methods, biological control provides an alternative means to contain *P. cinnamomi* (Pratt, 1971).

Certain areas in Australia are reported to be less prone than others to infestation, even when *P. cinnamomi* is present in adjacent areas, as was reported in Chapter 3. These differences in vulnerability to dieback due to *P. cinnamomi* have been attributed to the presence of soil microorganisms antagonistic to the pathogen (Newhook & Podger, 1972). Soil samples from roots of various native plants such as *Eucalyptus marginata*, *C. calophylla* and *Banksia grandis* have been found to harbour a myriad of microorganisms such as bacteria, actinomycetes and fungi and a significant proportion of these microorganisms were found to be antagonistic to various root pathogens, including *P. cinnamomi* (Broadbent *et al.*, 1971; Murray, 1987). These microorganisms, particularly the actinomycetes, were able to antagonise *P. cinnamomi*

by various modes of action such as inhibiting the growth of mycelium, suppressing the formation of sporangia and the germination of zoospores (Malajczuk *et al.*, 1977b; Malajczuk *et al.*, 1983; Murray, 1987). Although the presence of microbes (and sometimes their secretions) has been reported to be necessary to stimulate formation of sporangia, most of these sporangia aborted and did not release zoospores (Malajczuk *et al.*, 1983). Antagonistic microorganisms therefore create conditions unfavourable for the growth of *P. cinnamomi* and protect the roots from infection. Actinomycetes are found in greater abundance in soil associated with the rhizosphere, that is, soil that is directly influenced by plant roots, than in the bulk soil (Crawford *et al.*, 1993). Weste & Vithanage (1978; 1977) reported that the roots of plants tolerant of infection by *P. cinnamomi*, such as *Acacia pulchella*, frequently supported larger populations of microorganisms than roots of susceptible species such as *Banksia grandis*, and postulated that severe disease occurs in areas where populations of soil microflora are small. The population of soil microflora in the rhizosphere of native plants in South Australia has not been investigated.

The aims of this study were to isolate actinomycetes from the rhizosphere of tolerant *Acacia pycnantha* and susceptible *Xanthorrhea semiplana* plants from a *Phytophthora*-infested site. Actinomycetes isolated then would be screened for antagonistic activity against *P. cinnamomi in vitro*, then tested for their ability to protect susceptible plants from Phytophthora dieback in greenhouse experiments.

5.2 Materials and methods

5.2.1 Isolation of actinomycetes from soil

Soil dilution plating is one of the most commonly used methods to isolate and enumerate actinomycetes (Dhingra & Sinclair, 1995). Rhizosphere soils were collected from around the roots of three mature plants of the tolerant *Acacia pycnantha* and three young and healthy plants of *X. semiplana* growing near stumps of dead *X. semiplana* plants at Mount Bold Catchment Reserve (see Chapter 3) on 25 June 2009 (winter). Rhizosphere soil from these plants was collected following a method adapted from Miller *et al*. (1990). Selected plants were carefully uprooted to preserve as much of the fine root system as possible. All loose soil was shaken from the roots and the soil remaining was collected to represent the rhizosphere.

The soil samples were air dried on a tray at ambient temperature for 4 d before sieving with a mesh of size 2 mm. Soil samples were then subjected to one of the following pre-treatments : (i) no treatment; (ii) air-dried for 13 d (Dhingra & Sinclair, 1995); (iii) heated at 100°C for 1 h (Athalye *et al.*, 1981); or (iv) addition of calcium carbonate, $CaCO_3$ in moist conditions for 9 d (Tsao *et al.*, 1960). These soil pre-treatments were carried out to enhance isolation and enumeration of actinomycetes from soil and to reduce contamination of cultures by bacteria and fungi (Dhingra & Sinclair, 1995). Serial dilutions (10^{-1} to 10^{-6}) of the soil samples were prepared using half-strength Ringer's solution. Ten g of soil (oven-heated equivalent weight) was added to 90 mL of half-strength Ringer's solution and mixed well using a magnetic stirrer, instead of grit as used by Miller *et al*. (1990), for 30 min. One hundred µL aliquots of each of the dilutions 10^{-2} to 10^{-6} were plated onto the following four isolation media: (i) water-yeast agar (WYE); (ii) casamino acids-yeast extract-glucose

agar (YCED); (iii) humic acids-vitamin agar (HVA); and (iv) casein glycerol agar (CGM), with three replications each. These media have been used successfully to isolate actinomycetes from soil previously (Crawford *et al.*, 1993; Istifadah, 1997). Humic acid-vitamin agar has been recommended for isolation of specific actinomycetes of genera such as *Streptomyces, Micromonospora*, *Nocardia* and *Microbispora* (Hayakawa & Nonomura, 1987). All media were amended with cycloheximide (Sigma-Aldrich) and nalidixic acid (Sigma-Aldrich) at final concentrations of 50 mg L^{-1} and 20 mg L^{-1}, to inhibit fungal and bacterial growth, respectively (Weste *et al.*, 1976; Hayakawa & Nonomura, 1987). The antibiotics were filter-sterilised using Millex®-HA 0.45 µm filters (Millipore Corporation) and added to the media after cooling to 50-55°C. Each aliquot was spread on an agar plate using a sterile L-shaped glass spreader.

All plates were incubated at 25°C for 14 to 21 days to allow formation of actinomycete colonies. Actinomycete-like colonies formed (colony forming units, CFU) on each plate were counted and the means of the three CFU counts of the three no treatment replications were used to estimate the population of the actinomycetes in the rhizosphere. Actinomycete-like colonies were picked with a loop and streaked onto fresh International *Streptomyces* Project 2 (ISP2) medium for purification (Shirling & Gottlieb, 1966). Each actinomycete isolate was labelled according to plant species, plant and isolate number. For example, AC2.10 indicates the actinomycete isolate was obtained from *Acacia pycnantha*, plant number 2 and was isolate number 10 while XS3.4 indicates that the actinomycete was isolated from *Xanthorrhoea semiplana*, plant number 3 and was isolate number 4.

Plates having between 30 and 300 actinomycete colonies were enumerated to estimate the population size. The estimated populations of actinomycetes (CFU g^{-1}

dried soil) in the rhizosphere of *Acacia pycnantha* and *Xanthorrhoea semiplana* were log transformed in order for data to be normally distributed and then compared using ANOVA at $P \leq 0.05$. Colonies of actinomycete were transferred to ISP2 plates and incubated at 25°C for 7 days before being sub-cultured onto half-strength potato dextrose agar (PDA) slants in McCartney bottles and incubated at 25°C until they were required.

5.2.2 *In vitro* antagonism tests (Dhingra & Sinclair, 1995)

All actinomycete isolates were tested for antagonism of *P. cinnamomi* on ISP2 medium. A 5 mm-diameter agar disc, containing the actinomycete isolate was cut from a plate of the well-grown culture and placed on one side of each ISP2 plate, as shown in Figure 5.1. Another 5 mm-diameter disc, cut from the edge of a 5-day old culture of *P. cinnamomi,* was placed directly opposite, at a distance of 40 mm from the disc containing the actinomycete at the same time. *P. cinnamomi* isolate 71a was used throughout the experiment (see section 2.2.1). Three replicates of each isolate were prepared and all plates were incubated at 25°C for 14 d. Control plates contained *P. cinnamomi* and a blank agar disc without an actinomycete. After 14 d incubation, the width of the inhibition zone was measured between the agar discs containing *Phytophthora cinnamomi* and the actinomycetes in each of the three replicate plates per combination and the data averaged. The strength of antagonism was classified according to the mean width of the inhibition zone as follows: 0 mm, no antagonism; > 0 to 10 mm, weak antagonism; >10 to 18 mm, moderate antagonism; and > 18 mm, strong antagonism. Eight actinomycetes which showed strong, moderate and weak inhibitory effects towards *P. cinnamomi* were selected for further assessment of their

ability to protect susceptible plants from Phytophthora dieback in greenhouse experiments, and identification using molecular techniques.

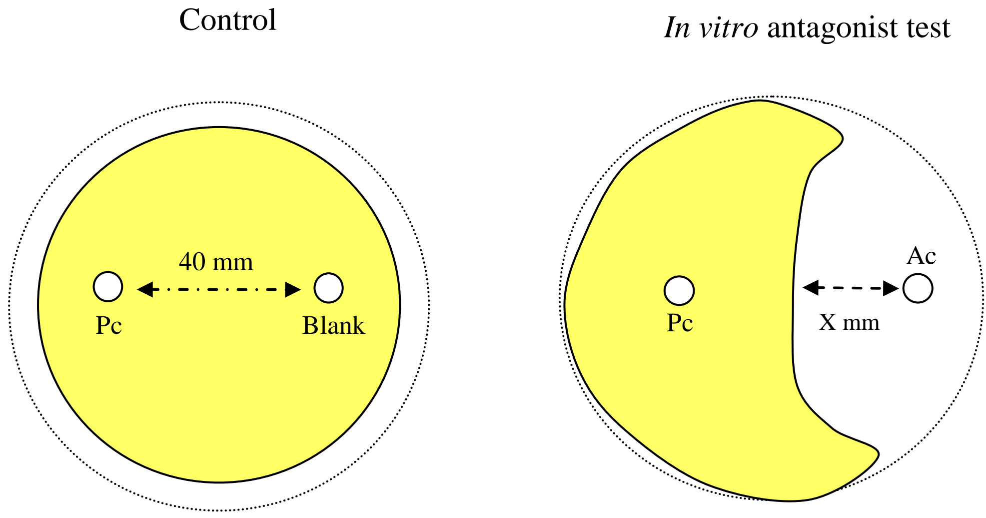

Figure 5.1 Method for testing antagonism of *Phytophthora cinnamomi* by actinomycete isolates on ISP2 medium. (Pc) *Phytophthora cinnamomi* isolate 71a; (Ac) antagonist; (x mm) width of inhibition zone measured between the discs after incubation at 25°C for 14 days. The inhibition tests were replicated three times for each isolate. The control is shown on the left.

In this study, antagonism of *P. cinnamomi* by an actinomycete isolate was estimated based on the width of the inhibition zone on ISP2 medium. However, as this does not necessarily reflect efficacy *in planta* representative weak, moderate and strong antagonists were selected for use in the greenhouse trial.

5.2.3 Testing actinomycetes for ability to protect plants in greenhouse trials

The eight actinomycete isolates selected for use in greenhouse experiments were as follows: strong antagonists AC2.10, AC2.33, AC3.26 and XS3.4; moderate antagonists AC2.25 and XS3.5; and weak antagonists AC2.39 and XS2.42.

5.2.3.1 Preparation of spores for inoculation

Spores for inoculation of plants were produced by culturing the selected actinomycete isolates on either PDA, oatmeal-agar (OA) or mannitol-soy (MS) plates. A loop-full of culture of the selected actinomycete isolates from agar slants (in McCartney bottles half-strength PDA slants) was transferred to the Petri dishes after which a drop of sterile distilled water was added. The mycelium and spores were then spread over the plate with a sterile L-shaped glass spreader. The plates were incubated at 25°C in the dark and observed every second day until spores were well developed.

5.2.3.2 Determination of density of harvested spores

Spores were harvested following the method described by Wellington & Toth (1996). Spores were removed from the mycelia by adding 5 mL half strength Ringer's solution to each plate, followed by gentle agitation with a sterilised glass spreader. The crude spore suspensions from the culture plates of the same isolates were poured into a sterilised 500-mL-glass beaker and then half strength Ringer's solution was added to produce a final volume of 300 mL of spore suspension. The spore suspension was stirred thoroughly with a magnetic stirrer for 20 min. The density of the spores in the suspension was determined following the method of Miles & Misra (1938). Serial dilutions (10^{-1} to 10^{-12}) of the spore suspension were made with sterile distilled water. A half-strength PDA plate, after drying at room temperature for 2 h in a laminar flow cabinet, was divided into six sectors and labelled 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} and 10^{-12}. Ten µL of each dilution was pipetted into the corresponding sector in triplicate as separate spots. The Petri dishes were incubated at 25°C in the dark for 5 to 10 days. The density of viable spores was estimated based on the number of countable colonies at one or two dilutions only.

