Interactions between *Phytophthora cinnamomi* and *Acacia pulchella*: consequences on ecology and epidemiology of the pathogen

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This thesis is presented for the degree of  
Doctor of Philosophy  
School of Biological Sciences and Biotechnology  
Murdoch University  
Perth, Western Australia  
May 2006
Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institution. To the best of my knowledge, the work performed by others, has been duly acknowledged.

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May 2006
Abstract

Phytophthora cinnamomi is an important pathogen of many plant species in natural ecosystems and horticulture industries around the world. In Western Australia, a high proportion of native plant species are susceptible to P. cinnamomi attack. Acacia pulchella, a resistant legume species native to Western Australia has been considered as a potential biological control tool against P. cinnamomi. To develop effective control methods, it is important to understand the interactions between the control agent and the different life forms of the pathogen. In this thesis the interactions are investigated between P. cinnamomi and varieties of A. pulchella which occur in jarrah (Eucalyptus marginata) forest and sand plain ecosystems.

The soil inoculum of P. cinnamomi was compared under the potted plants of the three common varieties of A. pulchella, var. pulchella, var. glaberrima and var. goadbyi. These were grown in infected jarrah forest soil in the glasshouse and in vitro in a sterilised soil-less mix aseptically. Acacia urophylla (a species non suppressive towards P. cinnamomi) was also included as a control. An isolate of the most commonly found clonal lineage of P. cinnamomi in the jarrah forest, A2 type 1 was selected for use in experiments after testing showed it reliably produced zoospores and chlamydospores both axenically and in non-sterile conditions, in comparison to several other isolates. The lowest survival of P. cinnamomi inoculum was found under A. pulchella var. goadbyi plants grown both in non sterile soil and in aseptic soil-less mix.
All the life forms of *P. cinnamomi* were affected by *A. pulchella* (Chapters 2, 3, 4 and 5). The soil leachates from potted plants of *A. pulchella* var. *goadbyi* reduced sporangial production (Chapter 2) and caused cytoplasm collapse of chlamydospores (Chapter 3). The confirmation was obtained that soil under *A. pulchella* was inhibitory to sporangial stage of *P. cinnamomi* and new evidence was obtained on chlamydospore inactivation. Cytoplasm collapse in the chlamydospores was observed both for chlamydospores on mycelial discs on Mira cloth exposed to the soil leachate and within infected roots buried in soils under the three varieties of *A. pulchella* plants. The effect was strongest under the plants of *A. pulchella* var. *goadbyi* and indicated that the chlamydospores of *P. cinnamomi* are unlikely to act as persistent structures under *A. pulchella* var. *goadbyi* plants.

In Chapter 4, bioassays were conducted with axenically produced mycelia, chlamydospores and zoospores to test the inhibitory effect of the root exudates collected from aseptically grown *A. pulchella* var. *goadbyi* plants. The zoospores of the same isolate used in the soil leachate tests were immobilised (became sluggish and encysted) within one to two minutes. When incubated for 24 h, zoospores predominantly clumped and germ tubes were observed only from the clumped ones. Chlamydospores produced by four isolates of the common A2 type 1 strain and the only one A2 type 2 strain available at the time were tested. A higher percentage of chlamydospores collapsed and a very low percentage germinated after 24 h. Chlamydospores of all the A2 type 1 isolates were inhibited by the root exudates whilst the A2 type 2 isolate remained viable. The findings showed that the suppressive effect must be due at least in part to substances exuded by the *A. pulchella* plants. However, it appeared that the A2 type 1 isolates were more vulnerable to this effect than the single A2 type 2 isolate.
In Chapter 5, the effect of season on sporangial suppression of *P. cinnamomi* was shown using field soils collected from three jarrah forest soil vegetation types and a *Banksia* woodland on Bassendean sand, collected in winter and summer. The effect of age of *A. pulchella* plants was demonstrated using the soils collected from rehabilitated bauxite mine pits. In all the locations soils were collected under *A. pulchella* plants and 5 m away from the nearest *A. pulchella*. An effect of soil type was evident as whilst the soil leachates made from the three lateritic jarrah forest soil types where *A. pulchella* is common in the understorey were suppressive to the sporangial stage of *P. cinnamomi*, this effect was not evident in the Bassendean sand under *A. pulchella*. *A. pulchella* soils collected in winter were less suppressive towards sporangial production than soils collected in summer. An effect of plant age was demonstrated as soil leachates from four year-old *A. pulchella* stands in rehabilitated bauxite mine sites were more suppressive for sporangia than leachates from one year-old stands.

Further information on the behaviour of the pathogen in soil and in potting mix with and without *A. pulchella* was obtained by infecting lupin radicles with an isolate of each A2 type, 1 and 2 strains of *P. cinnamomi* and burying them in the soil under the three varieties of *A. pulchella* plants. After a week, the chlamydospores were mostly collapsed and hyphae deteriorated. Oospores were observed and in significant numbers under the potted plants of *A. pulchella* var. *glaberrima*.

Isolates of all three clonal lineages of *P. cinnamomi* found in Australian soil were tested for the ability to produce oospores. Two isolates of the A1 and A2 type 2 and three isolates of the common A2 type 1 were screened. The two isozyme types of the A2
clonal lineage isolated in Australia varied in ability to self and produce oospores in planta in several soils from the jarrah forest. The isozyme type 2 of the A2 clonal lineage of *P. cinnamomi* produced oospores under these experimental conditions. This stimulation was not effective for most of the tested isolates of the A2 type 1 and the A1 clonal lineage. The *in planta* oospores were viable but dormant and the oogonial-antheridial associations were amphigynous both *in vitro* and *in vivo*. For the first time it was established that, the stimulus for selfing and oospore formation in the A2 type 2 of *P. cinnamomi* is available in some jarrah forest soils, with and without *A. pulchella* and also in the potting mix used. This raises important questions for the management of the pathogen.

Several factors were identified as potential stimuli for selfing. Among them, soil nutrient levels and essentially enhanced sulphur presence were found important. Temperature also played a key role. Oospores were produced abundantly at 21 – 25 °C but not over 28 °C.

The biology of *P. cinnamomi* has been studied for several decades but some important aspects remain un-researched. This thesis pioneers research into the *in planta* selfing aspect of the pathogen in soil. It also improved the understanding of the interactions between *P. cinnamomi* and *A. pulchella* which to some extent supports use of *A. pulchella* as a biological control tool against *P. cinnamomi*. However, attention is drawn to the natural mechanisms of this complex pathogen to survive *in planta* by producing oospores, the most persistent form of its life cycle.
TABLE OF CONTENTS

Declaration......................................................................................................................ii
Abstract.........................................................................................................................iii
Table of contents..........................................................................................................viii
Publications and presentations......................................................................................xvii
Acknowledgements.......................................................................................................xix

CHAPTER 1...............................................................................................................1

General Introduction

1.1 Phytophthora cinnamomi and its global impact..............................................1

1.2 Impact of Phytophthora cinnamomi in the Australian ecosystem..............2
  1.2.1 Variability in disease spread and impact......................................................4

1.3 Pathogenicity.........................................................................................................7

1.4 Biology of Phytophthora cinnamomi.................................................................8
  1.4.1 Asexual reproduction of Phytophthora cinnamomi..............................8
  1.4.2 Sexual reproduction of Phytophthora cinnamomi..............................10

1.5 Survival mechanisms of Phytophthora cinnamomi.......................................11
  1.5.1 Saprophytic phase of Phytophthora cinnamomi...............................12

1.6 Disease management..........................................................................................13
  1.6.1 Biological control......................................................................................14

1.7 Acacia pulchella and suppression of Phytophthora cinnamomi...............17
  1.7.1 Biology of Acacia pulchella .................................................................18
  1.7.2 Germination of Acacia pulchella..........................................................23
1.7.3 The role of fire ................................................................. 23
1.7.4 Plant effects in suppression of soil borne pathogens ........... 24
1.7.5 Saponins of Acacia pulchella ........................................... 28
1.7.6 Volatiles ....................................................................... 28
1.8 Source of the mechanism of suppression ............................. 29
1.9 Thesis objectives ............................................................... 31

CHAPTER 2 ............................................................................. 32

Varietal differences of Acacia pulchella in suppression of
Phytophthora cinnamomi in aseptic and non-aseptic substrates

2.1 Introduction ...................................................................... 32
2.2 Materials and Methods ..................................................... 35
  2.2.1 Experimental design for Experiments 1& 2 ...................... 35
  2.2.2 Plant material and soil for Experiments 1 & 2 ................. 36
  2.2.3 Inoculum and inoculation ............................................. 38
  2.2.4 Inoculum potential of soil .......................................... 40
  2.2.5 Non inoculated Experiment .......................................... 40
  2.2.6 Experimental design for Experiment 3 ......................... 42
  2.2.7 Establishment of Acacia pulchella plants in vitro .......... 42
  2.2.8 Soil collection ............................................................. 44
  2.2.9 Statistical analysis ....................................................... 44

2.3 Results ............................................................................. 44
  2.3.1 Inoculum potential of infected soil under the glasshouse plants 44
2.3.2 Inoculum potential of infected soil mix under the aseptic plants………..45

2.3.3 Sporangial production in soil leachates collected from the
glasshouse-grown Acacia pulchella var. goadbyi and
Acacia urophylla plants………………………………………………………46

2.3.4 Soil…………………………………………………………………………48

2.4 Discussion……………………………………………………………………49

2.4.1 Soil inoculum………………………………………………………………50

2.4.2 Sporangial production…………………………………………………………50

CHAPTER 3…………………………………………………………………...54

Effects of Acacia pulchella and Acacia urophylla on the production
and persistence of Phytophthora cinnamomi chlamydospores……….54

3.1 Introduction……………………………………………………………………54

3.2 Materials and Methods…………………………………………………………..56

3.2.1 Experimental Design – the effect of filter sterilized soil leachates on
clamydospores………………………………………………………………56

3.2.2 Experimental Design – soil inoculation of the Acacia plants with
Phytophthora cinnamomi colonised lupin roots……………………………57

3.2.3 Statistical analysis…………………………………………………………..59

3.3 Results…………………………………………………………………………. 61

3.3.1 Chlamydospore production and survival on the mycelial disks………… 61

3.3.2 Phytophthora cinnamomi sporangia in lupin root inoculum………………. 63
3.3.3 Recovery of *Phytophthora cinnamomi* from lupin root inoculum and soil under *Acacia* species.................................................................63

3.4 Discussion........................................................................................................65

CHAPTER 4............................................................................................................71

Effect of root exudates of *Acacia pulchella* on the asexual stages of *Phytophthora cinnamomi*

4.1. Introduction.....................................................................................................71

4.2 Materials and Methods..................................................................................73

   4.2.1 Experimental design..................................................................................73
   4.2.2 Growth containers....................................................................................74
   4.2.3 Maintenance of the seedlings.....................................................................75
   4.2.4 Root exudate collection.............................................................................75
   4.2.5 Bioassay of chlamydomspores with *Acacia* root exudates...............76
   4.2.6 Bioassays of zoospores with *Acacia* root exudates.............................77
   4.2.7 Bioassays of the mycelium with *Acacia* root exudates.....................77
   4.2.8 Evaluation of soluble sulphur in the root exudates...............................78
   4.2.9 Statistical analysis...................................................................................78

4.3 Results..............................................................................................................80

   4.3.1 Bioassay of chlamydomspores with *Acacia* root exudates...............80
   4.3.2 Bioassays of zoospores with *Acacia* root exudates.............................82
   4.3.3 Mycelial bioassay....................................................................................83
   4.3.4 Soluble sulphur.......................................................................................84
4.4 Discussion..................................................................................................................84

4.4.1 Effect on chlamydospores..................................................................................85

4.4.2 Effect on zoospores.........................................................................................85

4.4.3 Effect of soluble sulphur in the root exudates of *Acacia pulchella*

var. *goadbyi*..............................................................................................................86

CHAPTER 5....................................................................................................................88

Suppression of *Phytophthora cinnamomi* under *Acacia pulchella* in the native vegetation

5.1 Introduction..........................................................................................................88

5.2 Materials and Methods......................................................................................90

5.2.1 Experimental design......................................................................................90

5.2.2 Soil.....................................................................................................................90

5.2.3 *Acacia pulchella* varieties..............................................................................91

5.2.4 Preparation of soil extracts and inoculation with

*Phytophthora cinnamomi*.......................................................................................93

5.2.5 Effect of the soil filtrates on *Phytophthora cinnamomi* recovery.................94

5.2.6 Statistical analysis...........................................................................................95

5.3 Results..................................................................................................................95

5.3.1 Effect of the forest soils on *Phytophthora cinnamomi* recovery...............95

5.3.2 Effect on *Phytophthora cinnamomi* of extracts from soil collected

under one and four years-old *Acacia pulchella* stands on rehabilitated

bauxite mine pits.......................................................................................................98
5.3.3 Sporangial production of Phytophthora cinnamomi in soil filtrates……… 99
5.3.4 Soil chemical properties.................................................................100

5.4 Discussion..........................................................................................101
5.4.1 Sporangial production and zoospore release.................................102
5.4.2 Variation in the species complex of Acacia pulchella.......................103

CHAPTER 6..............................................................................................105

Oospore production of Phytophthora cinnamomi in the presence
of Acacia pulchella

6.1 Introduction.........................................................................................105

6.2 Materials and Methods.......................................................................108
6.2.1 Plant and soil material.................................................................108
6.2.2 Inoculum and inoculation.........................................................109
6.2.3 Viability of Oospores.................................................................112
6.2.4 Statistical analysis.................................................................113

6.3 Results...............................................................................................115
6.3.1 In planta oospore production..................................................115
6.3.2 Oospore viability........................................................................117
6.3.3 Soil chemical properties under the three Acacia pulchella varieties....118

6.4 Discussion.........................................................................................120
6.4.1 Selfing.........................................................................................120
6.4.2 Stress response.........................................................................121
6.4.3 Plant effect...............................................................................121
6.4.4 Soil chemical properties.................................................................123
6.4.5 Variability among isolates............................................................123
6.4.6 Variability of oospores.................................................................124
6.4.7 Niche effect of oospore formation...............................................125
6.4.8 Viability of oospores.................................................................125

CHAPTER 7.............................................................................................................128

Determination of the ability of the three clonal lineages of
Phytophthora cinnamomi present in Australia to produce
oospores in planta in several jarrah forest soils

7.1 Introduction...............................................................................................128

7.2 Materials and Methods...........................................................................129

7.2.1 Isolates...............................................................................................129
7.2.2 Soil.....................................................................................................130
7.2.3 Inoculum............................................................................................131
7.2.4 In vitro mating of A1 and A2 type isolates........................................131
7.2.5 Statistical analysis..............................................................................132

7.3 Results.................................................................................................132

7.3.1 Variation between isolates...............................................................132
7.3.2 Soil chemical properties.................................................................135
7.3.3 Correlation between the in planta oospore production and soil
    sulphur levels.........................................................................................136
7.3.4 In vitro mating of A1 and A2 type isolates........................................138
7.4 Discussion ........................................................................................................... 138

7.4.1 In vitro mating ................................................................................................. 139
7.4.2 Variation between isolates ................................................................................ 140
7.4.3 Effect of *Acacia pulchella* plants on selfing .................................................. 141

CHAPTER 8 ............................................................................................................. 143

Effect of elemental sulphur and influence of the soil temperature on *in planta* selfing and oospore formation of *Phytophthora cinnamomi*

8.1 Introduction ......................................................................................................... 143
8.2 Materials and Methods ......................................................................................... 144

8.2.1 Experimental design ....................................................................................... 144
8.2.2 Experiment 1 .................................................................................................... 145
8.2.3 Experiment 2 .................................................................................................... 146

8.3 Results ................................................................................................................ 146

8.3.1 Experiment 1 .................................................................................................... 146
8.3.2. Experiment 2 .................................................................................................. 148

8.4 Discussion .......................................................................................................... 149

8.4.1 Effect of sulphur ............................................................................................... 149
8.4.2 Influence of temperature .................................................................................. 151
CHAPTER 9.......................................................................................153

General Discussion

APPENDICES..................................................................................165

REFERENCES....................................................................................177
Publications and presentations

Publications

Jayasekera AU, McComb JA, Shearer BL, Hardy GE St J (2006) In planta selfing and oospore production of Phytophthora cinnamomi in the presence of Acacia pulchella. Mycological Research (Under review).


Poster presentations


Oral presentations

Acknowledgements

I appreciate the Murdoch University research studentship and the completion scholarship for this project. My sincere thanks to Prof. Jen McComb for support, excellent supervision and encouragement throughout the project. Her positive energy inspired me to stay focused in the ‘no funding environment’. I am grateful to A/Prof. Giles Hardy for his generosity in lending valuable reading material and constructive assessment of my work. I thank Dr. Bryan Shearer for the valuable comments and advice. I acknowledge A/Prof. Rob Trengove for the helpful discussions on exudate chemistry.

I thank Dr. Ian Colquhoun (Alcoa) and Mr Bruce MacMahon (Mt Barker) for providing the soil and appreciate Dr. Bruce Maslin (CALM) for identifying the Acacia varieties.

Special thanks are due to the technical staff of Biological Science - Gordon, Max, Ian and Kim and the office staff - especially Nan and Colleen. I also wish to thank Aaron Maxwell and Chris Dunne for inducting me to the Plant Path Lab. The friendship and therapeutic laughs with Penny Hollick and Julie Ellery from Lab 3.01 and long distance chats with my sister, Savithri made some of the PhD blues disappear.

I am indebted to all the people who helped me to choose right things in life - my parents, the special family I grew up in, friends and teachers. Finally, I thank my husband, Sunil and my two girls, Yovundhi and Aparna (my “Chief Editor”) from the bottom of my heart for their love, comfort, help and blessings - without them I could not have carried this through to the end.
CHAPTER 1

General Introduction

1.1 Phytophthora cinnamomi and its global impact

Phytophthora cinnamomi is a major plant pathogen in native forests and horticultural industries in many parts of the world. It was discovered by Rands in 1922 in cinnamon (Cinnamomum brumannii) trees in Sumatra. Zentmyer (1983) described P. cinnamomi as ‘the most widely distributed species of Phytophthora and the species with the largest host range’. Evidence of P. cinnamomi attack comes from records in natural vegetation, plantations and horticulture. Podger et al. (1965) first associated P. cinnamomi with the dieback of jarrah (Eucalyptus marginata). Since that time, P. cinnamomi and other Phytophthora spp. have dominated forest pathology in Australia (Davison and Shearer 1989). Similarly, in other parts of the world, diseases caused by P. cinnamomi have attracted significant attention.

In the eastern oak ecosystems of USA, the most frequently recovered Phytophthora species (90%) is P. cinnamomi (Balci et al. 2004). It is the cause of the little leaf disease of Pinus echinata in the south-eastern USA (Tainter 1997), ink disease of chestnuts (Castanea spp.) in Europe (Vannini and Vettriano 2002) and the USA (Erwin and Ribeiro 1996) of red oak (Quercus rubra) in south western France (Robin et al. 1992). P. cinnamomi is among other Phytophthora species identified as the cause of oak decline in southern and central Europe (Brasier and Jung 2001) and a major contributor to the ohia (Metrosideros collina ssp. polymorpha) tree decline in Hawaii (Kliejunas and Scharpf 1977) and the cause of oak mortality in Mexico (Tainter et al. 2000). In the Dominican Republic, little leaf disease in Pinus caribinea is caused by
*P. cinnamomi* (Jung *et al.* 2004) and identified as a key threat to forestry. In China, *P. cinnamomi* has been identified as the cause of death of a popular urban tree species; *Cedrus deodara* in the Nanjing area (Ho *et al.* 1984) and an important pathogen of *Eucalyptus* in South Africa (Linde *et al.* 2001).

Diseases caused by *P. cinnamomi* in horticulture have been well reported from around the world. Root rot of avocados (*Persea americana*) in California and Latin America (Zentmyer 1977), in New South Wales (NSW) and Queensland in Australia (Broadbent and Baker 1974) root and crown rot of pineapple (*Ananas comosus*) and ornamental plants around the world (Zentmyer 1983) are some important examples. An existing problem for the Christmas tree industry in the US is *P. cinnamomi* root rot in one of their premier species; Fraser fir (*Abies fraseri*) which causes significant loses (Frampton *et al.* 2001). *P. cinnamomi* outbreaks have recently been reported from Polish oak stands. The nurseries where the plant material originated have been implicated as the source of infection and spread (Oszako *et al.* 2004).

1.2 Impact of *Phytophthora cinnamomi* in the Australian ecosystems

In Australia, *P. cinnamomi* is one of the 13 key threatening processes to the biodiversity in the Australian ecosystems (Natural Heritage Trust and Environment Australia 1999). The native species susceptible to *P. cinnamomi* include many from jarrah forest, *Banksia* woodlands (Fig. 1.1) and northern and southern sand plains of the south west of Western Australia (Shearer 1994). These regions of south-western Australia have high biodiversity and there are many rare and endangered species, initially threatened by habitat restriction and now being pushed to extinction by the pathogen (Hardy *et al.* 1994). It is estimated that 40% of the 5710 described plant
species in the South-West of Western Australia are susceptible and 14% highly susceptible to the pathogen (Shearer et al. 2004).

![Figure 1.1 Banksia woodland, Northcliff, Western Australia affected with Phytophthora cinnamomi. A – dead Xanthorrhoea preissii (▼), B – Dying Banksia spp.](image)

The dry sclerophyll forest in the Brisbane Ranges and East and South Gippsland in Victoria (Weste 1993) are among the high impact areas in Eastern Australia. *P. cinnamomi* occurs in over 30% of the parks listed under the National Parks Act in Victoria (Cahill et al. 2003), but its impact is also evident in the north where the tropical rainforest at Garrawalt and Dalryple Heights in Queensland (Brown 1976).

In New South Wales and Tasmania, though *P. cinnamomi* is widespread it has for some time been regarded non-threatening to forest trees and other flora in national parks (Podger and Brown 1989; Davison and Shearer 1989). However, recent evidence suggests that *P. cinnamomi* is threatening susceptible rare species such as *Telopea speciosissima* and is causing decline of *Xanthorrhoea* in some national parks (McDougall et al. 2001).
1.2.1 Variability in disease spread and impact

Despite being widely distributed in a range of Australian soils (Pratt et al. 1971) in some areas *P. cinnamomi* causes no damage. This variability can be attributed to factors such as differences in susceptibility of plant species (Old et al. 1984), age and maturity of plants (Garrett 1970; Shea and Broadbent 1983), soil types and environmental conditions (Shearer and Tippett 1989).

**Susceptibility of plant species**

Some plant species withstand *P. cinnamomi* infection better than others. Species which die very quickly when the pathogen is present can be used as indicator species. Shearer and Tippett (1989) observed that *Banksia grandis* died more quickly in soil infected with *P. cinnamomi* than jarrah even though jarrah is susceptible. Wills (1992) rated plant species in the Stirling Range National Park in the south-west of Western Australia from low to highly susceptible or field resistant. The majority of the susceptible species belong to *Proteaceae, Epacridaceae* and *Papilionaceae*. Jarrah was rated as susceptible, while most of the eucalypts in the Stirling Range National Park were rated field resistant to *P. cinnamomi* (Wills 1992). Cho (1981, 1983) showed variability in *P. cinnamomi* susceptibility to one-month old seedlings of 29 *Banksia* species grown on vermiculite in a glasshouse study. There was a clear association between disease susceptibility of *Banksias* and their provenances. The susceptible species were from Western Australia whereas the resistant species were from eastern states of Australia. This finding supported Pratt and Heather’s (1973) argument that *P. cinnamomi* has been a long-term component in the soil of eastern Australia, resulting in the presence of resistant plant species in the ecosystems. *P. cinnamomi* resistance in the eastern
Australian Banksia species was confirmed by McCredie et al. (1985) under Western Australian plantation conditions.

**Age and maturity of plants**

Difference in susceptibility to Phytophthora spp. as with pathogenic fungi such as *Pythium* spp. and *Rhizoctonia solani* depends on the age and maturity of the plants (Garrett 1970). Shea and Broadbent (1983) made the observation that some plant species are susceptible to *P. cinnamomi* at the seedling stage, but resistant at later stages of development, for example; *Pinus* spp. (Newhook 1959). Experiments on the susceptibility or resistance of Banksia species to *P. cinnamomi* infection recorded some differences in the susceptibility of the plants depending on their age. McCredie et al (1985) found older plants (7-9 months) of several Banksia species (*B. candolleana, B. lindleyana* and *B. caleyi*) to be less susceptible to *P. cinnamomi* infection in the field than Cho’s (1983) one-month old seedlings in the glasshouse. The observed differences may have been due to age and maturity of the plants or the fact that one test was done in the glasshouse and the other in the field.

**Soil and environmental conditions**

Warm and moist conditions are high in the list of requirements for *P. cinnamomi* to multiply and spread. The suitable temperature range is 5-36°C with the optimum around 24-28°C (Zentmyer 1980). Maximum disease expression is observed in spring, late summer and autumn and minimum in winter in both European (Brazier and Jung 2001, Vettraino et al. 2004) and Australian forest and tree plantations (Marks et al. 1975; McCredie et al. 1985; Shearer and Tippett 1989).
Soil moisture level is regarded as crucial to *P. cinnamomi* reproduction (Duniway 1985). For zoospore dispersal, free water in the soil is absolutely necessary (Weste 1972; Shea 1975). The northern jarrah forest in Western Australia where the disease incidence is high has a Mediterranean climate. Within the forest there is a range of soil types and associated vegetation compositions (Havel 1975) and Shea (1975) recognised the moisture retention properties of these soil types as greatly influencing the potential for *P. cinnamomi* activity. Havel (1975) described the vegetation structure of the south-west of Western Australia extensively and designated vegetation types as A-Z. Type P with low fertility and poor drainage is associated with high impact of *P. cinnamomi*. Site type T, which is regarded as more fertile is associated with low *P. cinnamomi* disease impact (Havel 1975).

Weste and Marks (1987) found the soil characteristics that favour *P. cinnamomi* infestation of forest sites in the Brisbane ranges (Victoria) to be very similar to the situation in the northern jarrah forest. Infertile, lateritic soils with low organic matter that are poorly drained favour activity of *P. cinnamomi* (Weste and Marks 1987). In contrast wet sclerophyll forest soils with high organic matter and river valleys with red loams were less affected by *P. cinnamomi* (Shearer and Tippett 1989). Similar situations exist in the rain forests of Queensland and New South Wales. Despite its presence in the rain forest soils, *P. cinnamomi* has not caused widespread damage (Pegg 1977). Also, there is evidence from avocado groves in the south-eastern Queensland of suppression of *P. cinnamomi* by high organic matter content. The suppression of the pathogen on soils with high organic matter was associated with microbial activity (Broadbent *et al.* 1971; Broadbent and Baker 1974).
Soil microbiota

Interactions with soil microorganisms impact on the existence and influence the spread of *P. cinnamomi*. Whilst a range of soil microbes have been identified as being antagonistic towards *P. cinnamomi* (Malajczuk 1973; Murray 1983; Murray 1987) presence of metabolites from some organisms is essential in stimulating sporulation (Ayers 1971) of *P. cinnamomi* which is necessary for its spread.

1.3 Pathogenicity

Infection of *P. cinnamomi* is expressed primarily as root lesions, root and collar rots and subsequent death of plants (Weste 1975; Marks *et al.* 1983). Jarrah deaths were originally explained by Marks and Smith (1980) and Shea and Dell (1981) as a result of infection in short lateral and perennial roots. Shearer *et al.* (1981) and Dell and Wallace (1981) recovered *P. cinnamomi* in large roots of *E. marginata* and following this finding, the cause of jarrah deaths came to be associated with the infection of large roots. O'Gara (1988) showed that *P. cinnamomi* is capable of infecting the collar region of young jarrah plants where the stems are saturated by ponds of water after rain. There has not been a great deal of research done with regards to many other vulnerable species in the natural vegetation. The common phenomenon is the slow decline of susceptible understorey species (Shearer and Tippet 1989) and gradual deterioration of the trees (Podger 1972). However, the sudden collapse of trees and stands of understorey is not exceptional (Podger 1975). Shearer and Tippett (1989) suggested that the rapid death of trees could be due to climatic conditions, which favour high *P. cinnamomi* activity in the soil.
1.4 Biology of *Phytophthora cinnamomi*

*P. cinnamomi* is a soil-borne microscopic organism which is classified in the kingdom, Chromista and belongs to the Oomycetes. It has the characteristics of pseudofungi (Erwin and Ribeiro 1996) such as the presence of motile zoospores in its life cycle, wall composition with mainly β-glucans and cellulose (Bartnicki-Garcia and Wang 1983) rather than chitin (as in the true fungi) and diploidy in the major part of the life cycle (Erwin and Ribeiro 1996). In agar culture it grows as a whitish mass of fragile hyphae on the surface. The mycelium on agar has a characteristic coralloid form and chlamydosporas are attached mostly at the hyphal tips (Waterhouse 1965).

1.4.1 Asexual reproduction of *Phytophthora cinnamomi*

*P. cinnamomi* can reproduce asexually by producing motile zoospores or chlamydosporas (Zentmyer 1980). Zoospores are produced in sporangia, the asexual reproductive organs, and released when the conditions are favourable (Waterhouse 1963; Gisi 1983). Asexual sporangium development results in rapid reproduction (Erwin and Ribeiro 1996). The motile zoospore, which is the main infective agent of *P. cinnamomi* (Zentmyer 1980) is equipped to swim actively in the soil water towards plants (Zentmyer and Mircetich 1966; Tippett et al. 1976; Malajczuk and McComb 1977; Ho and Hickman 1967). This phenomenon known as positive taxis is caused by either the stimulus provided by the chemicals in the root exudates or the electrostatic effect surrounding the roots (West et al. 2002).

Chlamydosporas of *Phytophthora* develop by spherical expansion of hyphal tips or by localised swelling of hyphal tubes to produce terminal or intercalary spores. Most fully expanded chlamydosporas of *P. cinnamomi*, as in the other species of *Phytophthora* are
spherical (Hemmes 1983). They may be produced singly, in chains or in clusters. Chlamydospores, which can be intercalary or terminal, are delimited by septation from the mycelium (Erwin and Ribeiro 1996).

Weste and Vithanage (1979) reported that chlamydospores of *P. cinnamomi* form in soil gravel or plant tissue during dry periods. They germinate under favourable conditions and produce infective mycelia and sporangia, or more chlamydospores. Weste (1975) also indicated that the cycle may continue for at least five years provided there is a nutrient source (organic material) and a microbial population that is not highly competitive.

Chlamydospores of *Phytophthora* have either a thin or a thick wall depending on the species (Erwin and Ribeiro 1996). In *P. cinnamomi*, the chlamydospore wall is usually 0.5 μm, thinner than the other *Phytophthora* species (Zentmyer 1980; Alizadeh and Tsao 1985). According to Weste (1983), survival in antagonistic environments might depend on the thickness of the chlamydospore wall. However, it is not clear whether *P. cinnamomi* is capable of producing thick-walled chlamydospores. The small thick walled spores found by Cother and Griffin (1973) within decaying root tissues buried in non-sterile soil have not been confirmed to be propagules of *P. cinnamomi* by germinating them. Cother and Griffin (1973) inoculated the excised root pieces of New Zealand blue lupin (*Lupinus angustifolius*) seeds, germinated under sterile conditions with *P. drechsleri, P. parasitica, P. cinnamomi* and *P. syringae*. The infected root pieces were buried in moist field soils in glass vials, which were kept in sealed humid tins at room temperature. Small thick walled spores were observed within the lupin roots after three weeks in soil, which were not observed in the roots before burial or in
the roots buried for less than seven days. They also claimed not observing these spores within the roots buried in sterile (autoclaved) soil. However, the only confirmation was for *P. drechsleri* spores which were germinated on water agar and isolated on a selective medium. In this study the diameters for all four *Phytophthora* species were recorded. The small spores of *P. cinnamomi* ranged from 5.2-14.4 \( \mu \text{m} \) in diameter. However, the wall thickness of these small spores was recorded only for *P. drechsleri* which ranged from 1-1.5 \( \mu \text{m} \). Old *et al.* (1984b) examined *P. cinnamomi* chlamydospores within decaying *Eucalyptus sieberi* roots after burying them in several Australian forest soils and reported the occurrence of small thick walled spores. However, attempts to germinate these spores were not successful and no confirmation was obtained.

