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PHENOLIC AND LIGNIN CONCENTRATION AS AN INDICATOR OF RESISTANCE TO *PHYTOPHTHORA CINNAMOMI* IN *BANKSIA* SPECIES

Sandra Jane Nicoski

Thesis submitted in partial fulfilment

of the requirement for the award of

B. Sc.(Biological Science) Honours

Department of Applied Science

Edith Cowan University

December 1996

"Extinction undoubtedly is being played out as we gather our thoughts today." – S. Hopper 1994

ABSTRACT

Phytophthora cinnamomi Rands, a soilborne fungus, is regarded as one of the most devastating pathogens yet recorded in natural ecosystems. The disease, caused by *P. cinnamomi*, poses an enormous threat to the flora of the southwest. The Proteaceae, in particular, contribute considerably to species richness and provide the fundamental floristic structure of many south-western plant communities. The genus *Banksia*, a member of the Proteaceae, is very susceptible to disease caused by *P. cinnamomi*.

Three species, namely *B. attenuata, B. grandis and B. menziesii*, were established in an aeroponics system under glasshouse conditions. Trials were conducted to optimise growing conditions in these systems and to determine the most effective method of inoculation. Shoots were treated with a 0.5% phosphonate foliar spray and roots were dipped in *P. cinnamomi* zoospore suspensions.

The temporal, spatial and treatment changes in the concentrations of total soluble phenolics and lignin in roots, four and twelve days after inoculation, were measured. Total soluble phenolics decreased in inoculated treatments compared with controls. In addition, phenolic levels were even lower by twelve days after inoculation. In comparison, lignin concentrations did not

have a significant effect on these compounds in *B. menziesii*, however, it increased in inoculated plants above control levels. Phosphonate application *B. grandis* produced increased levels of total soluble phenolics with phosphonate application.

This study aimed at developing an understanding of the biochemical defences of *Banksia* species to *Phytophthora*, enables the resistance of these species to be compared and assessed.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by any other person except where due reference is made in the text.

Sandra Jane Nicoski

6 December 1996

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1. INTRODUCTION

1.1 The Genus *Banksia*

1.1.1 Origins and Distribution

The genus name, *Banksia*, commemorates the botanist Joseph Banks who accompanied Captain James Cook in 1770 to the east coast of Australia (Holliday and Watton, 1975). Of the 75 species of *Banksia* recognised, 58 are endemic to south-western Australia, and 14 occur in eastern Australia ranging from the Eyre peninsula in south Australia, to Tasmania and north Queensland (George, 1984). A single species, *B. dentata* L.f. occurs across tropical Australia and extends its range to Papua-New Guinea, Irian Jaya, and the Aru Islands (George, 1984). The age of the genus is uncertain, however, palynological studies have revealed *Banksia* or *Banksia*-like pollen from the Australian Palaeocene (George, 1981). This has allowed time for banksias to adapt to a variety of habitats ranging from true coastal species to those species growing in high-altitude, alpine conditions, high-rainfall forest, and inland sand heaths. They predominate, however, in poor sandy soils and are most diverse in the coastal sandy heathlands near the coast (Holliday and Watton, 1975).

All banksias are woody plants but vary considerably in habit. Those species of arborescent form have been recorded up to 25 m tall, with trunk diameters of up to 70 cm. They are more commonly shrubs, which range in size from large bushes down to prostrate forms with one or many lateral branches at ground level. In

all forms there are fire-tolerant and fire-sensitive species (George, 1981). Those species which are fire-tolerant possess either a lignotuber or a thick, rough-barked trunk from which epicormic shoots may sprout after fire. Fire-sensitive species lack these lignotubers or have thin bark, and as a result are killed by fire and regenerate by seed.

A characteristic of banksias is that they bear large, conspicuous and sometimes spectacular inflorescences (Cho, 1981). These vary in shape from spherical or globular to cylindrical and are made up of numerous tightly-packed, spirally arranged individual flowers. These mature to produce a fruiting cone comprised of woody follicles in which the seeds are encapsulated. Under natural conditions the seeds can remain enclosed in the follicles for many years until fire causes the release and the subsequent regeneration of new plants (George, 1984).

1.1.2 Ecological Value and Commercial Uses of *Banksia*

1.1.2.1 Ecological importance

The Proteaceae are a key element of south-western Australian ecosystems (James, 1991). Proteaceous species are a major component of the forest understorey, and in the heathlands of the southern coast they are dominant, accounting for up to 60% of the total species (Dell and Malajczuk, 1989). Wills and Keighery (1994) reported that after infection by *P. cinnamomi* the percentage cover of dominant proteaceous species may be reduced by 95%.