5.2.3.3 Inoculation of *Eucalyptus sieberi* seedlings with antagonists

Young *E. sieberi* seedlings were used to test the ability of the selected actinomycetes to prevent or reduce Phytophthora dieback. There were four treatments as follows: (i) antagonist only; (ii) antagonist and pathogen; (iii) pathogen only; and (iv) control, without antagonist or pathogen. Each treatment had 15 replications. Germination of *E. sieberi* was carried out in Jiffy-7 pots as described in section 2.3.1.2 and seedlings were then thinned to one per pot. Fifty days after sowing, 10 mL of the actinomycete spore suspension, with spore density ranging from 3.4×10^{6} to 2.8×10^{10} spores mL^{-1}, was pipetted into the compost in each Jiffy-7 pot close to the roots and then the Jiffy-7 pot was transplanted into 15 cm-diameter pot filled with UC mix (section 2.3), pH 6.5. No attempt was made to standardise the density of spores as the main objective of this experiment was to assess if the antagonist was able to protect the test plant from infection by *P. cinnamomi*. Inoculation holes were prepared as described in section 2.3.1.2. The seedlings were allowed to grow for 2 weeks to allow the spores to germinate and colonise the roots, after which the plants were inoculated with *P. cinnamomi* using infested pine plugs and maintained as described in section 2.3.3. Spores of actinomycetes are anticipated to have germinated and colonised roots within 2 weeks of inoculation (Zvyagintsev *et al.*, 2007). For the control, the pots were inoculated with blank agar discs. At the time of inoculation with *P. cinnamomi*, heights of all the plants with treatments (i) and (iv) were recorded. The potting mix was maintained at high water potential (> -10 kPa), as described in chapter 4, through the duration of experiment.

The test plants were arranged in randomised complete block design in the greenhouse at the University of Adelaide, Waite Campus. The plants were watered

daily and observed for disease symptoms daily. When a plant died, the date was recorded and root samples were collected and tested for the presence of *P. cinnamomi* following the protocol described in section 2.4. The efficacy of all eight actinomycete isolates in controlling Phytophthora dieback was compared using a Kaplan-Meier survival curve and Log-Rank test (Machin *et al.*, 2006) performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA). At the end of experiment, the heights of all living plants from treatment (i) and (iv) were recorded and populations of actinomycetes were estimated from three pots of each treatment using the soil dilution plating method, as above. The means of plant heights, at the beginning and at the end of experiment among the different treatments were compared using one-way analysis of variance (ANOVA) using the LSD ($P \leq 0.05$).

5.2.4 Molecular techniques for identifying actinomycete isolates

The eight isolates selected for the pot experiment were identified to the species levels as follows.

5.2.4.1 Preparation of actinomycete biomass for DNA extraction

Genomic DNA for use in sequencing of the 16S rRNA gene was extracted from the biomass (mycelium and spores) of the actinomycetes. In order to obtain clean actinomycete biomass, free of agar, each actinomycete isolate was cultured on PDA, OA and MS agar overlaid with a sterilised cellophane sheet (Kaewkla, 2009). A loop-full of mycelium of the selected actinomycete isolate from the McCartney bottle was transferred to the cellophane after which a drop of sterilised distilled water was added. The mycelium was then spread over the plate with a sterile L-shaped glass rod. The

plates were incubated at 25°C in the dark and observed for 10 to 14 d until the actinomycete sporulated.

5.2.4.2 DNA extraction

Extraction of DNA from the actinomycete biomass was carried out following the protocols described by Kaewkla (2009). A loopful of actinomycete biomass, from the plate with the best growth, were aseptically scraped from the surface of the cellophane and suspended in 500 µL of TE buffer (consisting of 10 mM Tris, 10 mM EDTA with pH 7.4) in a 1.5-mL microfuge tube. Ten µL of lysozyme (10 mg/mL) was added to the tube to digest the cell walls. The suspension was homogenised and incubated at 37°C for 60 min. Ten µL of proteinase K and 32.5 µL of 10% sodium dodecyl sulphate (SDS) were added to the suspension to further lyse the cells. The suspension was mixed thoroughly and incubated at 55°C for a further 60 min. A clear solution indicated that the actinomycete cells had been lysed. One hundred µL of 5M NaCl and 65 µL of CTAB/NaCl [4.1 g NaCl, 10 g hexadecyltrimethylammonium bromide (CTAB) to 100 mL reverse osmosis (RO) water] was added to the cell lysate, mixed by gentle vortexing and incubated at 55°C for 10 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was then added to the tube, mixed well and incubated at room temperature for 30 min. The suspension was centrifuged at 12,000 *g* for 15 min to pellet the cell debris. DNA in the aqueous phase was extracted by adding an equal volume of chloroform, mixed well and incubated at room temperature for 15 min. The suspension was centrifuged and the aqueous phase was collected and transferred to a fresh microfuge tube. Ten µL of 10 mg mL^{-1} RNase was added and incubated at 37°C for 60 min to remove any remaining RNA. An equal volume of chloroform was then added, mixed well and left at room temperature for 15

min. The suspension was centrifuged and the water phase transferred to a fresh microfuge tube in the same manner as the previous centrifugation step. The colourless DNA in the aqueous phase was precipitated out by adding 10 x volume of 3M sodium acetate and 2 x volume of 95% ethanol. The suspension was kept at -20°C overnight before centrifugation at 16,000 *g* for 15 min. The supernatant was discarded and the pellet was washed twice with 70% alcohol. The pellet was dried at 55°C in a heating block with the microfuge tube open. The dried pellet was resuspended with sterile nanopure water and stored at -20°C until required. The DNA was analysed by electrophoresis on an agarose gel (0.8%) and visualised by UV light. Loading dye of 6x was added to 5 µL of DNA sample and mixed well before dispensing the DNA into the wells in the agarose gel. The loading dye contained xylene cyanol to make the DNA sample visible as a purple colour. The genomic DNA obtained from this process was used as a template for amplification of the 16S rRNA gene by PCR.

5.2.4.3 PCR amplification

Genomic DNA was used as the template for 16S rRNA gene amplification. The primers for the PCR reaction were the “actinobacteria-biased” 16S rRNA primers which can amplify a partial product (750 bp) from the 16S rRNA of any bacterium [27f (5’-AGAGTTTGATCCT GGCTCAG-3’) and 765r (5’-CTGTTTGCTCCCCACGCTTTC-3’)] (Coombs & Franco, 2003). Amplification of the 16S rRNA gene was carried out in 50 µL volumes containing the following reagents: primers (5 µM), 2µL; 765r (5 µM), 2µL; 10X Taq buffer (1X Taq buffer: 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 5µL; dNTP (10 mM), 1 µL; sterile nanopure water, 37 µL; *Taq* polymerase (Biolabs) (5 units μL^{-1}), 1 µL; and DNA template, 2 µL. The PCR amplification was performed on a PC-960G Thermal Cycler

(Corbett Research) under the following conditions: initial denaturation of the RNA template at 94°C for 2 min, 40 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min, and a final elongation cycle of 72°C for 10 min and 27°C for 1 min.

After amplification, the PCR products were confirmed by 1% agarose gel electrophoresis. The PCR amplicons generated were prepared for sequencing using Mo Bio Ultra Clean™ PCR Clean-Up™ kit (Mo Bio Laboratories, Inc.) following the manufacturer's instructions. The PCR product of *Streptomyces griseus* DSM 40855 was also digested and amplified in similar conditions and used as a positive control. A molecular weight ladder (Bio Labs, 500-1500 bp) was loaded in lane one (Kaewkla, 2009).

The PCR products were sequenced at the Flinders Sequencing Facility, Flinders University, using the protocol described by Kaewkla (2009). The primer, 27f (5 µM) and purified PCR products at the final concentration of 10 ng/100 bp were combined with 1.0 M betaine, big dye terminator V3.1 (Applied Biosystems), 5x sequencing buffer (Applied Biosystems) and made up to 20 µL with water. Reactions were taken through a cycle sequencing PCR protocol on a MyCycler Thermal Cycler (Bio-rad Laboratories). PCR extension products were then resolved using the 3100 Genetic Analyser (Applied Biosystems). The resultant sequences were compared to the NCBI (National Center for Biotechnology Information) online database using Blastn (Altschul *et al.*, 1990) available at www.ncbi.nlm.nih.gov (accessed date: 25 June 2011). Accessions with closest sequence similarity were recorded. A phylogenetic tree, based on the neighbour-joining (NJ) method (Saitou & Nei, 1987), was constructed using MEGA 5 software (Tamura *et al.*, 2007). Two common species, *Streptomyces scabiei* and *S. europaeiscabiei,* were also included as references in the construction of the phylogenetic tree. *Micromonospora echinospora*, which belongs to a different

genus from the presumed group of the isolates was added as an outlier. These reference strains were included to determine the relatedness or phylogeny of the different isolates.

5.3 Results

5.3.1. Estimation of actinomycete populations

Colonies of actinomycetes formed on all culture plates. Representatives of the actinomycete colonies on the four media are shown in Figure 5.2. Contamination by other bacteria and fungi was uncommon. The average number of actinomycete colonies on the four media inoculated with untreated soil was used to quantify the population of actinomycetes in the rhizosphere of *A. pycnantha* and *X. semiplana*. The populations of actinomycetes ranged from 1.3 to 3.3 ± 1.8 x 10^5 CFU g^{-1} dry soil in the rhizosphere of *A. pycnantha* and from 2.0 to 13.0 ± 1.8 x 10^5 CFU g^{-1} dry soil in the rhizosphere of *X. semiplana* (Table 5.1).

Although the type of culture medium did not affect the number of actinomycete colonies that formed on plates (Figure 5.2), actinomycete colonies formed on CGM and YCED were large and more diverse, with some colonies producing red and brown pigments. All colonies of actinomycetes growing on HVA and WYE were small and homogenous. Colonies on these two media produced white powdery spores. Pre-treatment of soil had a significant effect on the populations of actinomycetes (Figure 5.3). Incubation of soil sample with $CaCO_3$ in moist conditions significantly increased the population of actinomycetes from, 10^5 to 10^8 CFU g^{-1} dried soil for all six samples when compared with the untreated soil ($P < 0.001$). There were significant plant and treatment interaction effects on the populations of actinomycetes (Appendix E).

Table 5.1 Actinomycete numbers (average number of CFU g^{-1} dried soil) in the rhizosphere of each plant. The plants are arranged in increasing order of CFU g^{-1} dried soil. Different letters indicate significant differences for each plant ($P \leq 0.05$). The mean of each plant was based on three replications. L.S.D (0.05) = 187,700.

Plant	Mean
Acacia pycnantha plant 1	139,583^{a}
Xanthorrhoea semiplana plant 1	203,333^{ab}
Acacia pycnantha plant 3	231,667^{ab}
Acacia pycnantha plant 2	334,167^{b}
Xanthorrhoea semiplana plant 2	712,500^{c}
Xanthorrhoea semiplana plant 3	1,300,000^{d}

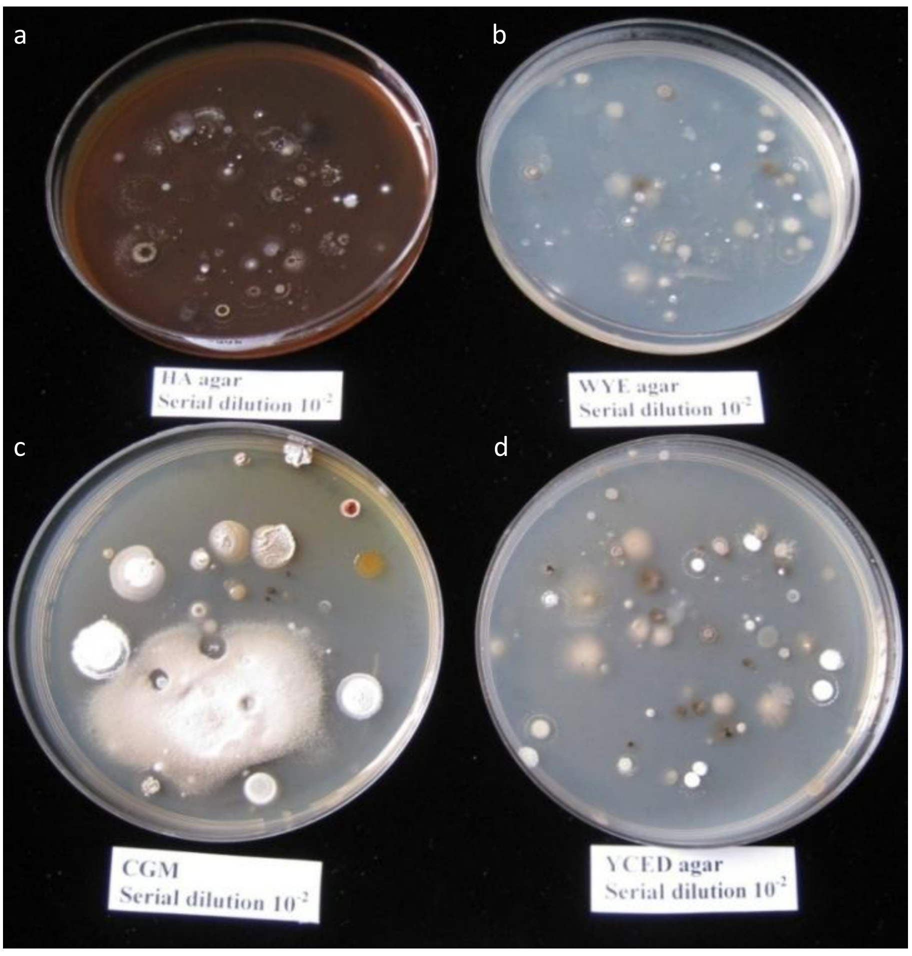

Figure 5.2 Representative actinomycete colonies from the rhizosphere soil of *Acacia pycnantha* formed on: (a) humic acid-vitamin B agar; (b) water-yeast extract agar; (c) casein glycerol agar; and (d) casamino acids-yeast extract-glucose agar.