### 1.4.2 Sexual reproduction of *Phytophthora cinnamomi*

Sexual reproduction of *P. cinnamomi* which results in oospore production is achieved by mating the two compatible types, A1 and A2 *in vitro* as normal for heterothallic species (Chang *et al.* 1974; Zentmyer 1983). However, there is evidence that *P. cinnamomi* is capable of selfing in the presence of certain stimuli such as volatiles of *Trichoderma viride* (Brazier 1971) or avocado root exudates (Zentmyer 1979). Selfing of *P. cinnamomi* was also induced by mechanical damage of the mycelium (Reeves and Jackson 1974).

*P. cinnamomi* is known to behave differently from other heterothallic *Phytophthora* species by producing both paragynous and amphigynous antheridia in the sexual process. Hüberli (2001) observed both amphigyny and paragyny *in vitro* in many Australian isolates of *P. cinnamomi*. Some authors associated paragyny with selfing
and amphigyny with heterothallism (Savage et al. 1968). Since evidence for hybridisation is lacking in many studies due to difficulties in germinating the oospores produced in vitro, the inevitable questions to be asked are, were all the gametangial associations in mating circumstances entirely between the two mating types? Or was there a proportion of selfing? Is the belief that it is normal to have amphigyny associated with sexual interactions based on circumstantial evidence?

In Australian forest soils the commonly isolated strain is the A2 type 1 (Old et al. 1988), though the A1 mating type has been recovered in the Northern Territory (Zentmyer 1976) and other areas (Old et al. 1984a) including Banksia woodlands of the south west of Western Australia (Dobrowolski et al. 2003). The other less frequently isolated type in Australia is the A2 type 2 (Old et al. 1984a) which however is found in jarrah forest (Hüberli 2001). There is little available information on its behaviour due to lack of experimentation.

1.5 Survival mechanisms of Phytophthora cinnamomi

Information on the methods of survival of P. cinnamomi in soil is essential for formulating control methods. P. cinnamomi is capable of producing a number of propagules such as mycelium, sporangia, zoospores and chlamydospores and oospores (Zentmyer et al. 1976; Weste and Vithanage 1978; Mitchell 1979). The oospores of P. cinnamomi are not considered common in the forests of the south-west of Western Australia (Shearer 1994; Hüberli 2001) where one mating type (A2) is dominant (Dobrowolski et al. 2001). The mycelium of P. cinnamomi survives within living tissues of host plants by parasitising them, causing disease and eventually death. Upon the death of plants, the pathogen enters the saprophytic phase. According to Hornby (1983) and Baker and Cook (1983), pathogen suppression takes place in the saprophytic
stages surviving in the soil. This can be exploited in formulating disease management strategies for *P. cinnamomi* as well as for true fungi.

### 1.5.1. Saprophytic phase of *Phytophthora cinnamomi*

The possible modes of survival for pathogenic root infecting fungi are, by saprophytic survival in dead tissues of the plants invaded during the parasitic phase, by competitive saprophytic colonisation of additional dead plant tissues and by production of dormant survival structures (Garrett 1970). Saprophytic survival of root-infecting fungi is defined as survival of mycelia in infected host tissues invaded during its parasitic phase (Garrett 1970). It is difficult to determine when some substrates are completely dead and when they still contain some living cells that might be available as a food base. According to Marks *et al.* (1975), *P. cinnamomi* has been shown to behave in ways which makes it eligible to be classified (Griffin 1972) as a saprophytic soil survivor.

Zentmyer and Mircetich (1966) demonstrated that *P. cinnamomi* can colonise sterile dead wheat straw and Broadbent and Baker (1974) confirmed that *P. cinnamomi* was able to invade through a 1.5 cm moist soil layer and colonise dead lucerne stems on the soil surface. It is possible that the colonisation might have been achieved through motile zoospores rather than the hyphae growing through the soil.

Zentmyer and Mircetich also showed (1966) that *P. cinnamomi* was able to survive in soil in the absence of a living host for six years which they believed was through inactive mycelia, chlamydospores and oospores in the small root pieces which were still available in the soil. Subsequently a discovery of oospores and chlamydospores in decaying avocado feeder roots was made (Mircetich and Zentmyer 1966) in a separate
experiment and confirmation of the ability of *P. cinnamomi* to form oospores in naturally infected host tissue was acquired for the first time. Hence, the oospores came to be identified as the long term survival structures. However, there is little understanding of survival mechanisms within the dead wood. There are also some questions about the competitive ability of *P. cinnamomi* in non-sterile soil situations compared with sterile laboratory based experiments.

Tsao (1969) showed that lysis of mycelium was associated with the formation of zoosporangia and chlamydospores. Several authors found a direct relationship between lysis of mycelium of *P. cinnamomi* and the number of sporangia produced in a range of different soil types (Nesbitt *et al.* 1979; Malajczuk 1983). Rapid reproduction can be considered important for survival and may be a mode of survival for *P. cinnamomi*. The fact that sporulation of *P. cinnamomi* took place in wet but adverse conditions such as in the presence of antagonistic bacteria or low nutrients supported this suggestion.

### 1.6 Disease management

The diseases caused by *P. cinnamomi* occur when the three components – pathogen, susceptible plant and a conducive soil are present. A significant change in any of these three components can result in a change in the disease occurrence. Baker (1974) equated the severity of disease to the mathematical product of inoculum potential and the disease potential of the pathogen. For *P. cinnamomi*, changes in either the soil environment or the host range or both would make a significant difference in the infection rate.
Although chemical control is widely accepted as the fastest means of eliminating *P. cinnamomi*, fungicides are expensive to use on a broad scale in native vegetation and their indiscriminate use can produce undesirable results. The widely used fungicide Phosphite can be harmful for the native flora causing leaf burn and reducing pollen fertility (Fairbanks *et al.* 2001; Fairbanks *et al.* 2002; Barrett *et al.* 2004). Also, there is evidence that several fungi may acquire resistance to fungicides. *P. cinnamomi* has been shown to acquire resistance to Phosphite (Dobrowolski *et al.* unpublished).

Biological control may be an environmentally sound way of combating this problem. However, the biological control methods remain under-utilised and under-researched in controlling *P. cinnamomi*. Research programs to provide convincing evidence of effect and lack of undesirable side effects have not been effectively implemented.

### 1.6.1. Biological control

Garrett (1965) defined biological control as “any condition under which, or practice whereby survival or activity of a pathogen is reduced through the agency of any other living organisms (except man himself), with the result that there is a reduction in incidence of the disease caused by the pathogen”. Several authors (Mitchell 1973; Papavizas 1979) attempted to redefine this concept giving it a broader aspect covering every treatment, even chemical treatments provided they do not have a simple direct eradicating effect. The aim of biological control is to prevent the harmful effect of the pathogen and reduce disease incidence so it is no longer a problem. The great virtue of biological control is that it makes use of the natural mechanisms by which damage to plants by pests and pathogens is kept at the low values usually found in natural populations (Wood and Way 1988).
Competition, antibiosis, parasitism and toxicity has been identified as useful tools in formulating biological control methods for *P. cinnamomi* since they limit the growth and development of soil pathogens. Lowering of the inoculum potential of a pathogen would be a good example of an effective biological control.

*Trichoderma* spp. effectively reduced damping-off caused by *Pythium* and *Rhizoctonia solani* through mycoparasitism (Lifshitz *et al.* 1984) and the potential of *Trichoderma* spp. as an effective biological control agent in many crops has been reported internationally (Sivan *et al.* 1984; Coley-Smith *et al.* 1991). Stimulation of oospore formation in *Phytophthora* by antagonistic species of *Trichoderma* was considered an effective biological control method (Brazier 1978). The oospores stay dormant (Mircetich and Zentmyer 1966) thus reducing *P. cinnamomi* activity. *Trichoderma* also replaces *Phytophthora* spp. in diseased root tissues, which also lead to disease suppression (Brazier 1978).

In the horticultural industry the possibility has been demonstrated of using composted hardwood bark in container media to suppress several *Phytophthora* species (Sivasithamparam 1988; Hardy and Sivasithamparam 1991). The aspect of enzymatic suppression of *P. cinnamomi* in organic mulches was investigated by Downer *et al.* (2001). The role of biofumigants in the suppression of *P. cinnamomi* in cut flower industry has also been demonstrated. For example, Dunne *et al.* (2003) demonstrated that biofumigants from *Brassica juncea* tissues suppressed colony growth of *P. cinnamomi in vitro.*
The role of suppressive soils was brought to wider attention by the studies of Broadbent and Baker (1974). They found the soils of two avocado groves in the south-eastern Queensland to be suppressive of *P. cinnamomi*. Pegg (1977) also found the rain forest soils of Queensland to be suppressive towards *P. cinnamomi*. The “Ashburner method” whereby the soil environment of the avocado groves was maintained as close as possible to that in the adjacent rain forest soils has been shown to be one of the best biological control models. Baker and Cook (1974) explained this phenomenon as a synergy between complex factors such as high organic matter, calcium, ammonium nitrogen content and a pH level of 6-7. Together, these factors create a favourable environment for a large and active lytic microbial population to combat *P. cinnamomi*. A number of soil microorganisms are considered antagonistic to *Phytophthora* mycelium and its propagules (Pratt *et al.* 1971; Shea and Malajczuk 1977; Malajczuk *et al.* 1984).

Several authors associated the understorey vegetation with *P. cinnamomi* disease spread. Inoculum build up of *P. cinnamomi* under susceptible species like *B. grandis* was found to be high (Shearer and Shea 1987) and the dead and dying plant parts act as a disease reservoir (Shearer and Tippett 1989). Shea *et al.* (1978) discussed manipulation of the understorey composition to encourage the presence of resistant species. The presence of resistant species inhibits disease spread and these species are of interest in the development of biological control.

Plant breeding and selection programs can contribute by the development of resistant clonal lines, which can be utilised as the understorey component in rehabilitated areas or in diseased sites to reduce the disease impact. Production of resistant clonal lines of
E. marginata in Western Australia (McComb et al. 1990; Stukely and Crane 1994) is continuing despite problems associated in production of large numbers for operational plantings (Stukely et al. 2001).

1.7 Acacia pulchella R. Br. and suppression of P. cinnamomi

A. pulchella is resistant to infection by P. cinnamomi. When invaded by the pathogen the roots show hypersensitive responses followed by necrosis at the site of infection and failure of the infection to proceed (Tippet and Malajczuk 1979; Cahill et al. 1985).

Shea and Malajczuk (1977) observed lower numbers of P. cinnamomi sporangia under A. pulchella stands than under Banksia grandis in jarrah forest. This was supported by the observations of Cary (1982). Murray et al. (1985) examined sporangial production and zoospore discharge of P. cinnamomi in soils dug from beneath A. pulchella, B. grandis and E. marginata stands. They observed a seasonal variation and confirmed that soils dug under A. pulchella in autumn and spring supported a significantly low sporangial formation compared to B. grandis. Zoospore release in the B. grandis or E. marginata soil was significantly greater than that in A. pulchella soil in autumn which was not apparent in spring.

Several Victorian Acacia and Eucalyptus species were studied in a pot experiment by Smith and Marks (1983) to assess their resistance to P. cinnamomi. The most promising of the Acacias, Acacia longifolia and two resistant Eucalyptus species, E. sideroxylon and E. globulus spp. pseudoglobulus were then tested along with Acacia pulchella to establish their ability to protect E. sieberi from P. cinnamomi attack. The plants were grown in coarse sand medium and inoculated with P. cinnamomi when they were 14-weeks-old and kept permanently waterlogged. Under these conditions which strongly
favour root infection, all the plants were substantially infected and none of the tested species protected *E. sieberi* from death. Water-logging predisposes plants to infection by altering their metabolism (Drew and Lynch 1980) and affecting phytoalexin production (Cruishank *et al.* 1971). In this experiment, lack of a control with plants waterlogged but not infected by *P. cinnamomi* makes it difficult to determine whether the plant deaths were due to water-logging or *P. cinnamomi* infection.

*A. pulchella* is found to provide ‘protection’ to the susceptible species such as *B. grandis* or *E. marginata* growing near by from the attacks of *P. cinnamomi* (Shea and Malajczuk 1977; D’Souza *et al.* 2004). The level of protection recorded by D’Souza *et al.* (2004) towards *B. grandis* in their bauxite pit trial was higher than in the glasshouse experiment. This protection has been implicated mainly as a result of low sporangial production of *P. cinnamomi* under *A. pulchella* due to an antagonistic microbial environment (Shea and Malajczuk 1977; Malajczuk 1983; Murray 1983; Murray 1987).

**1.7.1 Biology of Acacia pulchella**

*A. pulchella*, the shrubby legume ‘prickly Moses’ is a common understorey component of a number of forest and woodland ecosystems throughout the south-west of Western Australia. It is a shrub 0.5-2 (3) m tall, spindly and spiny. It has a relatively short life span in comparison with most of its companion shrubby species (Monk *et al.* 1981) ranging from 5-13 years. *A. pulchella* is described as undoubtedly the most variable member of the Pulchellae and perhaps also the most variable *Acacia* species in Western Australia and previously included six varieties (Maslin 1975). However, two of the six varieties; *A. pulchella* var. *fagonioides* and var. *subsessilis* have now been excluded and
are treated as distinct species. According to the latest classification there are four identified varieties of *A. pulchella* in the Western Australian forest ecosystems and the sand plains. (B. Maslin, *pers. comm.*, 2005). They are *A. pulchella* R. Br. var. *pulchella*, var. *glaberrima* Meisn, var. *goadbyi* (Domin) and var. *reflexa* Maslin. Some morphological differences and clearly distinguishable variational characteristics exist among these varieties. These variations are mainly in the plant height, presence or lack of hairs on the branches, number and length of the spines and the size of the leaves. Depending on the habitat there seems to be variations in characteristics among populations. Hybrids between the varieties have also been identified (Fig. 1.3 D).

*A. pulchella var. pulchella*

Usually the smallest of the three varieties (0.5-1m tall), var. *pulchella* (Fig. 1.3 A) is most common in areas north of Perth from near Bullsbrook northwards to near Coorow. In the south-west it extends from Moora to Augusta and Albany. It is commonly found in lateritic soil. The shrub is much branched and described as a variable taxon. Usually there are two spines per node with the spines being unequal in length (Fig. 1.4 A). Variants with one spine per node (Fig. 1.4 B) also exist. Leaves have 4-8 pairs, pinules often dark green above and lighter green below. The presence or absence of hair on the branches varies according to provenance.

*A. pulchella var. glaberrima*

*A. pulchella* var. *glaberrima* (Fig 1.3 B) is especially common on poor sandy soils of the Swan Coastal Plain between Yanchep and Busselton, loamy-clay soils near swamps in the Fitzgerald River area and on the lateritic soils of the Darling Range (Maslin 1975). The inland populations of var. *glaberrima* (from about Wongan Hills to Lake
King) grow as somewhat smaller and more compact shrubs than those occurring elsewhere. They usually have one long spine per node (Fig. 1.4 C). The branches of this variety are typically glabrous, but some pilose individuals occur particularly in areas south of Harvey.

*A. pulchella* var. *goadbyi* (Fig.1.3 C)

In the Albany and Esperance districts, this variety is recognised by its prominently ribbed branchlets (1.4 B), its relatively few, solitary axillary spines and its quite large leaves bearing 5-8 pairs of pinnules. However, in other areas, e.g. Stirling Range National Park, Manjimup, Boyup Brook, the leaves are smaller and the plants appear to be close to var. *glaberrima* and, to a lesser degree, *var. pulchella*. 
Figure 1.3 A – Acacia pulchella var. pulchella, B – Acacia pulchella var. glaberrima, C – Acacia pulchella var. goadbyi and D - the hybrid form Acacia pulchella var. pulchella x Acacia pulchella var. glaberrima.
Fig 1.4 A – E Varieties of Acacia pulchella. A – A. pulchella var. pulchella - two axillary spines per node (▼), B – A. pulchella var. pulchella variant 1- one axillary spine per node, peduncles ~ 20 mm long, globular heads (◄). C – A. pulchella var. goadbyi – no axillary spines, branchlets prominently ribbed, reddish (►). D – A. pulchella var. glaberrima, two axillary spines per node - one long and one short (►◄), branches glabrous, peduncles ~ 20 mm long. E – Hybridized form of the varieties glaberrima x reflexa. Branches hairy (►), two axillary spines both similar in length (block arrows).
1.7.2 Germination of *Acacia pulchella*

This species exhibits the characteristics of a fire weed (Shea 1975; Monk *et al.* 1981). In its natural habitat seeds germinate abundantly only after intensive fires (Monk *et al.* 1981). The hard seed coat contributes to the extended dormancy of *Acacia* species. Some species stored in artificial conditions were viable after sixty-eight years (New 1972). The natural softening mechanisms of the seed coat are thought to be the effects of soil microbes and diurnal temperature variations. Hot water treatment is the commonest pre-germination method used in nurseries. Other various artificial treatments include ‘chipping’ or scarifying, treatment with sulphuric acid or with dry heat (Clements *et al.* 1977; Cavanagh 1980).

1.7.3 The role of fire

Intense fire destroys the pathogen to a depth of 15 cm below the soil surface (Weste 1975). These fires stimulate the native legume germination through both the breaking of the hard seed coat and through an effect of smoke on germination (Dixon *et al.* 1995). In the absence of fire, legume stands decline after approximately 15 years (Shea *et al.* 1979). The germination of native legumes in Western Australia varies with the intensity and frequency of fire. In northern (dry sclerophyll) jarrah forest, native legume species rarely regenerate following prescription burning, which is of low intensity. In areas subjected to low intensity fires, legume species are replaced by proteaceous species which become the dominant components of the shrub and understorey layer (Shea *et al.* 1979). Several studies have shown that the proteaceous species are highly susceptible to *P. cinnamomi* attack. Replacement of the proteaceous species with an understorey dominated by leguminous species was suggested to be
beneficial to the jarrah forest ecosystem. Hence, fire plays an important role in *Phytophthora* disease in Western Australia.

1.7.4 Plant effects in suppression of soil borne pathogens

*Root exudation*

The movement of water-soluble organic molecules across the root tissues into the surrounding environment is termed root exudation. Root exudation of biologically active compounds plays an important role in the interaction between higher plants and the soil ecosystem. This includes the exudation of chemical signals to attract symbiotic microorganisms such as the soluble components in the root exudates of several plant species on the growth of vesicular-arbuscular mycorrhizal fungi (Bécard and Piché 1992) as well as the exudation of antimicrobial substances for plant defence against soil-borne pathogens (Park *et al.* 2004). Hale *et al.* (1978) stated that any soluble compound found within the plant could also be found in exudates from roots. However, the substances released by the plants as exudates may not be truly representative of the *in planta* compounds.

Root exudates have been grouped into those with low and high molecular weight compounds. The main constituents of root exudates of many plant species are compounds with low molecular weights such as amino acids, sugars, organic acids, proteins, polysaccharides, growth substances and phenolics whereas proteins, polysaccharides and mucilage make up the high molecular weight group (Walker *et al.* 2003). Depending on age, stage of development of plants and the environmental conditions, composition of root exudates and the quantities of the compounds present vary among and within species (Vancura and Hanzlikova 1972). This has been found to
influence the plant’s resistance or susceptibility towards soil borne diseases. Keeling (1974) observed variations in the soluble carbohydrate levels in root exudates of three soybean cultivars, and showed that this influenced plant susceptibility towards *Pythium* spp.

In some species the common compounds in the root exudates do not have a specific role in disease susceptibility/resistance. Malajczuk and McComb (1977) examined the root exudates of two eucalypt species, which differ in disease susceptibility to *P. cinnamomi*. Seedlings of *Corymbia calophylla*, which is field resistant and *Eucalyptus marginata*, which is susceptible were raised axenically in nutrient solution and root exudates were collected periodically. The total amino acids in the root exudates of *E. marginata* seedlings were twice as high as those of *Corymbia calophylla*. Proline and phenylalanine were present in higher quantities in the root exudates of older *E. marginata* seedlings. In addition, an unidentified amino acid was found in the root exudates of younger *E. marginata* seedlings. However, zoospores of *P. cinnamomi* showed a non-specific response in infecting the roots of the two species. They were chemotaxically attracted towards the roots of both species, encysted on the roots in similar numbers and germinated. Based on their observations, Malajczuk and McComb (1977) suggested that the root exudates *per se* do not directly influence resistance/susceptibility of the studied species to *P. cinnamomi* infection. Instead they play an indirect role by supporting a micro flora, which can inhibit *P. cinnamomi*. It is also possible that the microorganisms can function as elicitors in the production of specific inhibitory chemicals by the roots.
Some plant species contain specific substances in their root exudates in addition to the common compounds. Antifungal compounds (6R)-7,8-dihydro-3-oxo-α-ionone and (6R, 9R)-7,8-dihydro-3-oxo-α-ionone isolated from Zea mays root exudates were inhibitory towards F. oxysporum f.sp. melongenae (Park et al. 2004). Gurha and Srivastava (2002) reported inhibition of Fusarium oxysporum f.sp. in the presence of the root exudates of wheat (Triticum aestivum) and Indian mustard (Brassica juncea).

**Root extracts**

The widely used practice is extracting the compounds from different plant parts by various methods, bioassay and analysing the chemical composition.

There are few identified compounds isolated from plants that show activity against P. cinnamomi. Zaki et al. (1980) isolated borbonol from the roots of Persea borbonia and the bioassays showed that it inhibited mycelial growth at a minimum concentration of 1 μg/mL and sporangial production at 5-10 μg/mL. This compound is present in stems and roots of P. cinnamomi resistant avocado cultivars and plays a defensive role against P. cinnamomi infection.

Among the chemicals extracted from plants, saponins play an important role because of their diverse biological effects. Much of the work on saponins has been done in India and China on herbaceous species and the properties of saponins from several Indian species are well known. The termite and fungal resistance of the ancient woods in temples have been found to be due to the presence of saponins (Agarwal and Rastogi 1974).
Saponins

Endogenous saponins have been implicated in disease resistance in some plant species (Sang et al. 2001) Saponins, extracted from both plants and animals have been shown to have antimicrobial and antimycotic properties and considerable work was done on this topic between 1966 – 1972 (Agarwal and Rastogi 1974). The name saponin is derived from the soapwort plant (Saponaria spp.). Saponins get their individual names from the species from which they were originally extracted. For example, acacinine is extracted from the Indian Acacia concinna and avenacin is extracted from oats (Avena spp).

Detailed investigations of the potential role of saponins as determinants of disease resistance have been carried out in oats (Avena) and tomato (Lycopersicon) that produced avenacin and tomatin, respectively (Deacon et al. 1985; Osbourn et al. 1994, 1995). Osbourn et al. (1994) reported that composition of these saponins is relatively simple and included avenacin in the triterpenoid group. Toxicity of saponins to fungi is likely due primarily to their ability to complex with sterols, causing loss of membrane integrity (Gus-Mayer et al 1994). Avenacin and escin (Gossipium saponin) were found to inhibit the growth of various fungi including Gaeumannomyces graminis and Neurospora crassa. Plant pathogens such as Sclerotinia fructicola, Claviceps purpurea, Trichothecium roseum, Piricularia oryzae and Fomes officinalis were classed as sensitive towards saponins. Botrytis allii, Alternaria solani, Coniophora cerebella, Rhizoctonia solani, Schizophyllum commune and three Fusarium species were found to be resistant (Osbourn et al. 1994). Zoospores of Phytophthora spp. including P. cinnamomi are known to be sensitive to saponins (Zentmyer and Thompson 1967). Deacon and Mitchell (1985) demonstrated the toxicity of β aescin (a saponin extracted
from the leaves of *Aesculus hippocactanum*) to the zoospores of *Pythium* spp. In experiments to control *P. cinnamomi* on avocado using alfalfa meal in California it is possible that it was the saponins in the meal that reduced root rot (Zentmyer 1963).

### 1.7.5 Saponins of *Acacia pulchella*

Alexander (1978) isolated saponins from the roots of *A. pulchella*. The saponin fraction was obtained as a pale yellow gum from an aqueous alcoholic extract of the roots. Dried roots of *A. pulchella* were milled and extracted with 50% aqueous ethanol over a period of three days. Preliminary bioassays against *P. cinnamomi* showed that an aqueous solution of saponins at a concentration of 500 ppm caused considerable lysis of *P. cinnamomi* mycelium and some suppression of sporulation.

### 1.7.6 Volatiles

Volatiles from several plant species are known to be involved in suppression and stimulation of soil fungi (Linderman RG 1969; Hora and Baker 1972; Smith 1972). Owens *et al.* (1969) showed that certain volatile substances released from leguminous tissues during decomposition in soil triggered germination of propagules of *Sclerotium rolfsii* and *Verticillium dahliae*, but did not support their continuing growth which resulted in their death.

**Volatiles of *Acacia pulchella* roots**

The possible role of volatile components of *A. pulchella* roots was studied by Whitfield *et al.* (1981). Non-sterile roots of *A. pulchella* plants dug from a three-year-old stand in a jarrah forest were used in the extraction process using three methods; headspace vapour sampling technique, steam distillation method and low temperature vacuum distillation methods. The bioassays used an extract obtained by steam distillation and...
measured inhibition of sporangial production and zoospore germination of *P. cinnamomi*. The sporangial production was almost totally suppressed by the root extract at a concentration of 1400 mg/L. At lower concentrations, sporangial production was still significantly less than in the control. Sporangial abortion was caused at a concentration low as 20 mg/L and there was almost total abortion at 600 mg/L. Suppression of zoospore germination and mycelial growth was achieved at varying concentrations of the extract. Fractionation of the volatile extract was carried out using gas chromatography and mass spectrometry and a considerable number of compounds were identified. The complex mixture of compounds consisted mainly of alcohols, aldehydes, ketones, esters and acids. However, a specific chemical or a group of chemicals could not be directly implicated as the inhibitor of the pathogen. The characteristic smell of *Acacia* roots was associated with 1,2,4-trithiolane which has a strong sulphurous aroma.

### 1.8 Source and the mechanism of the suppression

There is no evidence that saponins, volatile compounds or any other antifungal compounds are present in the root exudates of *A. pulchella*. It is not known whether the suppression of *P. cinnamomi* under *A. pulchella* is a result of direct activity of the plants by secreting anti-fungal saponins or volatiles or an indirect effect by encouraging antagonistic soil microbes. It is possible that *A. pulchella* supports a microflora, which includes organisms that inhibit *P. cinnamomi* mycelial growth, zoospore discharge and spore germination or that roots of *A. pulchella* themselves produce an inhibitory compound(s) which cause suppression of the pathogen in all or some of these stages.
Few attempts have been made to assess the activity of the pathogen in soils under *A. pulchella*. The reduction of sporangial numbers and the ensuing low zoospore release of *P. cinnamomi* in the soil around *A. pulchella* plants have been regarded as sufficient to prevent effective invasions of susceptible plants. However, zoospores are not the only infective agent of *P. cinnamomi*. The total inoculum of *P. cinnamomi* consists of vegetative mycelium, chlamydospores, sporangia, motile zoospores and oospores (Zentmyer 1980; Erwin and Ribeiro 1996). All these stages have the potential to be infective. It has been established that the mycelium can readily produce chlamydospores in infected plant tissues (Weste and Ruppin 1977; Old *et al.* 1984b) and they are able to germinate directly (Weste and Vithanage 1978) or act as resting propagules (Zentmyer 1980). Since chlamydospores play a significant role in the persistence of *P. cinnamomi* in the jarrah (*Eucalyptus marginata*) forest (Schild 1995), it is important to establish what effect *A. pulchella* has on chlamydospores before the mechanism of suppression of *P. cinnamomi* by *A. pulchella* can be fully understood.
1.9 Thesis Objectives

Disease suppression by resistant plant species such as *A. pulchella* has unexplained aspects. Is this common but very variable legume species effective in suppressing all the life forms of *P. cinnamomi*? Are all the identified varieties of *A. pulchella* equally suppressive of the pathogen? Also the root exudates of *A. pulchella* and their potential antifungal qualities towards *P. cinnamomi* is an unexplored area of research. There is a pressing need to implement environmentally sound methods in maintaining healthy soil environments around the world and more knowledge of suppressive species is needed.

The main objectives of this thesis were to

- determine whether the most common varieties of *A. pulchella* in Western Australian forest ecosystems are similarly suppressive towards *P. cinnamomi*.
- gain knowledge of the survival strategies of *P. cinnamomi* in the presence of three varieties of *A. pulchella*.
- establish aseptic systems where the effect of root exudates of *A. pulchella* can be studied without influences from soil microorganisms.
- determine the possible mechanisms by which *P. cinnamomi* is suppressed in soils under *A. pulchella*. 
CHAPTER 2

Varietal differences of *Acacia pulchella* in suppression of *Phytophthora cinnamomi* in aseptic and non-aseptic substrates

2.1 Introduction

*A. pulchella* has been described as the most variable member of the Pulchellae and the most variable *Acacia* species in Western Australia and previously included six varieties (Maslin 1975). In the latest classification (Maslin 2001) there are four identified varieties of *A. pulchella* in the Western Australian forest ecosystems and the sand plains (described in Section 1.7.2). The identified varieties of this species: var. *pulchella*, var. *goadbyi*, var. *glaberrima* and var. *reflexa* display some clear morphological differences. The varietal differences are clearly distinct. There are also a number of informal variants within varieties and some hybrids between varieties. It has been emphasised that detailed studies are required to clarify the great variation in this species complex and reassess the status of the varieties. Although reduced inoculum of *P. cinnamomi* was reported under *A. pulchella* consistently in previous studies, none of the studies defined the variety and it is not clear whether all the varieties are equally suppressive to this pathogen or whether any particular variety is more effective than others.

Among the widely encountered plant species in native vegetations of Western Australia, *B. grandis* is susceptible to *P. cinnamomi*, and the inoculum build up of *P. cinnamomi* under this species occurs at high levels (Shea and Shearer 1980). However, the native legume *A. pulchella* not only prevents the spread of *P. cinnamomi*
in its tissues (Tippett and Malajczuk 1979; Cahill et al. 1989) it also reduces the inoculum of *P. cinnamomi* in the soil (Shea and Malajczuk 1977; D’Souza et al. 2004).

D’Souza et al. (2004) found a range of inoculum potentials of *P. cinnamomi* in soil under sympatric *Acacia* species in a glasshouse pot trial. The soil under 9-month-old plants were inoculated with *Banksia grandis* plugs infected with four A2 type isolates of *P. cinnamomi*. Inoculum potential under each species was determined by direct plating of 5 g of the infested soil on NARPH (Hüberli et al. 2000) selective agar. The resulting colony numbers were counted. The lowest was observed under *A. pulchella* and was five times less than under *B. grandis*. Under *A. alata*, *A. extensa*, *A. drummondii*, and *A. latericola*, inoculum potentials were lower than under *B. grandis*, but still higher than the inoculum potential under *A. pulchella*. *A. urophylla* was found to have a high inoculum of *P. cinnamomi* which was similar to *B. grandis* (D’Souza et al. 2004).