The species removed are often keystone species which act as food, shelter and nesting resources for associated animals. For example, banksias flower in summer, autumn and winter (George, 1984). As a result many native marsupials and birds rely on the pollen and nectar provided by these species. Species such as the honey possum (*Tarsipes rostratus*) rely entirely on a specialised diet of pollen and nectar mainly from *Banksia* species (Wilson *et al.*, 1994). Removal of dominant species also alters the light regimes necessary to support associated plants, resulting in a loss of diversity and structure in a complex plant community.

1.1.2.2 Commercial uses

The Proteaceae family are an important resource for the wildflower export industry both in Australia and overseas (Cho, 1981). In 1991 Australian Proteaceae flower exports achieved a wholesale return of A$818 500 (Batt, 1993). The *Banksia* species are of particular importance because of their large, spectacular inflorescences and several species, *B. baxteri* R.Br., *B. coccinea* R. Br. and *B. grandis* Willd. are heavily exploited for their foliage and seed as well as flowers (Wills and Robinson, 1994).

The Proteaceous flora of south-west Australia also contributes to one of the highest rates of honey production in the world. Wills (1989) found that almost the entire honey crop is gained from native plant communities and that virtually all species of *Hakea*, *Grevillea* and *Banksia* were utilised by honey bees. In Western Australia 2 264 tonnes of honey was produced in 1992, worth A$2.25 million

(Kelly, 1993). Western Australia is the second smallest producer of honey in Australia. However, the rates of production are some of the highest in Australia and one of the highest in the world, making the Proteaceae family a valuable resource (Wills, 1993).

1.2 The Genus *Phytophthora*

1.2.1 Classification

Phytophthora species are soil-borne pathogens belonging to the class Oomycetes, order Peronosporales and family Pythiaceae (Irwin *et al.*, 1995). This is a relatively primitive group that differs structurally, biochemically and genetically from the more familiar fungal taxa such as the Ascomycetes and Basidiomycetes (Hemmes, 1983). Some features which distinguish *Phytophthora* species from other fungi include motile zoospores which are able to encyst within minutes of release from the sporangium, cell walls which contain predominantly cellulose rather than chitin and a lysine biosynthesis pathway which differs from that of higher fungi.

In evolutionary development the genus *Phytophthora* is believed to represent a transitional group between entirely aquatic and completely terrestrial fungi (Barr, 1983). This is evident in their complex life cycles dependant upon moist conditions for survival, sporulation, dispersal and infection.

1.2.2 Morphology and Reproduction

The complicated life cycle of *Phytophthora* (Figure 1.1) includes an asexual and sexual reproductive phase. In the asexual phase the fungus produces chlamydospores which act as resting spores. When soil conditions are suitable (15°C or above) the chlamydospores germinate to form mycelia and sporangia. The sporangia can either germinate directly by forming a germ tube, or most commonly differentiate up to 50 biflagellate zoospores. Zoospores do not normally remain mobile for more than six hours (Irwin *et al.*, 1995). If within this period they come into contact with a root, they immediately form a thick-walled spore and encyst upon the epidermal layer of the root. If the zoospore does not come into contact with a root it still encysts and germinates to form mycelium which in turn can produce sporangia.