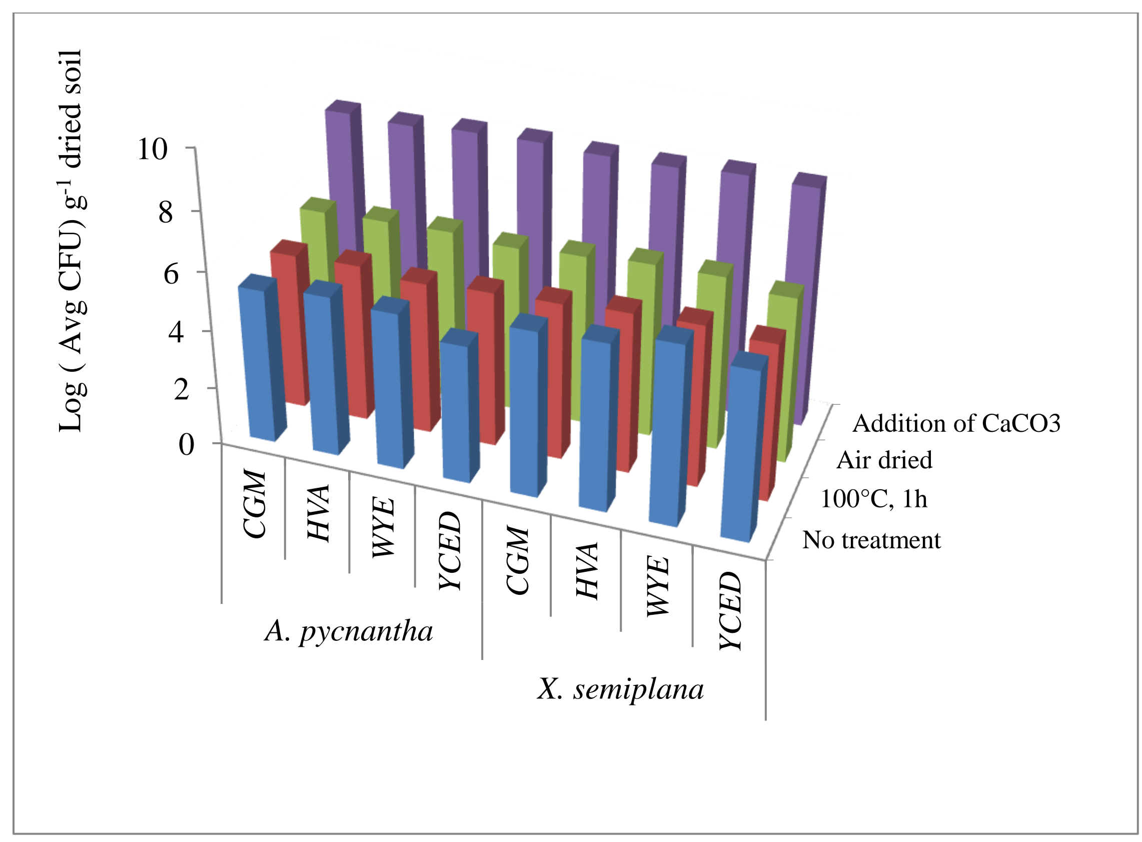

Figure 5.3 Actinomycete numbers (average number of CFU g^{-1}dried soil) in the rhizosphere of *Acacia pycnantha* and *Xanthorrhoea semiplana* plants on four different media: casein glycerol medium (CGA), humic acids-vitamin agar (HVA), water-yeast extract agar (WYE) and casamino acids-yeast extract-glucose-agar (YCED) and four soil pre-treatments: addition of $CaCO_3$; air dried at ambient temperature (17-25°C) for 9 d; heated at 100°C for 1 h; and no pre-treatment. Number of plant samples of each species, n = 3. LSD ($P \leq 0.05$) for plant and treatment interaction = 0.3.

5.3.2 *In vitro* antagonism of actinomycete isolates

A total of 127 actinomycete isolates (68 from *A. pycnantha* and 59 from *X. semiplana*) were successfully purified on ISP2 medium and tested for *in vitro* antagonism of *P. cinnamomi*. After incubation at 25°C for 7 to 10 d, *P. cinnamomi* grew across the plate in the pathogen-alone controls. When co-cultured with isolates of actinomycetes,

inhibition zones of various widths were observed (Figure 5.4). A continuum of inhibition zones, ranging from 0 to 25 mm was observed (Figure 5.5). Out of 68 actinomycetes isolates from *A. pycnantha* tested, 64 isolates or 94.1% were found to be antagonistic to *P. cinnamomi* while 86% or 51 out of 59 isolates from *X. semiplana* were antagonistic. The complete results for each antagonist are presented in Appendix F. The mean inhibition of 11.7 ± 0.9 mm caused by the actinomycetes from *A. pycnantha* was significantly greater ($P < 0.001$) than the mean inhibition of 7.6 ± 0.8 mm caused by the actinomycetes from *X. semiplana* (Figure 5.6).

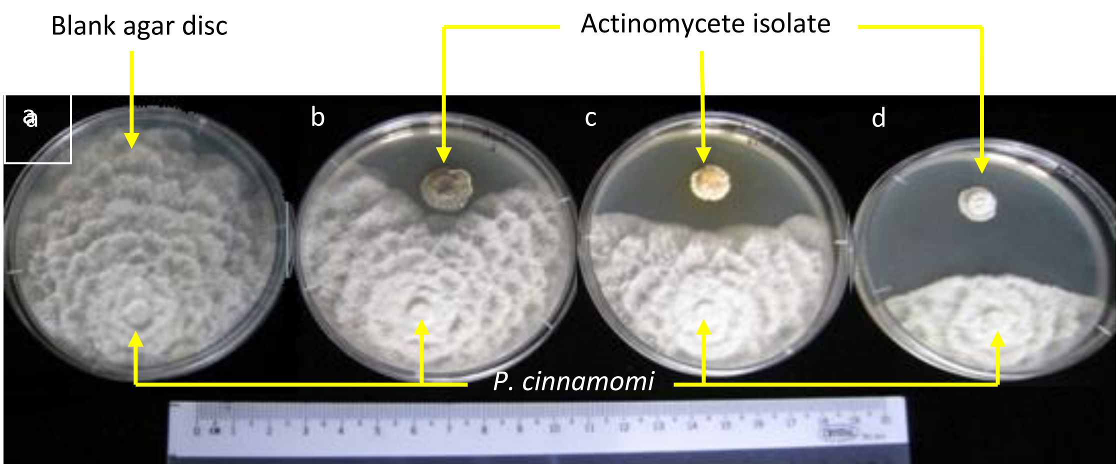

Figure 5.4 Dual cultures on International *Streptomyces* Project medium 2 incubated at 25°C for 14 days representing the following responses, from left to right: (a) control; (b) weak; (c) moderate; and (d) strong inhibition (section 5.2.3).

5.3.3 Greenhouse testing of potential control agents

None of the control plants sham-inoculated with sterile water and sterile agar discs, instead of *P. cinnamomi*, died 55 days after inoculation, as shown by the black line in Figure 5.7. Three plants inoculated with antagonists but no *P. cinnamomi*, one with antagonist XS2.42 and two with antagonist AC2.10, died 35 d after inoculation.

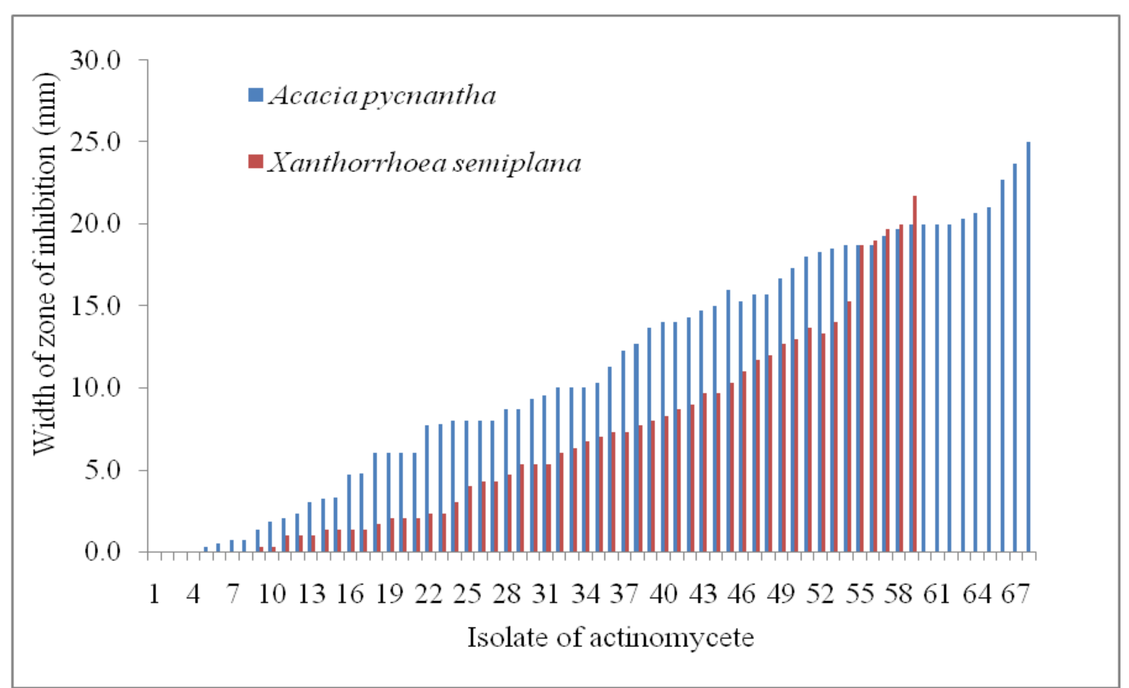

Figure 5.5 *In vitro* antagonism of *Phytophthora cinnamomi* isolate 71a by actinomycetes on ISP2 plates. A total of 68 actinomycete isolates from the rhizosphere soil of *Acacia pycnantha* and 59 isolates from *Xanthorrhoea semiplana* were tested for antagonism of *P. cinnamomi*. Actinomycete isolates are arranged in order of increasing antagonism.

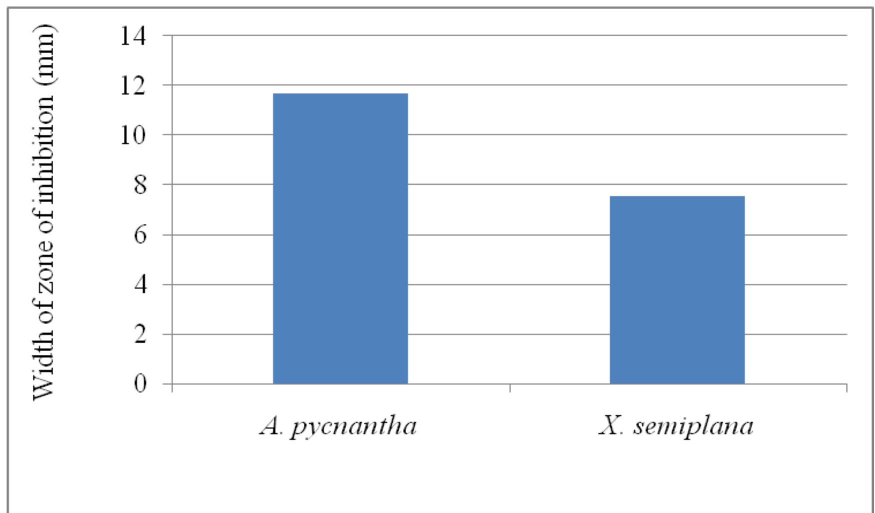

Figure 5.6 Comparison of means of width of zone of inhibition (mm) of *Phytophthora cinnamomi* between antagonists from the rhizosphere of *Acacia pycnantha* and *Xanthorrhoea semiplana*. LSD ($P \leq 0.05$) = 2.38. Number of isolates tested was 127, 68 isolates from *Acacia pycnantha* and 59 from *Xanthorrhoea semiplana*.