The low inoculum of *P. cinnamomi* under *A. pulchella* has been attributed mainly to the low sporangial production in the soil (Shea and Malajczuk 1977). This phenomenon is believed to be caused mainly by soil microflora, which includes organisms that are inhibitory to *P. cinnamomi* (Shea and Malajczuk 1977; Murray et al. 1982; Malajczuk et al. 1984) or the soil physical properties unfavourable for *P. cinnamomi* sporulation (Shea et al. 1978). However, low sporangial production could also be due to compounds produced by the plant such as saponins (Alexander et al. 1978) or volatiles (Whitfield et al. 1981). It is not known whether the suppression is a result of direct activity of antagonistic soil microbes or the effect of root exudates. It is possible that *A. pulchella* supports a microflora, which includes organisms that inhibit *P. cinnamomi*.
mycelial growth, zoospore discharge and spore germination or that roots of *A. pulchella* themselves produce an inhibitory compound(s) which cause suppression of the pathogen in all or some of these stages.

The ability of root exudates of *A. pulchella* plants and the associated soil microflora in suppression of *P. cinnamomi* was investigated to determine whether this trait was common to several varieties of the species or whether any particular variety is more effective than others. The suppressive effects of three commercially available varieties of *A. pulchella*; var. *pulchella*, var. *goadbyi* and var. *glaberrima* on *P. cinnamomi* soil inoculum was studied in experiments conducted under aseptic conditions and in the glasshouse.

Three experiments were conducted. The first determined whether there are varietal differences of *A. pulchella* in suppression of *P. cinnamomi* under glasshouse conditions. The second determined whether the leachate from pots of uninfected *A. pulchella* is inhibitory to *P. cinnamomi*. The third tested the root exudates of *A. pulchella* could suppress *P. cinnamomi* in the absence of soil microbes. *A. urophylla* was used as a control. (A preliminary experiment using potted plants with soil inoculated with *P. cinnamomi* infected *B. grandis* plugs showed the inoculum densities under *A. urophylla* and *B. grandis* to be high and comparable – data not shown) and as *A. urophylla* was easier to grow it was selected as a control for the glasshouse experiments.
2.2 Materials and Methods

2.2.1 Experimental design for Experiments 1 (inoculated) & 2 (non-inoculated)

The pots with and without *P. cinnamomi* in the soil were arranged on separate glasshouse benches in a randomised block design. Each block contained five replicate pots of the three varieties of *A. pulchella*, *A. urophylla* (non suppressive – D’Souza *et al.* 2004) and five replicate pots with soil and no plants.

**Inoculated experiment**

The independent variables were the plants of the three varieties of *A. pulchella* and *A. urophylla*, inoculated with *P. cinnamomi*, and the control soil without plants also infected with *P. cinnamomi*. The dependent variable was the number of *P. cinnamomi* colonies that grew out of 1 g of soil. The experiment was repeated once.

**Non-inoculated experiment**

The independent variables were the leachates collected from the *Acacia* and soil only control pots with non-sterilised and filter sterilised treatments. The dependent variable was the number of sporangia produced on the mycelial mats incubated in the leachates. The experiment was repeated once.

2.2.2. Plant material and soil for Experiments 1 & 2

**Plant material**

Seeds of the three commercially available varieties of *A. pulchella*; var. *pulchella*, var. *goadbyi* and var. *glaberrima* were purchased from the Nindathana Nursery of Western Australia and seeds of *A. urophylla* were purchased from the seed nursery of
Department of Conservation and Land Management (Table 2.1). Provenances of the varieties are shown in Fig.1.2.

Table 2.1 Localities, where the seeds were collected

<table>
<thead>
<tr>
<th>Variety</th>
<th>District</th>
<th>Site</th>
<th>Vegetation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. pulchella</em></td>
<td>Boyanup</td>
<td>8 km south of Boyanup town</td>
<td>Predominantly jarrah</td>
</tr>
<tr>
<td>var. pulchella</td>
<td></td>
<td>from the state forest on</td>
<td>and marri</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gravely lateritic soil</td>
<td><em>(C. calophylla)</em></td>
</tr>
<tr>
<td><em>A. pulchella</em></td>
<td>Mt. Barker</td>
<td>5 km south/south east of</td>
<td>Stunted jarrah, marri</td>
</tr>
<tr>
<td>var. goadbyi</td>
<td></td>
<td>Mt. Barker, off St Werburghs Road</td>
<td>with incursions of karri</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>(E. diversicolor)</em></td>
</tr>
<tr>
<td><em>A. pulchella</em></td>
<td>Boddington</td>
<td>Mount Saddleback reserve</td>
<td>Scrub with mainly</td>
</tr>
<tr>
<td>var. glaberrima</td>
<td>32° 50’ S, 116° 30’ E</td>
<td>south/south east of Boddington</td>
<td>acacias</td>
</tr>
<tr>
<td></td>
<td></td>
<td>on sandy gravelly soil</td>
<td></td>
</tr>
<tr>
<td><em>A. urophylla</em></td>
<td>Manjimup</td>
<td>Torbay</td>
<td>Predominantly karri</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36° 15’ S, 116° 0° E</td>
<td></td>
</tr>
</tbody>
</table>

*Jarrah forest soil*

The top-soil from a *P. cinnamomi* free cleared jarrah forest, about to be mined at Alcoa World Alumina, Australia’s Huntley mine in Dwellingup, Western Australia was used (Fig. 1.2). The soil was sieved through a mesh with 4 mm holes to remove rocks and gravel. Preliminary tests were also conducted to confirm that the soil was conducive to *P. cinnamomi* sporangial production. Soil texture was examined by sifting through a set of sieves (Test Sieve for Precise Particle Analysis, Endecotts Ltd, England) with pore sizes of 2 mm, 0.7 mm, 0.5 mm and 0.1 mm and by checking the ability of wetted soil to be formed into a ball or a noodle shape. The following chemical properties were
analysed by CSBP Ltd. Bibra Lake Western Australia with a measurement accuracy of ± 0.15 according to the ASPAC (Australasian Soil & Plant Analysis Council) standards; pH, conductivity (Rayment and Higginson 1992), organic carbon by the Walkley and Black method (Rayment and Higginson 1992) nitrate and ammonium nitrogen (Searle 1984), sulphur (Blair et al. 1991), phosphorus and potassium by the Colwell method and reactive iron by the Tamm method (Rayment and Higginson 1992).

Figure 1.2 Locations of the southwest of Western Australia where seeds of the three varieties of *Acacia pulchella* (▼) and soils (▲) for experiments (Chapters 2 - 8) were collected.
**Baiting for Phytophthora cinnamomi**

The soil was baited with rose petal discs. This was a novel bait material, first time used to bait *P. cinnamomi*. Samples of 100 g soil and 250 mL of distilled water were placed in 1000 mL plastic containers (Plastic product of Bonson Industries Co Ltd). Unblemished rose petals were stacked on a cutting board and cut with a 6 mm diameter cork borer. Fifty petal discs were sprinkled on the water and left on the bench in the glasshouse at ~ 25°C for 24 h. The soil was free of *P. cinnamomi* or other *Phytophthora* spp. The validity of this baiting method was checked by infecting some soil with *P. cinnamomi*, baiting with rose petal discs and subsequently recovering *P. cinnamomi*. Soil was also direct plated on NARPH selective medium and incubated at 25°C for three to four days and microscopically examined. All tests were negative.

Soil was placed into 150 mm free-draining polyvinyl chloride pots. Seeds of the three varieties of *A. pulchella* and *A. urophylla* were pre-treated with boiling water for 1 min then sown in the pots and thinned to one per pot. Plants, seven-months-old were used in the experiment. Plants were watered twice a day for 5 min by overhead sprinklers. During the 10 weeks of the trial the maximum and minimum daily temperatures in the glasshouse were 35 °C and 18 °C, respectively.

**2.2.3 Inoculum and inoculation**

*Isolate MP 97-16 of Phytophthora cinnamomi*

Isolate MP 97-16 of the *Phytophthora* collection of Murdoch University was originally isolated from jarrah and belongs to the A2 isozyme type 1 (Hüberli 2000) which is the most commonly isolated *P. cinnamomi* strain. Cultures of this isolate were grown on V8 juice agar (Miller 1955) and maintained at 25°C. Preliminary experiments showed
that MP 97-16 was able to produce sporangia axenically more reliably than several other A2 type isolates.

**Inoculum**

Inoculum of *P. cinnamomi* was prepared *in vitro* by infecting young roots of aseptically germinated lupin (*Lupinus angustifolius*) seeds with zoospore suspension of *P. cinnamomi*. The lupin seeds were surface sterilised with 5% NaOCl for 10 min and left in sterile Petri dishes on moist filter paper for three days under light at 25°C. Mycelial discs (6-mm-diameter) of *P. cinnamomi* bearing sporangia, produced using the axenic method of Byrt and Grant (1979) were placed in 3-cm-diameter Petri dishes containing 5 mL sterile distilled water. The dishes were chilled for 30 min at 4°C to synchronise zoospore release and zoospore density was determined to be 10 – 20 per μL. Lupin seedlings with ~ 1-cm-long radicles were placed on the rim of the Petri dishes so the root tips were in contact with the zoospore suspension (Fig. 2.1 A). After 2 h the lupin seedlings were removed and the roots were excised close to the cotyledons. Infected root segments of 1-cm were plated on water agar (0.7%) and incubated at 25°C in the dark for seven days for lesion development (Fig. 2.1 B). Microscopic examination of unstained, randomly chosen infected root segments revealed fully formed chlamydsospores within the roots.

Each infected lupin root was enclosed in a sachet (2 cm x 2 cm) made with nylon fabric and tied with a string. Five pots of 7-months-old *A. pulchella*; var. *pulchella*, var. *goadbyi*, var. *glaberrima* and *A. urophylla* grown in the glasshouse as described above and five pots without plants (control) were inoculated. A clean spatula was used to insert slits into the soil at 10 positions around the plants to a depth of 10 cm. Ten inoculum sachets were gently pushed into the slits and were filled with small amount of
sterilised sand. The plants were hand watered daily and kept in a glasshouse with the temperature controlled at 25°C ± 5°C.

2.2.4 Inoculum potential of soil

The inoculum potential in the soil under the acacias and in the soil without plants was measured by assessing the resulting colony formation on agar from the soil samples collected from the pots. Samples of 5 g moist soil were collected from the bottom of the inoculated pots with and without plants, seven days after inoculation using a 1 cm-diameter corer and plated directly onto NARPH agar Petri dishes. The dishes were incubated at 24°C in the dark for 24 h, then the soil was washed off with distilled water and the number of individual *P. cinnamomi* colonies was determined with a light microscope. After 48-72 h of incubation the number of *P. cinnamomi* colonies formed was recounted.

2.2.5 Non inoculated experiment

*Leachate collection*

Leachates were collected from 6-months old plants of *A. pulchella* var. *goadbyi*, *A. urophylla* and the control pots without plants. The bottoms of the pots were sealed with tape (Gaffer’s 357), and were flooded with tap water to container capacity. The tape was removed and leachates were collected from each pot. The leachates were filtered through Whatman No. 1 papers to remove dirt and leaves and each pH was recorded. Samples of 20 mL from each leachate were sterilised through 0.22 μm Millipore filter membranes and collected into 9-cm-diameter Petri dishes.
Both filter sterilised and non sterilised leachates were bioassayed for sporangial production of *P. cinnamomi* with five replicates per treatment. Aliquots of potting mix (comprised of composted pine bark, coarse river sand and coco peat at the ratio of 2: 2: 1) extract prepared by shaking 100 g potting mix in 250 mL tap water for 2 h and filtrate was added to the filter sterilised leachates to induce sporangial production. Preliminary experiments showed the potting mix of pH 5 was highly conducive to sporangial production of the isolate MP 97-16 of *P. cinnamomi*.

**Preparation of mycelial discs**

Miracloth discs (6 mm in diameter) were autoclaved three times at 121°C then plated on V8 juice agar at equal distance from the centre. A square (1 cm²) of *P. cinnamomi* (MP 97-16) was cut from the edge of a growing colony and placed in the middle. The plates were incubated at 22°C in the dark for seven days until the colony grew over the discs. Mycelial discs were gently lifted from V8 agar plates and five discs were placed submerged in each leachate. Treatment dishes were incubated at 25°C under light. After 24 h one mycelial disk per leachate was harvested and mounted on slides and stained with aniline blue for optical microscopy. The procedure was repeated on day 3.

**Microscopy**

Numbers of viable sporangia produced on each of the five mycelial discs after 72 h incubation in the leachates were counted under x 100 magnification in five fields of view. The sporangial counts of five discs were averaged per treatment.
2.2.6 Experimental design for Experiment 3

The independent variables were the three varieties of *A. pulchella* soil inoculated with *P. cinnamomi* and the control, soil mix without acacias also inoculated with *P. cinnamomi*. The dependent variable was the number of *P. cinnamomi* colonies that grew out of 1g of the soil cores on NARP H selective agar. The treatments of acacias in *in vitro* units were replicated 3 times. The experiment was repeated once.

2.2.7 Establishment of *Acacia pulchella* plants *in vitro*

Seeds of the three varieties of *A. pulchella* and *A. urophylla* were surface sterilised with 2.5% NaOCl for 15 min and rinsed in sterile distilled water three times. The seeds were pre-treated by immersing in boiling water for one minute and then immediately transferring them to pre-cooled sterile distilled water for a further minute. The seeds were dried on sterilized paper towel and plated on Petri dishes containing 0.7% water agar. The Petri dishes were incubated in the dark at 15°C for 14 days until the seeds germinated. Seedlings were transferred to plastic tubs containing ½ strength Murashige & Skoog medium (Appendix 1) and placed under light at 25°C for four weeks for the seedlings to produce the first pair of true leaves (Fig. 2.1 D).

The seedlings were removed from the tubs and transplanted to 150 g of sterilised *in vitro* soil-less (IVS) mix which comprised of sphagnum peat, coarse river sand and perlite at a ratio of 0.5:2:2 (Newell *et al*. 2003) in polyurethane punnets (13 x 7 x 5 cm) under aseptic conditions. The punnets containing the IVS mix were autoclaved twice prior to transplanting. The punnets with five seedlings in each and the ones with only IVS mix (control) were placed in sterile polypropylene containers (1000 mL, Plastic
product of Bonson Industries Co Ltd) with a layer of sterilized plastic beads in the bottom (Fig. 2.1 E). A second container of similar size was placed over the first one as a lid and sealed with plastic wrap. Each unit was labeled and kept in a growth cabinet illuminated with fluorescent and incandescent light (675 μE m⁻² sec⁻¹) with a 14/10 h light/dark regime. This design ensured that the roots were adequately aerated and protected from waterlogging. Punnets with IVS but without plants were included as the control. The units were watered weekly with ½ MS (Murashige and Skoog 1962) basal mineral solution for eight weeks before inoculation with *P. cinnamomi*. The IVS mix in each unit was inoculated by burying five infected lupin roots enclosed individually in autoclaved nylon sachets (2 cm x 2 cm), under each plant.

Figure 2.1 A - Inoculation of lupin roots with *Phytophthora cinnamomi* zoospore suspension. B – Infected lupin roots plated on agar. C – Direct germination of sporangium incubated in the *Acacia urophylla* leachate. D – E *In vitro Acacia*, D – on agar, E – in IVS mix. Bar A, B, D, E = 1 cm. C = 5 μm.
2.2.8. Soil core collection

Samples of 1g of moist soil were collected using a sterilized cork borer near the inoculation zone and plated directly onto NARPH agar dishes. The plates were incubated at 24°C in the dark for 24 h. The soil was washed off with distilled water and the colony formation was observed with a light microscope. After 48-72 h of incubation the plates were again observed under a light microscope and individual *P. cinnamomi* hyphal colonies were counted.

2.2.9. Statistical analysis

Experiments were repeated once. Analysis of variance was carried out on the data collected from one experiment using SPSS 14.0 for Windows statistics package (SPSS Inc., USA). The ANOVA assumptions of normality were screened by subjecting residuals to standard tests; normal Q-Q and detrended normal Q-Q plots. The data were normally distributed. Post hoc comparisons of all significant main effects and interactions were compared using Tukey multiple comparison test among means.

2.3 Results

2.3.1. Inoculum potential of infected soil under the glasshouse plants

Soil with the three varieties of *A. pulchella* plants inoculated with *P. cinnamomi* yielded less colonies on agar compared to the colonies formed by *P. cinnamomi* infected soil with *A. urophylla* plants (Fig. 2.2). The highest numbers of *P. cinnamomi* colonies were found in the soil under *A. urophylla* plants and the lowest in the soil only control. One-way ANOVA between groups showed a significant (P < 0.05) difference between groups at F = 3.892, df = 4, 20 and P < 0.05 (Appendix 2). A post hoc multiple comparison among means done using the Tukey test showed significant (P < 0.05)
differences the *P. cinnamomi* colony numbers in the soil samples under *A. pulchella* var. *goadbyi*, soil only control and the highest *A. urophylla*. The other two varieties, var. *pulchella* and *glaberrima* failed to reach the significance at 0.05 level compared to *A. urophylla*.

![Figure 2.2](image)

**Figure 2.2** Number of *Phytophthora cinnamomi* colonies formed on NARPH selective agar from 1g soil from under the glasshouse grown plants of the three varieties of *Acacia pulchella* (*Ap. pulch* = var. *pulchella*, *Ap. goad* = var. *goadbyi*, *Ap. glab* = var. *glaberrima* and *A. uroph* = *Acacia urophylla* and the no plant control. All varieties and controls were infected with *P. cinnamomi* 7 days before collection of soil samples. Bars indicate standard error of the means. Means with different letters are significantly (P < 0.05) different.

**2.3.2. Inoculum potential of infected soil mix under the aseptic plants**

Among the three varieties, *A. pulchella* var. *goadbyi* showed the least inoculum potential of *P. cinnamomi* (Fig. 2.3) when plants were grown under aseptic conditions. One-way ANOVA between groups showed a significant (P < 0.05) difference at F = 4.293, df = 3, 8 and P < 0.05) (Appendix 2). A post hoc multiple comparison between means done using the Tukey test showed significant (P < 0.05) differences between the
*P. cinnamomi* colony numbers under *A. pulchella* var. *goadbyi* and the IVS mix control. Both of the other groups failed to reach significance at 0.05 level. The *A. urophylla* plants died and were not included in the statistical analysis.

![Graph showing colony numbers](image)

**Figure 2.3** Number of *Phytophthora cinnamomi* colonies formed on NARPH selective agar from 1g IVS mix from under the three varieties of *Acacia pulchella* (*Ap. pulch* = var. *pulchella*, *Ap. goad* = var. *goadbyi*, *Ap. glab* = var. *glaberrima* and the no plant control under aseptic conditions. All varieties and controls were infected with *P. cinnamomi* 7 days before collection of soil samples. Bars indicate standard error of the means. Means with different letters are significantly (*P* < 0.05) different.

### 2.3.3. Sporangial production in soil leachates collected from the glasshouse-grown *Acacia pulchella* var. *goadbyi* and *Acacia urophylla* plants

No sporangia were observed on mycelial discs at Time 0. The number of sporangia produced on the mycelial discs of *P. cinnamomi* in leachates from *A. pulchella* var. *goadbyi* pots was significantly (*P* < 0.05) lower than that of *A. urophylla* and soil without plants when the leachate was not filter sterilized (Fig. 2.4 A). No sporangia were formed.
on the mycelial discs of any of the filter sterilised leachates without induction using the extract of potting mix. When this was done the mycelial discs of *P. cinnamomi* incubated in *A. pulchella* var. *goadbyi* leachate yielded the lowest number of sporangia compared with *A. urophylla* leachate or the leachate from soil without plants (Fig. 2.4 B). In the non sterile *A. urophylla* leachate some direct germination of sporangia were observed (Fig. 2.1 C).

Univariate ANOVA between groups test showed a significant (P < 0.05) difference in sporangial counts for the two main effects; three leachate treatments at F = 18.534 df = 2, 18 P < 0.01 and two sterilisations at F = 18,819 df = 1, 18 P < 0.01. A post hoc multiple comparison between means done using the Tukey test showed a significant (P < 0.05) difference in sporangial production between the two species and the soil only control.

Figure 2.4 Number of *Phytophthora cinnamomi* sporangia produced on the mycelial discs after 72 h in A - non filter sterilized and B - filter sterilized leachates from *Acacia pulchella* var. *goadbyi* and *Acacia urophylla* plants and soil only control. Bars represent standard error of the means.
2.3.4 Soil

Texture

Soil was easily pressed into a ball when wetted but could not be rolled between two palms to make a noodle shape. The soil description and particle sizes after sieving through 4 mm mesh are shown in Table 2.2. The relative proportion of the very fine sand (the particle size = 0.1 – 0.5 mm) was the highest texture class at 40%.

<table>
<thead>
<tr>
<th>Soil Description</th>
<th>Particle size %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jarrah forest soil</td>
<td>0.7-2 mm 0.5-0.7 mm 0.1-0.5 mm &lt; 0.1 mm</td>
</tr>
<tr>
<td>Brownish</td>
<td>36 12 10 40 2</td>
</tr>
</tbody>
</table>

Under the regular watering regime the soil in the “soil only” pots became compacted.

Chemical properties

The soil pH values of both Acacia species were slightly acidic at 6.4 and 6.6 while it was 7.2 for the soil only treatment (Table 2.3). Under A. pulchella var. goadbyi all the soil nutrient properties were higher than A. urophylla and the soil only control.
Table 2.3 Chemical properties of the soils

<table>
<thead>
<tr>
<th>Jarrah Forest Soil</th>
<th>pH</th>
<th>Cond (dS/m)</th>
<th>Org C (mg/Kg)</th>
<th>Nitr. N (mg/Kg)</th>
<th>Amm. N (mg/Kg)</th>
<th>Sulphur (mg/Kg)</th>
<th>P (mg/Kg)</th>
<th>K (mg/Kg)</th>
<th>Iron (mg/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil without plants</td>
<td>7.2</td>
<td>0.062</td>
<td>2.08</td>
<td>1</td>
<td>1</td>
<td>7.86</td>
<td>1</td>
<td>25</td>
<td>857</td>
</tr>
<tr>
<td>A. pulchella var. goadbyi/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil</td>
<td>6.4</td>
<td>0.109</td>
<td>3.08</td>
<td>5</td>
<td>5</td>
<td>11.40</td>
<td>3</td>
<td>86</td>
<td>1589</td>
</tr>
<tr>
<td>A. urophylla/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil</td>
<td>6.6</td>
<td>0.240</td>
<td>2.32</td>
<td>1</td>
<td>1</td>
<td>9.60</td>
<td>2</td>
<td>43</td>
<td>1388</td>
</tr>
</tbody>
</table>

2.4 Discussion

Among the three varieties of *A. pulchella* the most suppressive in both non sterile soil and the sterile soil mix was *A. pulchella* var. *goadbyi*. The other two varieties, var. *pulchella* and *glaberrima* failed to show significant differences when compared with the non-*P. cinnamomi* suppressive *A. urophylla*. Previous finding of inhibition of sporangial production and zoospore release in the soil under *A. pulchella* plants (Shea and Malajczuk 1977; Murray *et al.* 1985) were confirmed by the results of all the experiments conducted in this chapter. In addition, it was shown for the first time that the sterile and non sterile leachates from the non-infected soils from *A. pulchella* pots were inhibitory to
sporangial production of *P. cinnamomi*. Suppression of *P. cinnamomi* by aseptically grown *A. pulchella* too was shown for the first time.

2.4.1 Soil inoculum

Proliferation of *P. cinnamomi* was suppressed under all three varieties of *A. pulchella* compared to *A. urophylla*. Recovery of *P. cinnamomi* from the soil samples collected from inoculated glasshouse soil only pots was the lowest despite the fact that the soil was conducive to sporangial production as shown with the non sterile soil leachate. This indicates *P. cinnamomi*’s difficulty to proliferate in the absence of plant material. It could also have been due to soil compaction and poor drainage in the pots under the regular watering regime, which would have lead to an anaerobic environment in these pots. In the pots with plants, root growth ensured good aeration. The pore spaces in the soil which was composed of relatively high proportion of very fine sand may not have been adequate for supporting good aeration or zoospore movement in the soil only pots. Sporangial production and zoospore release of *P. cinnamomi* strongly depend on aeration of the substrate (Duniway 1983) and the matric potential of the substrate (Hardy and Sivasithamparam 1991).

Recovery of *P. cinnamomi* from the sterile soil mix with and without *Acacia* plants was higher than that of the glasshouse soil. The reason may have been the sphagnum peat present in the IVS mix which provided the organic matter for *P. cinnamomi* to survive.

2.4.2 Sporangial production

Suppression of the pathogen may have been fully or at least partly through reduction of sporangial production in the soil under *A. pulchella* plants. As the sporangial production
was significantly low in the soil leachate collected from *A. pulchella var. goadbyi* pots it can be hypothesised that low sporangial numbers also occurred in the soil which caused the reduced inoculum of *P. cinnamomi* in the soil under *A. pulchella* plants.

These results indicate the presence of inhibitory compound(s) in the soil and the leachates from under *A. pulchella* plants grown in the glasshouse. It is possible that the *A. pulchella* plants exude an inhibitory compound(s) into the soil/leachates. It is equally possible that soil microorganisms secrete antifungal metabolites into the soil that washed out with the leachates.

Nesbitt and Glenn (1979) found that the zoospore production of *P. cinnamomi* was markedly reduced in sterile and non sterile jarrah leaf litter leachates compared to the leachates from lateritic soils, which they initially associated with the microbial effect. In a subsequent experiment Nesbitt and Glenn (1981) found a similar pattern of results in sterile and non sterile leachates derived from *P. cinnamomi* suppressive soils and concluded that the sporangial abortion and prevention of zoospore release was possibly due to a common chemical property present in leachates prepared with jarrah leaf litter and the two suppressive soils. The soil analysis of Nesbitt and Glenn’s first experiment (1979) showed a high organic matter content and higher levels of all the nutrients in the soil amended with jarrah leaf litter than the non amended lateritic soil. Similarly, higher soil C levels and nutrient levels under the *A. pulchella var. goadbyi* plants were observed in the experiments here indicating that the chemical properties of the soil under *A. pulchella var. goadbyi* could have been responsible for the low sporangial production of *P. cinnamomi*. 
It was shown in this study the root exudates from IVS with *A. pulchella* growing under aseptic conditions and exposed to *P. cinnamomi* inhibited the pathogen. This proves that *A. pulchella* plants are capable of producing inhibitory factors in the absence of soil microbes. The soil pH values were similar between the two *Acacia* species indicating no effect on the suppression of stimulation of sporangial production in their leachates.

Knowledge of the antifungal properties of *A. pulchella* root exudates is very limited. It has been shown that *A. pulchella* roots contained *P. cinnamomi* suppressive saponins (Alexander *et al.* 1978) and volatiles (Whitfield *et al.* 1981). These compounds were extracted from the roots and no information is available on whether they are released into the soil. The root exudates of *A. pulchella* may or may not essentially contain saponins and volatiles.

As described earlier (Section 1.7.5) root exudates vary in composition and quantity between species and among varieties (Vancura and Hanzlikova 1972; Keeling 1974). It is likely that the antifungal activities of the root exudates of *A. pulchella* can also differ from variety to variety.

**Conclusions**

*P. cinnamomi* suppression differs among the varieties of *A. pulchella*. This information is valuable for using *A. pulchella* as an effective control tool against *P. cinnamomi* and suggests that a further search for genotypic variation in level of suppression in the most effective variety, var. *goadbyi* could lead to discovery of plants with even higher levels of suppression.
Previous work on suppression of *P. cinnamomi* by *A. pulchella* does not state the variety, and these studies may have used a highly suppressive variety. The focus of the previous studies had mainly been on determining the difference of *P. cinnamomi* suppression between the resistant *A. pulchella* and the susceptible *E. marginata* and *B. grandis* (Shea et al. 1978) and on comparing inoculum potentials of *P. cinnamomi* under *A. pulchella* and other legumes (D'Souza 2001). Although these studies consistently found low inoculum levels of *P. cinnamomi* under *A. pulchella* plants, it is important to consider possible differences between the *A. pulchella* varieties in *P. cinnamomi* suppression when using it as a biological control method.

The experiments in this Chapter also provided valuable knowledge about isolate MP 97-16 of *P. cinnamomi*. This isolate readily produced sporangia axenically and in non sterile soil leachates. It also produces abundant chlamydospores on V8 juice agar and was used for the subsequent bioassays in this thesis. The experiments also showed the suitability of *A. urophylla* as a non-suppressive control for further experiments.
CHAPTER 3

Effects of *Acacia pulchella* and *Acacia urophylla* on the production and persistence of *Phytophthora cinnamomi* chlamydospores

3.1 Introduction

Chlamydospores and oospores of *P. cinnamomi* are considered to be the main survival structures in the soil (Zentmyer and Erwin 1970). However, since only the A2 mating type of *P. cinnamomi* is predominantly present in jarrah forest (Old et al. 1984a; Dobrowolski et al. 2001) it is unlikely that sexual reproduction will occur. Oospores are not common in the Western Australian jarrah forest soils (Shearer et al. 1994; Huberli 2001) while chlamydospores play a significant role in the persistence of *P. cinnamomi* in the northern jarrah forest soils (Schilds 1995).

A few studies have been conducted on survival of *P. cinnamomi* within plant tissues. Old et al. (1984b) compared survival of *P. cinnamomi* in *Eucalyptus seiberi* L. Johnson root fragments after burying them for varying periods in soils obtained from several Australian forest sites, including lateritic soil and krasnozem from jarrah forest and grey brown solodic soil from NSW. Petri dishes containing soil with *P. cinnamomi* infected root fragments were kept in the laboratory at different moisture regimes. In order to control contact with soil microflora, root fragments were enclosed in sealed packets with specific pore sizes. High recovery rates of *P. cinnamomi* were recorded from the root fragments which were enclosed in the microflora proof packets and buried in the two jarrah forest soils; laterite soils with and without *Acacia* (unspecified species) in the understorey and krasnozem. Microscopic examination of roots revealed chlamydospores and sporangia to be the prominent propagules. In contrast, recovery of
*P. cinnamomi* under similar conditions was lower in the grey brown solodic soil from NSW which was attributed to the presence of a diffusible substance in this soil suppressive to *P. cinnamomi*.

At the time the above experiment was conducted there was little information on the possible role of secondary metabolites produced by soil microflora present in the jarrah forest soils in terms of their toxicity towards *P. cinnamomi*. Murray (1987) identified several micro-organisms in the rhizosphere of *A. pulchella* which caused restriction of *P. cinnamomi* colony growth on nutrient agar medium via the production of diffusible secondary metabolites.

There are many possible reasons as to why *P. cinnamomi* propagules were not affected within the root fragments in the experiments conducted by Old *et al.* (1984b) using jarrah forest soil. The relevant microorganisms may not have been present in the two jarrah forest soils used or the soil may not have been dug from under *A. pulchella* but from other *Acacia* species. Furthermore, since the varieties of *A. pulchella* show varying levels of suppression towards *P. cinnamomi* (Chapter 2), the chemical environment and the soil microorganisms under these varieties could also differ from one another.

The work reported in this chapter relates to two aspects of the behaviour of chlamydospores of *P. cinnamomi* in relation to the effects of *A. pulchella* and *A. urophylla*. The response of chlamydospores of the pathogen to soil leachate from the two *Acacia* species was studied in two environments. Firstly, when the pathogen was growing on a nutrient deficient sterile substrate and secondly when grown on a food
base in the presence of non sterile soil leachate. It was hypothesised that chlamydospor e production and persistence were important for prolonging the life of *P. cinnamomi* and ascertaining the effect of *A. pulchella* at chlamydospores stage was an essential component in the suppression study.

### 3.2 Materials and Methods

#### 3.2.1. Experimental Design – the effect of filter sterilized soil leachate on chlamydospores

Independent variables were the two *Acacia* species, *P. cinnamomi* suppressive *A. pulchella* var. *goadbyi* and non-suppressive *A. urophylla*. Treatments included filter-sterilised soil leachates collected from five replicate pots with *Acacia* plants and control pots without plants. The dependant variable was the number of chlamydospores produced on mycelial discs measured microscopically over a period of five days recording intact, collapsed and germinating chlamydospores.

**Leachate collection and inoculation with mycelial discs**

Leachates were collected from six month-old plants as done in Chapter 2.2.5. The leachates were filtered through Whatman No 1 filter paper, pH was measured and 20 mL of each leachate were sterilised through 0.22 μm Millipore filter membranes and collected into 9-cm-diameter Petri dishes.

For inoculation of these leachates Miracloth discs (6 mm in diameter) prepared as in Section 2.2.5 were used. Mycelial discs were gently lifted from V8 agar plates and five discs were placed submerged in each leachate. Each leachate was replicated five times.
The number of chlamydospores present on the mycelial discs before incubation (at Time 0) was recorded by examining five randomly selected discs from each agar plate. Treatment dishes were incubated at 25°C under light. After 24 h one mycelial disk per leachate was harvested and mounted on a slide and stained with aniline blue for optical microscopy. The procedure was repeated on days three and five. The experiment was repeated once.

*Microscopy*

The numbers of intact, collapsed and germinating chlamydospores (defined below) on each mycelial disk were counted under x 100 magnification in five fields of view.

**Intact chlamydospores:** fully expanded chlamydospores that had the cytoplasm occupying the total space within the cell wall (Fig. 3.1 A),

**Collapsed chlamydospores:** the cytoplasm had detached from the outer wall leaving a gap between the outer wall and the plasmalemma (Fig. 3.1 B).

**Germinating chlamydospores:** that had germ tubes initiating from the spore (Fig. 3.1 C).

3.2.2 Experimental Design – soil inoculation of the *Acacia* plants with *Phytophthora cinnamomi* colonised lupin roots

Independent variables were the soil inoculated plants of the three varieties of *P. cinnamomi* suppressive *A. pulchella*; var. goadbyi, var. glaberrima and var.
pulchella, non-suppressive *A. urophylla* and the soil only control. The dependant variables were the numbers of *in planta* chlamydospores microscopically recorded in three categories; intact, collapsed and germinating. Additional roots were buried for staining with Fluorescein diacetate.

**Inoculum and inoculation**

The inoculum was prepared using isolate MP 97-16 of *P. cinnamomi* and the three varieties of *A. pulchella* were inoculated as described in Section 2.2.3. At time 0, randomly selected inoculum were cleared, stained and microscopically examined to confirm the *in planta* chlamydospore production. Each infected lupin root was enclosed in a nylon sachet and tied with a string. Five pots of each variety of *A. pulchella*; var. *pulchella*, var. *goadbyi*, var. *glaberrima* and *A. urophylla* were inoculated with 10 sachets each and arranged in a randomised block design.

**Retrieval of the roots and microscopic examination**

Five roots were retrieved from all the pots seven days after inoculation and the other five were left for a further seven days. The roots were surface sterilised with 0.2% NaOCl solution, washed in three changes of sterile distilled water and longitudinally halved with a sterile scalpel. One half of each root was plated on NARPH selective agar and incubated at 24ºC in the dark for three days. The other half was fixed with acetic alcohol, cleared by autoclaving in 10% KOH and stained with aniline blue. The stained root material was examined under a light microscope and the numbers of full, collapsed and germinating chlamydospores were recorded.
The additional root segments were retrieved from soil under *A. pulchella* var. *goadbyi*, surface sterilised as above and spread on a microscope slide with the aid of two needle points. These root segments with collapsed chlamydospores were stained with 0.1 mL of Fluorescein diacetate solution (Widholm 1972) and examined using a compound microscope with fluorescence illumination (Olympus BX-1, Japan). The wavelength of the excitation filter was 460 – 490 nm. The emission filter transmitted light > 520 nm.

The colonies which grew out from the root halves on NARPH selective agar were observed after two days under a light microscope for positive identification of *P. cinnamomi*. The morphological characteristics of *P. cinnamomi* the coralloid hyphae, hyphal swellings and chlamydospores were observed.

### 3.2.3 Statistical analysis

All the experiments were repeated once. Analysis of variance was carried out using SPSS 14.0 for Windows statistics package (SPSS Inc., USA) and the data from one experiment is presented. The ANOVA assumptions of normality were screened by subjecting residuals to standard tests; normal Q-Q and detrended normal Q-Q plots. As there was no incidence where normality was considered to be violated, it has not been reported. Post Hoc comparisons of all significant main effects and interactions were compared using Tukey multiple comparison test between means.
Figure 3.1 Chlamydospores of *Phytophthora cinnamomi*. A – D Chlamydospores produced on mycelial discs. E – I *In planta* chlamydospores. A - Fully formed chlamydospores. B – Collapsed, C – Partially plasmolysed chlamydospore which could be mistaken for a thick walled chlamydospore. Cytoplasm has shrunk (►) leaving a gap (◄►) between the outer wall and the cytoplasm. Empty hyphae (◄) around the collapsed chlamydospores. D – Germinating chlamydospore, germ tube (◄). E – Intact chlamydospore within lupin root. F – I Collapsed chlamydospores within lupin root tissue which had been in the soil under *A. pulchella* for 7 days. F – stained with aniline blue. G – non-stained. H – Collapsed chlamydospores in teased out tissues of lupin root and stained with Fluorescein Diacetate (►) still viable but collapsed cytoplasm (◄). I – Oospore (▲) next to a collapsed chlamydospore (►). Bar A = 50 μm. B, C = 10 μm. D - G = 20 μm.
3.3 Results

3.3.1 Chlamydospore production and survival on the mycelial discs

At time 0 there were ~ 10 chlamydospores present on the mycelial discs and these were mainly in the range of 40 - 50 μm in diameter (Fig. 3.1 A). After 24 h incubation in the Acacia leachates a two-fold increase in the numbers of intact chlamydospores compared to the control was recorded (Fig. 3.2 A). No significant (P > 0.05) increase in intact chlamydospores was observed in the soil only treatment. In A. pulchella leachates the number of intact chlamydospores started to decline steadily with time and by day five the number was significantly (P < 0.05) less compared to day one (Fig. 3.2 B). In comparison the A. urophylla treatment showed a different trend with a maximum number of chlamydospores on day three and then a sharp drop by day five (Fig. 3.2). The ANOVA tests found all the interactions were to be significant (P < 0.01) (Appendix 2).

Declines in the number of intact chlamydospores with time of incubation in the two Acacia leachates can be linked to collapse and germination (Fig. 3.1 B, C and D). Figs. 3.3 A, B show a pattern for this occurrence.
Figure 3.2 Chlamydospore production of *P. cinnamomi* on mycelial discs incubated in leachates obtained from pots with *Acacia pulchella* var. *goadbyi* ♦, *Acacia urophylla* ■ and ▲ soil without plants (control). A – The total number of chlamydospores per field of views (0.02 cm$^2$) B – The percentage of intact chlamydospores.

Figure 3.3 Chlamydospore survival of *Phytophthora cinnamomi* on mycelial discs incubated in leachates obtained from pots with *Acacia pulchella* var. *goadbyi* ♦, *Acacia urophylla* ■ and ▲ soil without plants ▲. A - Percentage of collapsed chlamydospores of *Phytophthora cinnamomi*. B - Percentage of germinating chlamydospores

**Chlamydospore collapse**

The only significant increase (P < 0.05) in the number of collapsed chlamydospores from the initial value for the soil only control was recorded on day five (Fig. 3.3 A). In contrast, the *A. pulchella* leachate treatment caused a significant (P < 0.05) spore
collapse from day one through to day five. The *A. urophylla* leachate showed a similar trend to the soil only leachate with a slight increase in collapsed chlamydospires only on day three.

**Chlamydospore germination**

The highest number of germinating chlamydospires was observed in *A. urophylla* leachate compared to the *A. pulchella* or the soil only control. Nearly 25% germinated after 24 h incubation and increased to nearly 80% by day five. Between 10 -20% was observed in soil only control and *A. pulchella* leachate on day three and by day five the number decreased slightly for *A. pulchella* while it stayed same for the soil only control (Fig.3.3 B).

**3.3.2 Phytophthora cinnamomi sporangia in lupin root inoculum**

Viable sporangia were observed in all the replicate lupin root samples from *A. urophylla* and soil-only treatments but was either not observed or less frequent under the plants of the three varieties of *A. pulchella* (data not shown).

**3.3.3 Recovery of Phytophthora cinnamomi from lupin root inoculum and soil under Acacia species**

The number of roots yielding *P. cinnamomi* colonies on agar was lowest (50%) in the roots retrieved from soil under *A. pulchella* var. goadbyi compared to the 100% recovery in the other treatments (Table 3.1). These observations can be directly linked to the deteriorating condition of the pathogen within the lupin roots. Microscopic examination of the lupin roots after seven days in soil revealed the presence of
chlamydospores and mycelium in varying conditions from mostly healthy in the *A. urophylla* treatment to mostly deteriorating mycelium and cytoplasmic collapse of chlamydospores in the soil with *A. pulchella* var. *goadbyi* present (Figs. 3.1. F-G and 3.4). A univariate ANOVA was conducted on the number of collapsed chlamydospores from lupin roots buried under the three varieties of *A. pulchella*, *A. urophylla* and soil only control showed a significant (P < 0.05) difference between the subjects (at F = 9.05, df = 4, 20 and P < 0.01) (Appendix 2). A multiple comparison between means using Tukey test showed a significant (P = 0.05) difference with highest in the soil under *A. pulchella* var. *goadbyi* compared to all the other treatments. Between the three *A. pulchella* varieties the lowest was under var. *pulchella*. *A. urophylla* and soil only control had no chlamydospores collapsing in the lupin roots buried for seven days.

Table 3.1 Percentage recovery of *Phytophthora cinnamomi* from infected lupin roots after burial for 7 days in the soils under the Acacias and soil without plants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of recovery of <em>P. cinnamomi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil without plants</td>
<td>100</td>
</tr>
<tr>
<td><em>Acacia pulchella</em> var. <em>pulchella</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. pulchella</em> var. <em>goadbyi</em></td>
<td>50</td>
</tr>
<tr>
<td><em>A. pulchella</em> var. <em>glaberrima</em></td>
<td>100</td>
</tr>
<tr>
<td><em>A. urophylla</em></td>
<td>100</td>
</tr>
</tbody>
</table>

64
Figure 3.4 Mean number of viable □ and collapsed ■ chlamydospores of *Phytophthora cinnamomi* per field of view in fragments of lupin roots retrieved 7 days after burial in the soil with the three varieties of *A. pulchella*; var. *pulchella* (*Ap. pulch.*), *goadbyi* (*Ap. goad.*) and *glaberrima* (*Ap. glab.*), *A. urophylla* and soil without plants. Means are of 5 replicates and bars represent SE of means where large enough to be shown.

The roots buried for 14 days were too deteriorated to quantify the chlamydospores. Small spores in a diameter range of 8 – 9 µm with thick walls were observed within the root tissues buried for 14 days. Since there is not sufficient evidence to confirm that they were propagules of *P. cinnamomi* no further attempt was taken to record them. Also infrequent oospores were observed in the lupin roots buried under *A. pulchella* var. *glaberrima* plants.

### 3.4 Discussion

The degradation of significant numbers of *P. cinnamomi* chlamydospores by *A. pulchella* has been shown for the first time in this study. It was also shown that the non-suppressive *A. urophylla* stimulated chlamydospore germination of *P. cinnamomi*. These differences in chlamydospore behaviour were observed both when mycelium and...
chlamydospores were incubated in soil leachates or within infected lupin roots buried in the soil under the plants.

The increase in number of chlamydospores on the mycelium in leachates of *A. pulchella* var. *goadbyi* and *A. urophylla* in the first 24 h in comparison to the control leachate indicates that the leachates had a stimulatory effect on chlamydospore production or a survival response of *P. cinnamomi* to the change in the environment.

After 24 h a higher proportion of chlamydospores exposed to *A. pulchella* var. *goadbyi* leachate showed plasmolysis of the cytoplasmic contents while those in the *A. urophylla* leachate predominantly germinated. Chlamydospores exposed to the leachate from soil without plants persisted for several days but eventually deteriorated possibly due to lack of a food base.

Continual exposure to *A. pulchella* var. *goadbyi* leachates over five days resulted in collapse of nearly 80% of the chlamydospores whilst exposure to *A. urophylla* leachates resulted in germination of a similar percentage. However, prolonged exposure to the soil-only control did not cause immediate lysis or germination of chlamydospores suggesting their ability to persist for several days on a substrate devoid of nutrients. However prolonged exposure resulted in a decline in their viability and an upward trend in collapsed chlamydospores in the soil only leachate suggests a limited life span for these propagules.

Weste (1983) suggested that the survival of chlamydospores might depend on the thickness of their wall. The notion of wall thickness in chlamydospores is still an area
where convincing evidence is lacking. Evidence of Zentmyer (1980), Alizadeh and Tsao (1985) suggests that the chlamydospores of *P. cinnamomi* have 0.5 µm thick wall, thinner than in chlamydospores of other *Phytophthora* species. This was observed in the fully formed chlamydospores produced on the nutrient agar. Despite the fact that some chlamydospores seemed to be thick walled after being incubated in the *A. pulchella* leachate treatments for 24 h and buried in the soil under *A. pulchella* for seven days it was difficult to distinguish between the wall thickening and the gap created between the outer wall and the cytoplasm in the first stages of cytoplasmic shrinkage. A definite conclusion could not be reached and it was decided to include all the chlamydospores with the gap between the outer wall and cytoplasm in the lysed category.

A limitation of this work is that exposure of mycelial mats to leachates is not a natural situation and that lack of a food source may affect chlamydospore production and health. This situation was tested by burying the infected lupin roots under the *Acacia* plants.

Collapse of chlamydospores occurred within the lupin root tissues after being buried under the three varieties of *A. pulchella* indicating their inability to persist even within plant debris. This finding also showed the suppressive effect of *A. pulchella* plants towards *P. cinnamomi*. *A. pulchella* var. *goadbyi* plants were more effective in inactivating chlamydospores than the other two varieties.
Lupin roots recovered from the soil after seven days under non-suppressive *A. urophylla* contained cortical cells with full, germinating chlamydospores. This explains the increased inoculum found under *A. urophylla* plants.

These experiments show that conditions under *A. pulchella*, and especially *A. pulchella* var. *goadbyi* are unfavourable for the survival of chlamydospores of *P. cinnamomi* whilst the rhizosphere of *A. urophylla* is not suppressive to these spores and they germinate readily. They also showed that the presence of living microbes is not necessary as the effect was obtained using filter sterilised soil leachates. Thus, substances secreted by the *A. pulchella* roots or microbes individually or combined in the rhizosphere must have caused the lysis of the chlamydospores. The inactivation of chlamydospores would have contributed to the low inoculum of *P. cinnamomi* under *A. pulchella*.

Discovery of some oospores within the lupin root tissues recovered from under *A. pulchella* var. *glaberrima* plants in small numbers was an interesting finding in this study. Although the small thick walled spores observed in this study were not confirmed to be a propagule of *P. cinnamomi* it should be mentioned that similar structures have been reported by others (e.g. Cother and Griffin 1973). However, most of the studies indicated that for *P. cinnamomi*, chlamydospores and sporangia are the prominent propagules within root tissues (Old *et al.* 1984b; McKay *et al.* 1985). There were only a few sporangia found on the roots buried under *A. pulchella*. This can be attributed to the inhibition in this stage of the life cycle of the pathogen by the *A. pulchella* plants as supported by the observations made on *P. cinnamomi* mycelium.
on inert substrates previously achieved by Shea et al. (1982) and the experiments in Chapter 2.

Conclusions

The experiments of this chapter have shown suppression of chlamydospores of *P. cinnamomi* in soil leachates from *A. pulchella* and no suppression in soils from under *A. urophylla*. *P. cinnamomi* is described as a parasite, which possess saprophytic abilities (Zentmyer 1980). Saprophytic survival of root-infecting fungi is defined as survival of mycelia in infected host tissues invaded during its parasitic phase (Garrett 1970). Garrett (1970) also described the possible modes of survival for pathogenic root infecting fungi to be, by saprophytic survival in dead tissues of the plants invaded during the parasitic phase, by competitive saprophytic colonisation of additional dead plant tissues and by production of dormant survival structures. Marks et al. (1975) acknowledged that the behaviour *P. cinnamomi* qualifies it to be called a saprophytic survivor according to the characteristics defined by Griffin (1972). The display of these saprophytic qualities in *P. cinnamomi* in the presence of *A. pulchella* var. *goadbyi* plants were either poor or totally absent.

It is not understood whether the oospores observed infrequently within lupin roots buried under *A. pulchella* var. *glaberrima* for seven days were products of selfing of hyphae by some chemical stimulus under the *A. pulchella* plants or from the presence of specific microorganisms. For example, *P. cinnamomi* is capable of producing oospores when exposed to volatiles of *Trichoderma viride* (Brasier 1971). It can be speculated that under stress due to the diminishing food base *P. cinnamomi* may respond by producing the thick walled survival structures. It could also be that the
pathogen is in a hyperactive mode. This phenomenon will be examined with more isolates of *P. cinnamomi* in Chapters 6, 7 and 8.
CHAPTER 4

Effect of root exudates of *Acacia pulchella* on the asexual stages of

*Phytophthora cinnamomi*

4.1 Introduction

*Phytophthora* species may change their morphological characteristics and growth rate when exposed to toxic environments. Metalaxyl was reported to cause variation in colony type and growth rate in *P. cactorum* (Chang and Ko 1990) and exposure to chloroneb altered the colony morphology in *P. cinnamomi* (Zeng and Ko 1997). Differences have been recorded among A2 type isolates of *P. cinnamomi* in response to *in vitro* phosphite treatment. Wilkinson et al. (2001) found some isolates to be tolerant to phosphite whilst others were sensitive. This observation was made in determining colony growth of *P. cinnamomi* on agar amended with phosphite in different dosages. Some isolates were inhibited by phosphite incorporated into agar medium whilst the tolerant ones thrived. How *P. cinnamomi* responds when exposed to toxic factors in the natural environment is still an under-researched area.

*P. cinnamomi* is capable of producing a number of propagules such as mycelium, sporangia, zoospores, chlamydospores and oospores (Zentmyer 1980; Weste and Vithanage 1978; Mitchell 1979). According to the findings of previous chapters (Chapter 2) the mycelium of *P. cinnamomi* produced low sporangial numbers when either incubated in leachates from *A. pulchella* plants or when infected roots were buried under *A. pulchella* plants (Chapter 3).
Some plants contain substances which are toxic to fungi (Kuhn and Hargreaves 1987). A wide range of secondary metabolites grouped into different classes according to their chemical structures are antifungal agents. These compounds are distributed in different parts of the plants and play a role in defence mechanisms against diseases (Kuhn and Hargreaves 1987). Toxins in roots and shoots of oats (Avena sativa) have been implicated in the resistance of this crop to several fungal pathogens (Turner 1953). One toxin is avenacin, a saponin present in the root tips at a concentration of 8 µg per root tip (Crombie et al. 1986). Saponins are known to be toxic to zoospores of Phytophthora species including P. cinnamomi (Zentmyer and Thompson 1967). Saponins extracted from roots of A. pulchella with 50% aqueous ethanol caused considerable lysis of P. cinnamomi mycelium and some suppression of sporulation at a concentration of 500 ppm (Alexander et al. 1978). A. pulchella roots also contain volatiles suppressive to P. cinnamomi (Whitfield et al. 1981). Bioassays using an extract obtained by steam distillation of A. pulchella roots inhibited sporangial production, zoospore germination and mycelial growth of P. cinnamomi (Whitfield et al. 1981). However, a specific chemical or a group of chemicals could not be directly implicated as the inhibitor of the pathogen. Among the complex mixture of isolated volatile compounds from steam distillates of A. pulchella roots, two volatile sulphur compounds were believed to be responsible for the characteristic sulphurous aroma of A. pulchella roots. Although there is no evidence that saponins or sulphurous compounds are exuded from the roots of A. pulchella plants into the surrounding soil, it can be hypothesised that the water soluble root exudates from A. pulchella may contain compounds which inhibit sporangial production and mycelial growth of P. cinnamomi. Root exudates present in the soil leachates obtained from field or glasshouse grown plants are not only very low in concentration but also altered by many external factors. Due to this reason, water-soluble
exudates are most frequently obtained for experimental purposes from root systems of the plants immersed in aerated trap solutions (Neumann and Römheld 2001). Most of the existing models have been developed based on this principle.

Experiments in this chapter are designed to test the hypothesis that root exudates toxic to \( P. \text{cinnamomi} \) zoospores, chlamydospores and/or mycelium are produced by \( A. \text{pulchella} \) plants in the absence of soil microflora.

### 4.2 Materials and Methods

Root exudates were collected for bioassays from plants grown in two ways. For bioassays with \( P. \text{cinnamomi} \) zoospores and chlamydospores, water-soluble root exudates were collected from the model designed with root systems immersed in nutrient solution (4.2.2). For the bioassays with the mycelium of \( P. \text{cinnamomi} \) where greater volumes were required, root exudates collected from acacia plants grown aseptically in the IVS mix (Chapter 2) were used.

#### 4.2.1 Experimental design

Seedlings of \( A. \text{pulchella var. goadbyi} \) and \( A. \text{urophylla} \) were produced aseptically on agar as described in Section 2.2.7. When the seedlings produced the first pair of green leaves some were transferred into the specially designed growth containers (Fig. 4.1 A) and the rest were grown in IVS soil mix.

Plants in beads (propylene beads - Thai Polypropylene Co.) with one plant per tub maintained as described below (Section 4.2.4) had five replicates per each acacia species. The control tubs with beads and no plants, was also replicated five times.
Plants of *A. pulchella* var. *goadbyi* and *A. urophylla* in IVS mix with five plants per punnet were replicated three times. The control punnets with IVS mix and no plants, was also replicated three times. All the bioassays were repeated twice.

**4.2.2 Growth containers**

Twenty grams of poly-propylene beads (Thai Polypropylene Co.) were placed in screw cap poly-propylene containers, 105 mm x 44 mm (Starstedt, South Australia). A hole of 5 mm in diameter was drilled in each screw cap and sealed with an antimicrobial tape. Into each container 20 mL distilled water was added, autoclaved at 121°C for 20 min, and the water was drained off. The containers were left to cool down in a laminar flow unit prior to transplanting the aseptically grown seedlings.

The growth containers were designed to provide the plants with a sterile, undisturbed environment with a reasonable level of mechanical support and for the roots to be in contact with the nutrient solution (Fig 4.1 A). The *Acacia* seedlings with their first pair of green leaves were gently removed from the agar tubs and replanted in the beads without damaging the roots and fed with 5 mL of ½ strength Murashige and Skoog (MS) medium. The screw caps were replaced and the bottom part of the container wrapped with aluminum foil to reduce light exposure of roots. The containers were placed in a growth cabinet illuminated with fluorescent and incandescent lamps with a 14/10 h light/dark regime and the temperature maintained at 24 °C. The light intensity was 130 μE m⁻² sec⁻¹ throughout the experiment.
4.2.3 Maintenance of the seedlings

The seedlings were kept in the original containers for four to six weeks, supplied weekly with ½ strength MS medium. As the plants matured, the volume of nutrient solution was increased to 10-15 mL. As the plants grew, the containers were extended by removing the screw caps and placing a second container of the same size over the first one and sealing the join with antibacterial tape. This method ensured aeration and least disturbance to the seedlings. Control tubs without plants were also established.

4.2.4 Root exudate collection

When the plants were four months old, the solution from each growth container was pipetted out and discarded. Once the nutrient solution was removed, 5 – 10 mL sterile distilled water was added into each container and left for 24 h to prevent mineral salt build up on the beads. After 24 h the sterile distilled water with remnants of mineral salts were removed and fresh sterile distilled water was added and left for 48 h or so. This solution was pipetted out, pH value measured then sterilised through 0.22 µ Millipore filter. Tubs without plants were irrigated in the same way and the solution collected. All the solutions were stored in sample bottles at 2-4°C before being used in bioassays. The weekly nutrient cycle then recommenced and collections of solutions were carried out monthly until the plants were seven months old. For convenience, the solutions collected from the containers are referred to as root exudates even though it is appreciated that the solution is a mixture of sterile distilled water, plant exudates and any remaining nutrients not flushed out from the beads by the above treatment.
4.2.5 Bioassay of chlamydospores with *Acacia* root exudates

Chlamydospore suspensions of *P. cinnamomi* were made by using the method described by Darling (1978) (Appendix 1). Four A2 type isolates of *P. cinnamomi*; MP–62, MP–80 and MP 97-16 of the A2 type 1 and one A2 type 2; MP–125 were grown on V8 juice agar and incubated at 25 ºC in the dark until the mycelium covered the agar surface. Ribeiro’s liquid nutrient medium was amended with cholesterol dissolved in di-ethyl ether (1ppm) and left under a laminar flow until the ether evaporated. Using sterilised needles, mycelium was lifted off the agar and incubated in the above solution dispensed into 9 cm-diameter Petri dishes. After 2 weeks, the mycelial mats were washed in three changes of sterile distilled water and transferred into vials with 100 mL sterile distilled water and macerated with a hand held grinder. The chlamydospores were separated from mycelial fragments by straining through four layers of gauze and concentrated by pipetting under a dissecting microscope.

Bioassays were conducted with the exudates solution collected from four and seven month old *A. pulchella* var. *goadbyi* and *A. urophylla* plants. Using a micro pipette, aliquots of 10 µL of the concentrated chlamydospore suspension (~ 25 chlamydospores) were pipetted into wells of cavity slides and 30 µL of root exudate was added separately. For each *Acacia* species and the control, four replicate slides were prepared and were incubated in 14.5 cm diameter Petri dishes lined with moist filter paper for 24 h. Each of the four isolates was separately bioassayed. After 24 h, the numbers of intact, collapsed and germinating chlamydospores on each slide were counted under a light microscope using 100 x magnification.
4.2.6 Bioassay of zoospores with *Acacia* root exudates

Zoospore suspensions of *P. cinnamomi* were made using isolate MP 97-16 as described in Section 2.2.3. Mycelial disks bearing sporangia were placed in 3-cm-diameter Petri dishes containing 5 mL sterile distilled water. The dishes were chilled for 30 min at 4°C to synchronise zoospore release and zoospore concentration was determined. Aliquots of 1 μL of the zoospore suspension (10 – 20 per μL) were pipetted into wells of cavity slides and the behaviour of the zoospores was observed under the microscope at 50 x magnification with addition of 10 μL of filter sterilized root exudates collected from the seven-month-old *Acacia* plants in beads. Four slides were made per treatment. The length of time taken for the first 50% of the swimming zoospores to encyst was recorded with a timer starting at the addition of exudates. The time taken for all the zoospores to encyst was recorded next. Once all the zoospores encysted the slides were incubated in large Petri dishes (14.5 cm in diameter) lined with moist filter paper for 2 h and numbers of germinating zoospores were counted. In order to prevent zoospores sticking to the surfaces they contacted, all the equipment used was immersed in 1N HCl solution for 24 h prior to washing in distilled water for a further 24 h as done by Byrt and Grant (1979).

4.2.7 Bioassay of the mycelium with *Acacia* root exudates

Leachates collected from *Acacia* plants grown aseptically in the IVS mix (Chapter 2) were sterilized using a 0.22 μm Millipore filter and used for the bioassays with the mycelium of *P. cinnamomi*. It is appreciated that the leachate solution is a mixture of nutrient solution and plant exudates. A series of dilutions of the root exudate solutions were made by incorporating the filter sterilized leachates from *A. pulchella* var. *goadbyi*, *A. urophylla* and the control solution from tubs without plants into Ribeiro’s basal medium in Petri dishes (5 cm in diameter). The concentrations ranged from 10-50%
leachates in 10 mL of solution. There were two replicates for each concentration. A series of solutions were also made with 10-50% of sterile distilled water and Ribeiro’s basal liquid medium. This allowed discrimination between the effect of diluting the nutrients in the Ribeiro’s medium and the added effect of any compounds in the root exudates. A mycelial disc of *P. cinnamomi* (isolate 97-16) prepared as in Section 3.2.1 was placed in each dish and incubated at 22 °C in the dark for five days. After five days the solution in each dish was drained through a pre-weighed Whatman No 1 filter paper (9 cm diameter). The filter papers, each with a mycelial mat were dried at 60°C in an oven for 24 h then weighed.

### 4.2.8 Evaluation of soluble sulphur in the root exudates

Soluble sulphur in the root exudates was determined using the inductively coupled plasma combined with atomic emission spectroscopy (ICP-AES) method (Marine and Freshwater Research Laboratory, Murdoch University). Samples of exudate solutions were diluted ten times prior to analysis. The detection limit was 0.05 mg/L.

### 4.2.9 Statistical analysis

The data were analysed using SPSS 14.0 for Windows statistics package (SPSS Inc., USA). Multivariate analysis of variance tests were conducted for the bioassays with chlamydomspores and zoospores. A univariate ANOVA test was done for the mycelial bioassay. The ANOVA assumptions of normality were screened for by subjecting residuals to standard tests; normal Q-Q and detrended normal Q-Q plots. As there were no incidences where normality was considered to be violated, it has not been reported. Post hoc comparisons of all significant main effects and interactions were compared using Tukey test between means.
Fig 4.1  A – *In vitro Acacias*, the plants on the left are growing on agar and those on the right on plastic heads. B – Axenic chlamydomspore suspension of *P. cinnamomi*, C– Releasing zoospores from the sporangium D – Lysed chlamydomspore after incubation in *A. pulchella* for 24 h, E – Germinating chlamydomspore after incubating in *A. urophylla* root exudate for 24 h, F – Encysted zoospores clumped and with one germ tube in *A. pulchella* root exudate, G – Zoospores incubated in *A. urophylla* root exudate for 2 h, germ tubes (two arrow heads) producing microsporangium (▼), H – Mycelium of *P. cinnamomi* with swollen hyphal tips (►) incubated in *A. pulchella* var. goadbyi root exudates. Scale B = 100 µM. C, F = 10 µM. D, E = 50 µM. G, H = 20 µM.
4.3 Results

4.3.1 Bioassay of chlamydospores with *Acacia* root exudates

A.

B.

C.