The low mortality in the control contrasted sharply with the high mortality in plants inoculated with *P. cinnamomi* either with antagonists or sterile water. Death occurred in plants inoculated with actinomycete isolates AC2.39, XS2.42, AC3.26, XS3.5, AC2.33, AC2.25 and XS3.4 as early as 8 days after inoculation with *P. cinnamomi*. All the plants showed sudden wilting and rapid death. No leaf chlorosis was observed prior to plant death. In all cases, massive loss of fine roots was observed and severe necrosis had occurred at the collar region, from which *P. cinnamomi* was readily reisolated. After 22 days, mortality had increased to more than 50% in plants treated with isolates AC2.39, XS2.42, XS3.5, AC2.33 and XS3.4. At this stage, plants treated with two antagonists, AC3.26 and AC2.25, had the same mortality as the positive control, at 40%. Only plants treated with antagonist AC2.10, a strong antagonist on agar, had lower mortality, at 33% compared to the positive control (40%). However, none of the antagonists ultimately protected *E. sieberi* from Phytophthora dieback (Figure 5.7). The Log-Rank test carried out at the end of the experiment showed that there were no significant differences in the mortality among plants treated with antagonists compared with the positive control. Actinomycete populations estimated from the potting mix at the end of the experiment ranged from 10^2 to 10^3 CFU g^{-1} dried soil.

The mean plant height at the time of inoculation and at the end of experiment was 6.13 – 7.5 ± 0.11 cm and 60.2 – 74.5 ± 1.4 cm respectively (Figures 5.8a and b). There was no significant difference in the means of plant height among all the groups at the beginning and at the end of the experiment.

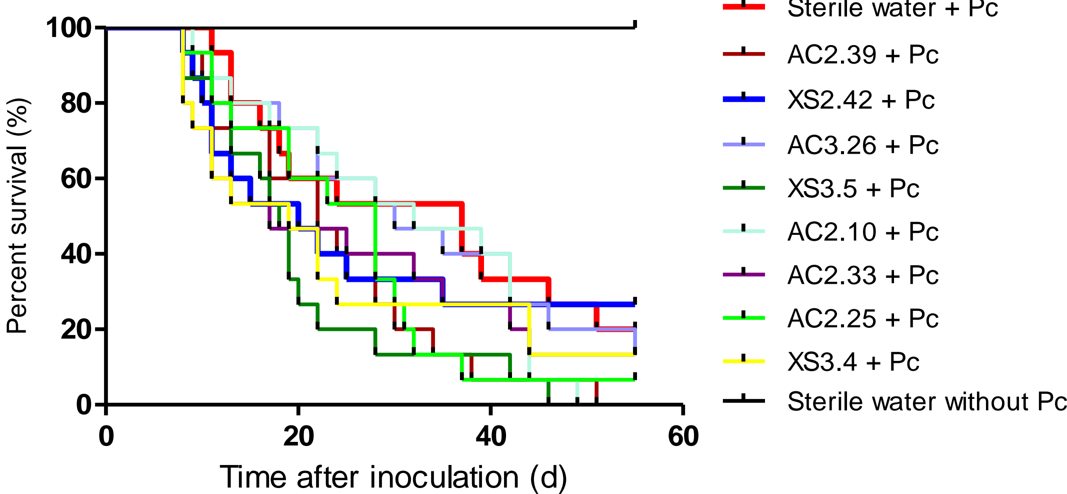

Figure 5.7 Kaplan-Meier survival curves of *Eucalyptus sieberi* inoculated with *Phytophthora cinnamomi* and eight selected Actinomycetes. Log-Rank test showed that there were no significant differences between the eight survival curves and the control at $P < 0.05$.

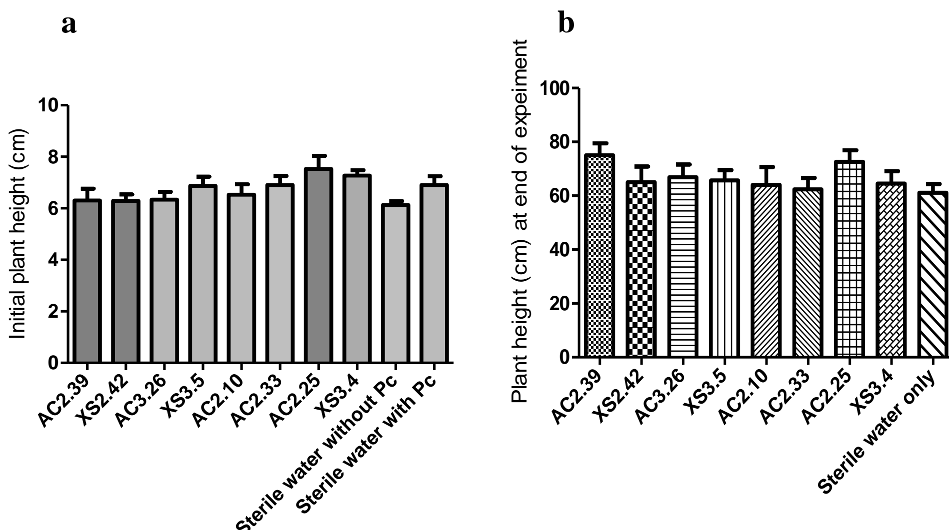

Figure 5.8 Means height of plants of *Eucalyptus sieberi* for each treatment group inoculated with actinomycetes or sterile water (a) at the time of inoculation and (b) at the end of experiment. There were 15 plants in each group. The bar in each column represents SEM.

5.3.4 16S rRNA gene sequencing

Lysis of cells and spores by lysozyme and proteinase K yielded genomic DNA for all the eight actinomycete isolates, as visualised by a broad band on agarose gels. After amplification of the genomic DNA, the PCR products were obtained for all eight isolates (Figure 5.9). The partial corrected sequence of 16S rRNA genes of the eight isolates are shown in Appendix G. Comparison with the NCBI online database using Blastn showed that all the actinomycetes isolated were of the genus *Streptomyces* (Table 5.2). The eight actinomycete isolates formed five phylogenetic clusters (Figure 5.10). Isolates AC2.10, XS3.5 and XS3.4 were closely related to *Streptomyces sanglieri*. AC2.39 was likely to be *S. scabrisporus* (100% identity) and AC2.33 was likely to be *S. cavourensis* subsp. *washingtonensis* (99% identity). The other three isolates, AC2.25, AC3.26 and XS2.42, were identified as most similar to *S. puniceus* (99% identity) and *S. xanthochromogenes* (98% identity).

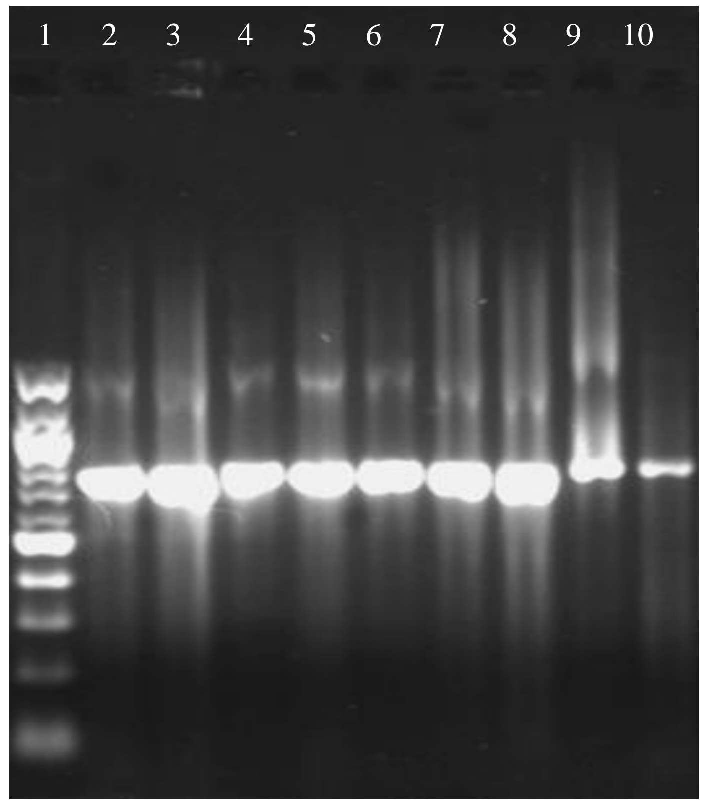

Figure 5.9 PCR products of the 16S rRNA genes of eight selected actinomycete isolates, from left to right: lane 1 Molecular weight ladder (500-1500 bp); lane 2 AC2.10; lane 3 AC2.25; lane 4 AC2.33; lane 5 AC2.39; lane 6 AC3.26; lane 7 XS2.42; lane 8 XS3.4; lane 9 XS3.5; and lane 10 PCR product (750 bp) from *Streptomyces griseus* as positive control.

Table 5.2 Identification of eight actinomycete isolates based on partial sequencing of the 16S rRNA gene. Match 1 and 2, in order of decreasing similarity, are two accessions of type strains with valid names from the online NCBI Blastn search deemed most closely related to the actinomycete isolate.

Isolate	Match 1	Strain No.	Max. Identity	Match 2	Strain No.	Max. Identity
AC2_10	*Streptomyces sanglieri* AB249945.1	NBRC 100784	99%	*Streptomyces gelaticus* DQ026636.1	NRRL B-2928	98%
AC2_25	*Streptomyces puniceus* GU383219.1	DSM 40083	99%	*Streptomyces floridae* GU383177.1	DSM 40938	99%
AC2_33	*Streptomyces cavourensis* ssp. *washingtonensis* DQ026671.1	NRRL B-8030	99%			
AC2_39	*Streptomyces scabrisporus* AB249946.1	NBRC 100760	100%	*Streptomyces griseoplanus* AB184138.1	NBRC 12779	95%
AC3_26	*Streptomyces xanthochromogene*s AB184176.1	NBRC 12828	98%	*Streptomyces mauvecolor* AB184532.1	NBRC 13854	98%
XS2_42	*Streptomyces chattanoogensis* AB184119.1	NBRC 12754	99%	*Streptomyces lydicus* AB184281.1	NBRC 13058	99%
XS3_4	*Streptomyces xanthochromogenes* DQ442559.1	NRRL B-5410T	99%	*Streptomyces michiganensis* AB184153.1	NBRC 12797	99%
XS3_5	*Streptomyces sanglieri* AB249945.1	NBRC 100784	99%	*Streptomyces pulveraceus* AB184806.1	NBRC 3855	98%

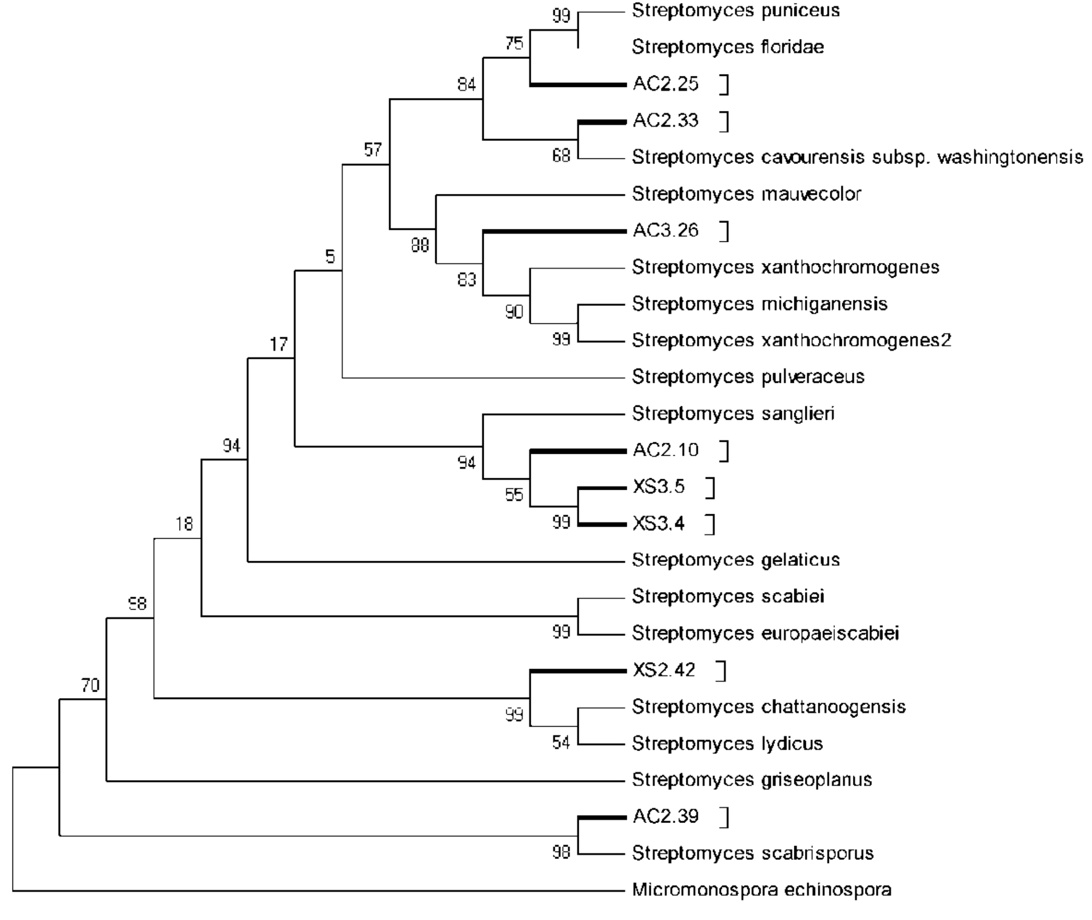

Figure 5.10 Phylogenetic tree based on partial 16S rRNA gene sequences showing the relationship between the eight actinomycete isolates and the closely related actinomycete type strains recovered from NCBI online database. Online sequence accession numbers are given in Table 5.2. Two common *Streptomyces* species, *S. scabiei* (Accession number: HQ143590.1) and *S. europaeiscabiei* (Accession number: FJ546725.1), were included as reference strains. The partial 16S rRNA gene sequence of *Micromonospora echinospora* (Accession number: JN180159.1) was used as an outgroup. The tree was generated by using the neighbour-joining method (Saitou & Nei, 1987).