Figure 4.2 Effect of the root exudates solutions collected from the growth containers with no plant control □, *Acacia urophylla* ■ and axenically grown *Acacia pulchella* var. *goadbyi* ■ on chlamydospores of the four *Phytophthora cinnamomi* isolates after 24 h incubation. Percentage of intact chlamydospores (A), collapsed chlamydospores (B) and germinating chlamydospores (C). Bars represent standard error of means.
Of the four *P. cinnamomi* isolates tested, the best chlamydosporic producers were MP 97-16 and MP-125. Chlamydosporic used in the bioassays were approximately 45 μm in diameter (Fig. 4.1 B) except for isolate MP 62 which produced smaller chlamydosporic (~30 μm in diameter).

There was no difference between numbers of intact, collapsed and germinating chlamydosporic in leachates from four months old *A. pulchella*, *A. urophylla* and control (data not shown). Leachates from seven months old *A. pulchella* var. *goadbyi* plant had a significant (P < 0.05) effect on the chlamydosporic of three isolates of *P. cinnamomi*. The MANOVA test (Appendix 2) showed a significant (P < 0.001) interaction between the isolates and the treatments. The comparison done on intact, collapsed and germinating chlamydosporic showed a significant (P < 0.01) differences between *A. pulchella* var. *goadbyi* and the control treatment. After 24 h incubation in the root exudates of seven months old *A. pulchella* var. *goadbyi* plants a large number of chlamydosporic of isolates MP 97-16, MP- 80 and MP-62 collapsed (Figs. 4.1 D, 4.2 B) and only a small percentage germinated. In contrast, chlamydosporic of isolate MP-125 were less damaged by the presence of *A. pulchella* root exudates. There were over 40% intact chlamydosporic and nearly 50% germinated with less than 10% collapsed after 24 h. The number collapsed was the lowest in this isolate compared to the other three.

In contrast, the root exudates from *A. urophylla* plants had a stimulatory effect on chlamydosporic germination (Fig.4.2 C) in all the isolates.
4.3.2 Bioassay of zoospores with *Acacia* root exudates

Zoospores which were released into the sterile distilled water in the Petri dishes were able to swim without encysting for at least two hours. However, when pipetted into glass cavity slides the zoospores encysted within 30 min. Exposure to exudates from either *Acacia* reduced the period of mobility. The MANOVA tests of between subject effects (Appendix 2) showed a significant (P < 0.05) difference among the three treatments for causing encystment of the zoospores in the time taken for 50% to encyst at F = 26.557 df = 2,8 P < 0.01 and for 100% at F = 18.932, df = 2,8 and P < 0.01).

<table>
<thead>
<tr>
<th>Species</th>
<th>Time taken (min) For 50% to encyst</th>
<th>Time taken (min) For 100% to encyst</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. pulchella</em> var. <em>goadbyi</em></td>
<td>1.25 ± 0.5</td>
<td>2.75 ± 1.2</td>
</tr>
<tr>
<td><em>A. urophylla</em></td>
<td>6.0 ± 2.0</td>
<td>17.3 ± 5.3</td>
</tr>
<tr>
<td>No plant control</td>
<td>15.3 ± 1.8</td>
<td>30.3 ± 2.3</td>
</tr>
</tbody>
</table>

*A. pulchella* var. *goadbyi* root exudates resulted in the fastest encystment of zoospores and in the presence of the solution they also clumped. Examination after two hours showed almost half of the encysted zoospores clumped and produced a single germ tube from the clump (Fig. 4.1 F). Zoospores incubated in *A. urophylla* root exudates took longer to encyst (Table 4.1) than when incubated in *A. pulchella* var. *goadbyi* root exudates and almost all the zoospores produced single germ tubes with a terminal
microsporangium (Fig. 4.1 G). All the encysted zoospores in the control solution from the tubs without plants produced single germ tubes and no clumping was observed.

**4.3.3 Mycelial bioassay**

Mycelial growth of *P. cinnamomi* was suppressed in the presence of *A. pulchella* var. *goadbyi* root exudates in the liquid medium (Fig. 4.3). The dry mass of the *P. cinnamomi* colonies was significantly (*P < 0.005*) less (*F = 173.131 df = 3 and *P < 0.01*) when *A. pulchella* var. *goadbyi* soil leachates were incorporated into the medium compared to that of *A. urophylla* or the control without plants. Total inhibition of the colony growth was observed when the medium contained 50% *A. pulchella* var. *goadbyi* root exudates.

![Graph showing mycelial growth of P. cinnamomi (isolate 97-16) in liquid nutrient broth amended with the leachates of in vitro grown A. pulchella and A. urophylla plants. The control leachates of the IVS mix without plants and sterile distilled water incorporated in the same concentrations as the leachates from Acacia plants. Bars represent the SE error of means.](image-url)
Microscopic examination of *P. cinnamomi* colonies exposed to *Acacia* leachates revealed that the hyphal tips were unusually swollen. This did not occur in the control and increased with the concentration of the exudates in the medium (Fig. 4.1 H).

### 4.3.4 Soluble sulphur

There was a higher level of soluble sulphur in the root exudate solutions from the *A. pulchella* var. *goadbyi* plants both from the beads and IVS mix compared to the control solutions or the root exudate solution from the *A. urophylla* plants (Tables 4.2).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble sulphur mg/L</th>
<th>Leachates from beads</th>
<th>IVS mix</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. pulchella</em> var. <em>goadbyi</em></td>
<td>19.0</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td><em>A. urophylla</em></td>
<td>4.0</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>No plant control</td>
<td>3.4</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Nutrient medium</td>
<td>-</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

### 4.4 Discussion

The bioassays using aseptically grown *Acacia* plants showed for the first time that the root exudates *per se* have the potential to inhibit *P. cinnamomi*. The inhibition was not observed in leachate from four month old seedlings, but root exudates of seven month old aseptically grown *A. pulchella* var. *goadbyi* suppressed mycelial growth and chlamydosore germination of *P. cinnamomi*. The hypothesis that the water soluble root
exudates from *A. pulchella* plants may contain inhibitory compounds in the absence of soil microorganisms was proven.

4.4.1 Effect on chlamydospores

Root exudates of *A. pulchella* var. *goadbyi* caused cytoplasmic collapse of chlamydospores and reduced germination of those that remained intact. Chlamydospores of all the isolates belonging to the A2 isozyme type 1 showed inhibition. The A2 type 2 isolate, MP-125 showed no inhibition. This isolate is the only one amongst those tested that is resistant to phosphite. It would be worth testing other isolates with resistance to phosphite to see if the link between this resistance and resistance to *A. pulchella* root exudate is consistent.

4.4.2 Effect on zoospores

Root exudates of *A. pulchella* var. *goadbyi* resulted in zoospores encysting faster than in the sterile distilled water control and in increased clumping. Zoospores of *P. cinnamomi* are considered the main infective agent of plant roots. Swimming zoospores chemotactically respond to both organic and inorganic stimuli exuded by roots in aqueous solutions (Hickman 1970). With encystment, the mobility of the zoospores is reduced. Thus they lose efficiency in reaching a target.

Germ tubes were produced less frequently in *A. pulchella* var. *goadbyi* than in *A. urophylla* root exudates. So was the tendency to produce a single germ tube from few clumped spores. When the rate of germ tube production is low it can lead to a slowing of the infection process.
Increased clumping was observed simultaneously with encystment of the zoospores. A variety of amino acids and sugars have been shown to attract zoospores of *P. cinnamomi* and other *Phytophthora* species, whilst other substances have the ability to repel them. The latter phenomenon known as negative chemotaxis, is caused by several cations. Allen and Harvey (1974) demonstrated that the zoospores of *P. cinnamomi* were repelled by HCl, KCl, NH$_4$Cl and NaCl. It may be possible that the high soluble sulphur levels or some other substance in the root exudates of *A. pulchella* var. *goadbyi* plants might have caused the observed clumping of the zoospores of *P. cinnamomi*.

Deacon and Mitchell (1985) observed the responses of zoospores of *P. cinnamomi*, *Allomyces arbuscula* and *Aphanomyces* species to root exudates of various plant species and recorded how close the zoospores swam against roots. The attempts taken to adopt the methodology of Deacon and Mitchell (1985) and observing zoospore behaviour towards *A. pulchella* roots was not successful because of the difficulty in observing and recording high numbers of zoospores and the root tip under the microscope at different optical planes at one time.

**4.4.3 Effect of soluble sulphur in the root exudates of *Acacia pulchella* var. *goadbyi***

The soluble sulphur detected in the root exudates of *A. pulchella* var. *goadbyi* may have contributed towards inhibition of the asexual stages of the pathogen. Although the presence of volatile sulphurous compounds in the steam distillate of *A. pulchella* roots was shown by Whitfield *et al.* (1981), its presence in the root exudates was demonstrated for the first time in the present study. The bioassays by Whitfield *et al.* (1981) showed the suppression of *P. cinnamomi* by the volatile compounds *en masse*, nevertheless no specific compound/s were implicated as the active compound.
Sulphur exists in elemental form in planta in several plant taxa and acts as an antifungal agent against invading fungi (Cooper and Williams 2004). Although the antifungal qualities of sulphur have been known for a long time, there is a paucity of research in Australia in the area of its effects on oomycetes. The only available evidence at present was provided by elemental sulphur causing reduction of *P. cinnamomi* disease in pineapple (Pegg *et al.* 1977).

**Conclusions and implications**

It has been known for some time that the inoculum potential of *P. cinnamomi* under *A. pulchella* is low (Shea and Malajczuk 1977; D’Souza *et al.* 2004), but the mechanism(s) of action had not been fully described. Further, it was unclear as to whether the *A. pulchella* plants itself produced inhibitory substances in root exudates, or if these were metabolised by soil microflora before becoming inhibitory. This study provides the evidence that the root exudates of *A. pulchella* var. *goadbyi* per se have the potential to reduce pathogen population. The inhibitory compounds are produced by aseptically grown plants in vitro from plants older than four months. Root exudation and microbial colonisation have both been shown to change with plant age and stage of development (Prikryl and Vancura 1980; Brimecombe *et al.* 2001). Therefore, more research into the impact of the interactions between soil microflora and *A. pulchella* plants on *P. cinnamomi* inoculum in non-sterile environments is required to fully understand the inhibition observed in the field.
CHAPTER 5

Suppression of *Phytophthora cinnamomi* under *Acacia pulchella* in the native vegetation

5.1 Introduction

*Acacia pulchella* is a common understorey component of a variety of forest ecosystems throughout the south-west of Western Australia (Monk *et al.* 1981) including the most prominent, jarrah forest (Shearer and Tippett 1989). Jarrah forest includes several vegetation types outlined by Havel (1975). In the upland jarrah forest the prevailing site types are designated T, S, O and P. Site type T is more fertile and is associated with low *P. cinnamomi* disease impact. Type P sites with low soil fertility and poor drainage are associated with high impact of *P. cinnamomi*. Type S includes both high and low *P. cinnamomi* impacts. Type Q is considered as one of the best from the point of view of fertility and moisture levels however, little information of *P. cinnamomi* impact is available. In addition, *A. pulchella* grows on Bassendean sand on the Swan Coastal Plain (Monk *et al.* 1981), and has been re-established on rehabilitated mine sites. Very little information is available on whether it has a similar impact on *P. cinnamomi* across each of these soil types.

The presence of *P. cinnamomi* resistant plant species in the under-storey can have a limiting impact on the pathogen directly and indirectly. An example of the direct action is the presence of *P. cinnamomi* resistant *A. pulchella* plants which reduce sporangial production of the pathogen in the soil (Shea *et al.* 1978) and cause low soil inoculum of *P. cinnamomi* (Shea and Shearer 1980; D’Souza 2004). The low inoculum is a result of the reduced biomass of the pathogen owing to the inhibition in the sporangial and
chlamydospore stages of its life cycle (Chapter 3). However, soil under *A. pulchella* plants can induce oospores of *P. cinnamomi* (Chapters 3, 6 and 7), which can stay dormant within infected plant material (Chapter 6). Oospores may persist for longer than other propagules as they have a thick wall and the inherent ability of endogenous dormancy (Zentmyer and Mircetich 1966).

In contrast, non-suppressive plant species such as *Acacia urophylla* can stimulate sporulation of *P. cinnamomi* (Chapter 2), and may be associated with the spread of the pathogen. Also by stimulating chlamydospore and zoospore germination (Chapters 3 and 4) these species can increase the biomass of the pathogen and aid its inoculum potential.

It is not known whether the direct suppressive effect of *A. pulchella* is the same with plants growing in different soil types, or if the effect can vary due to an effect of the soil type of the *Acacia* plants and the associated soil microflora. To investigate this, the effect of extracts from soil under *A. pulchella* plants growing in various soil types was compared with extracts from the same soils in areas but without *A. pulchella* plants. Also there is no information available on whether or not the age of field grown *A. pulchella* stands plays a role in *P. cinnamomi* suppression.

Experiments in this chapter tested

- the effect of field grown *A. pulchella* from different soil types on *P. cinnamomi* and the interaction of time of the year the soils were collected, and
- the effect of age of the field grown *A. pulchella* plants on *P. cinnamomi* suppression.
5.2 Materials and Methods

5.2.1 Experimental design

The independent variables were the soils collected in summer (February) and winter (July) beneath *A. pulchella* plants and away from *A. pulchella* plants (control) growing in forest soils and the Bassendean sands. The dependent variables were recovery of *P. cinnamomi* from the soil extracts made from three replicate samples of each soil type and sporangial numbers produced on the mycelial discs incubated in the soil extracts.

The independent variables in the experiment with mine site soil were the soils under two different aged stands of *A. pulchella* and control soils without *A. pulchella* plants collected in summer. The dependent variable was recovery of *P. cinnamomi* from the soil extracts.

5.2.2 Soils

Soil samples were collected from beneath three *A. pulchella* stands and 5 m away from *A. pulchella* stands from several different Havel forest vegetation site types, coastal sand and rehabilitated mine pits. One and four-year-old rehabilitated mine pits with *A. pulchella* were selected and soil samples were collected from beneath and away from *A. pulchella* stands.

At each site, three replicate soil samples were collected as close as possible to each plant in the root zone to a depth of 10 – 15 cm and also at each site, at locations 5 m away from the nearest *A. pulchella*. The soils were brought to the laboratory in plastic bags and stored in 2-4°C for total of one week. The soil extracts were prepared 24 h after collection and repeated after one week storage.
Chemical analysis

Chemical analysis was done only on the undisturbed forest soil and the Bassendean sands. The soils from rehabilitated mine pits were heavily fertilised at revegetation (C. Wilkinson, Alcoa World Alumina, personal communication, D’Souza et al. 2004) but no chemical analysis was carried out.

Soil samples were oven dried at 40°C for 24 h prior to analysis. Soil chemical properties of all the soils other than the mine soils were analysed commercially (CSBP LIMITED, Bibra Lake, Western Australia). The following chemical properties were analysed as done in Chapter 2; pH and conductivity (Rayment and Higginson 1992), organic carbon by the Walkley and Black method (Rayment and Higginson 1992) nitrate and ammonium nitrogen (Searle 1984), sulphur (Blair et al 1991), phosphorus and potassium by the Colwell method and reactive iron by Tamm method (Rayment and Higginson 1992).

5.2.3 Acacia pulchella varieties

Samples of the A. pulchella plants were collected from each site and the variety was identified (Table 5.1) with the aid of botanical keys (Flora of Australia) and in consultation with Dr. Bruce Maslin of WA Herbarium (personal communication). The specimens have been deposited in the Murdoch University Herbarium.
Table 5.1 Soil types and vegetation characteristics of the field soils

<table>
<thead>
<tr>
<th>Soil</th>
<th>Location</th>
<th>Havel vegetation type</th>
<th>Dominant species</th>
<th>Common understorey species</th>
<th>A. pulchella variety</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forest soils</td>
<td>Glen Eagle State Forest Armadale</td>
<td>P</td>
<td>Allocasuarina fraseriana and Banksia grandis</td>
<td>Xanthorrhoea preissii, Persoonia longifolia, Adenanthis barbigera and Loxocarya flexuosa</td>
<td>Variant 1 of A. pulchella var. pulchella (Fig. 1.4 B)</td>
</tr>
<tr>
<td></td>
<td>32º 15’ S, 116º 15’ E</td>
<td>Q</td>
<td>Eucalyptus patens</td>
<td>Trymalium sp., Xanthorrhoea preissii, Eucalyptus marginata</td>
<td>A. pulchella var. pulchella (Fig. 1.4 A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>Eucalyptus patens</td>
<td>Pteridium esculentum, Acacia urophylla, Clematis pubescence and Leucopogon verticillatus</td>
<td>A. pulchella var. goadbyi (Fig. 1.4 C)</td>
</tr>
<tr>
<td>Bassendean sand</td>
<td>Wireless Hill Nature Reserve Ardross</td>
<td>Banksia</td>
<td>Banksia attenuata, Banksia menziesii, Banksia grandis</td>
<td>Xanthorrhoea preissii, Acacia pulchella, Macrozamia riedlei and sedges</td>
<td>A hybridised form of varieties pulchella and glaberrima (Fig. 1.4 D)</td>
</tr>
<tr>
<td></td>
<td>31º 57’ S, 115º 51’ E</td>
<td>Woodland</td>
<td>Banksia spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rehabilitated Mine pits</td>
<td>Dwellingup</td>
<td>The pits were revegetated with a seed mix to include native plant species* which had been present in the sites before the sites were disturbed. However, no information was available of the site vegetation types. Several legume species were dominant with Eucalyptus marginata, Corymbia calophylla and Banksia spp. growing in between.</td>
<td>A hybridised form of varieties glaberrima</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>32º 43’ S, 116º 02’ E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Personal communication, C. Wilkinson, Alcoa World Alumina
5.2.4 Preparation of soil extracts and inoculation with *Phytophthora cinnamomi*

Root material and gravel were removed from each soil sample by sieving through a 2 mm mesh. Samples of 100g were placed in conical flasks and 250 mL of distilled water was added. The flasks were placed on a shaker for 2 h and contents were filtered through Whatman No 1 filter paper. Potting mix was used as the control. The soils and the potting mix used to induce sporangia were tested for the presence of *P. cinnamomi* by baiting with rose petal discs (Chapter 2) and proved free of the pathogen. The filtrates were collected and sterilised using 0.22 µm Millipore filters. Twenty mL sterile samples from each filtrate were placed into five 9- cm-diameter Petri dishes and aliquots of non-sterile potting mix extract (as described in Chapter 2) were added to induce sporangial production.

To each dish five 6-mm-diameter mycelial discs inoculated with *P. cinnamomi* prepared as described in Chapter 2 were added. Before incubation no sporangia were observed on the mycelial discs. These discs sank to the bottom of the dishes so there was no direct contact between the inoculum and the petal baits (see below). There were three replicate dishes per soil. The dishes were incubated at 25 ± 1°C for 24 h during which sporangia formed on the mycelial mats. After 24 h the dishes were chilled at 4°C for 30 min and baited with rose petal discs (see below).

**Baiting**

Twenty rose petal discs were floated on the surface of the soil filtrate and incubated at 25 ± 1°C under light (Fig. 5.1). Every 10 h the petals were removed, dried on paper towels and plated on NARPH selective agar and incubated at 24°C for 48 – 72 h. A
fresh batch of petal discs was placed in the dishes and this procedure was carried out four times over 40 h.

![Image of Petri dishes with rose petals and mycelial discs]

**Figure 5.1** Baiting the soil filtrates with rose petal discs. The mycelial discs of *Phytophthora cinnamomi* (►) incubated in the bottom of the Petri dishes.

### 5.2.5 Effect of the soil filtrates on *Phytophthora cinnamomi* recovery

It was assumed that the zoospores released from the mycelial disc inoculum in the bottom of the dishes would swim to the surface and infect the rose petal baits. These baits were then plated on NARPH selective agar to determine the recovery of *P. cinnamomi*, which yielded colonies after 48 h. The suppression of the soil filtrate towards *P. cinnamomi* was determined by the number of petal baits that yielded *P. cinnamomi* colonies expressed as a percentage. Recovery of *P. cinnamomi* and suppression of the soil filtrate were inversely correlated by adopting a rating system of 0 – 100% (Table 5.2).
Table 5.2 Rating system of recovery of *P. cinnamomi* adopted to express the suppression

<table>
<thead>
<tr>
<th>Recovery</th>
<th>Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10%</td>
<td>Highly suppressive</td>
</tr>
<tr>
<td>10–25%</td>
<td>Suppressive</td>
</tr>
<tr>
<td>25–50%</td>
<td>Less suppressive</td>
</tr>
<tr>
<td>&gt; 50%</td>
<td>Conducive</td>
</tr>
</tbody>
</table>

*Sporangial production of Phytophthora cinnamomi in soil filtrates*

After removing the petal baits the mycelial discs were removed from the dishes, mounted on slides and stained with aniline blue. The number of sporangia per field of view under x 100 was scored in five fields of view using a light microscope.

5.2.6 Statistical analysis

The experiments were repeated once. Data collected from one experiment were analysed using SPSS 12.0.1 for Windows (SPSS Inc., USA). The ANOVA assumptions of normality were screened by subjecting residuals to standard tests; normal Q-Q plots and detrended normal Q-Q plots. No violations of normality were found. No statistical analysis was performed on the soil chemical properties.

5.3 Results

5.3.1 Effect of the forest soils on *Phytophthora cinnamomi* recovery

*Effect of soil extracts made from soils collected in winter*

Among the controls, the highest recovery of *P. cinnamomi* was from the inoculum incubated in the Bassendean sand extract, where 100% of the petal baits yielded
*P. cinnamomi* colonies whilst, extracts from soil types P, T and Q yielded 10%, 50% and 80% respectively. In contrast, extracts made from soil under *A. pulchella* were significantly (*P < 0.05*) low. No *P. cinnamomi* was recovered after 24 h incubation in Q soil type extract (Fig. 5.2 A). According to the rating system adopted (Table 5.2) the Bassendean soil extract was conducive to *P. cinnamomi* whereas, the Q soil type was the most suppressive. Soil types P and T were also suppressive with *P. cinnamomi* recovery within the range of 10 – 20%. A Univariate ANOVA showed a significant (*F = 4.731, df = 3,16 and P < 0.05*) 2-way interaction between the two independent variables; the four soil types and *A. pulchella* soil/control soils at the 0.05 level (Appendix 2). The main effects between the four soil types was significant (*F = 18.582 df = 3,16 P < 0.01*) and *A. pulchella* soil and the control for each soil type were also significantly (*F = 26.285, df = 1, P < 0.01*) different. The Post Hoc multiple comparison test between means done on all the soils using Tukey HSD showed significant (*P < 0.05*) differences between Bassendean sand and all the forest soils in the recovery of *P. cinnamomi*. The same test showed a significant (*P < 0.05*) difference between the two forest soils P and Q in the recovery of *P. cinnamomi* yet the differences were non significant between P and T or Q and T soils.

**Effect of soil extracts made from soils collected in summer**

Recovery of *P. cinnamomi* from the controls was significantly (*P < 0.05*) higher than the *A. pulchella* soils. No recovery was recorded for *A. pulchella* soil in type T and recovery was less than 10% for the soil types P, Q and BS indicating that the soils under *A. pulchella* in all the soil types were suppressive to *P. cinnamomi* (Table 5.2). The Univariate ANOVA test between subjects was significant (*F = 3.329 df = 3,16 and P < 0.05*) for the four soil types (Appendix 2). The effect for the *A. pulchella* soil and
the control for each soil type was also significant (F = 25.293 df = 1,16 and P < 0.05). The test failed to find a significant (P < 0.05) interaction between the two independent variables.

Figure 5.2 Recovery of Phytophthora cinnamomi from soil extracts made from soils collected from Havel vegetation site types in the jarrah forest P, T, Q and Bassendean sand (BS) in winter (A) and summer (B). Soil extracts made from soil collected away from Acacia pulchella plants (control) □ and beneath Acacia pulchella plants ■, were incubated with P. cinnamomi mycelial mats for 24 h and baited with rose petal discs. Bars represent the standard error of means.

In summer, soil extracts from beneath A. pulchella in all the soil types showed high level of suppression towards P. cinnamomi incubated in them with less than 10% of the baits yielding P. cinnamomi colonies. The most suppressive soil was the Havel type T where no recovery of P. cinnamomi was recorded in the soil extracts from the soil beneath A. pulchella plants and only 20% of the baits yielding P. cinnamomi from the control soil. There was no significant difference in suppression of P. cinnamomi in the Havel P type A. pulchella soils and control soils between the two times of collection. The effect of the presence of A. pulchella was most evident on the T and Q sites, and least of P site. In Bassendean sand there was a significant effect of the presence of A. pulchella in summer.
5.3.2 Effect on *Phytophthora cinnamomi* of extracts from soils collected under 1 and 4-years-old *Acacia pulchella* stands on rehabilitated bauxite mine pits

There was no significant (P < 0.05) difference in the recoveries of *P. cinnamomi* from mycelium incubated in extracts from the soils under one-year-old *A. pulchella* compared to the control whilst no *P. cinnamomi* was recovered from under four-year-old stands in both sites (Fig 5.3). Univariate ANOVA test performed between *P. cinnamomi* recoveries from soil extracts made with the soils from one and four-year-old *A. pulchella* stands showed a significant (F = 16.094, df = 1,20 and P < 0.01) difference. The soil extracts made with soil from the four year-old *A. pulchella* stands were highly suppressive whilst the control soils away from *A. pulchella* were conducive towards the zoospores of *P. cinnamomi*. The univariate test confirmed the significance difference at (F = 16.094, df = 1,20 and P < 0.01) (Appendix 2).

![Figure 5.3 Recovery of *Phytophthora cinnamomi* from soil extracts from one and four-year-old *Acacia pulchella* stands in rehabilitated bauxite mine sites](image)

Figure 5.3 Recovery of *Phytophthora cinnamomi* from soil extracts from one and four-year-old *Acacia pulchella* stands in rehabilitated bauxite mine sites ■. Controls were extracts from soil collected 5 m away from *A. pulchella* □. Bars = SE of the means.
5.3.3 Sporangial production of *Phytophthora cinnamomi* in soil filtrates

The number of sporangia produced on the mycelial mats after 24 h incubation in soil filtrates made from the soils collected from different soils beneath and away from *A. pulchella* plants revealed varying levels of sporangial suppression (Fig. 5.4).

![Graph A](image)

**Figure 5.4** Effects of soil extracts on the sporangial production of *Phytophthora cinnamomi*. Mycelial mats were incubated for 24 h in soil extracts prepared from the soil collected in winter (A) and summer (B), away from *Acacia pulchella* plants (control) □ and beneath *Acacia pulchella* plants ■. Bars represent the SE of means.
In both summer and winter the highest sporangial production was observed in the soil filtrates from the Bassendean soils but this was significantly (P < 0.05) less in samples collected under *A. pulchella* plants (Appendix 2). In winter, low numbers of sporangia were produced in P soil filtrate and there was no difference between soils from under and away from *A. pulchella*. However, in winter on soil type T and Q, and in summer on soil types T, Q and P there was a significant suppression.

5.3.4 Soil chemical properties

Soils from both beneath and away from *A. pulchella* plants were slightly acidic with soil of T type under *A. pulchella* var. *goadbyi* plants being the most acidic at pH 5.6 (Table 5.3).

<table>
<thead>
<tr>
<th>Soil type</th>
<th><em>A. pulchella</em></th>
<th>pH</th>
<th>Cond dS/m</th>
<th>Org C %</th>
<th>Nitr. N mg/Kg</th>
<th>Amm.N mg/Kg</th>
<th>Sulphur mg/Kg</th>
<th>P mg/Kg</th>
<th>K mg/Kg</th>
<th>Iron mg/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>+</td>
<td>6.2</td>
<td>0.076</td>
<td>4.66</td>
<td>1.00</td>
<td>4.00</td>
<td>6.3</td>
<td>8.0</td>
<td>117</td>
<td>1104</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>6.1</td>
<td>0.036</td>
<td>0.73</td>
<td>1.00</td>
<td>1.00</td>
<td>5.0</td>
<td>2.0</td>
<td>28</td>
<td>566</td>
</tr>
<tr>
<td>Q</td>
<td>+</td>
<td>6.3</td>
<td>0.044</td>
<td>4.78</td>
<td>1.00</td>
<td>4.00</td>
<td>6.4</td>
<td>8.0</td>
<td>52</td>
<td>1527</td>
</tr>
<tr>
<td>Q</td>
<td>-</td>
<td>6.0</td>
<td>0.044</td>
<td>3.06</td>
<td>1.00</td>
<td>1.00</td>
<td>5.4</td>
<td>2.0</td>
<td>39</td>
<td>707</td>
</tr>
<tr>
<td>T</td>
<td>+</td>
<td>5.6</td>
<td>0.029</td>
<td>5.28</td>
<td>1.00</td>
<td>3.00</td>
<td>7.1</td>
<td>7.0</td>
<td>81</td>
<td>2506</td>
</tr>
<tr>
<td>T</td>
<td>-</td>
<td>6.0</td>
<td>0.036</td>
<td>2.54</td>
<td>3.08</td>
<td>1.36</td>
<td>5.2</td>
<td>5.38</td>
<td>37</td>
<td>759</td>
</tr>
<tr>
<td>BS</td>
<td>+</td>
<td>6.4</td>
<td>0.031</td>
<td>1.41</td>
<td>3.04</td>
<td>3.70</td>
<td>3.4</td>
<td>5.32</td>
<td>24</td>
<td>71</td>
</tr>
<tr>
<td>BS</td>
<td>-</td>
<td>6.2</td>
<td>0.015</td>
<td>0.91</td>
<td>2.00</td>
<td>2.00</td>
<td>2.1</td>
<td>4.0</td>
<td>15</td>
<td>292</td>
</tr>
</tbody>
</table>
This soil also had the highest organic carbon and sulphur levels. The *A. pulchella* soils of types P and Q had similar organic carbon, sulphur levels and the same ammonium and nitrate nitrogen and phosphorus levels. Nutrient levels other than the available nitrogen in the Bassendean sands were lower than for the forest soils.

### 5.4 Discussion

*P. cinnamomi* suppression under *A. pulchella* was dependent on the age of the plants. In the rehabilitated mine sites young, one-year-old plants did not suppress the pathogen whilst four year-old stands were highly suppressive. Although it was not possible to assess the age of the *A. pulchella* plants in the forest locations accurately, soil collections were carried out beneath the seemingly mature plants. The suppressive ability of *A. pulchella* plants also depended on the variety and the season in which the soils were collected. Soils of different Havel site vegetation types where different varieties or variants were found, showed varying degrees of suppression towards *P. cinnamomi*. The soils from P, Q, and T Havel site types collected beneath *A. pulchella* in winter and summer ranged from suppressive to highly suppressive towards *P. cinnamomi*. In contrast in the Bassendean sand extracts there was no suppression of *P. cinnamomi* in winter with no significant difference between the *A. pulchella* soil and the control without *A. pulchella*. The soil collected from beneath *A. pulchella* from Havel Q site type in winter was the most suppressive with no *P. cinnamomi* recovery from baits.

This also varied between the two seasons with higher suppression in summer. No *P. cinnamomi* was recovered from soil filtrates made with the soil collected under *A. pulchella* plants in Q vegetation type in winter and T soils in summer.
5.4.1 Sporangial production and zoospore release

Total suppression of sporangial production of *P. cinnamomi* was recorded in mycelial discs incubated in soil filtrates made with Q soil collected in winter and the T soil collected in summer. This result is in accord with no *P. cinnamomi* recovered from the soil filtrates of the same soils. No *P. cinnamomi* recovery from the baits is a direct indication of zoospore inactivity on them not being produced or released. Nesbitt *et al.* (1979) showed that high organic matter caused sporangial abortion. Havel T soils where the highest organic carbon and high ammonium nitrogen was found, yielded no sporangia in its leachates. Sporangium development and zoospore release is associated with rapid reproduction (Erwin and Ribeiro 1996) of the pathogen. When this stage of the life cycle is suppressed it directly impacts the inoculum of the pathogen. Low *P. cinnamomi* impact has been associated with the Havel T vegetation type which was related to high fertility (Havel 1975). The findings of this chapter showed that the soil under the *A. pulchella* var. *goadbyi* plants, collected from Havel T vegetation type in summer were the most suppressive. This soil was also high in sulphur among other nutrients. It can be hypothesised that the soil extracts of this soil contained water soluble sulphur which affected the sporangial production. Although Havel vegetation type Q has been identified as the most fertile, the soil nutrient levels were not markedly different to Havel types P and T soils. Both P and Q soils collected in winter were suppressive towards sporangial production of *P. cinnamomi*.

Several studies found seasonal variations in the suppressive or stimulatory effects of field soils on sporangial formation of *P. cinnamomi* (Broadbent and Baker 1974; Shea *et al.* 1978; Murray *et al.* 1985; Malajczuk 1983). Most of these studies focused on the soil microflora, soil physical characteristics or the soil organic content. The present study has
broadened the existing knowledge by showing the direct effect of *A. pulchella* on sporangial stage of *P. cinnamomi*.

**5.4.2 Variation in the species complex of *Acacia pulchella***

*A. pulchella* has been identified as the most variable member of the Pulchellae and the most variable *Acacia* species in Western Australia (Maslin 1975). This study also showed the species to be very variable and identified two informal hybrids, *A. pulchella* var. *pulchella* x *A. pulchella* var. *glaberrima* in the Bassendean sandy location and *A. pulchella* var. *glaberrima* x *A. pulchella* var. *reflexa* in the rehabilitated mine pits which have not been documented previously. The documented variant 1 of *A. pulchella* var. *pulchella* were identified in the Havel type P site. The two other forest locations inspected which were only a few kilometres apart had two different varieties of *A. pulchella*. In Havel vegetation type T where *A. pulchella* var. *goadbyi* plants were an understorey component, the soil beneath these plants were more suppressive to sporangial production and zoospore release of *P. cinnamomi* in summer. *A. pulchella* var. *pulchella* and its variant found in Havel vegetation types P and Q had similar levels of suppression towards *P. cinnamomi*. Soil beneath the hybridised form of four-year-old *A. pulchella* var. *glaberrima* and var. *reflexa* from the rehabilitated mine sites were highly suppressive. These data support earlier observations (Chapter 2).

As described in Section 1.8.3, in other plant species, root exudates can vary in composition and quantity between species and between varieties. Vancura and Hanzlikova (1972) observed differences in amino acid quantities exuded by barley (*Hordeum distichum* L) var. ‘Branišovský C’, wheat (*Triticum vulgare* Vill) var. ‘Remo OA’, cucumber (*Cucumis sativus* L.) var. ‘Bliska’ and bean (*Phaseolus vulgaris* L.) var.
Veltruská Saxa’. In the root exudates of three soybean cultivars, significant differences in soluble carbohydrate levels were observed by Keeling (1974) which influenced their susceptibility to *Pythium* spp.

**Conclusions**

The most suppressive sites had mature *A. pulchella* plants. It can be hypothesised that the concentration of inhibitory compounds in the root exudates under the mature plants to be higher with increased root mass or the chemical composition changing with age and maturity of *A. pulchella* plants. The suppression of *P. cinnamomi* by *A. pulchella* also varies depending on the Havel site vegetation type and the time of the year. Overall the suppression is more evident in summer than in winter. This can be due to the fact that the water soluble compounds in the root exudates are leached out faster in winter than in summer. Similarly, they may leach faster from the Bassendean sand than from the lateritic soils of jarrah forest. Based on the different soil nutritional capacities observed, it is therefore possible that either the antifungal activities of the root exudates of *A. pulchella* may differ from variety to variety or each variety encourages specific soil microflora under them. Further work is required to address these questions.
CHAPTE R 6

Oospore production of *Phytophthora cinnamomi* in the presence of

*Acacia pulchella*

6.1 Introduction

There are few reports of *P. cinnamomi* producing oospores within plant roots in the soil so the occurrence of oospores in lupin roots recovered from the soil with *A. pulchella* var. *glaberrima* (Chapter 3) is of particular significance. Although *P. cinnamomi* oospores were first reported in avocado roots and mycelial mats by Mircetich and Zentmyer (1966) there has been only one subsequent report. Reeves and Jackson (1972) observed *P. cinnamomi* oospores on nylon mesh mats adhered to small pieces of *Castanea sativa* root, which had been buried for six to eight days in soil.

In contrast, there are many reports on the production of oospores by *P. cinnamomi* in *vitro*. Oospores of *P. cinnamomi* are generally produced by pairing A1 and A2 mating types (Chang *et al.* 1974; Zentmyer 1982). They can also be produced by chemical stimuli (Brasier 1971) or mechanical damage (Reeves and Jackson 1974). An unidentified volatile chemical produced by *Trichoderma viride* was shown to be an effective stimulus for *P. cinnamomi* (Brasier 1971; Pratt *et al.* 1972) and other *Phytophthora* species (Reeves and Jackson 1972). Oleic acid present in root extracts of avocado initiated oospores in the A2 mating types of *P. cinnamomi*, *P. drechsleri* and *P. capsici*, but was not effective on the A1 type (Zentmyer 1979). Lecithin extracted from soybean stimulated oospore production in *P. cactorum*, *P. parasitica* (Ko and Ho 1983), *P. capsici* (Ko 1985; 1986) and in *P. boehmeriae* and *P. sojae* (Wu *et al.* 2003).
*P. cinnamomi* was considered by several authors to be homothallic and hermaphroditic prior to being shown to be heterothallic with the A1 and A2 mating types (Chang et al. 1974). The demonstrated ability of the A2 type to produce oospores under different conditions has made it possible to overcome the confusion and controversy regarding its sexuality. Zentmyer et al. (1979) described *P. cinnamomi* as being functionally heterothallic but potentially homothallic under special conditions. Chang et al. (1974) suggested that the diploid mycelium of *P. cinnamomi* can produce apomictic oospores. According to Chang et al. (1974) oospores of *P. cinnamomi* can be three types: homothallic, heterothallic and selfed, depending on their origin and method of formation. Selfed oospores are produced by a single mating type thallus of heterothallic species under special conditions.

*P. cinnamomi* oospores, produced in avocado roots and mycelial mats in soil (Mircetich and Zentmyer 1966) could have been stimulated by either the oleic acid found in the avocado roots or through chemical stimulus from *T. viride* in the soil or both. Reeves and Jackson (1972) suggested that the presence of *T. viride* and root material were imperative for *P. cinnamomi* to produce oospores in soil. *T. viride* and other oospore inducing *Trichoderma* species are present in several Australian soils (Pratt et al. 1972; Johnson and Heather 1982) including the Western Australian jarrah forest soils (Pratt et al 1972; Malajczuk and McComb 1979), but there are no reports in the literature of *P. cinnamomi* oospores either in jarrah forest soils or in soils of other native vegetation types of Australia. The only information on *P. cinnamomi* survival within plant tissue in Australian forest soils was obtained from studies conducted in laboratory situations (Malajczuk and Theodorou 1972; Cother and Griffin 1973; Old et
al. 1984b; Mackay 1984). The artificial environment makes it difficult to extrapolate these observations to the natural systems.

There is no indication of sexual interaction for *P. cinnamomi* in jarrah forest (Old *et al.* 1984a; 1988; Dobrowolski 1990) or other parts of Australia. In most areas only the A2 mating type is present, but both A1 and A2 mating types were isolated from the same site at two locations in New South Wales, in the central coastal Ourimbah and the south coastal Kioloa (Old *et al.* 1984a). Dobrowolski (1990) found the two mating types in Gull Rock, Western Australia in the same sampling site within a 1 m area. Nevertheless, no sexual recombinant genotypes were recovered. It was suggested that the two mating types are incompatible due to differences in their ploidy or karyotype (Dobrowolski 1990).

When brought together in Petri dishes the two *P. cinnamomi* mating types appear to overcome this incompatibility factor. Oospore production is triggered at the junction where the two mating types come into contact (Chang *et al.* 1974). However, there is no clear indication that oospore production under pairing circumstances is a result of the association between the two opposite types *per se*. The gametangial interactions between A1 and A2 types can be manifold (Brasier 1972). The resulting *P. cinnamomi* oospores in the Petri dishes therefore can be products of A2 oogonia and A1 antheridia or *vice versa*. They can also be formed by A2 oogonia and A2 antheridia. According to Brasier (1972), the compatibility system probably is a chemical mechanism, which initiates sexual differentiation within or between isolates. Ko (1978) demonstrated that this stimulant may pass through a polycarbonate membrane. Given the observation that many generally heterothallic *Phytophthora* species may self, it is reasonable to hypothesise that *P. cinnamomi* may also can self and produce oospores.
Although temperature, light, oxygen tension and composition of the medium have all been shown to influence sexual reproduction in *Phytophthora in vitro* (Elliot 1982), currently, very little information is available on *in planta* oospore production of *P. cinnamomi*. Since the oospores of *P. cinnamomi* occurred only within root material buried under *A. pulchella* var. *glaberrima* (Chapter 3), it was necessary to determine whether this phenomenon was restricted to this variety of the species or whether the other two varieties, *goadbyi* and *pulchella* can also stimulate oospore production of *P. cinnamomi*. The following experiments were designed, firstly to test the hypothesis that the rhizosphere environment of all varieties of *A. pulchella* induces oospore production of *P. cinnamomi*. Secondly, to compare *in planta* oospore production of isozyme types 1 and 2 of the A2 clonal lineage of *P. cinnamomi*, two isolates of which were found to vary in the response of their chlamydospores towards *A. pulchella* root exudates (Chapter 4).

### 6.2 Materials and methods

Independent variables were the three varieties of *A. pulchella*; *A. pulchella* var. *pulchella*, var. *goadbyi* and var. *glaberrima* and the two isolates of *P. cinnamomi* (MP 97-16 and MP-125). Control was the soil without plants. The dependant variable was the number of oospores produced in lupin root segments buried in the soil for seven days.

#### 6.2.1 Plant and soil material

Plants of the three varieties of *A. pulchella*, var. *pulchella*, var. *goadbyi* and var. *glaberrima*, grown on jarrah forest soil (Chapter 2) and on a potting mix were used. The potting mix of initially pH 5 comprised of composted pine bark, coarse river sand
and coco peat at the ratio of 2: 2: 1. Both the soil and the potting mix were baited with rose petal discs as done in Chapters 2 and 3, and also plated onto NARPH selective agar and confirmed to be free of *Phytophthora*. The validity of the method was checked by infection of a soil with *P. cinnamomi* before baiting and its subsequent recovery. All the plants were 12 months old and were in 150 mm free-draining polyvinyl chloride pots, one plant per pot. Each *A. pulchella* variety in the potting mix or in soil was inoculated with lupin roots infected with isolate MP-125 or MP 97-16. Pots of soil or potting mix without plants were also inoculated with each isolate as controls. There were five replicates of each treatment. Pots were arranged in a randomised block design on the bench in the glasshouse at 25°C and watered daily.

**Soil chemical properties**

Samples of 100 g soil were taken from the root zone from each replicate pot. All the samples for each *A. pulchella* variety were bulked and mixed thoroughly. The resulting 500 g per variety were used for the analysis as described in Chapter 2.

6.2.2 Inoculum and inoculation

The inoculum was prepared as described in Chapter 2 using two isolates of *P. cinnamomi* from the *Phytophthora* collection of Murdoch University (Table 6.1). The isolates were passaged through lupin roots and cleaned by growing on NARPH selective medium for three to four days and subcultured on V8 juice medium. The clean cultures were maintained at 25°C in the dark with regular sub-culturing.

The biology of the two isolates has been examined in previous chapters. More insight into MP 97-16 has been achieved in this thesis as it belongs to the widely encountered
isozyme type A2 type 1 of *P. cinnamomi* in Western Australia. Both isolates produce abundant chlamydospores *in vitro* (Chapter 4) both on agar and in liquid culture. The axenically produced chlamydospore of MP 97-16 either collapsed or had low germination whilst chlamydospores of the MP–125 were not inhibited in the root exudates of the most suppressive *A. pulchella* var. *goadbyi* (Chapter 4). The mycelial growth of isolate MP 97-16 was inhibited by root exudates of *A. pulchella* var. *goadbyi* (Chapter 4).

**Table 6.1 Details of the isolates of Phytophthora cinnamomi**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isozyme type</th>
<th>DAWA accession no</th>
<th>Host</th>
<th>Location</th>
<th>Collector and Year isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP 97-16</td>
<td>A2 type 1</td>
<td>12877</td>
<td><em>E. marginata</em></td>
<td>Jarrahdale, W.A.</td>
<td>N D’Souza 1997</td>
</tr>
<tr>
<td>MP-125</td>
<td>A2 type 2</td>
<td>12878</td>
<td><em>E. marginata</em></td>
<td>Jarrahdale, W.A.</td>
<td>G. Hardy 1993</td>
</tr>
</tbody>
</table>

DAWA Department of Agriculture Western Australia

Mycelium of MP–125 was found to be tolerant to phosphite (Wilkinson, 2000) whilst no information on responses to phosphite was available for MP 97-16. Both isolates readily produced sporangia axenically (Chapter 4). When washed mycelial mats bearing mature sporangia produced as described in Chapter 2 were placed in a small volume of sterile distilled water, zoospores of both isolates were readily discharged. The zoospore numbers were found to be of 10 – 20 zoospores per µL.
As done in Chapter 2 and 3, lupin radicles (1 cm long) were placed with the root tips in contact with the zoospore suspensions (Fig. 2.1 A) of both isolates and left undisturbed for a few hours. The infected radicles were then excised from seeds and plated on water agar and incubated at 25°C in the dark for seven days (Fig. 2.1 B). Each infected lupin root (1 cm long) was enclosed in a sachet made with nylon material and tied with a string (Chapters 2 and 3) for easy retrieval. A clean spatula was inserted into the soil at five places around the plants to the depth of 10 cm in each pot (Chapters 2 and 3) and inoculum sachets were gently pushed down these slits and the soil was closed in. Each variety of *A. pulchella* growing in the potting mix and in the soil was inoculated separately using the lupin roots infected with the two isolates. Un-infected lupin radicles enclosed in separate sachets and tied with different colour strings for identification, were also buried alongside the infected ones in each pot. The treatments were replicated five times and the experiment was repeated twice.

The inoculum was retrieved after seven days. Root pieces were first washed in 0.25% NaOCl solution for 1 min then gently rinsed repeatedly in three washes of distilled water. Each 1 cm root segment was mounted on microscope slides in a drop of distilled water. Using two needles the root pieces were spread into a thin layer and examined under x100 magnification. Numbers of oospores were counted under 10 fields of view. The measurements of the diameters were done using an image analysis program (Olysia-BioReport Soft Imaging Systems, 2002 connected to Olympus BX 51 microscope and IMAGING MicroPublisher 3.3 RTV camera).
**Axenic oospore production**

The two isolates were grown on modified Ribeiro’s medium (Ribeiro 1978) for oospore production (Appendix 1). Petri dishes containing the 20 mL of the above medium and mycelial mats produced as described in Chapter 2 were incubated at 25°C in the dark for 30 days. The mycelia were washed three times in sterile distilled water and macerated in 10 mL using a hand held macerator. The suspensions were strained through four layers of sterile gauze material and examined under the microscope.

6.2.3 Viability of oospores

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

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

In order to determine oospore viability, 10 fully developed oospores were dislodged from the root tissues under a dissecting microscope. The protocols included dissecting root tissue with a sharp scalpel, as close as possible to the oospores followed by dislodging them with the aid of two needles. A drop of distilled water was added to the separated oospores. By using a micropipette, the separated oospores were transferred onto water agar and incubated at 25°C in the dark for 30 days.

**Oospore dormancy**

Upon accomplishment of the above tasks, the remaining slide specimens of root segments containing oospores were placed in 15 cm-diameter Petri dishes with bottoms lined with moist filter paper to create a humid environment. The Petri dishes were sealed with tape to prevent moisture loss and placed in the dark at 10-15°C for 60 days. The slides were inspected weekly to examine any changes in the oospores and observations recorded and photographed.

**6.2.4 Statistical analysis**

The experiments were repeated twice. A univariate analysis of variance between subjects was performed using SPSS 14.0 for Windows statistics package (SPSS Inc., USA) and data from one experiment is presented. The ANOVA assumptions of normality were screened by subjecting residuals to standard tests; normal Q-Q and detrended normal Q-Q plots. Statistical differences were expressed at the 95% confidence level. All significant main effects and interactions were compared using Tukey multiple comparison test between means.
Figures 6.1-9 Selfed oospores of *Phytophthora cinnamomi*. Figures 1-8 Oospores of (isolate MP-125) produced in lupin root pieces. 1-Many amphigynous oospores concentrated into an area with vacuolated hyphae (►), 2-Spherical oospore, 3- Elongated oospore, 4 - Tapering oogonium, 5 - Comma shaped antheridium, 6 - Loosely organized oogonial wall, 7 - Paired (Twin) oospores, 8 - Dormant and viable oospore stained with tetrazolium bromide, 9 - Axenically produced oospore of isolate 97-16, 10 - After 10 day on water agar, 11 – Dead (autoclaved oospores stained with tetrazolium bromide, 12 – Live and fluorescing oospores. Bars: 2-7 = 20 µm, 9, 12 = 40 µm. 10 = 10 µm.
6.3 Results

Oospores of *P. cinnamomi* were distinguished within root tissues by their distinct golden brown colour and thick walls (Fig. 6.1-8). They were found concentrated into areas with vacuolated mycelium and collapsed chlamydospores (Fig.6.1.) predominantly in the parenchyma. Perforated or swollen hyphae could be seen close-by which was not evident before burial (Fig.6.1). Both isolates produced oospores axenically. The oospores produced axenically were also golden brown in colour and with thick walls and the dimensions were similar (Fig.6.9).

*P. cinnamomi* was not found within the non-infected lupin roots which were buried in the same pots and the root pieces were intact and not macerated like the infected ones.

6.3.1 In planta oospore production

Both isolates of *P. cinnamomi* produced oospores within lupin root pieces after seven days in the soil and potting mix under *A. pulchella* var. *glaberrima* plants. However, isolate MP-25 produced a greater number of oospores compared to isolate 97-16 (Fig 6.13). No oospores were produced by isolate 97-16 in the lupin root tissues under *A. pulchella* var. *goadbyi*. In contrast, oospore production by isolate MP-125 occurred under all three varieties and in potting mix without plants. The highest numbers of oospore were from MP-125 under *A. pulchella* var. *glaberrima* in potting mix (Fig 6.13). A univariate analysis of variance showed a significant (F = 45.463 df = 1,144 and P < 0.01) difference in oospore production between the two isolates and among the *A. pulchella* varieties (F = 6.586, df = 7,144 and P < 0.01). The interaction between the two main effects was significant (F = 3.981, df = 7,144 and P < 0.01) (Appendix 2).
Tukey multiple comparison test showed significant (P < 0.05) differences of the means between MP-125 in *A. pulchella* var. *glaberrima/potting mix* treatment and all the rest.

Figure 6.13 Mean number of oospores per field of view for A2 isolates; MP 97-16 (A2 Type 1) and MP-125 (A2 Type 2) of *Phytophthora cinnamomi* within lupin roots buried under three varieties of *Acacia pulchella* plants growing in potting mix (□) or jarrah forest soil (■) in the glasshouse. Controls are potting mix or jarrah forest soil without plants. Means are of 5 replicates and bars represent SE of means where large enough to be shown.

Different stages of gametangial development were observed for both isolates. Oospores of *P. cinnamomi* isolate MP 97-16 were mainly in the range of 30 µm to 40 µm and all with smooth thick walls and amphigynous antheridial attachments. In contrast, the oospores produced by isolate MP-125 were numerous. They varied in size from 13 µm to 39 µm with an average of 26 µm (Fig. 6.14) and not all were at the same level of maturity. Among the fully formed oospores with the antheridial attachment, there were smaller ones lacking an antheridium that were markedly aplerotic.
Oospores of *P. cinnamomi* isolate MP 97-16 were mainly spherical, 30 µm to 40 µm in diameter and all had smooth thick (~2 µm) walls and amphigynous antheridia (Fig. 6.9). In contrast, the oospores produced by isolate MP-125 were either spherical (Fig. 6.2) or elongated (Fig. 6.3). Some oogonia had a tapered base (Fig. 6.4). Among the fully formed ones some antheridia were comma-shaped (Fig. 6.5). Some were with slightly to highly ornamented or loosely organised oogonial walls (Figs. 6.6). There were oospores which were markedly aplerotic. Paired oogonia were present infrequently (Fig. 6.7).

### 6.3.2 Oospore viability

All the oospores within root tissues were viable as they stained magenta pink after 48 h incubation in 0.1% solution of tetrazolium bromide (MTT) (Fig. 6.8) while the autoclaved oospores stained black (Fig. 6.11). The live oospores in the root tissues
stained with fluorescein diacetate, fluoresced under a compound microscope with fluorescence illumination at 400 nm (Fig. 6.12), whilst the autoclaved ones did not.

**Oospore germination**

Oospores did not germinate. Separation of oospores from root tissues proved to be a labour intensive task. Only one isolated oospore of MP-125 remained uncontaminated for 30 days. The rest had to be discarded due to contamination. Under the given conditions the single oospore stayed unchanged for 20 days but showed deterioration towards Day 30 (Fig. 6.10).

**Oospore dormancy**

The *in planta* oospores stayed intact during the 60 day incubation period. The antheridia were observed to be diminishing. Ooplasts remained largely unchanged. There were some oospores with contracted or plerotic ooplasts.

### 6.3.3. Soil chemical properties under the three *Acacia pulchella* varieties

Table 6.3 presents the soil chemical properties under the three varieties. High levels of nitrate and ammonium nitrogen, phosphorus, potassium and sulphur contents were found under *A. pulchella* var. *glaberrima* plants compared to that of *A. pulchella* var. *goadbyi* and var. *pulchella*. The ammonium nitrogen under *A. pulchella* var. *glaberrima* plants was found to be the highest at 6 mg/Kg compared to 1 mg/Kg in soil without plants and 2 mg/Kg and 3 mg/Kg for *A. pulchella* var. *goadbyi* and var. *pulchella* respectively. The highest nitrate nitrogen level was also observed in the soil under *A. pulchella* var. *glaberrima* plants. Iron levels were high under all *A. pulchella* varieties.
Table 6.3 Soil chemical properties of the soil under the three Acacia pulchella varieties and oospore production of Phytophthora cinnamomi

<table>
<thead>
<tr>
<th>Soil and species</th>
<th>pH</th>
<th>Cond dS/m</th>
<th>Org. C %</th>
<th>Nitr. N mg/Kg</th>
<th>Amm. N mg/Kg</th>
<th>Sulphur mg/Kg</th>
<th>P mg/Kg</th>
<th>K mg/Kg</th>
<th>Iron mg/Kg</th>
<th>Mean no of oospores per field of view MP 97-16 MP-125</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jarrah forest soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control - no plants</td>
<td>7.2</td>
<td>0.062</td>
<td>2.08</td>
<td>1</td>
<td>1</td>
<td>7.86</td>
<td>1</td>
<td>25</td>
<td>857</td>
<td>0.0</td>
</tr>
<tr>
<td>Ap var. pulchella/Soil</td>
<td>7.7</td>
<td>0.088</td>
<td>2.48</td>
<td>1</td>
<td>3</td>
<td>11.70</td>
<td>1</td>
<td>28</td>
<td>1069</td>
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</tr>
<tr>
<td>Ap var. godbyi/Soil</td>
<td>7.7</td>
<td>0.127</td>
<td>2.90</td>
<td>6</td>
<td>2</td>
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<td>2</td>
<td>29</td>
<td>1039</td>
<td>0.0</td>
</tr>
<tr>
<td>Ap var. glaberrima/Soil</td>
<td>7.6</td>
<td>0.091</td>
<td>2.71</td>
<td>30</td>
<td>6</td>
<td>16.00</td>
<td>12</td>
<td>63</td>
<td>1166</td>
<td>0.9</td>
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<td>Potting mix</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control - no plants</td>
<td>6.9</td>
<td>0.291</td>
<td>4.10</td>
<td>1</td>
<td>2</td>
<td>9.20</td>
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<td>85</td>
<td>516</td>
<td>0.0</td>
</tr>
<tr>
<td>Ap var. pulchella/Pot.mix</td>
<td>5.4</td>
<td>0.113</td>
<td>2.71</td>
<td>1</td>
<td>8</td>
<td>19.00</td>
<td>2</td>
<td>111</td>
<td>1209</td>
<td>0.4</td>
</tr>
<tr>
<td>Ap var. godbyi/Pot.mix</td>
<td>5.5</td>
<td>0.844</td>
<td>6.60</td>
<td>7</td>
<td>6</td>
<td>39.50</td>
<td>5</td>
<td>95</td>
<td>979</td>
<td>0.0</td>
</tr>
<tr>
<td>Ap var. glaberrima/Pot.mix</td>
<td>5.5</td>
<td>1.395</td>
<td>6.81</td>
<td>5</td>
<td>6</td>
<td>53.10</td>
<td>14</td>
<td>105</td>
<td>919</td>
<td>0.8</td>
</tr>
</tbody>
</table>
6.4 Discussion

The experiments confirmed that *P. cinnamomi* oospores, could be consistently produced within root tissues in a jarrah forest soil under controlled conditions. The ability of *P. cinnamomi* to produce oospores *in planta* in a potting mix was also demonstrated. This is the first evidence of *P. cinnamomi* oospores formed *in planta* in an Australian soil and also in the presence of a resistant plant species. The oospores were viable but dormant. Furthermore, a significant variation between the two A2 type isolates, in oospore production was highlighted. The hypothesised plant influence on oospore production was demonstrated for both isolates in soil. For isolate MP 97-16 this effect was clearly expressed with the oospore production occurring in both soil and potting mix strictly under *A. pulchella var. glaberrima* plants.

6.4.1 Selfing

Although the abundance of axenic oospores was less than the *in planta* ones their occurrence *per se* is a proof that they are produced by selfing. The stimulus for selfing *in vitro* could have been initiated by composition of the medium, temperature or the age of the culture. These factors could have influenced selfing individually or in combination. Oospore formation therefore is dependent on the substrate and is more likely to occur in plant tissue. Ribeiro *et al* (1978) also obtained fewer oospores of *P. cinnamomi* in liquid medium than on agar medium.

Previous work has shown that several extrinsic factors can induce selfing of the A2 mating type of *P. cinnamomi*. Stress response, *Trichoderma* effect and plant effect were regarded likely to be the contributing factors towards initiating this phenomenon in the
present study. It may be possible that these factors perform singly or in synergy. Although presence of *Trichoderma* spp. was not tested in this study, the *Trichoderma* effect can not be ruled out in the experiments in soil and potting mix which contained pine bark as *Trichoderma* species are common in the jarrah forest soil (Malajczuk and McComb 1979) and *Trichoderma* volatiles were found on the roots of some *Pinus* species (Johnson and Heather 1982). However, although stress and *Trichoderma* may be involved in stimulating oospore production it is likely that some factor(s) associated with *A. pulchella*, is also important.

6.4.2 Stress response

As previously discussed (Chapter 3), after burial for more than seven days under *Acacia* plants the mycelium within the lupin root pieces became largely deteriorated. Also, the root pieces were highly macerated. Either the diminishing food base or the dying mycelium may have prompted the oospore formation as a stress response. If this was the case it produced significantly different results in the two isolates, with isolate MP 97-16 producing far fewer oospores than MP-125. This finding suggests that the stress response of *P. cinnamomi* varied between the two isozyme types A2 type 1 and 2 in production of the ultimate survival forms. Further investigation is needed with more isolates, which differ in their response towards substrate nutrition availability.

6.4.3 Plant effect

Presence of an organic nitrogen source was a requirement for sexual reproduction of some *Phytophthora* species (Ribeiro 1975). Chang *et al.* (1974) demonstrated that a high concentration of V-8 juice (50 g/L) resulted in abundant oospores of *P. cinnamomi*
in vitro relative to a low one (20 g/L). Selfing also may be dependent on the nutrition levels of the substrate, especially on organic nitrogen or another chemical. The potting mix which contained pine bark stimulated oospores. Pine bark is widely used in nursery mixes. The other commonly used hardwood bark products in nursery industry of Western Australia are marri (C. calophylla) and karri (E. diversicolor) and all have been shown to suppress P. cinnamomi (Sivasithamparam 1981; Sivasithamparam et al. 1981; Hardy and Sivasithamparam 1991). It is a possibility that pine bark or other plant material present in nursery mixes also can provide the stimulus for selfing and oospore formation of P. cinnamomi in Australia and elsewhere.

**Acacia effect**

Soil nutrient levels under the A. pulchella var. glaberrima plants in pots where abundant oospores were formed, were markedly higher than the other two varieties and soil. Presence of an organic nitrogen source was a requirement for sexual reproduction of some Phytophthora species (Ribeiro 1978). Chang et al. (1974) demonstrated that a high concentration of V-8 juice (50 g/L) resulted in more abundant oospores of P. cinnamomi in vitro than a low one (20 g/L). Under A. pulchella var. glaberrima plants in potting mix where highest numbers of oospores were recorded, the highest level of sulphur was recorded. Two volatile sulphur compounds have been identified from the steam distillate of A. pulchella roots and the strong sulphurous aroma of the A. pulchella roots were believed to be associated with them (Whitfield et al. 1981). It is possible that the high levels of sulphur in the soil are a result of A. pulchella root exudates that contain water soluble sulphur compounds (Chapter 4).
6.4.4 Soil chemical properties

The soil chemical properties under the three varieties of *A. pulchella* were directly related to the quantities of oospores produced by the two isolates of *P. cinnamomi*. Especially for isolate MP 97-16, which produced oospores only under the plants of *A. pulchella* var. *glaberrima*. Since the root exudates vary in composition and quantity depending on the species, varieties or the maturity of plants (Vancura and Hanzlikova 1972; Keeling 1974) the root exudates of the three *A. pulchella* varieties can be directly implicated as the agents responsible for differences in soil chemical properties.

The high levels of iron in the soils with *A. pulchella* did not appear to be correlated with the production of oospores as all varieties had high iron, and the highest levels were not associated with the highest numbers of oospores.

6.4.5 Variability among isolates

The two A2 type isolates performed significantly differently in *in planta* oospore production. This finding is in agreement with the results of the *in vitro* study of Chang *et al.* (1974), where oospore production proved to be an innate characteristic of isolates.

Results of the present study indicted that isolate MP-125 was more readily selfed than MP 97-16 under the controlled conditions. For MP 97-16 similar stimulus provided by any of the above factors or their combined effect, was insufficient for habitual selfing and abundant oospore formation. There may be other requirements of this particular isolate to initiate selfing more regularly. Some knowledge can be acquired from the *in vitro* studies, which showed that temperature, light, oxygen tension and composition of the medium influenced sexual reproduction in *Phytophthora* (Elliot 1983).
The other important factor to be considered is the difference in their isozyme types. MP 97-16 belongs to the isozyme type 1 where as MP-125 is type 2 (Dobrowolski et al. 2006). It can be hypothesised that isozyme type 2 isolates of A2 type of *P. cinnamomi* may be more readily selfed than the type 1. More research is needed to test this hypothesis. Similarly MP- 25 is resistant to phosphite (Wilkinson et al. 2001) and this correlation should be further tested.