5.4 Discussion

The association of areas with less severe disease due to *P. cinnamomi* with high populations of soil microflora has been well documented (Weste & Vithanage, 1977; 1978). Many soil microflora, particularly the actinomycetes, are antagonistic to *P. cinnamomi* and this creates an environment unfavourable for *P. cinnamomi* to survive. In this study, populations of actinomycetes in the rhizosphere of *A. pycnantha* and *X. semiplana* were estimated in the range of 10^5 to 10^6 CFU g^{-1} dried soil. These populations were comparable to many published reports on the populations of actinomycetes in rhizosphere of Australian native plants (Walchhuetter, 2002; Weste & Vithanage, 1977; Broadbent *et al.*, 1971). About 89% of the 127 actinomycete isolates was found to be antagonistic to *P. cinnamomi in vitro*.

There were significant differences in the population of rhizosphere actinomycetes from plant to plant, ranging from 2.0 to 13 x 10^5 CFU g^{-1} dried soil (Table 5.1). However, whether these differences were due to random variations or due to species of the plants was not clear as the number of plants sampled from each species was too small to make any statistically valid conclusion. Although the type of medium used to isolate the actinomycetes did not affect the number of colonies formed, the treatment applied to soil prior to culture did, in that incubation of soil samples with $CaCO_3$ in moist conditions significantly increased the number of actinomycetes isolated compared to the untreated soil. This result was in accordance with Istifadah (1997), who reported that addition of $CaCO_3$ to rhizosphere soil of capsicum seedlings significantly increased the number of actinomycetes isolated by a factor of 10 on CGM, YCED and WYE media.

A clear zone of inhibition in the dual culture test suggested that the production of antibiotics was a likely mechanism of antagonism (Broadbent *et al.*, 1971; Murray, 1987). There were appreciable differences in the *in vitro* antagonism of actinomycetes from *A. pycnantha* and *X. semiplana*. More actinomycetes from the rhizosphere of *A. pycnantha* were found to be antagonistic to *P. cinnamomi* than from X. *semiplana*. More significantly, stronger antagonism of *P. cinnamomi* was exhibited by actinomycetes from the rhizosphere of *A. pycnantha* than from the rhizosphere of *X. semiplana*. These data indicate that actinomycetes present in the rhizosphere of *Acacia* antagonistic to *P. cinnamomi* might be able to protect the roots from infection by the pathogen.

That the rhizosphere of *Phytophthora*-tolerant plants such as *Acacia pulchella* supports a larger microbial population, including fungi and bacteria, than the rhizosphere of susceptible species like *Banksia grandis* is well documented in the literature and the presence of these microbes and their exudates may contribute to a decreased ability of *P. cinnamomi* to attack the roots of these two species (Weste & Vithanage, 1978; Broadbent *et al.*, 1971; Pratt, 1971; Halsall, 1982). The larger microbial population found in the rhizosphere of tolerant species could be associated with exudates secreted by that plant species. Plant root exudates contain sugars, amino acids, organic acids, fatty acids and nucleotides which attract a variety of soil microorganisms (Rovira, 1956; Smucker, 1993). This variety of nutritional compounds is species specific and therefore likely to selectively enrich the plant's rhizosphere for microorganisms that are well adapted to the utilisation of the specific organic exudates (Strap, 2006). Interestingly, Noble (1989) attributed the greater resistance to *P. cinnamomi* of species of *Eucalyptus* subgenus *Symphyomyrtus* than that of subgenus *Monocalyptus* to the presence of mycorrhizae in the roots of *Symphyomyrtus* spp.

All eight actinomycete isolates were identified as species of *Streptomyces* based on partial sequences of the 16S rRNA gene (Table 5.2). At least two isolates, AC2.39 and AC2.33, were identified to species level. Although the small number of soil samples analysed in this study did not allow a conclusive assessment of the prevalence of this genus in native soil, a more extensive study conducted by Zvyagintsev (1994) showed that this genus of actinomycetes predominated in the soil of a forest ecosystem in Russia. Other studies conducted by Broadbent *et al*. (1971) and Murray (1987) also indicated that *Streptomyces* is the most prevalent genus in Australian soil and many isolates antagonised *P. cinnamomi in vitro*. For example, Broadbent *et al*. (1971) tested 426 isolates of *Streptomyces* obtained from avocado, wheat, pine forest and jarrah forest soil and found that 80% of the isolates inhibited *P. cinnamomi* by antibiotic production. The strongest antibiotic-producing actinomycetes were obtained from jarrah soils from Western Australia.

Streptomyces species are prolific synthesisers of antibiotics and other bioactive metabolites (Strap, 2006), suggesting they may be potential biocontrol agents for *P. cinnamomi*. However, none of the isolates tested in the greenhouse experiment showed an ability to protect *E. sieberi* from infection by *P. cinnamomi*. Only plants treated with isolate XS2.42 appeared to survive longer than the control, though the difference was not statistically significant (Figure 5.7). The lack of biological control by the antagonistic actinomycetes in this study could be attributed to several factors. In the greenhouse experiment, the water potential of the potting mix was deliberately kept at high constant water potential of $\geq$ -10 kPa to provide a conducive environment for the production of zoospores and infection by *P. cinnamomi* (Sterne *et al.*, 1977). However, this wet condition was not conducive for the growth and production of antibiotics by *Streptomyces*. The small populations of actinomycetes, only 10^2 to 10^3 CFU g^{-1} of

dried soil, estimated at the end of the experiment confirmed that the moist potting mix was not favourable for the growth of actinomycetes. Wong and Griffin (1974) reported that optimum growth of *Streptomyces* occurred at water potential of between -500 and -1,000 kPa while maximum amount of antibiotics was produced at a water potential of between -2,000 and -3,500 kPa, a low water potential compared with the optimum for production of zoospores by *P. cinnamomi*. Thus conducting the greenhouse experiment at water potential higher than -10 kPa, in order to conform with the method developed for susceptibility testing, is likely to have promoted infection by *P. cinnamomi* but suppressed the growth of actinomycetes.

If, on the contrary, the experiment had been conducted at low water potential, of -2,000 to -3,500 kPa, growth of *P. cinnamomi* in the pot might have been inhibited so that no zoospores would be produced. Such a low water potential, however, may promote the production of antibiotics. This assumption can be tested by investigating the effect of a range of water potentials on the growth and production of antibiotics at different water potentials in the soil using *Phytophthora*-free as control. Detection of antibiotics using ethyl acetate extraction method (Bonsall *et al.*, 1997) or high pressure liquid chromatography or mass spectrometry (Raaijmakers *et al.*, 1999) techniques could be adapted for the quantification of antibiotics produced *in situ*.

The growth of antagonists in the greenhouse experiment could also be affected by other factors. In this test, antagonists were isolated from their natural habitats, that is, from the rhizosphere of *A. pycnantha* or *X. semiplana,* and transferred to the rhizosphere of *E. sieberi*. The antagonists might not be capable of colonising the roots of plants from another species (Strap, 2006). Certain rhizosphere microorganisms such as *Streptomyces* are species-specific, only attaching themselves to the roots of specific plant species due to the exudation of unique chemical compounds by those species.

Examples of this specificity include *Streptomyces lydicus* WYEC108, an isolate from the rhizosphere of linseed (Crawford *et al.*, 1993) and *Streptomyces* strain RG, an isolate from the rhizosphere of sagebrush (Basil *et al.*, 2004). *S. lydicus* readily colonises the roots of a variety of linseed plants (Crawford *et al.*, 1993), but is a poor coloniser of sagebrush (Basil *et al.*, 2004). Thus confirmation that an isolate obtained from a rhizosphere soil is actually rhizosphere competent or possesses the ability to colonise the roots of inoculated plants (Campbell, 1989) is important before assessing its potential for biological control.

In future experiments, one modification that can be made to the above experiment to increase the growth of actinomycetes is to add an organic food source such as oatmeal to the potting mix and to inoculate the plant in free draining pots. It is important to strike a balance in term of water potential so that both the actinomycetes and *P. cinnamomi* can grow.

This study has shown that numerous actinomycetes that produce antibiotics active against *P. cinnamomi* are present in the rhizosphere of two native plant species. However, the tendency of actinomycetes to produce larger amounts of antibiotic at low water potentials (Wong & Griffin, 1974), which are unfavourable for *P. cinnamomi*, may limit their antagonism to drier areas. Therefore, other genera of actinomycetes or other groups of microorganisms, such as bacteria and fungi, which share similar ecological requirements as *P. cinnamomi* in terms of the water requirement, should be explored for potential biological control. For example, *Micromonospora*, which is found in inland lakes, should be screened for antagonism against *P. cinnamomi* as it can tolerate high water potentials. Fungi, which can tolerate a wide range of water potentials (Metting, 1992), have also been revealed to exhibit stronger antagonism towards *P. cinnamomi* than do actinomycetes (Walchhuetter, 2002).

A few endophytes isolated from the root tissue of rhododendron exhibited strong *in vitro* antagonism against *P. cinnamomi*. By residing inside the tissues of plants, these endophytic antagonists are not exposed to the wet conditions in the soil if wet weather prevails, and therefore may be better suited to protect the plants from infection by *P. cinnamomi*. Actinomycetes have also been found to exist as endophytes in the root tissue of Australian native plants (Kaewkla, 2009). Pioneering work carried out by Kaewkla (2009) on endophyte actinobacteria revealed that Australian native plants are a novel and rich source of endophytic actinomycetes. More than 576 isolates of actinomycetes from 17 genera were isolated, many of which produced antifungal compounds including those active against *Phytophthora palmivora*. These isolates were tested against seven microorganisms, including: one yeast, *Candida albicans*; one fungus and one oomycete plant pathogens, *Fusarium oxysporum* and *Phytophthora palmivora*; and four bacteria associated with humans and animals, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Micrococcus luteus* and *Staphylococcus aureus*. However, *P. cinnamomi* was not included in the tests.

The findings reported in this chapter suggested that the potential for biological agents to control *P. cinnamomi* in Australia exists. The large populations of actinomycetes antagonistic to *P. cinnamomi* may, perhaps, contribute to the continued survival of the highly susceptible *X. semiplana* in the *P. cinnamomi*-infested site at Mount Bold Catchment Reserve. Further research should be conducted to examine microbial antagonism for management of *P. cinnamomi* in South Australia.

Chapter 6 General discussion

Phytophthora dieback, a soil borne disease caused by *Phytophthora cinnamomi*, is a key threat to native ecosystems in Australia and, if left unchecked, has the potential to drive populations and species to extinction. This study has generated empirical data and information to support a new Phytophthora management strategy in South Australia, a State where native vegetation has already been extensively affected by the disease. The strategy will aim to prioritise threatened plant species and plant communities for *in situ* conservation and protection from the disease. The risk posed by the disease to threatened plant species and plant communities in South Australia can now be assessed based not only on the proximity to *P. cinnamomi* and conservation status but also on the knowledge of susceptibility of a species to *P. cinnamomi*.

In particular, the aims of this study were achieved as follows:

(i) Susceptibility testing in the greenhouse of 37 native plant species, including 15 threatened species known to be at risk or vulnerable to Phytophthora dieback, revealed that a high proportion (90%) was susceptible to the disease. The response, particularly of the threatened species, was very variable, ranging from highly susceptible to fully resistant (non-host).

(ii) No spread of Phytophthora dieback was detected at the monitoring site at Mount Bold Catchment Reserve from 2008 to 2010, which may reflect the slow spread of the pathogen through root to root contact. Environmental factors, in particular, soil temperature and moisture and the sandy loam texture at the site did not provide conditions conducive for the spread of *P. cinnamomi* through zoospores.

(iii) Large populations of actinomycetes were found in the rhizosphere of three *A. pycnantha* and three *X. semiplana* plants. A diversity of actinomycetes was isolated and purified, many of which (90% of 127 isolates) exhibited antibiosis against *P. cinnamomi in vitro*. Eight isolates, all of which belonged to the genus *Streptomyces*, ranging from weakly to strongly antagonistic failed to protect susceptible plants from Phytophthora dieback in moist potting mix in a greenhouse.

6.1 Determining susceptibility of plant species to Phytophthora dieback

Understanding host susceptibility to infection by *P. cinnamomi* is fundamental to selecting conservation options (Shearer *et al.*, 2004a; Barker & Wardlaw, 1995) especially given that many threatened species are susceptible to Phytophthora dieback. The susceptibility of 37 South Australian native plant species to *P. cinnamomi* in terms of disease symptoms and survival was tested in the greenhouse where environmental conditions could be controlled. As individual plants within a population vary in susceptibility to Phytophthora dieback (Shearer *et al.*, 2004a), survival analysis is deemed an appropriate method to assess and compare the response of plant species to infection by a pathogen (Machin *et al.*, 2006). Ranking of species according to level of susceptibility to *P. cinnamomi* then allows prioritisation of threatened species according to risk from the pathogen (Shearer *et al.*, 2007).

In this study, plant species were classified into broad groups based on susceptibility, ranked according to percentage of mortality. Other researchers, likewise, have classified susceptibility into broad categories (Podger & Batini, 1971; McCredie

et al., 1985; Peters & Weste, 1997; Tynan *et al*., 1998). Plant species with mortality below 20% were classified as slightly susceptible, between 20 and 80% moderately susceptible and above 80% highly susceptible. However, even though rating susceptibility based on percentage mortality can identify which species is susceptible or which species is resistant, this classification has its weaknesses. For example, a species with 70% mortality at 3 weeks after inoculation is definitely more susceptible than a species with 70% mortality at one year after inoculation. Classification of susceptibility into such broad groups based on mortality alone would, therefore, make it difficult to decide which species should be prioritised for protection from the disease. This weakness was highlighted by Podger & Batini (1971) when comparing the relative susceptibility of 36 species of *Eucalyptus*.

This difficulty can be overcome by using logistic equations to analyse the percentages of plant mortality, from which three mortality parameters, the upper asymptote (K_{max}), median survival time ($t_{1/2}k$) and rate of infection (r), can be derived. Van der Plank (1965) first described the dynamics of disease progress in a field crop by using logistic equations. Shearer *et al*. (2007) applied the logistic equation to analyse the susceptibility of Australian native plant species to Phytophthora dieback following experiments conducted in a greenhouse. These mortality parameters describe the susceptibility of a species to the disease in greater detail than percentage of mortality alone. The parameter, *r*, in particular describes how quickly mortality occurs over time for the test species. In greenhouse experiments where environmental conditions for infection and disease development are optimised, the rate of infection would reflect the intrinsic susceptibility of the test species to the disease. Thus, logistic equations represent a more comprehensive and precise description for rating the susceptibility of a species than percentage mortality alone (Shearer *et al*., 2007; Barrett *et al*., 2008).

However, a large number of replicates, up to several hundred, are required for both the inoculated and the non-inoculated treatments in order to obtain reliable mortality parameters for the use of logistic equations (Machin *et al.*, 2006). This requirement is not compatible with research on disease susceptibility of threatened species because such plants are rare, difficult to propagate and they generally do not grow well, even in greenhouse conditions. Many Australian native plant species are difficult to propagate. For example, seeds of the following South Australian threatened plant species; *Brachyscome ciliaris* var. ciliaris, *Leionema microphyllum*, *Derwentia derwentiana* ssp. *derwentiana* and *Euphrasia collina* ssp. *osbonii* were germinated in the greenhouse in winter 2009 but all the seedlings died with the arrival of spring. In this study, mortality rate was therefore not analysed using logistic equations because the relatively small number of replicates, particularly the control, would make such analysis dubious. Further research to determine the optimum condition for the germination and growth of threatened species is, therefore, warranted. An effective propagation method would make available sufficient propagules for susceptibility experiments to be carried out with adequate replication so that data could be analysed using appropriate statistical analysis.

Threatened species are particularly vulnerable to local extinction due to *P. cinnamomi* because they generally have small, disjunct populations. If all the plants are in one place, such as *Wollemia nobilis* which is located in two separate valleys near Sydney, New South Wales, only (Woodford, 2005), then there is a risk that disease will kill the entire population due to a stochastic event, or the population may be drastically reduced to a non-viable number for the population to persist in the long-term due to inbreeding depression, genetic drift or low reproduction rate (Primack, 2006).

The ranking of susceptibility should, therefore, not be the sole criterion for prioritising species for protection, and other factors such as rarity and biology of a taxon should be taken into account. This is particularly the case in Australia. Although families of many of the Australian flora are widely distributed elsewhere in the world, such as in South America and New Zealand, the uniqueness (or endemism) of the Australian flora is principally at the level of genus and species (Morcombe, 1970). Loss of species endemic to Australia would lead to global extinction. Therefore, rarity of a species should be assessed on an international basis for prioritisation for management (Hambler, 2004).

Because many Australian species have a naturally fragmented and geographically restricted distribution, much genetic differentiation occurs between populations and population groups (Hopper & Coates, 1990). Conservation units should, therefore, be based not only on the level of taxonomic entity, but also at the level of the population (Hopper & Coates, 1990; Coates, 2000). To conserve diversity at the taxon level might lead to loss of genetic resources. As such, all populations of threatened species should be surveyed and tested for the presence of *P. cinnamomi*. When considering a population of a species for protection, location of the population is of importance equal to susceptibility for prioritisation of the species for conservation. A population located on a hilltop, for example, is less vulnerable to infection by *P. cinnamomi* than a population located at the bottom of a hill, as observed in the field monitoring study (see Chapter 3).

In this study, only two isolates of *P. cinnamomi*, 71a from Kangaroo Island and SC4 from Scott Creek were used to inoculate plants in susceptibility testing. As the pathogenicity among Australian isolates of *P. cinnamomi* may be different (Dudzinski *et al.*, 1993) and plants of the same species from different populations might be

genetically different from each other, further research should use more isolates and plant seeds of the same species from different parts of Australia to ensure that the susceptibility rating of the species reflects the natural variations among different populations. In particular, susceptibility testing should include plant species from Mount Bold areas using isolates of *P. cinnamomi* collected from the field site at Mount Bold as they are in close proximity with each other and results generated from such experiments would be more relevant for the management of Phytophthora dieback in the area. In addition, actinomycetes from Mount Bold should be tested against the isolates of *P. cinnamomi* from Mount Bold and if found to be sensitive could further explain why there is no detectable spread of disease in that areas.

6.2 Managing areas infested by *Phytophthora cinnamomi*

An important aspect of an effective disease management is our ability to detect and map the distribution of *P. cinnamomi*. Currently, most laboratories in Australia rely solely on baiting techniques to detect *P. cinnamomi* in soil and root samples. The problem with baiting techniques is that they often failed to detect *P. cinnamomi* in infested soil (Eden *et al.*, 2000; Hüberli *et al.*, 2000), thus there is a need for a more reliable method of detection. Even the use of DNA-based assays for detection of *P. cinnamomi* in soil are liable to produce false negative results (section 1.11.3). Thus, several institutions in Australia have conduct research to increase the species-specificity of DNA-based assays (Hardham, 2005; Drenth *et al.*, 2006; O'Brien, 2008). The recent success in the development of a nested-PCR technique, which is many times more sensitive than baiting for the detection of *P. cinnamomi* in soil, at CPSM will greatly help in the identification and diagnosis of Phytophthora dieback in native vegetation (Williams *et al.*, 2009). A DNA-based assay can also help to overcome

uncertainties associated with identification of *P. cinnamomi* based on morphological features. A collaborative effort by The University of Adelaide and the Root Disease Testing Service of SARDI at Waite Campus is developing a real time PCR based using TaqMan® minor groove blinder (MGB) (Applied Biosystems, 2010) for identification and quantification of *P. cinnamomi* in soil, with high throughput (A. McKay, pers. com., 2011). Such a rapid assay would help in the production of a detailed distribution map of *P. cinnamomi* in the State thereby enhancing management of the disease. In particular, the DNA-based assay would also be an invaluable tool at cryptic sites where indicator species are not present (Williams *et al.*, 2009).

The need for an ecologically friendly approach for disease control in the native ecosystem has encouraged the development of alternative management measures such as biological control. The rhizosphere contains a complex community of microflora which could suppress soil-borne pathogens via competition, antagonism or hyperparasitism (Raaijmakers *et al.*, 2009). Studies have provided concrete evidence that antagonistic microorganisms may produce a range of antimicrobial metabolites, such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, pyoluteorin, phenazines, cyclic lipopeptides and hydrogen cyanide (Raaijmakers *et al.*, 1999), which bring about natural biological control of soil-borne pathogens. Thus, the ability of the actinomycetes isolated during the present study, or other rhizosphere microorganisms, to produce antibiotics effective against *P. cinnamomi* in the rhizosphere merits further study to explore if they could be used as a root treatment for seedlings prior to planting in revegetation programs.

In this study, populations of actinomycetes in the rhizosphere were found to be large, in the range of 10^5 CFU g^{-1} dried soil, an estimate similar to that reported by other researchers to be suppressive to *P. cinnamomi* (Broadbent *et al.*, 1971; Weste &

Vithanage, 1977; Walchhuetter, 2002). Failure of the eight actinomycete isolates to suppress Phytophthora dieback in the greenhouse was likely to be due to high moisture potential in the soil, which has been reported to be harmful to actinomycetes (Wong & Griffin, 1974). The use of microorganisms for control of Phytophthora dieback caused by *P. cinnamomi* has been successfully employed in avocado plantations in Queensland and Western Australia, where organic much is applied in mounds around plants to promote the growth of microorganisms (You *et al.*, 1996). The organic mulch promotes the growth of actinomycetes which control Phytophthora dieback (You *et al.*, 1996). In future biological control experiments involving actinomycetes, the experiments should be carried out using a soil moisture potential that is suitable for the growth of actinomycetes and *P. cinnamomi*. There is also a need to evaluate other groups of microorganisms such as fungi, bacteria, endophytes and mycorrhizae as these groups of microorganisms have been found to be antagonistic to *P. cinnamomi*. Any microorganisms selected as biocontrol agents should preferably be able to protect plant species in natural vegetation on different soil types across different climatic conditions.

6.3 Future of Phytophthora control in South Australian native ecosystems

The goal of many conservation programs in South Australia is primarily to preserve and conserve existing populations *in situ* by reducing key threats to these species and attending to their specific management needs (Davies, 1995). The preferred first option for the conservation of plant biodiversity is to protect plants *in situ* because this is the best way to conserve the species with all its diversity while maintaining its evolutionary capacity (Henry, 2010). Many rare plant species survive in areas with

unique habitats (Hambler, 2004). Protection of these areas is probably the only option for conservation of these species. As observed at the field monitoring site at Mount Bold Catchment Reserve, certain areas, due to their topographic features, are protected from spread of *P. cinnamomi* from neighbouring areas, which might make those areas suitable for *in situ* conservation. However, one significant future threat to *in situ* conservation could be climate change.

In the near future, a sound risk assessment strategy, together with hygiene measures, would be expected to play an increasingly important role in mitigating the impact of Phytophthora dieback in South Australia. Information on the susceptibility and tolerance (cryptic infection) of South Australian native plant species established in this study can be used to prioritise protection of endangered plant species from Phytophthora dieback. For example, *Allocasuarina robusta*, which is endangered in the state and nationally, was found to be highly susceptible to Phytophthora dieback in this study. This information has been conveyed to the Department of Environment and Natural Resources, South Australia, and as a result has been given priority for immediate protection from the disease (Velzeboer, pers. com., 30 May 2011). Locations of natural populations of *A. robusta* should be considered as dieback priority protection areas. If such an area is found to be free of *P. cinnamomi*, access to the area may need to be restricted temporarily or permanently and hygiene measures put in place to protect the area from infestation. However, if the area is already infested by *P. cinnamomi*, and the populations are vulnerable to infection by *P. cinnamomi*, then phosphite spray should be considered to protect the plants and, if necessary, *ex situ* conservation be carried out simultaneously. In this way, the species can be better protected from extinction due to Phytophthora dieback.

The identification of threatened plant species as susceptible to Phytophthora dieback in this study can form the basis for further research on threatened plant species and biological agents. For example, if phosphite spray has to be applied to protect wild populations of these threatened plant species, then its efficacy on these plant species, its potential phytotoxicity effect and effect on biological control agents should be evaluated.

Many rare and endangered species, such as *Acacia spooneri* and *A. enterocarpa*, were found to be resistant but are hosts of *P. cinnamomi*. As there are many revegetation or recovery programs involving threatened species in the state, staff involved in revegetation programs should be made aware that threatened plants may harbour cryptic infection. Ensuring that only seedlings free of *P. cinnamomi* are planted in the field is important.

In conclusion, information generated from this study fills a gap in the knowledge that is needed for the implementation of the new Phytophthora management strategy in South Australia. In particular, this study has provided data for the assessment of risk and assists in prioritisation of management areas to mitigate the devastating impact of Phytophthora dieback on plant biodiversity in the State.