### 6.4.6 Variability of oospores

The diameters of the *in planta* oospores produced by islolate MP-125 ranged from 13 µm to 39 µm, which is consistent for this species (Stamps et al. 1990). However, the measurements and descriptions provided in the key of Stamps et al. (1990) were from the oospores produced by mating two compatible types on agar. There is no existing measurements for the selfed oospores for *P. cinnamomi* or for oospores of *P. cinnamomi* produced *in planta*. Oospores of isolate MP 97-16 were all amphigynous and within the same size range. The variability in oospore sizes and the gametangial development observed for isolate MP-125 within root tissue is suggestive of selfing and oospore formation occurring not at the same time but at various times during the seven days of soil incubation. For isolate MP 97-16 the uniformity of the oospore sizes suggests that selfing might have occurred at the beginning of soil incubation and the resulting oospores reached their maximum diameter by the seventh day. On the other hand, oospore size might have nothing to do with timing of their production, they might just be inherently smaller or bigger than the normal. Absence of various stages of gametangial development indicate that for isolate MP 97-16 continued oospore production did not occur.
For the two A2 type isolates tested in the present study, the most common oogonial-antheridial association *in planta* was amphigynous. Some authors associated paragyny with selfing and amphigyny with heterothallism (Savage *et al.* 1968). The present study demonstrated that *P. cinnamomi* is an exception to this rule. A mix of gametangial associations has been reported from *in vitro* studies of compatible mating types (Hüberli 2000) and such studies cannot exclude the possibility of selfing alongside heterothallism.

**6.4.7 Niche effect of oospore formation**

It was observed that the well-developed oospores were concentrated in pockets in the parenchymal tissues of the lupin roots. These niches can be explained by either enhanced stimulus in these areas or the few potent hyphae being concentrated into the last available nutrient bases.

**6.4.8 Viability of oospores**

Staining with tetrazolium bromide (MTT) or fluorescein diacetate indicated that oospores were viable but germination was not observed. The corresponding staining colour of viable and dead *P. cinnamomi* oospores was established in the present study. This observation was consistent with the viable and dead oospores of *P. megasperma* f. sp. *glycinea* (Sutherland and Cohen 1983) and *P. citricola* (Bunny 1996), which were magenta and black respectively.

A workable technique of dislodging oospores without damage from root tissues and transferring them onto agar medium has been devised in this study. However, due to contamination problems associated with non-sterile roots, a quantifiable number was
not accomplished. It was not within the scope of this study to refine the method, thus the following recommendations are offered. Surface sterilising with a weak hypochlorite solution of the separated oospores prior to transferring onto agar medium or incubating the oospores in a medium amended with antibiotics such as NARPH selective medium for several days followed by their transfer onto an agar medium. From the point of view of their tolerance to adverse conditions (Ribeiro 1983) the oospores should be able to withstand the above process.

Oospores of oomycetes are, in general endogenously dormant and capable of long-term survival (Ribeiro 1983). According to Mircetich and Zentmyer (1966) oospores of _P. cinnamomi_ can survive up to six years. There is no information in literature on survival and infectivity of _P. cinnamomi_ oospores produced _in planta_. Difficulties encountered in germinating the oospores in the present study held back further investigations into this area. However, findings of this study confirmed that the oospores of both isolates of _P. cinnamomi_ were dormant.

**Conclusion**

This is the first evidence of _P. cinnamomi_ oospores formed _in planta_ in an Australian soil, a potting mix and also in the presence of a resistant plant species, under controlled conditions. This study also demonstrated that the two A2 isolates can self _in vitro_ for the first time. It was not within the scope of this study to identify all the factors contributing to _in planta_ oospore production of _P. cinnamomi_. However, the factors discussed above are the most plausible and Chapter 8 examines the role of sulphur and temperature. Although the controlled conditions of the glasshouse are non-representative of nature it cannot be ruled out that these conditions are encountered in
natural forest situations. Furthermore, other *P. cinnamomi* resistant plant species or other legume species where substantial amounts of sulphur or ammonium, nitrate nitrogen exist, might also have conducive conditions for oospore production of *P. cinnamomi*. Root extract of avocado which stimulated oospore production reduced sporangial production in *P. cinnamomi* (Zentmyer 1979). Similarly leachates from soils of the potted *A. pulchella* that stimulated oospore production have been shown to reduce sporangial production, and cause collapse of chlamydospores of *P. cinnamomi* (Chapter 2 and 3). Hence, Zentmyer’s (1979) suggestion of higher plants providing the stimulus for the pathogen to produce oospores as a defence mechanism is supported in this study with *A. pulchella*.

Absence of sexual reproduction of *P. cinnamomi* in Australian forest soils (Old et al. 1984a; 1988; Dobrowolski 1990) does not totally discount the fact that oospores can be formed in Australian soils in the presence of *P. cinnamomi* resistant plant species such as *A. pulchella*. Although the infectivity of *P. cinnamomi* oospores produced *in planta* was not ascertained, it is highly possible that they can function as long term survival structures.
CHAPTER 7

Determination of the ability of the three clonal lineages of 

*Phytophthora cinnamomi* present in Australia to produce oospores *in planta* in several jarrah forest soils

7.1 Introduction

Selfing of *P. cinnamomi* in the presence of *A. pulchella* exudates was demonstrated for the first time (Chapter 6) with amphigynous antheridia as the most common oogonial-antheridial association *in planta*. It was shown that the two A2 type isolates of *P. cinnamomi* varied significantly in selfing and producing oospores *in planta*. The A2 type 2 isolate, MP-125 repeatedly produced abundant oospores and was identified as a habitual oospore producer. It was also shown that both A2 type isolates had the potential to self and produce oospores in nutrient broth in small numbers (Chapter 6).

*P. cinnamomi* populations in Australia, consist of three isozyme genotypes with low genetic variation, one A1 type and two A2 types (Old *et al.* 1984a, 1988). These genotypes represent the three clonal lineages of *P. cinnamomi* (Dobrowolski 1999; Dobrowolski *et al.* 2003). In Australia, the most commonly isolated one is the A2 type 1 (Old *et al.* 1988a; Dobrowolski 1999; Dobrowolski *et al.* 2003).

A2 type 2 isozyme genotype of *P. cinnamomi* is less frequently isolated in Australia and there is little information on its behaviour available. There is a need to examine this genotype more, since one isolate of this clonal lineage from the jarrah forest was shown to produce abundant selfed oospores *in planta* (Chapter 6). It was also shown to be less inhibited than the A2 type 1 isolates by *A. pulchella* root exudates (Chapter 4). In
addition, this isolate has shown resistance to phosphite treatment (Wilkinson et al. 2001).

Although the A1 clonal lineage of *P. cinnamomi* has been acknowledged universally as being difficult to induce oospores, there is evidence that it can self *in vitro* (Linde et al. 2001). Occurrence of this clonal lineage in Australian forests is rare. However, given the ability of the A2 type in general to behave in homothallic manner under stimulation, it can be hypothesised that isolates of both types may possess the ability to self and produce oospores *in planta* in soil. It was also hypothesised that the stimulus for selfing and oospore production of the A2 isolates of *P. cinnamomi* is higher under *A. pulchella* plants.

Experiments in this chapter were conducted to test these hypotheses with two isolates of each clonal lineage. Isolate MP-125 of A2 type 2, was included since it was identified as a habitual selfer (Chapter 6).

### 7.2 Materials and methods

#### 7.2.1 Isolates

All the isolates (Table 7.1) were passaged through lupin roots, cleaned by growing on NARPH selective medium for three to four days and subcultured on V8 juice medium. The clean cultures were maintained at 25°C in the dark with regular sub-culturing.
Table 7.1 Details of the isolates of *P. cinnamomi* used in this study

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isozyme type</th>
<th>DAWA accession no</th>
<th>Host</th>
<th>Location</th>
<th>Collector and Year isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 15</td>
<td>A1</td>
<td>12873</td>
<td><em>E. marginata</em></td>
<td>Kelmscot W.A.</td>
<td>K.Old (CSIRO) No date</td>
</tr>
<tr>
<td>DP55</td>
<td>A1</td>
<td>12874</td>
<td><em>Banksia baxterii</em></td>
<td>Fitzgerald River National Park, W.A.</td>
<td>CALM W.A. No date</td>
</tr>
<tr>
<td>MP80</td>
<td>A2 type 1</td>
<td>12875</td>
<td><em>E. marginata</em></td>
<td>Jarrahdale W.A.</td>
<td>G. Hardy 1993</td>
</tr>
<tr>
<td>MP62</td>
<td>A2 type 1</td>
<td>12876</td>
<td><em>E. marginata</em></td>
<td>Jarrahdale W.A.</td>
<td>G. Hardy 1993</td>
</tr>
<tr>
<td>MP125</td>
<td>A2 type 2</td>
<td>12878</td>
<td><em>E. marginata</em></td>
<td>Jarrahdale W.A.</td>
<td>G. Hardy 1993</td>
</tr>
<tr>
<td>A26</td>
<td>A2 type 2</td>
<td>12879</td>
<td><em>C. cunninghamiana</em></td>
<td>Barton ACT</td>
<td>K.Old (CSIRO) No date</td>
</tr>
</tbody>
</table>

DAWA Department of Agriculture Western Australia. CALM Conservation and Land Management, W.A., CSIRO Commonwealth Scientific & Industrial Research Organisation., ACT Australian Capital Territory.

7.2.2 Soil

Soils were collected from provenances of the three varieties of *A. pulchella* growing in jarrah forest of the South-west of Western Australia (Fig. 1.2). The site descriptions and locations were given in Table 2.1. Soil from Mt. Barker was collected in spring (September) while the others were collected in summer (December). Soil was dug to a depth of 15 cm under the *A. pulchella* stands and from approximately 5 m from the nearest *Acacia*, transported in polythene bags and stored at 4°C. Chemical properties
were analysed (CSBP Ltd, Bibra Lake, Western Australia) and the soils were screened to ascertain that they were free of *P. cinnamomi* as done in Chapter 2. Using a sieve with 4-mm holes, rocks, gravel and plant material were removed from the soil and samples of ~250 g were weighed into 1000 mL polypropylene containers with drilled drainage hole (Plastic product of Bonson Industries Co. Ltd.).

### 7.2.3 Inoculum

The inoculum was prepared by infecting lupin radicles with *P. cinnamomi* as done in Chapters 2 and 3. Two inoculum sachets of each isolate were buried in the containers with each soil type and replicated five times. Soil was watered to the container capacity and kept in a growth cabinet in the dark with the temperature controlled at 22 ± 1°C for seven days. The moisture levels were calculated from the difference of wet and dry weights, which were in the range of 15 - 20%.

After seven days the inoculum was retrieved, washed first with 0.25% NaOCl for 1 min and then in three washes with distilled water. The root pieces were mounted on microscope slides in a drop of distilled water. Using two needles the root pieces were spread into a thin layer and examined at 100 x magnification under 10 fields of view as discussed in Chapter 6 for the presence of oospores.

### 7.2.4 In vitro mating of A1 and A2 type isolates

Mating of the two types were carried out according to Chang *et al.* (1974) with modifications. Disks of 6-mm-diameter were cut out from colonies of the A1 isolates (DP 55 and A 15) and A2 isolates (MP-80, MP-62, MP-125 and A-26) grown on ½ PDA. One disk of each A2 type isolate was placed 3 cm apart from a disk of the A1
type on V8 juice agar medium and incubated at 25°C ± 1°C in the dark. Plates were microscopically observed after 96 h every second day for three-four weeks. Further observations were done weekly for four more weeks.

7.2.5 Statistical analysis

The experiment was repeated once and the data from one experiment presented. A univariate analysis of variance between subjects was performed using SPSS 14.1 for Windows (SPSS Inc., USA). on the oospore production. The ANOVA assumptions of normality were screened by subjecting residuals to standard tests; normal Q-Q and detrended normal Q-Q plots and no evidence of violation was found. All significant main effects and interactions were compared using Tukey multiple comparison test between means. A linear regression analyses were conducted to test the interaction of soils sulphur levels and the oospore production.

7.3 Results

7.3.1 Variation between isolates

Only the A2 type 2 isolates produced oospores. No oospores were discovered in roots infected with A2 type1 or A1 isolates (Table 7.2). The two A2 type 2 isolates, MP-125 and A-26 produced oospores in planta in the soils collected from under all three varieties of A. pulchella and in soils from some distance from A. pulchella var. goadbyi after seven days (Figs. 7.1 and 7.2).
Table 7.2 *In planta* oospore production in the three clonal lineages of *Phytophthora cinnamomi* after 7 days in the jarrah forest soils under the three varieties of *Acacia pulchella*. Controls were the soils collected 5 m away from the *A. pulchella* plants. Oospores present (✓) and oospores absent (x).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Clonal lineage</th>
<th><em>A. pulchella</em> var. goadbyi soil</th>
<th><em>A. pulchella</em> var. glaberrima soil</th>
<th><em>A. pulchella</em> var. pulchella soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Under</td>
<td>Away</td>
<td>Under</td>
</tr>
<tr>
<td>A 15</td>
<td>A1</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>DP 55</td>
<td>A1</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>MP-80</td>
<td>A2 type 1</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>MP-62</td>
<td>A2 type 1</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>MP 125</td>
<td>A2 type 2</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>A 26</td>
<td>A2 type 2</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Figure 7.1 - 2 *In planta* oospores of the A2 type 2 isolates of *Phytophthora cinnamomi*. 1 – Oospores in the parenchymal tissues within lupin root (►). 1 – Several oospores concentrated into an area, 2 – Amphigynous antheridium (◄).
MP 125 and A-26 operated similarly in the soils collected from under all three varieties of *A. pulchella*. A univariate ANOVA between subjects failed to find a significant (*F* = 3.09, df = 1,48 *P* > 0.05) difference between the two A2 type 2 isolates in oospore production. The test found a significant (*F* = 6.246, df = 5,48 and *P* < 0.01) difference between the soils (Appendix 2). The highest numbers were recorded from soils from *A. pulchella* var. *goadbyi*, and some oospores were also observed in soil collected 5 m from the nearest *A. pulchella* var. *goadbyi*. Tukey multiple comparison test based on the means showed a significant (*P* < 0.05) difference between the soils under *A. pulchella* varieties *goadbyi* and *pulchella* for *in planta* oospore production for both isolates.

The oospores produced by both A2 type 2 isolates within root tissues were similar and had amphigynous antheridial attachments (Fig. 7.1). Both isolates produced the highest numbers of oospores in lupin root tissues which were buried in soil collected from under *A. pulchella* var. *goadbyi* (Fig.7.2). A clear association can be seen between the soil sulphur levels and oospore formation (Table 7.3) for both isolates.

Small spores with thick walls (2 µm) and diameters in the range of 7-10 µ were present in all the treatments. Since no antheridial or hyphal attachments were present, it was decided not to include them in the selfed oospore category.
Figure 7.2 Mean number of oospores per field of view for A2 type 2 isolates of Phytophthora cinnamomi; MP-125 □ and A-26 ■ produced within lupin roots after 7 days in the jarrah forest soils from under the three varieties of Acacia pulchella var. goadbyi (Ap goad), var. pulchella (Ap pulc) and var. glaberrima (Ap glab). Controls were the soil 5 m away from the A. pulchella plants. Means are of five replicates and bars represent the standard error of the means. Isolates of A1 and A2 type 1 strains produced no oospores so data are not shown.

7.3.2 Soil chemical properties

The soil chemical properties showed higher sulphur levels under the A. pulchella plants compared to the control soils with no A. pulchella plants (Table 7.3). However, the differences between sulphur levels in soil under A. pulchella var. goadbyi plants and ~ 5 m away from them were less evident. The organic nutrient levels of these two soils were also similar with ammonium nitrogen contents and organic carbon levels being close.
7.3.3 Relationship between the *in planta* oospore production and soil sulphur levels.

![Graph showing the effect of soil sulphur levels on oospore production of the two isolates of *Phytophthora cinnamomi* MP-125 (■) and A-26 (♦). Y axis = Mean number of oospores per field of view (0.02 cm²), X axis = the amount of sulphur present in the soils.]

There was a clear correlation between the soil sulphur levels and the production of selfed oospores *in planta*, for both A2 type 2 isolates of *P. cinnamomi* (Fig 7.3) at $R^2 = 0.81$ and $P = 0.014$. The higher the sulphur levels in the soil the greater the abundance of oospores in both isolates.
Table 7.3 Comparison of the chemical properties of jarrah forest soils with and without *A. pulchella* in which the two A2 type
2 isolates of *P. cinnamomi*, MP-125 and A-26 produced oospores *in planta* within seven days.

<table>
<thead>
<tr>
<th>Soil</th>
<th>pH</th>
<th>Cond dS/m</th>
<th>Org. C %</th>
<th>Nitr. N mg/Kg</th>
<th>Amm. N mg/Kg</th>
<th>Sulphur mg/Kg</th>
<th>P mg/Kg</th>
<th>K mg/Kg</th>
<th>Iron mg/Kg</th>
<th>Oospores per field of view (0.02 cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A26</td>
</tr>
<tr>
<td>Under Ap</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>goad</td>
<td>5.2</td>
<td>0.088</td>
<td>3.99</td>
<td>1.00</td>
<td>5.00</td>
<td>8.7</td>
<td>50.0</td>
<td>67</td>
<td>367</td>
<td>0.66</td>
</tr>
<tr>
<td>Control</td>
<td>6.1</td>
<td>0.070</td>
<td>5.56</td>
<td>1.00</td>
<td>4.00</td>
<td>8.0</td>
<td>4.0</td>
<td>210</td>
<td>3177</td>
<td>0.20</td>
</tr>
<tr>
<td>Under Ap</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>glab</td>
<td>6.1</td>
<td>0.055</td>
<td>4.96</td>
<td>1.00</td>
<td>14.00</td>
<td>6.3</td>
<td>12.0</td>
<td>174</td>
<td>1346</td>
<td>0.20</td>
</tr>
<tr>
<td>Control</td>
<td>6.0</td>
<td>0.070</td>
<td>5.36</td>
<td>1.00</td>
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<tr>
<td>pulch</td>
<td>5.6</td>
<td>0.078</td>
<td>2.95</td>
<td>1.00</td>
<td>5.00</td>
<td>7.4</td>
<td>5.0</td>
<td>58</td>
<td>330</td>
<td>0.38</td>
</tr>
<tr>
<td>Control</td>
<td>6.2</td>
<td>0.040</td>
<td>3.98</td>
<td>1.00</td>
<td>6.00</td>
<td>5.4</td>
<td>8.0</td>
<td>75</td>
<td>1268</td>
<td>0.00</td>
</tr>
</tbody>
</table>
7.3.4 In vitro mating of A1 and A2 type isolates

Oospores were obtained in only one pairing of A1 and A2 strains (Table 7.4). After four weeks only the A-15 A-26 pairing on agar yielded oospores. All the oospores were fully formed with amphigynous antheridial attachments. The oospores were concentrated into an area (2 cm-diameter) towards the edge of the Petri dish, at the junction of two colonies on the A-26 side. None of the other pairings yielded oospores on agar in this time or later.

Table 7.4 Pairings of A1 and A2 types of *P. cinnamomi* on V8 juice agar. Oospores present ✔, oospores absent ✗.

<table>
<thead>
<tr>
<th>A1/A2</th>
<th>A-26 (A2 type 2)</th>
<th>MP-125 (A2 type 2)</th>
<th>MP-80 (A2 type 1)</th>
<th>MP-62 (A2 type 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-15 (A1)</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>DP-55(A1)</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>

7.4 Discussion

This study identified for the first time the variations between the three clonal lineages of *P. cinnamomi* found in Australia in producing selfed oospores in soil. The A2 type 2 clonal lineage produced selfed oospores in soils under *A. pulchella* plants. The findings of this study proved the hypothesis that the stimulus for *P. cinnamomi* to produce selfed oospores was higher in the soil under *A. pulchella* plants. The two A2 type 2 isolates operated similarly and all the oogonial antheridial associations were amphigynous. Oospore production was clearly associated with the chemical properties of the soil. Increased oospore production was associated with the soils with higher levels of sulphur and organic nutrients. These soils were collected mainly from under the three varieties
of *A. pulchella* with the exception of the soil away from the *A. pulchella* var. *goadbyi* plants. The similar nutrient levels and no marked difference of the sulphur levels in this soil could be explained by the fact that the water soluble compounds may have been dispersed more easily in the soil from under the *A. pulchella* plants in the spring when the soils were collected.

The hypothesis that A1 isolates may also have the potential to self and form oospores within root tissue in the soil under the same conditions was disproved by the absence of oospores after seven days. The available stimulus also failed to induce selfing and oospore production in the two A2 type 1 isolates used for this study. These isolates may require other mechanisms to produce selfed oospores. It can also be hypothesised that under the given stimulus these isolates may take longer than seven days to produce selfed oospores. However, with fast decomposition occurring in the roots buried in soil it was difficult to extend the period beyond seven days in this study.

7.4.1 *In vitro mating*

Oospores were obtained in only one pairing of A1 and A2 strains and in a small area at the junction. Brasier (1972) suggested that, the compatibility system probably is a chemical mechanism, which initiates sexual differentiation within or between isolates. None of the other tested matings proved productive and it could be hypothesised that they were either incompatible or the stimulus was not strong enough. Although the oospores were detected on the A-26 side, there is no indication that the oospores were hybrids or selves. As there was a clear zone between the two colonies and the A2 type 2 isolate (A-26) can produce selfed oospores *in planta* it is possible these oospores were selfed. A molecular study using random amplified polymorphic DNA (RAPD) primers...
as done by Linde et al. (2001) is needed to establish this fact. The other A2 type 2 isolate, M-125 which was identified as a high potential selfer in planta in Chapter 6 and this study did not yield any oospores under the pairing circumstances. It can be hypothesised that the stimulation provided in the pairing may not have been sufficient for this isolate to produce selfed oospores.

7.4.2 Variation among isolates

This study for the first time highlighted variations in the ability of isolates belonging to the A2 clonal lineage of P. cinnamomi found in Australian soils to produce selfed oospores in planta or in stimulating the opposite type on agar. These variations may also be explained by genotypic variations that have possibly arisen through mitotic crossing over as proposed by Dobrowolski (1999) to explain the phenotypic traits among P. cinnamomi isolates of the same clonal lineage.

Phenotypic variations among the isolates from different populations of P. cinnamomi have been recorded. Hüberli (2000) observed differences in colony morphology and growth rate on agar among isolates of A2 type 1 populations from the south-west of Western Australia. Differences were also expressed in pathogenicity which was determined by lesion formation on detached branches of jarrah and marri and deaths of inoculated jarrah plants in glasshouse trials (Hüberli 2000). Dudzinski (1995) also found variations in pathogenicity among isolates of the three clonal lineages. Differences have also been recorded among isolates of the A2 clonal lineage in response to in vitro phosphite treatment. Both MP-125 and A-26 appear relatively resistant to phosphite at 50 µg, although Wilkinson et al. (2001) only commented that MP-125 was an isolate tolerant to phosphite. Further isolates differing in response
to phosphite should be tested for ability to produce oospores, and the responses of the oospores to phosphite.

There are neuter or sterile isolates for *Phytopthora* spp. including *P. cinnamomi* (Shepherd 1978; Shepherd and Cunningham 1978) which can not be induced to produce oospores either by mating or other stimulation. Thus the inability for mating and sexual reproduction on agar or selfing under the given conditions for the two A2 type 1 isolates or the A1 type isolate, DP-55 used in this study may have been due to their sterility.

7.4.3 Effect of *Acacia pulchella* plants on selfing

The A2 type 2 isolate, MP-125 produced higher numbers of selfed oospores within lupin root tissue when buried for the same time period in the soil under the living *A. pulchella* plants, especially under *A. pulchella* var. *glaberrima* plants (Chapter 6) than it did in soils collected from beneath field grown *A. pulchella* plants. Although the increased level of sulphur found under those glasshouse grown *A. pulchella* var. *glaberrima* plants (Table 6.2) in containers was not observed under the *A. pulchella* var. *glaberrima* in the native vegetation (Table 7.3), the available amounts were still sufficient to stimulate selfing in both isolates in varying levels.

Conclusions and Implications

This study confirmed the finding of Chapter 6, that *P. cinnamomi* is able to produce selfed oospores in soils under *A. pulchella*. This ability to produce selfed oospores has been clearly demonstrated for the A2 type 2 of *P. cinnamomi* while it was not seen much in the widely occurring A2 type 1 and absent in A1 isolates. The association between phosphite tolerance and the ability to produce selfed oospores was highlighted for the first time in this study but more isolates need to be tested.
The relationship which exists between the potential to produce more oospores and higher stimulus may be present in only the A2 type of *P. cinnamomi* in general and expressed more vigorously in the isozyme type 2 of the A2 clonal lineage. Since the occurrence of this type in Australian forests is less frequent than the A2 type 1 the chances of oospores being formed in soil may not be very frequent.
CHAPTER 8

Effect of elemental sulphur and influence of the soil temperature on in planta selfing and oospore formation of Phytophthora cinnamomi

8.1 Introduction

In Chapters 6 and 7, it was established that the sulphur levels in the soils appear to have a direct relationship to selfing and oospore production of the A2 type of P. cinnamomi. Significant variations between two A2 type isolates in producing oospores in planta were observed with the A2 type 2 isolate MP-125 selfing more readily than the A2 type 1 MP 97-16 under the controlled conditions in Chapter 6. For MP 97-16, similar stimulus provided by any of the factors individually or combined was insufficient for consistent selfing and abundant oospore formation. Chapter 7 revealed that both A2 type 2 isolates readily produced selfed oospores. The results in Chapter 6 also showed that oospore production by the two isolates of P. cinnamomi is associated with soil chemical properties under the three varieties of A. pulchella. Especially isolate MP 97-16, which produced oospores only in the soil under the plants of A. pulchella var. glaberrima. Under A. pulchella var. glaberrima plants, sulphur content in the soil was the highest among the three A. pulchella varieties. The soluble sulphur in the leachates from aseptic plants of A. pulchella var. goadbyi was higher than the other varieties or the control (Chapter 4). Sulphur exuded by the roots of A. pulchella plants may have stimulated selfing and oospore production of P. cinnamomi.

The temperatures under which in planta oospore formation was observed in the soil under potted A. pulchella in Chapter 6 and the field soils in containers in Chapter 7
were 25°C and 22°C ± 1°C, respectively. Temperature plays an important role for *in vitro* oospore formation of *P. cinnamomi* (Zentmyer et al. 1979). However, an optimum temperature for *in planta* oospore formation has never been recorded before. During the months from August to November when abundant *in planta* oospores were observed in potted *A. pulchella* var. *glaberrima* in a potting mix, the glasshouse temperatures fluctuated between 20°C – 25°C while during the summer months of January – February it rose to between 28°C – 36°C. This temperature fluctuation could be present in jarrah forest environment under natural conditions at certain periods. Since there is no information available for *in planta* selfing and oospore formation it was important to know whether an increase in soil temperature would affect oospore formation.

Experiment 1 was designed to test the hypothesis that presence of elemental sulphur in the substrate is required as the stimulus for selfing and oospore production of *P. cinnamomi* while Experiment 2 observed the influence of increased soil temperature on *in planta* oospore formation.

**8.2 Materials and methods**

**8.2.1. Experimental design**

*Experiment 1 – In planta selfing and oospore formation of A2 type 2 isolates of P. cinnamomi in sand amended with sulphur*

The experiment consisted of two isolates of *P. cinnamomi* and five treatments per isolate. Each treatment was replicated three times. Independent variables were the two isolates and different quantities of elemental sulphur added to the substrate. The control was un-amended sand. The dependant variable was the presence/absence of
oospores and the number of oospores produced per field of view in 1 cm lupin root segments buried in the sand for seven days.

**Experiment 2 – Influence of increased temperatures on in planta selfing and oospore formation of *P. cinnamomi***

Independent variables were the six jarrah forest soils infected with isolate MP-125 of *P. cinnamomi*. Each treatment was replicated five times. The containers were arranged on the bench in a randomised block design. The dependant variable was presence/absence of oospores and the number of oospores as above.

Both experiments 1 and 2 were repeated once.

**8.2.2. Experiment 1**

Polypropylene containers (1000 mL, Plastic product of Bonson Industries Co. Ltd.) were filled with 500 g coarse river sand moistened and autoclaved at 121°C for 20 min. The containers with the sand were weighed and the wet weight was recorded. Elemental sulphur was added to sterile sand, chemical properties of which were determined as in Chapter 6 (6.2.1) prior to addition of sulphur in measured quantities (Table 8.1). The sulphur was thoroughly mixed in the sand. Lupin roots infected with two isolates of *P. cinnamomi* (MP 97-16 and MP-125) were enclosed separately in nylon sachets (Section 6.2.2) and buried in the sand. The containers were covered with aluminium foil and incubated in a growth cabinet at 25°C for seven days. After the inoculum was removed the containers with sand were oven dried at 40°C for 24 h and weighed. The moisture levels were calculated from the difference of wet and dry weights, and were in the range of 15 - 20%.
8.2.3. Experiment 2

Lupin roots infected with isolate MP-125 were buried in jarrah forest soils as done in Chapter 7. The containers were watered to the container capacity and lids with holes drilled for aeration were loosely placed. The containers were arranged in a randomised block design on the bench in the glasshouse with no temperature control (Glasshouse 1). The daily maximum and minimum temperatures were recorded using a maximum-minimum thermometer (Brannan, England), which had been placed on the glasshouse bench next to the containers during the seven days. A second set of containers with the same soils and infected lupin roots were placed in the glasshouse with temperature controlled at 24 ± 1 °C (Glasshouse 2) and the moisture levels were maintained at ~ 20%.

The inocula in both experiments were retrieved after seven days. Root pieces from experiment 1 were gently rinsed repeatedly in three washes of distilled water whilst the ones from experiment 2 were surface sterilised as done in Chapter 6 and 7. Each 1 cm root segment was mounted on microscope slides in a drop of distilled water. Using two needles the root pieces were spread into a thin layer and examined under x100 magnification. Presence/absence and the numbers of oospores per field of view were recorded under 10 fields of view.

8.3 Results

8.3.1. Experiment 1

Isolate MP-125 produced oospores in planta after seven days in sterilized sand amended with sulphur at 10 – 20 mg/Kg (Table 8.1). There were approximately five oospores per field of view (0.02 cm²). No oospores were observed in the control with
sand un-amended with sulphur or with 5 mg/kg of sulphur added to the sand. The extractable sulphur content in the sterilised sand as measured by ICP (CSBP LIMITED) was 1 mg/kg. The stimulus was provided by higher doses starting at 10 mg/kg. However, further increase in the concentration did not affect the frequency of oospore formed within lupin root pieces for this isolate.