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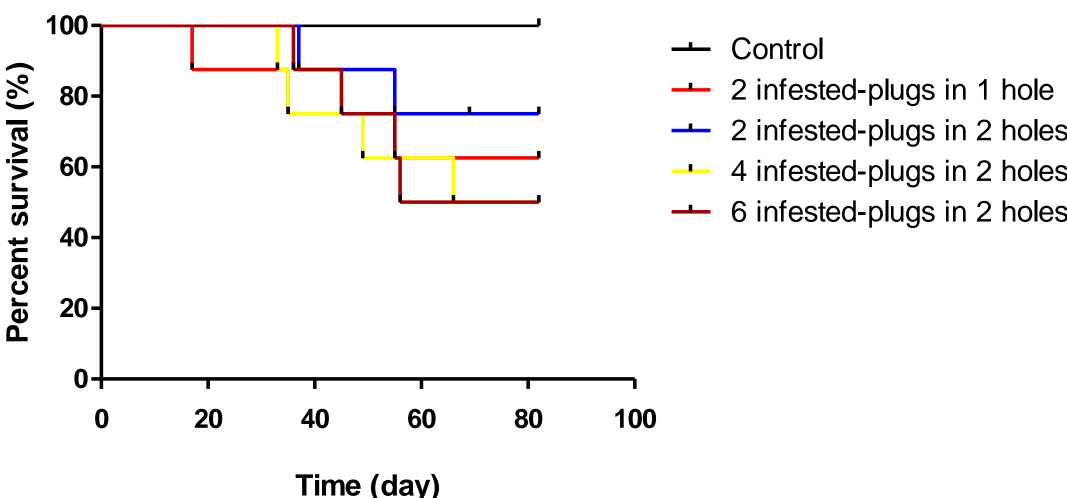
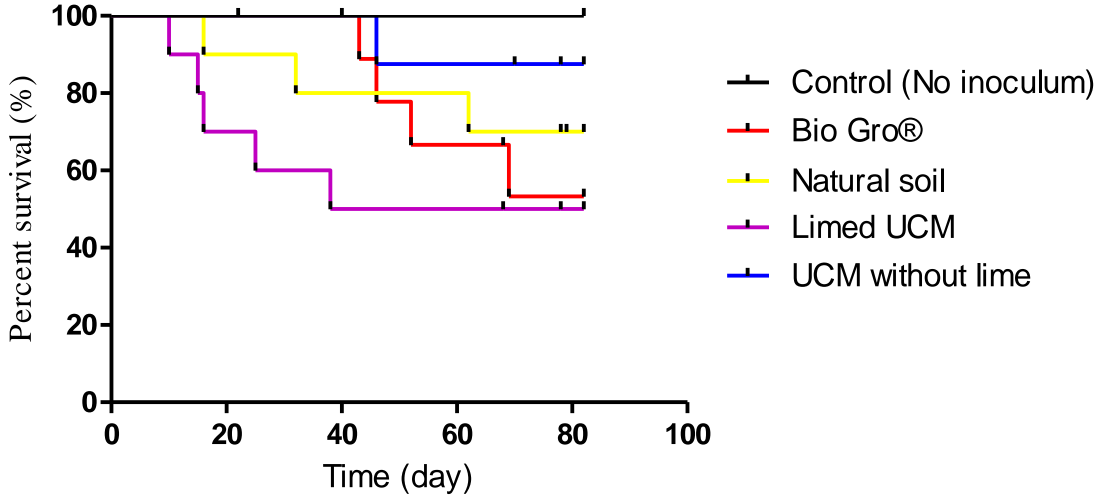
Appendices

Appendix A1 Kaplan Meier survival curves of eight plants (consisting of 4 *Eucalyptus sieberi* and 4 *Xanthorrhoea semiplana* plants) inoculated with different dosages of inoculum.



Log-rank test shows no significant differences between the survival curves P ≤ 0.05.

Appendix A2 Kaplan-Meier survival curves of 10 plants (consisting of 5 *Eucalyptus sieberi* and 5 *Xanthorrhoea semiplana* plants) grown on four different types of soil.



Log-rank test shows significant differences between the five survival curves at P ≤ 0.05.

Appendix B Preparation of University of California potting mix

University of California (UC) potting mix was prepared by Plant Growth Services, Waite Campus. Two bins of Waikerie (river) sand (equivalent to 1,200 L) were steamed at 100°C for a minimum of 1 hour. Three bales of peatmoss (about 340 L) were then added to the steaming Waikerie sand and mixed thoroughly. Water was added at this stage to facilitate mixing, if required. The UC mix was allowed to cool for 20 min. A mixture of calcium hydroxide and calcium carbonate (1:2.17) was used to adjust the pH to about 6.5. After the mixture had cooled to ambient temperature, 4.5 kg of a slow-release fertiliser, Osmocote Exact 'MINI' (16N:3P:9K+ Trace elements) manufactured by Scotts Australia Pty Ltd, was added.

Appendix C: List of plant species found at Mount Bold field site.

No	Species	Common name	Family
1	*Xanthorrhoea semiplana*	Yacca	Liliaceae
2	*Isopogon ceratophyllus*	Cone bush	Proteaceae
3	*Acacia pycnantha*	Golden wattle	Fabaceae
4	*Acacia myrtifolia*	Myrtle wattle	Fabaceae
5	*Acacia verniciflua*	Varnish wattle	Fabaceae
6	*Platylobium obtusangulum*	Common flat pea	Fabaceae
7	*Correa decumbens*	Spreading correa	Rutaceae
8	*Correa reflexa*	Common correa	Rutaceae
9	*Eriochilus cucullatus*	Parson's bands	Orchidaceae
10	*Eucalyptus cosmophylla*	Cup gum	Myrtaceae
11	*Eucalyptus obliqua*	Messmate stringybark	Myrtaceae
12	*Banksia marginata*	Silver banksia	Proteaceae
13	*Hakea carinata*	Erect hakea	Proteaceae
14	*Hakea rostrata*	Beaked hakea	Proteaceae
15	*Sypridium parvifolium*	Dusty miller	Rhamnaceae
16	*Hybanthus floribundus*	Shrub violet	Violaceae
17	*Davesia leptophylla*	Grose bitter pea	Fabaceae
18	*Pterostylis pedunculata*	Maroon-hood	Orchidaceae
19	*Thelymitra antennifera*	Rabbit ears	Orchidaceae
20	*Tetratheca pilosa*	Pink-eyed susan	Tremeandraceae
21	*Leucopogon virgatus*	Common bearded heath	Epacridaceae
22	*Hibbertia virgata*	Twiggy guinea flower	Dilleniaceae
23	*Drosera* sp.	Sundew	Drosceraceae
24	*Exocarpus cupressiformis*	Native cherry	Santalaceae
25	*Pinus radiata*	Radiata pine	Pinaceae
26	*Leptospermum continentale*	Tea tree	Myrtaceae
27	*Cassytha* sp.	Downy dodder	Lauraceae
28	*Brachyscome* spp.	Daisy	Asteraceae
29	*Phyllota pleurandroides*	Heathy phyllota	Fabaceae
30	*Acrotriche impressa*	Mount Lofty ground berry	Epacridaceae
31	*Hibbertia* spp.	-	Dilleniaceae
32	Various sedges	Grasses or rushes	Cyperaceae
33	*Spyridium* spp.	-	Rhamnaceae
34	*Pultenaea daphnoides*	Large-leaf bush pea	Fabaceae

Appendix D Baiting results of random soil and water samples collected from within and outside the experimental plot from 2009 and 2010

No	Date of sampling	Source	Position	Result of soil baiting
1	3 May 2009	*Isopogon ceratophyllus*	Inside grid	-
2	3 May 2009	*Platylobium obstusangulum*	Inside grid	-
3	25 June 2009	Asymptomatic *X. semiplana* Plant 1	TAQ4	+
4	25 June 2009	Asymptomatic *X. semiplana* plant 2	TAQ4	-
5	25 June 2009	Asymptomatic *X. semiplana* plant 3	TAQ4	+
6	25 June 2009	*Acacia pycnantha* Plant 1	Outside grid	-
7	25 June 2009	*Acacia pycnantha* Plant 2	Outside grid	-
8	25 June 2009	*Acacia pycnantha* Plant 3	Outside grid	-
9	12 August 2009	Creek water	Mt. Bold Reservoir	+
10	12 August 2009	Symptomatic *X. semiplana* Plant 1	T1Q2	-
11	12 August 2009	Symptomatic *X. semiplana* plant 2	T1Q2	-
12	12 August 2009	Symptomatic *X. semiplana*	T1Q3	-
13	12 August 2009	Symptomatic *X. semiplana*	T5Q2	-
14	12 August 2009	Symptomatic *X. semiplana* plant 1	T5Q3	-
15	12 August 2009	Symptomatic *X. semiplana* plant 2	T5Q3	-
16	12 August 2009	Symptomatic *X. semiplana* plant 1	T5Q6	-
17	12 August 2009	Symptomatic *X. semiplana* plant 2	T5Q6	-
18	13 August 2009	Water	Reservoir	+*
19	13 August 2009	Water	Reservoir	-
20	13 August 2009	Water	Creek	-
21	13 August 2009	Water	Creek	+*
22	13 August 2009	Symptomatic *X. semiplana* Plant 1	TAQ1	-
23	13 August 2009	Symptomatic *X. semiplana* plant 2	TAQ1	-
24	13 August 2009	Symptomatic *X.semiplana* plant 1	TAQ2	-
25	13 August 2009	Symptomatic *X. semiplana* plant 2	TAQ2	-
26	13 August 2009	Symptomatic *X. semiplana* plant 1	TAQ3	-
27	13 August 2009	Symptomatic *X. semiplana* plant 2	TAQ3	-
28	13 August 2009	Symptomatic *X. semiplana* plant 1	TAQ4	-
29	13 August 2009	Symptomatic *X. semiplana* plant 2	TAQ4	-
30	13 August 2009	Symptomatic *X. semiplana* plant 1	TAQ5	-
31	13 August 2009	Symptomatic *X. semiplana* plant 2	TAQ5	-
32	16 April 2010	Dead *X. semiplana*	Near reservoir	-
33	16 April 2010	Dead *X. semiplana*	Near reservoir	-
34	16 April 2010	Asymptomatic *X. semiplana*	Near reservoir	-
35	16 April 2010	Symptomatic *P. obtusangulum*	TAQ2	+
36	16 April 2010	Dead *X. semiplana*	Near reservoir	+
37	3 November 2010	Soil sample	Outside grid	-
38	3 November 2010	Symptomatic *X. semiplana*	Outside T2Q1	-
39	3 November 2010	Symptomatic *X. semiplana*	Outside T4Q6	-
40	3 November 2010	Dead *X. semiplana*	Outside T6Q1	-
41	3 November 2010	Symptomatic *X. semiplana* plant 1	Outside T6Q3	-
42	3 November 2010	Symptomatic *X. semiplana* plant 2	Outside T6Q3	-
43	3 November 2010	Healthy *X. semiplana*	Outside T6Q4	-
44	3 November 2010	Dead *X. semiplana*	Outside T6Q5	-
45	3 November 2010	Dead *X. semiplana*	Outside T5Q1	+
46	3 November 2010	Dead *X. semiplana* plant 1	Roadside	-
47	3 November 2010	*X. semiplana* plant 2	Roadside	-
48	3 November 2010	Asymptomatic *P. daphnoides* plan1	Near reservoir	-
49	3 November 2010	Asymptomatic *P. daphnoides* plant1	Near reservoir	-
50	3 November 2010	Asymptomatic *P. daphnoides* plant 2	Near reservoir	-
51	3 November 2010	Asymptomatic *P. daphnoides* plant 2	Near reservoir	-
52	3 November 2010	Dead *X.semiplana*†	Near reservoir	+

P. cinnamomi present (+); *P. cinnamomi* not detected (-); * suspected to be *Phytophthora cinnamomi*

Appendix E Analysis of variance of the number of actinomycete colonies (log average CFU g^{-1} dried soil) formed on four media: casein glycerol agar; water yeast agar; casamino-yeast extract-glucose agar; and humic acid vitamin agar with four soil pre-treatments: no treatment; air dried at ambient temperature for 9 d; heat at 100°C for 1 h; and incubation with $CaCO_3$ for 13 d.

Variate; log average CFU g^{-1} dried soil

Source of variation	d.f	s.s	m.s	v.r	F pr.
Plant	1	0.8236	0.8236	6.56	0.013
Media	3	0.5232	0.1744	1.39	0.254
Treatment	3	126.1794	42.0598	334.96	< 0.001
Plant.Media	3	0.1753	0.0584	0.47	0.707
Plant.Treatment	3	1.3505	0.4502	3.59	0.018
Media.Treatment	9	0.6199	0.0689	0.55	0.833
Plant.Media.Treatment	9	0.5439	0.0604	0.48	0.822
Residual	64	8.0362	0.1256		
Total	95	138.2519			

Appendix F Antagonism of *Phytophthora cinnamomi* by actinomycetes. Inhibition defined as no hyphal growth in area of the plate where growth of *P. cinnamomi* was retarded by actinomycetes. A total of 127 actinomycete isolates, 68 from rhizosphere soil of *Acacia pycnantha* and 59 from *Xanthorrhoea semiplana* were tested for antagonism of *P. cinnamomi*.

Inhibition zone (mm)					
	Source			Source	
Actinomycetes isolate No	*Acacia pycnantha*	*Xanthorrhoea semiplana*	Actinomycetes isolate No	*Acacia pycnantha*	*Xanthorrhoea semiplana*
1	0.0	0.0	35	10.3	7.0
2	0.0	0.0	36	11.3	7.3
3	0.0	0.0	37	12.3	7.3
4	0.0	0.0	38	12.7	7.7
5	0.3	0.0	39	13.7	8.0
6	0.5	0.0	40	14.0	8.3
7	0.7	0.0	41	14.0	8.7
8	0.7	0.0	42	14.3	9.0
9	1.3	0.3	43	14.7	9.7
10	1.8	0.3	44	15.0	9.7
11	2.0	1.0	45	16.0	10.3
12	2.3	1.0	46	15.3	11.0
13	3.0	1.0	47	15.7	11.7
14	3.2	1.3	48	15.7	12.0
15	3.3	1.3	49	16.7	12.7
16	4.7	1.3	50	17.3	13.0
17	4.8	1.3	51	18.0	13.7
18	6.0	1.7	52	18.3	13.3
19	6.0	2.0	53	18.5	14.0
20	6.0	2.0	54	18.7	15.3
21	6.0	2.0	55	18.7	18.7
22	7.7	2.3	56	18.7	19.0
23	7.8	2.3	57	19.3	19.7
24	8.0	3.0	58	19.7	20.0
25	8.0	4.0	59	20.0	21.7
26	8.0	4.3	60	20.0	
27	8.0	4.3	61	20.0	
28	8.7	4.7	62	20.0	
29	8.7	5.3	63	20.3	
30	9.3	5.3	64	20.7	
31	9.5	5.3	65	21.0	
32	10.0	6.0	66	22.7	
33	10.0	6.3	67	23.7	
34	10.0	6.7	68	25.0	

Appendix G Partial corrected sequence of 16S rRNA genes of eight actinomycetes isolates

Isolate AC2_10

1 CATGCAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGT 50
51 GAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAAC 100
101 GGGGTCTAATACCGGATAACACTCTGTCCCGCATGGGACGGGGTTGAAAG 150
151 CTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTG 200
201 ATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC 250
251 ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG 300
301 AATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT 350
351 GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGA 400
401 CGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTA 450
451 ATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGT 500
501 AGGCGGCTTGTTGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCT 550
551 GCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTG 600
601 GTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGC 650
651 GGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAA 700
701 CAGA 704

Isolate AC2_25

1 ATGAAGCCTTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGG 50
51 CAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCG 100
101 GATAACACTCTGTCCTGCATGGGACGGGGTTGAAAGCTCCGGCGGTGAAG 150
151 GATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGG 200
201 CGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAG 250
251 ACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG 300
301 GGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT 350
351 TGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAA 400
401 GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCA 450
451 AGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCAC 500
501 GTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGG 550
551 CTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAA 600
601 TGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCA 650
651 TTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAACAG 689

Isolate AC2_33

1 TGCAGTCGACCGATGAAGCCTTTCGGGGTGGATTAGTGGCGAACGGGTGA 50
51 GTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGG 100
101 GGTCTAATACCGGATAACACTCTGTCCCGCATGGGACGGGGTTAAAAGCT 150
151 CCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAAT 200
201 GGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC 250
251 ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA 300
301 TATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGA 350
351 CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACG 400
401 GTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT 450
451 ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG 500
501 GCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGC 550
551 ATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGT 600
601 GTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGG 650
651 ATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAACA 700
701 GA 702

Isolate AC2_39

```
1      CGCTGAAGCCCTTCGGGGTGGATGAGTGGCGAACGGGTGAGTAACACGTG      50
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151    AGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAGTGGCCTACCAA      200
201    GGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTG      250
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351    GTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAG      400
401    AAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG      450
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551    GGCAGGCTAGAGTTCGGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGA      600
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651    CGATACTGACGCTGAGGAGCGAAAGCGTGGG                         680
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AC3_26

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501    GTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGG      550
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601    TGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCA      650
651    TTACTGACGCTGAGGAGCGAAAGCGTGGG                           679
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XS2_42

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551    TGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCT      600
601    GGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGG      650
651    CGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGG             694
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XS3_4

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151	CCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAG	200
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351	CAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTA	400
401	ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGA	450
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551	GGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCA	600
601	GGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGA	650
651	GGAGCGAAAGCGTGGGGAGCAAACAGAG	694

XS3_5

1	TGCAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGA	50
51	GTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGG	100
101	GGTCTAATACCGGATAACACTCTGTCCCGCATGGGACGGGGTTGAAAGCT	150
151	CCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGAT	200
201	GGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCAC	250
251	ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA	300
301	TATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGA	350
351	CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACG	400
401	GTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT	450
451	ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG	500
501	GCGGCTTGTTGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGC	550
551	AGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGT	600
601	GTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGG	650
651	ATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAACA	700
701	GA	702

Appendix H Media and solutions for isolation of actinomycetes from rhizosphere soil and culture of actinomycetes

A. Antibiotic stock solutions

1. Cycloheximide stock solution

Cycloheximide (Sigma-Aldrich)	1.0 g
Sterile distilled water	100 mL

Add 1 g of cycloheximide to 100 mL sterile distilled water in a sterile glass bottle, stir well and store the solution at 4°C.

2. Nalidixic acid stock solution

Nalidixic acid (Sigma-Aldrich)	0.4 g
Sterile distilled water	100 mL

Add 1g of nalidixic acid to 100 mL sterile distilled water in a sterile glass bottle, stir well and store at 4°C.

B. Salt stock solutions

1. **Ringer's solution (McLean & Ivimey-Cook, 1952)**

NaCl	7.5 g
KCl (Sigma-Aldrich)	0.075 g
$CaCl_2.H_2O$	0.1 g
$NaHCO_3$	0.1 g

Distilled water	1.0 L
pH adjusted to	7.2

Add all the ingredients to 1 L sterile distilled water in a sterile glass bottle and stir well. Autoclave the solution and store on a bench at ambient temperature (ca. 17-22°C).

2. Vitamin B complex stock solution (Hayakawa & Nonomura, 1987)

Thiamine –HCL (Sigma-Aldrich)	0.5 mg
Riboflavin (Sigma-Aldrich)	0.5 mg
Niacin (Sigma-Aldrich)	0.5 mg
Pyridoxin-HCl (Sigma-Aldrich)	0.5 mg
Inositol (Sigma-Aldrich)	0.5 mg
Ca-pantothenate (Sigma-Aldrich)	0.5 mg
p-aminobenzoic acid (Sigma-Aldrich)	0.5 mg
Biotin (Sigma-Aldrich)	0.25 mg
Sterile distilled water	10 mL

Add all the ingredients in 10 mL of sterile distilled water in a sterile glass bottle. Stir well and store the solution 4°C.

3. Trace salts stock solution

$FeSO_4.7H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g

$ZnSO_4.7H_2O$	0.1 g
Sterile distilled water	100 mL

Add all the ingredients to 100 mL sterile distilled water and stir well. Store the solution on a bench at ambient temperature (ca. 17-22°C)

C. Culture media

1. Water-yeast extract agar (WYE) (Crawford *et al.*, 1993)

Yeast Extract (Sigma-Aldrich)	0.25 g
K_2HPO_4	0.5 g
Agar (Sigma-Aldrich)	18.0 g
Distilled water	1 L
pH (adjust with NaOH or HCl)	7.2

Add all the ingredients to 1 L of distilled water in a glass bottle. Adjust the solution to pH 7.2 with NaOH and HCl. Autoclave at 121°C for 20 min. When the medium has cooled down to 50-55°C, add 5 mL each of filter sterilised cycloheximide and nalidixic acid stock solutions to the medium to give a final concentration of 50 mg and 20 mg L^{-1} respectively. Stir well using a sterile magnetic stirrer before dispensing into Petri dishes.

2. Casamino acids-yeast extract-glucose agar (YCED) (Crawford *et al.*, 1993)

Yeast extract (Sigma-Aldrich)	0.3 g

Casamino acids	0.3 g
D-glucose anhydrous	0.3 g
K_2HPO_4	2.0 g
Agar (Sigma-Aldrich)	18.0 g
Distilled water	1 L

Add all the ingredients to 1 L of distilled water in a glass bottle. Adjust the solution to pH 7.2 with NaOH and HCl. Autoclave at 121°C for 20 min. When the medium has cooled down to 50-55°C, add 5 mL each of filter sterilised cycloheximide and nalidixic acid stock solutions each to the medium to give a final concentration of 50 mg and 20 mg L^{-1} respectively. Stir well using a sterile magnetic stirrer before dispensing to Petri dishes.

3. Casein glycerol medium (CGM) (Küster & Williams, 1964)

$CaCO_3$	0.02 g
Glycerol (Sigma-Aldrich)	10 g
KNO_3 (Chemical Supply)	2.0 g
NaCl	2.0 g
K_2HPO_4	2.0 g
$FeSO_4.7H_2O$	0.01 g
$MgSO_4.7H_2O$	0.05 g
Distilled water	1 L

Add all the ingredients to 1 L of distilled water in a glass bottle. Adjust the solution to pH 7.2 with NaOH and HCl. Autoclave at 121°C for 20 min. When the medium has cooled down to 50-55°C, add 5 mL each of filter sterilised cycloheximide and nalidixic acid stock solutions to the medium to give a final concentration of 50 mg and 20 mg L-1 respectively. Stir well using a sterile magnetic stirrer before dispensing to Petri dishes.

4. Humic acids vitamin agar (Hayakawa & Nonomura, 1987)

Humic acids (Sigma-Aldrich)	1.0 g dissolved in 10 mL of 0.2 M of NaOH
$MgSO_4.7H_2O$	0.05 g
KCl_2	1.71 g
Na_2HPO_4	0.5 g
$CaCO_3$	0.02 g
$FeSO_4.7H_2O$	0.01 g
Distilled water adjusted to	1 L

Add all the ingredients to 1 L of distilled water in a glass bottle. Adjust the solution to pH 7.2 with NaOH and HCl. Autoclave at 121°C for 20 min. When the medium has cooled down to 50-55°C, add 5 mL each of filter sterilised cycloheximide and nalidixic acid stock solutions to the medium to give a final concentration of 50 mg and 20 mg L^{-1} respectively. Add 5 mL of filter sterilised vitamin B complex stock

solution to the medium. Stir well using a sterile magnetic stirrer before dispensing to Petri dishes.

5. International *Streptomyces* Project 2 (ISP2 medium) (Shirling & Gottlieb, 1966)

Malt extract (Sigma-Aldrich)	10 g
Yeast extract (Sigma-Aldrich)	4.0 g
Glucose	4.0 g
Agar (Sigma-Aldrich)	20 g
Distilled water	1 L

Add all the ingredients to 1 L of distilled water in a glass bottle. Adjust the solution to pH 7.2 with NaOH and HCl. Autoclave at 121°C for 20 min. When the medium has cooled down to 50-55°C, dispense the medium into Petri dishes.

6. Half-strength Potato Dextrose Agar (PDA) (Dhingra & Sinclair, 1995)

PDA (Difco)	19.5 g
Agar (Sigma-Aldrich)	7.5 g
Distilled water	1 L

Add all the ingredients in a glass bottle. Add distilled water to 1 L volume. Autoclave at 121°C for 20 min. When the medium has cooled to about 50°C, dispense the agar into Petri dishes.

7. Mannitol-Soy agar (MS agar) (Kaewkla, 2009)

Mannitol	20.0 g
Agar (Sigma-Aldrich)	15.0 g
Soy flour	20.0 g
Distilled water	1.0 L

Mix 20 g of mannitol and 15 g of agar in a container before adding 500 mL of distilled water. In a separate container, weigh 20 g of soy flour and add 500 mL of distilled water. Mix the soy flour well using a magnetic stirrer and adjust the pH to 7.2 using NaOH and HCl. After autoclaving, allow the solutions to cool to about 55°C before pouring the mannitol solution into the soy solution. Mix well using a sterile magnetic stirrer. Dispense the mannitol-soy agar into Petri dishes.

8. Oatmeal Agar (Dhingra & Sinclair, 1995)

Oat grain	20.0 g
Agar (Sigma-Aldrich)	18.0 g
Distilled water	1.0 L
Trace salts stock solution	1 mL

Add 20 g of oat grain to 900 mL of distilled water and stir thoroughly before bringing the oat suspension to boil for 20 min. Filter the solution with a piece of cheese cloth before adding 18 g of agar. Adjust the volume to 1 L by adding distilled water and autoclave the solution at 121°C for 20 min. Add 1 mL of filter sterilised trace salt stock to molten agar once cooled to 50-55°C and stir thoroughly before dispensing the agar into Petri dishes.