Sulphur levels used in this experiment did not induce isolate MP 97-16 to self and produce oospores. However, presence of sulphur appeared to have caused collapse of chlamydospore and vacuolation of the mycelium of this isolate as well as isolate MP-125 (Fig. 8.1).
Table 8.1 Effect of sulphur on oospore production in two isolates of *P. cinnamomi*. (+) oospores present, (-) oospores absent.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Concentration of Sulphur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/kg</td>
</tr>
<tr>
<td>MP-125</td>
<td>-</td>
</tr>
<tr>
<td>MP 97-16</td>
<td>-</td>
</tr>
</tbody>
</table>

8.3.2. Experiment 2

The daily maximum – minimum temperatures in the glasshouse with no climate control, ranged from 26.7°C and 28.5°C to 30.7°C and 36.8°C (Fig 8.2) during the two week experimental period. The average maximum – minimum temperatures were 33.8°C – 27.6°C. No oospores were observed in any of the lupin roots buried in any of the soils tested in the glasshouse without climate control. In contrast, oospores were observed in all the lupin roots buried in the four oospore inducing soils (Table 7.2) after seven days in the evaporatively cooled glasshouse where temperature was maintained at 24 ± 1°C.
Fig 8.2 Temperatures in the non climate controlled glasshouse temperatures during the two week period of experiment measured with a maximum-minimum thermometer. Maximum temperatures ♦, minimum temperatures ■ and average temperatures ▲.

8.4 Discussion

8.4.1 Effect of sulphur

Sulphur in its elemental form provided the stimulus for selfing in isolate MP-125 of *P. cinnamomi*. This was shown by the absence of oospores within root tissues in the control not amended with elemental sulphur opposed to their presence in the sulphur treated sand. The stimulus appeared to be dependent on the sulphur concentration in the substrate. For isolate MP-125, selfing and oospore formation was initiated by the stimulus provided by 10 mg/kg elemental sulphur in the substrate. Further increases in sulphur did not have an effect on the frequency of oospores.

For isolate MP 97-16, the stimulus provided by the given sulphur levels may not have been sufficient to initiate oospore formation. In Chapter 6, the variation between the two A2 type isolates was discussed, in oospore production. The A2 type 2 isolate more
readily selfed than the A2 type 1 isolate. It was interesting however, to observe
collapsed chlamydospores and vacuolation of the mycelium within root tissue with
higher levels of sulphur in both isolates.

Presence of sulphur in planta, occurs in the form of sulphates, sulphides, thiosulphates
and elemental sulphur (Cooper and Williams 2004). Sulphur is present in most
phytoalexins and plays an important role in plant defence against invading fungi. Most
plant families produce organic phytoalexins of diverse chemistry as a defence
mechanism against invading pathogens and elemental sulphur is the only inorganic
phytoalexin and the only phytoalexin produced by so many different taxa (Cooper and
Williams 2004). The families, which have been identified by Williams and Cooper
(2003) to contain elemental sulphur in planta are Sterculiaceae, Solanaceae,
Malvaceae and Leguminosae in response to xylem invading fungi such as Verticillium
dahliae or Fusarium oxysporum. Cooper and Williams (2004) also found that the
production of elemental sulphur in planta was more rapid and intensive in disease-
resistant genotypes. These plant families are encountered in most Australian forest and
sandplain ecosystems and there is a possibility the soil under some or most of the
species would have increased sulphur levels.

Elemental sulphur was effectively used to control root rot caused by P. cinnamomi
(Pegg 1977) who attributed this to the pH level and low sporangial production.
Evidence of the present study showed that elemental sulphur causes P. cinnamomi
reduction by causing chlamydospore collapse and can provide the stimulus for
oospores which can stay dormant (Chapter 6) for prolonged periods (Zentmyer and
Mircetich 1966).
8.4.2. Influence of temperature

Increased temperatures were disadvantageous for selfing and oospores production of *P. cinnamomi*. Once temperatures reached 28 °C, oospore ceased to be induced in the A2 type 2 isolate, MP-125 of *P. cinnamomi*. This isolate produced abundant oospores at 24 ± 1 °C and also at 22 ± 1°C (Chapter 7). Reeves and Jackson (1972) observed oospores in *Castanea sativa* roots buried in soil for six to eight days at 22 ± 1°C. It thus seems the optimum temperature range for *in planta* selfing and oospore formation for *P. cinnamomi* in soil would include 21 – 25°C. According to Zentmyer et al. (1979) optimum temperature range for *in vitro* oospore production for *P. cinnamomi* was 15 – 24°C. Since there is a minimum, optimum and maximum temperatures for each species in producing reproductive organs, 15°C could be the minimum temperature for *in planta* selfing and oospore formation of *P. cinnamomi*. Further studies will confirm this hypothesis.

Conclusions

Among other possible mechanisms, sulphur and temperature proved to have a significant role in producing selfed oospores of the A2 type 2 of *P. cinnamomi*. Sulphur in its elemental form has been detected in xylem of several plant taxa (Williams and Cooper 2003) and it is possible that these species may have increased sulphur levels in the soil and may stimulate selfing and oospore formation by *P. cinnamomi*. Further investigation is required to determine the capacity of other Australian plant species resistant to *P. cinnamomi* in stimulating oospore production. Furthermore, the widely used chemical control methods against *P. cinnamomi* may have similar implications e.g. chloroneb (2, 5-dichloro-1, 4-dimethoxybenzene)
stimulated oospore production of *P. capsici*, *in vitro* (Noon and Hickman 1974). Therefore, any chemical use against *P. cinnamomi* should be thoroughly investigated prior to implementation.
CHAPTER 9

General Discussion

This thesis examined several aspects of the *Phytophthora cinnamomi* and *Acacia pulchella* interaction and revealed for the first time that *P. cinnamomi* is able to self and produce oospores in the presence of *A. pulchella*. The observation of *P. cinnamomi*'s ability to produce oospores in several jarrah forest soils and a potting mix raises a number of important questions with regards to the life cycle and management of the pathogen in horticulture, forestry and natural ecosystems. *P. cinnamomi* is clearly a versatile soil borne pathogen that survives under hostile conditions encountered in its natural environment. Without detailed knowledge of its biology, it is difficult to formulate effective control measures against *P. cinnamomi*.

The *P. cinnamomi* and *A. pulchella* interactions were studied in both aseptic and non-sterile environments, *in vitro* and *in planta*. It was revealed that, while *A. pulchella* suppressed the asexual stages of *P. cinnamomi*, it may stimulate the pathogen to self and produce oospores *in planta*. The main findings of this study were that *A. pulchella* can disrupt the normal life cycle of *P. cinnamomi* by:

- Reducing sporangial production in the soil and soil leachates.
- Reducing zoospore mobility.
- Suppressing chlamydospores either by damaging them or by reducing germinability.
- Reducing mycelial growth.
The study also highlighted the differences between the varieties of *A. pulchella* in *P. cinnamomi* suppression and established *A. pulchella* var. *goadbyi* as the most suppressive one. The suppression was due at least in part to the root exudates and the plants were not suppressive until at least seven months-old. The root exudates immobilised the main infective agent - the motile zoospores, caused mycelial damage and cytoplasmic collapse of the chlamydospores. It was demonstrated that *A. pulchella* appears to reduce the soil inoculum potential of the A2 type 1 strain of *P. cinnamomi* which was isolated most frequently from the jarrah forest (Old *et al.* 1988; Dobrowolski 1999; Dobrowolski *et al.* 2003). However, more isolates of both isozyme types (A2 type 1 and A2 type 2) need to be screened to confirm this observation. In contrast, the non suppressive *A. urophylla* contributed to the inoculum potential of *P. cinnamomi* by stimulating sporangial production and zoospore release. The varieties of *A. pulchella* and the maturity of the plants also played a role in reduction of *P. cinnamomi* inoculum and both these aspects should be considered in attempting to produce faster and more effective control. However, the suppressive effect was not observed in sandy soils which may be due to the fact that the water soluble compounds in the root exudates leach out faster in these soils.

*Bioglogy of Phytophthora cinnamomi*

The persistence of *P. cinnamomi* was examined on an inert substrate exposed to soil leachates and in infected lupin roots buried in soils in the presence and absence of *A. pulchella* plants. *P. cinnamomi* was able to survive in dying plant material by adopting the ‘transient mode of survival’ as it continues to change from one life form to
the next or from one survival strategy to the next. It was observed that when the mycelial stage lost its vigor with diminishing food base it changed to either the sporangial stage which ensured rapid reproduction through zoospore release or the production of chlamydospores. When this adjustment was not profitable in situations where sporangial production was a non option and chlamydospores become vulnerable as observed in the soil under *A. pulchella* plants, *P. cinnamomi* moved on to the most secure survival phase, oospore production, as the ultimate attempt for survival. However, for this process to occur many factors seemed to work in unison. During the process of switching from the parasitic phase in the living lupin root to the saprophytic stage within the rotting material, changes obviously took place in the mycelium of *P. cinnamomi* in the soil environment. The mycelium was seen to conserve energy by restricting into a few viable sections. Also the observation made in this study of the oospores being concentrated into limited areas shows that the stimulus or the necessary energy for selfing and oospore production was not ubiquitous. This may be an apparent symptom of *P. cinnamomi* suffering from stress.

Evidence of studies conducted in Petri dish/laboratory environments (Cother and Griffin 1973; Old *et al.* 1984b; Mackay *et al.* 1985) indicated that for *P. cinnamomi*, chlamydospores and sporangia to be the prominent propagules within root tissues. Asexual reproduction may be a short-term survival technique while survival for prolonged periods may depend primarily on oospore production.

It was found that the soil leachates of both the resistant and the susceptible *Acacia* species stimulated the mycelium to produce chlamydospores in increased numbers. However, their persistence varied with prolonged exposure to these leachates.
Chlamydospores collapsed in the resistant *A. pulchella* soil leachates with time while they mostly germinated in the susceptible *A. urophylla* leachates. In addition, the soil leachate without the *Acacia* species did not support their prolonged persistence either. The chlamydospores persisted for several days but eventually deteriorated possibly due to lack of a food base. They persisted within the lupin root tissue buried in moist soil without the *Acacias*. This observation indicates that those chlamydospores were not very effective survival structures on their own and needed a host for survival although chlamydospores are defined as survival structures. In summary, chlamydospores of the A2 type 1 of *P. cinnamomi* failed to act as survival structures in the presence of *A. pulchella*. The root exudates effect on chlamydospore inactivation was shown with *A. pulchella* var. *goadbyi* plants grown in environments without the soil microflora.

**Variations among isozyme types**

Another significant finding of this thesis was how the two isozyme types of the A2 strain of *P. cinnamomi* varied in their ability to survive within plant tissues under hostile conditions. This study was the first to examine this phenomenon and the observation was done under controlled conditions. In reality it is not always possible to examine the behaviour of a soil pathogen *in situ*. However, it is important to reinforce this finding by screening a significant number of isolates of the three clonal lineages of *P. cinnamomi* found in Australia under different conditions, including in the field.

The chlamydospores of the common A2 type 1 strain of *P. cinnamomi* examined in this thesis were more vulnerable to hostile environments thus, indicated poor survival ability. The saprophytic survival of a root-infecting fungi as defined by Garrett (1970) is survival of mycelia in infected host tissues invaded during its parasitic phase by
adopting the possible survival modes such as competitive colonisation of additional dead plant tissues and production of dormant survival structures. The A2 type 1 showed poorer saprophytic survival within root tissues with predominantly collapsed mycelium and chlamydospores. In contrast, the A2 type 2 isolates demonstrated more robustness and may be better equipped for survival and could possibly with time supersede the A2 type 1 strain of *P. cinnamomi*. This type was the better selfer and produced oospores which varied in numbers, sizes and shapes. All the *in planta* oospores were dormant and had amphigynous antheridial attachments. Although the oospores of *P. cinnamomi* are known to survive for long periods (Miretich and Zentmyer 1966) it is important to investigate their germinability and infectivity in future studies.

The A2 type 2 is less frequently isolated in Australia (Old *et al.* 1988; Dobrowolski 1999; Dobrowolski *et al.* 2003) and all the isolates that could be accessed were included in the experiments. While it is possible that they have not been identified or maintained in culture collections, it is also possible that this situation was created by the infrequency in isolations. The A2 type 2 strain of *P. cinnamomi* is infrequently isolated in Australia may be because the employed baiting methods or direct plating is not adequate to isolate this type. Even though only a few isolates were available for this study the evidence obtained undoubtedly indicates that this type of *P. cinnamomi* is able to exist in plant debris as oospores under the right conditions. For the first time this study showed the appropriate temperatures and nutritional conditions for *in planta* selfing and oospore production for the A2 type 2 of *P. cinnamomi* in soil and these conditions do exist in Australian vegetation (Christensen 1975).
The A2 type isolates of *P. cinnamomi* that are able to self might possibly be escaping detection through baiting and through direct plating onto selective agars. This ‘escape’ could well be due to the oospores being dormant, and not germinating during the baiting and plating procedures. This would have obvious implications with regards to quarantine and management of the pathogen. Staying dormant in plant debris can be the best form of life insurance for *P. cinnamomi* in jarrah forest situations, which is notable for the fluctuating soil moisture and temperature (Christensen 1975). And once the conditions become favourable this type of *P. cinnamomi* may also be able to regenerate itself fast.

*Is A. pulchella an effective biological control tool against P. cinnamomi?*

On the one hand *A. pulchella* suppresses the pathogenic or infective stages of *P. cinnamomi* while on the other it stimulates the persistent structures, the oospores, which can lay dormant in plant tissue for a long time under moist conditions (Mircetich and Zentmyer 1966). However, whether or not the soil moisture regimes present in the *P. cinnamomi* susceptible jarrah forest and the *Banksia* woodlands where *A. pulchella* is a common component is adequate to support the longevity of the oospores remains unknown and more work is required.

*A. pulchella* has been thought to play a biological control role for *P. cinnamomi*. The findings of this thesis have posed a number of questions - which of the two functions of *A. pulchella* on the pathogen plays a more significant role - the suppression of the infective stages or the stimulation of the dormant phase? and - whether the advantages of *A. pulchella*’s ability to depress the asexual and pathogenic stages of *P. cinnamomi* are compromised by its ability to induce selfing and oospore formation? The most important fact is that they both keep the pathogen in check. Studies in the natural
environment have indicated that *A. pulchella* does reduce the inoculum potential of *P. cinnamomi* (Shea 1977; D’Souza *et al.* 2004). Hence, the balance would appear to be in favour of destruction of the pathogen and reduction of mycelium, zoospores and chlamydomspores with oospores playing a lesser role in this environment.

The suppression is more evident when the plants are mature or entered the reproductive stage. Older plants have greater root biomass and it can be speculated more than not that the chemical properties become both concentrated and strong with an increased antifungal activity. This study revealed for the first time the important fact of how the natural soil sulphur levels participate in reducing the various infective stages of the life cycle of *P. cinnamomi*. Although definitive conclusions were not reached it gave an important insight into the possible role of water soluble sulphur levels in the root exudates and the soil under *A. pulchella* plants.

Thus, *A. pulchella* does exert a high degree of control over *P. cinnamomi*. This control can be either direct or indirect (Fig 9.1). The direct effect is the reduction in the infective stages of the pathogen. The protection of *A. pulchella* plants toward other susceptible species against *P. cinnamomi* invasion have been shown in other studies (Shea and Malajzcuk 1977; D’Souza *et al.* 2004) which can also be explained by this direct effect. The invading zoospores, if at all present are inactivated before they reach the targets, whilst the chlamydomspores are unable to survive for long as shown in this work. The high levels of sulphur found in the soil under *A. pulchella* plants can diffuse into the surrounding soil as water soluble sulphur. This may well be a key factor in the direct toxic effect towards the asexual propagules of *P. cinnamomi*. The high sulphur levels under *A. pulchella* plants also appear to induce selfing and oospore formation of
*P. cinnamomi*. Oospores being the dormant stage do not pose an immediate threat and are not considered as pathogenic.

There are several indirect effects that *A. pulchella* plants exert on *P. cinnamomi*. It encourages an antagonistic soil microflora as observed in other studies (Murray 1985; Malajczuk *et al.* 1984) or promotes a soil physical environment which is unfavourable for sporangial production of *P. cinnamomi* (Shea *et al.* 1978). By suppressing the pathogen *A. pulchella* plants provides a healthy soil environment for the adjacent susceptible species. Some of the plant species which are more susceptible towards diseases at seedling stage and show resilience when they are mature are *Pinus* spp. (Newhook 1959), *B. candolleiana*, *B. lindleyana* and *B. caleyi* (McCredie *et al.* 1985). The protective role of *A. pulchella* towards the two most common plant species in the Western Australian jarrah forest and *Banksia* woodlands, *E. marginata* and *B. grandis* from *P. cinnamomi* attack were shown at seedling stage (Shea and Malajzcek 1977; D’Souza 2004).

![Fig 9.1 Flow diagram of *Acacia pulchella* and *Phytophthora cinnamomi* interactions.](image-url)
Mechanism of suppression

This thesis confirmed the previous reports of sporangial inhibition (Shea et al. 1978; Murray 1987) under A. pulchella and showed that chlamydosporic viability is also reduced. Inactivation of P. cinnamomi chlamydosporic forms by A. pulchella is a major contribution towards suppression. These structures within the plant debris would otherwise be eligible contenders in increasing the soil inoculum. Hence, the mechanism of suppression of P. cinnamomi under A. pulchella plants is achieved predominantly by mycelial collapse, sporangial suppression and inactivation of chlamydosporic forms in addition to the stimulation of the dormant oospores.

Predictions and current trends in P. cinnamomi control

Excessive usage of chemical control methods e.g. use of phosphite (phosphonate) can be harmful for the native flora causing leaf burn and reducing pollen fertility (Fairbanks 2002; Barrett et al. 2004) and has been shown to lead to resistance in pathogen populations (Dobrowolski et al. unpublished). Other chemicals such as chloroneb (2,5-dichloro-1, 4-dimethoxybenzene) stimulated oospore production of P. capsici in vitro (Noon and Hickman 1978). The widely used fungicide phosphite (phosphonate) may have similar implications with P. cinnamomi. It still remains to be disproved that phosphite application may also induce the formation of oospores. The short term benefits of chemical control methods should be balanced with possible long term harmful effects.

The benefits of this study

This thesis will be of interest not only to Phytophthora researchers, but to a wider community of scientists in the disciplines of soil and rhizosphere chemistry and
ecology or environmentalists and forest managers with biological control interests. *A. pulchella* is a native legume of Western Australia which occurs widely in many forest woodland and heathland ecosystems. The idea of using it as a biological control tool was conceived previously. However, the benefit of this method has not been explored effectively. Findings of this study moves the 20\textsuperscript{th} century concept of utilising *A. pulchella* as a biological control tool forward into the new millennium.

Findings of this thesis also apply to the horticulture and plantation industries. It is possible that some nursery potting mixes may harbour *P. cinnamomi* oospores and eventually spread the disease into horticulture and from plantations into the native vegetation.

Inevitably there always will be speculation from the advocates of chemical control regarding the ineffectiveness of biological control when the problem does not simply get eradicated. Instances where the disease strikes after suppressed periods is a good example. In natural vegetation *A. pulchella* germinates after high intensity fires and lives for 5-13 years. If viable oospores remain in the soil after the death of the *A. pulchella* plants, they could trigger a new disease cycle. However, the germinability or infectivity of *in planta* oospores is still an unknown area and further research is needed to verify this possibility.

It should be pointed out that “the aim of biological control is to prevent the harmful effect of the pathogen and reduce disease incidence so it is no longer a problem”. Also the great virtue of biological control is that it makes use of the natural mechanisms by which damage to plants by pests and pathogens is kept at the low values usually found
in natural populations (Wood and Way 1988). It was not the scope of this thesis to combat the arguments on the virtues of biological control, but to contribute to the understanding of the mechanism(s) of *P. cinnamomi* suppression in soil under *A. pulchella*.

**Limitations encountered**

The commonly occurring strain of *P. cinnamomi* in Western Australian forest and sand plain ecosystems is the isozyme type 1 of the A2 clonal lineage (commonly identified as A2 type 1) and its impact can be reduced by *A. pulchella*. However, *A. pulchella* may be less effective in reducing the inoculum of the less frequently isolated type, the isozyme type 2 of A2 (A2 type 2). It is important to reinforce this finding by screening a large number of the isolates of the three clonal lineages of *P. cinnamomi* from different geographic regions of Australia and to conduct some trials under plants *in situ*. A few isolates were available for experiments in this thesis. A study equipped with more resources is needed to investigate how all the types of *P. cinnamomi* present in Australian ecosystems respond to *A. pulchella*. The availability of all the A2 type 2 isolates found in Australia should occupy the top of the list for any future study prospects. The imbalance in resource distribution created by predominance given to research on chemical control should be addressed and more resources should be allocated to biological control.
**Future Research**

This study identified several areas into which more research should be directed in order to formulate effective control of *P. cinnamomi* and thus provided the basis for several future research topics. These include

- Investigating new methods for isolating the A2 type 2 from soil and plant material as it is possible that the current methods may not be adequate.
- Identifying the inhibitory compound/s in the root exudates of *A. pulchella* in aseptic systems.
- Select plants of *A. pulchella* var. *goadbyi* that produce high levels of the inhibitory compound.
- Adopting the chlamydosporre bioassay designed in this thesis to test any commercial products which can be used as control agents against *P. cinnamomi*.
- Examining ways to germinate oospores formed *in planta*.
- Investigating whether the soil under other *P. cinnamomi* resistant plant species induce selfing and oospore formation of *P. cinnamomi*.
- Investigating the potential of potting mixes with pine bark and other plant material in inducing selfing and oospore production of *P. cinnamomi*.
- Studying whether the *Trichoderma* effect in selfing and oospore formation observed by Brasier (1978) is present in the jarrah forest soil.

Findings of this study also confirmed the presence of a definite biological mechanism in the soil under the *A. pulchella* plants and at the same time provided evidence to untangle the role of plant effect from the microbe effect under *A. pulchella*.
Appendices

Appendix 1 Media

Murashige and Skooge nutrient medium (½ strength)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Molecular weight (g)</th>
<th>Stock solution (g/L)</th>
<th>Quantities mL/L</th>
</tr>
</thead>
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<td>KNO₃</td>
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<td>95</td>
<td>10</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>80.08</td>
<td>82.5</td>
<td>10</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>246.5</td>
<td>18.5</td>
<td>10</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>237.56</td>
<td>0.863</td>
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</tr>
<tr>
<td>MnSO₄.4H₂O</td>
<td>169.01</td>
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<td>5</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>249.68</td>
<td>0.0025</td>
<td>5</td>
</tr>
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<td>5</td>
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</tr>
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<td>KH₂PO₄</td>
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<td>H₃BO₃</td>
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<td>0.618</td>
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<td>Na₂MoO₄.2H₂O</td>
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<td>NaFeEDTA</td>
<td>367.05</td>
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<tr>
<td>myo-inositol</td>
<td>180.16</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>123.11</td>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>337.28</td>
<td>0.01</td>
<td>10</td>
</tr>
<tr>
<td>Pyradoxione HCl</td>
<td>205.64</td>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>Glycine</td>
<td>75.07</td>
<td>0.2</td>
<td>10</td>
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</table>

All the salts, except FeEDTA, were stirred in 1 L of distilled water and autoclaved for 20 min at 121° C. FeEDTA was filtered through 0.22 µm Millipore filter and added to the cooled salt solution.
Mineral salt solution (Chen and Zentmyer 1970) for sporangial production

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity (g)</th>
</tr>
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<tbody>
<tr>
<td>Ca(NO₃)₂</td>
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</tr>
<tr>
<td>KNO₃</td>
<td>0.51</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
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</tr>
<tr>
<td>Deionised water</td>
<td>1 L</td>
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<tr>
<td>FeEDTA solution</td>
<td>1 mL</td>
</tr>
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</table>

FeEDTA

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (Ethylene Diaminetetra acetic acid)</td>
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</tr>
<tr>
<td>KOH</td>
<td>7.5</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>24.9</td>
</tr>
<tr>
<td>Deionised water</td>
<td>1 L</td>
</tr>
</tbody>
</table>

The salts were added to deionised water and autoclaved for 20 min at 121° C. FeEDTA was filtered through 0.22 µm Millipore filter and added to the cooled salt solution.

*Phytophthora* isolation medium NARPH

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (g/L)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn meal agar (CMA)</td>
<td>17</td>
<td>(DIFCO) Laboratories, USA</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.1</td>
<td>CSL Ltd., Australia</td>
</tr>
<tr>
<td>Nystatin (Nilstat)</td>
<td>1</td>
<td>Wyeth Ayerst Australia</td>
</tr>
<tr>
<td>Rifampicin (Rifadin)</td>
<td>0.5</td>
<td>Hoechst Marion Roussel, Australia</td>
</tr>
<tr>
<td>Hymexazol</td>
<td>0.05</td>
<td>Sankyo Co. Ltd. Japan</td>
</tr>
<tr>
<td>Pentachloronitrobenzene (PCNB)</td>
<td>0.1</td>
<td>Uniroyal Australia Pty. Ltd.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 L</td>
<td></td>
</tr>
</tbody>
</table>

CMA was added to distilled water and autoclaved for 20 min at 121° C. Rest of the ingredient were dissolved in ~ 5 mL sterile distilled water and added to the cooled CMA solution.
Modified Ribeiro’s medium for oospore production (Ribeiro, OK 1978)

Microelement stock solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂MoO₄.H₂O</td>
<td>41.1</td>
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<tr>
<td>ZnSO₄.7H₂O</td>
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<td>CuSO₄.5H₂O</td>
<td>7.85</td>
</tr>
<tr>
<td>MnSO₄.H₂O</td>
<td>15.4</td>
</tr>
<tr>
<td>Na₂B₄O₇</td>
<td>0.5</td>
</tr>
<tr>
<td>Deionised water</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

Ingredients were dissolved in 100 mL deionised water

Ferric stock solution

FeCl₃.6H₂O 52.0
EDTA 2.6
KOH 1.5
Deionised water 100 mL

EDTA and KOH were dissolved in 100 mL DI water followed by addition of FeCl₃.6H₂O

Basal medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4.5</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>0.1</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.15</td>
</tr>
<tr>
<td>KH₂PO₄</td>
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<tr>
<td>MgSO₄.7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1</td>
</tr>
<tr>
<td>Deionised water</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

Ingredients were dissolved in 100 mL DI water followed by additions of 1 mL of each of the above stock solutions and made up to a final volume of 1 L. The pH was adjusted to 6.2 with 1M KOH and the solution was autoclaved at 121º C for 20 min.

Thiamine stock solution

Thiamine-HCl 0.1
Deionised water 100 mL
Thiamine solution was filtered through a 0.22 µm Millipore filter and 1 mL was added to the cooled solution.

**Cholesterol solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity (mg)</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>50</td>
</tr>
<tr>
<td>CH₃OCH₃</td>
<td>100 mL</td>
</tr>
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</table>

Twenty mL of Ribeiro’s medium was dispensed to Petri dishes and 5 mL of the Cholesterol solution was added. The solutions were left in a laminar flaw unit for 2 h for the CH₂Cl₂ to evaporate.
Appendix 2 Data analysis

Chapter 2

ANOVA Table for *Phytophthora cinnamomi* colony numbers in the glasshouse experiment

Tests of Between-Subjects Effects

Dependent Variable: Colonies

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>4</td>
<td>8.317</td>
<td>3.892</td>
<td>.017</td>
</tr>
<tr>
<td>Intercept</td>
<td>1</td>
<td>50.519</td>
<td>23.644</td>
<td>.000</td>
</tr>
<tr>
<td>Taxa</td>
<td>4</td>
<td>8.317</td>
<td>3.892</td>
<td>.017</td>
</tr>
<tr>
<td>Error</td>
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<tr>
<td>Total</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected Total</td>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ANOVA Table for *Phytophthora cinnamomi* colony numbers - *in vitro* experiment

Tests of Between-Subjects Effects

Dependent Variable: Colonies

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<tr>
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</tr>
</thead>
<tbody>
<tr>
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<td>Taxa</td>
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<td>4.293</td>
<td>.044</td>
</tr>
<tr>
<td>Error</td>
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<tr>
<td>Total</td>
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<tr>
<td>Corrected Total</td>
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ANOVA Table for sporangial numbers in leachates

Tests of Between-Subjects Effects

Dependent Variable: Sporangia

<table>
<thead>
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<th>Sig.</th>
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<tr>
<td>Total</td>
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<tr>
<td>Corrected Total</td>
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Multiple Comparisons

Tukey HSD

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<tr>
<th>Dependent Variable</th>
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<th>(J) time</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
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Based on observed means.
* The mean difference is significant at the .05 level.

Chlamydospores within lupin roots

Tests of Between-Subjects Effects

Dependent Variable: Collapsed chlamydospores

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## Chapter 4

### ANOVA Table for chlamydomspore bioassay with root exudates

Tests of Between-Subjects Effects

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### ANOVA Table for zoospore bioassay

Tests of Between-Subjects Effects

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Chapter 5
ANOVA Table Effect of the forest soils on Phytophthora cinnamomi recovery in winter

Tests of Between-Subjects Effects

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ANOVA Table Effect of the forest soils on Phytophthora cinnamomi recovery in summer

Tests of Between-Subjects Effects

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ANOVA Table for different aged stands of *Acacia pulchella*

Tests of Between-Subjects Effects

Dependent Variable: Recovery of *Phytophthora cinnamomi*

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ANOVA Table for sporangial numbers in forest soils in winter

Tests of Between-Subjects Effects

Dependent Variable: Sporangia

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### ANOVA Table for sporangial numbers in forest soils in summer

Tests of Between-Subjects Effects

Dependent Variable: Sporangia

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### Chapter 6

### ANOVA Table for oospore counts

Tests of Between-Subjects Effects

Dependent Variable: oospores

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Chapter 7

ANOVA Table for oospore production in jarrah forest soils

Tests of Between-Subjects Effects

Dependent Variable: Oospores

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REFERENCES

*Phytochemistry* 13, 2633-2645.


*Phytopathology* 72, 956.


Carry JL (1982) *Native legumes of the Eucalyptus marginata Don ex Sm. forest in relation to nitrogen and Phytophthora cinnamomi Rands*. BSc (Honours) Thesis, Murdoch University, Western Australia.


Murray DIL (1983) Stimulation of sporangium production in Phytophthora cinnamomi by Penicillium spp. from the jarrah forest of Western Australia. Australasian Plant Pathology 12, 55-56.


Pratt BN, Heather WA (1973) Recovery of Phytophthora cinnamomi from native vegetation in a remote area of New South Wales. Transactions of the British Mycological Society 60, 197-204.


Reeves RJ, Jackson RM (1972) Induction of Phytophthora cinnamomi oospores in soil by Trichoderma viride. Transactions of the British Mycological Society 59, 156-159.


Sivasithamparam K (1981) Some effects of extracts from tree barks and sawdust on

fresh sawdust and composted tree-bark on *Phytophthora cinnamomi* Rands.

Smith AM (1972) Biological control of fungal sclerotia in soil. *Soil Biology and
Biochemistry* 4, 131-134.

Smith IW, Marks GC (1983) Influence of *Acacia* spp. on the control of *Phytophthora
cinnamomi* root rot of *Eucalyptus sieberii*. *Australian Forest Research* 13, 231-240.

Smith IW, Marks GC, Featherston GR, Geary PW (1989) Effect of inter-planted
wattles on the establishment of eucalyptus planted on forest sites affected by
*Phytophthora cinnamomi*. *Australian Forestry*, 74-81.

*Fusarium oxysporum* f. sp. *cucumerinum* as affected by fluorescent and lytic bacteria
from *Fusarium*-suppressive soil. *Phytopathology* 74, 1115-1124.

Stamps DJ, Waterhouse GM, Newhook FJ, Hall GS (1990) Revised tabular key to the


Williams JS, Cooper RM (2003) Elemental sulphur is produced by diverse plant families as a component of defence against fungal and bacterial pathogens. *Physiological and Molecular Plant Pathology* **63**, 3-16.