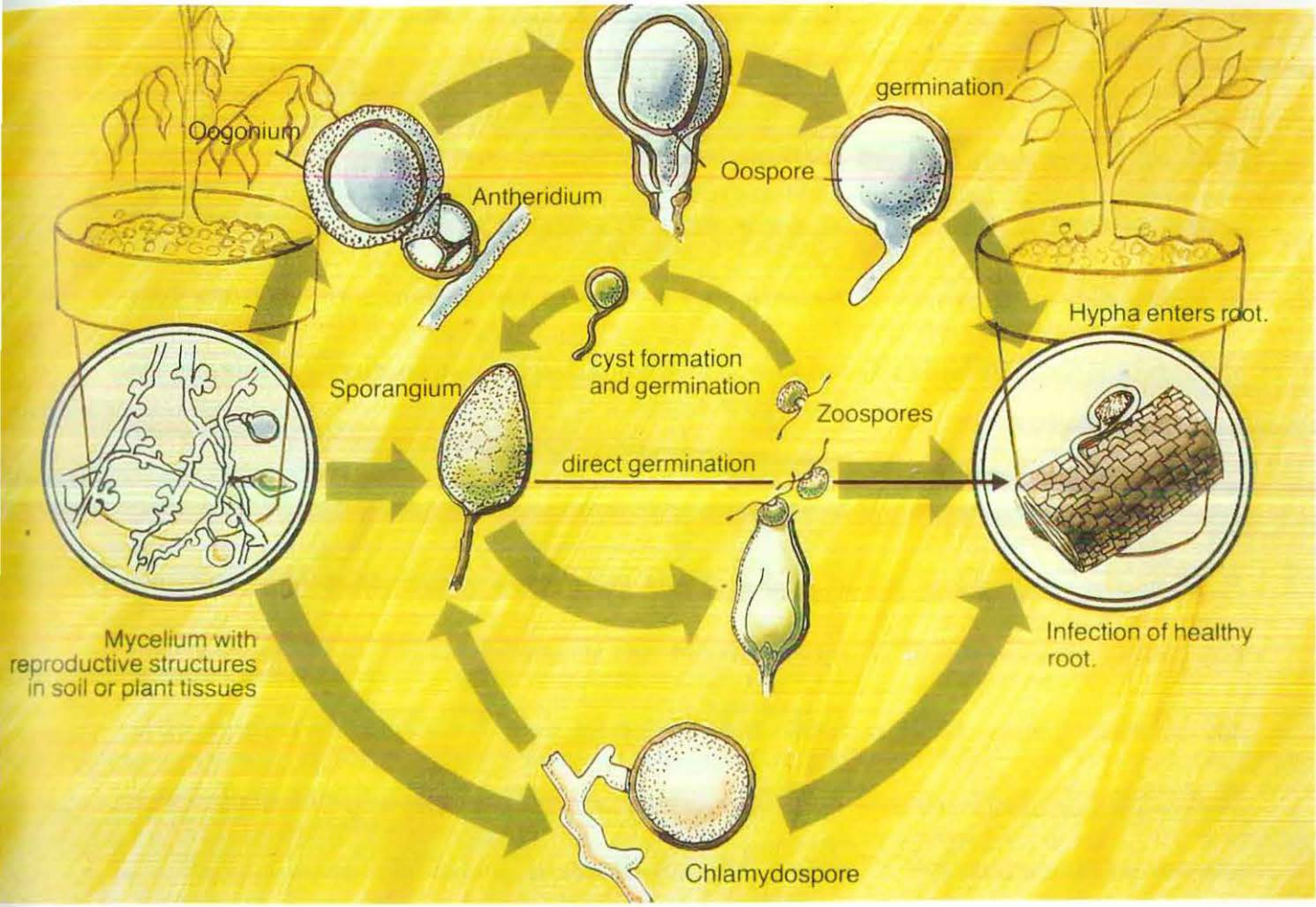

Figure 1.1 Life cycle of *P. cinnamomi* (sourced from Sivasithamparam and Goss, 1980). Sporangia are produced under conditions of high soil moisture and temperature. When the zoospores are released from the sporangia they encyst on contact with the root and germinate to produce a hypha which infects the host.

1.3 *Phytophthora cinnamomi*

1.3.1 Origin and Distribution in Australia

P. cinnamomi is regarded as one of the most devastating pathogens recorded in natural ecosystems. It was first discovered in 1922 by R.D. Rands on cinnamon trees in the mountains of tropical Western Sumatra (Dell and Malajczuk, 1989) and was first recorded as an agent of plant disease in Australia on pineapple in Queensland (Irwin *et al.*, 1995). However, it was not till the late 1960s and early 1970s that *P. cinnamomi* gained recognition.

Considerable controversy surrounds the question of the geographic origin of *P. cinnamomi*. The climate in which the fungus occurs is tropical, subtropical and mild temperate regions which suggests that the centre of origin may be the south-east Asian area (Zentemeyer, 1980). Little is known about when and how the fungus established itself in south-western Australia but there are few doubts that it was introduced some time after the first European settlers in 1827. While there are some indications of resistance in a range of species having developed in north-east Australia, the large number of susceptible hosts in Western Australia indicates that the fungus was introduced, probably within the past 70 to 80 years (Zentemeyer, 1980).

By 1972 dieback disease, caused by *P. cinnamomi*, was scattered extensively throughout dry sclerophyll vegetation in the east, west and south of the Australian mainland. Today the disease centre of *P. cinnamomi* in the south-west

is bounded by Eneabba north of Perth, east of Dryandra near Popanyinning, and Cape Arid east of Esperance on the south coast (see Figure 1.2). The highest incidence of *P. cinnamomi* occurs in the northern and southern jarrah forests, probably due to human activity (Shearer and Tippett, 1989). It also occurs frequently on acidic leached sands of the Bassendean Dune System of the Swan Coastal Plain, Gavin Sands of the Leeuwin-Naturaliste Ridge, laterite soils and winter flats of the d' Entrecasteaux and Walpole-Nornalup National Parks. Incidence is high in other parks of the south coast such as Stirling Range National Park, Cape Arid National Park and Two Peoples Bay Nature Reserve (Shearer, 1994).

1.3.2 Host Range

Phytophthora cinnamomi is the species which causes disease on the largest scale in Western Australia from the genus *Phytophthora*. This is because the fungus does not have a high degree of host specifity, as do most *Phytophthora* species (Whisson *et al.*, 1993). *P. cinnamomi* has been described in 67 countries and has over 2 000 hosts, of which, a large number are Australian native plants (Zentmeyer, 1980; Wills, 1989).

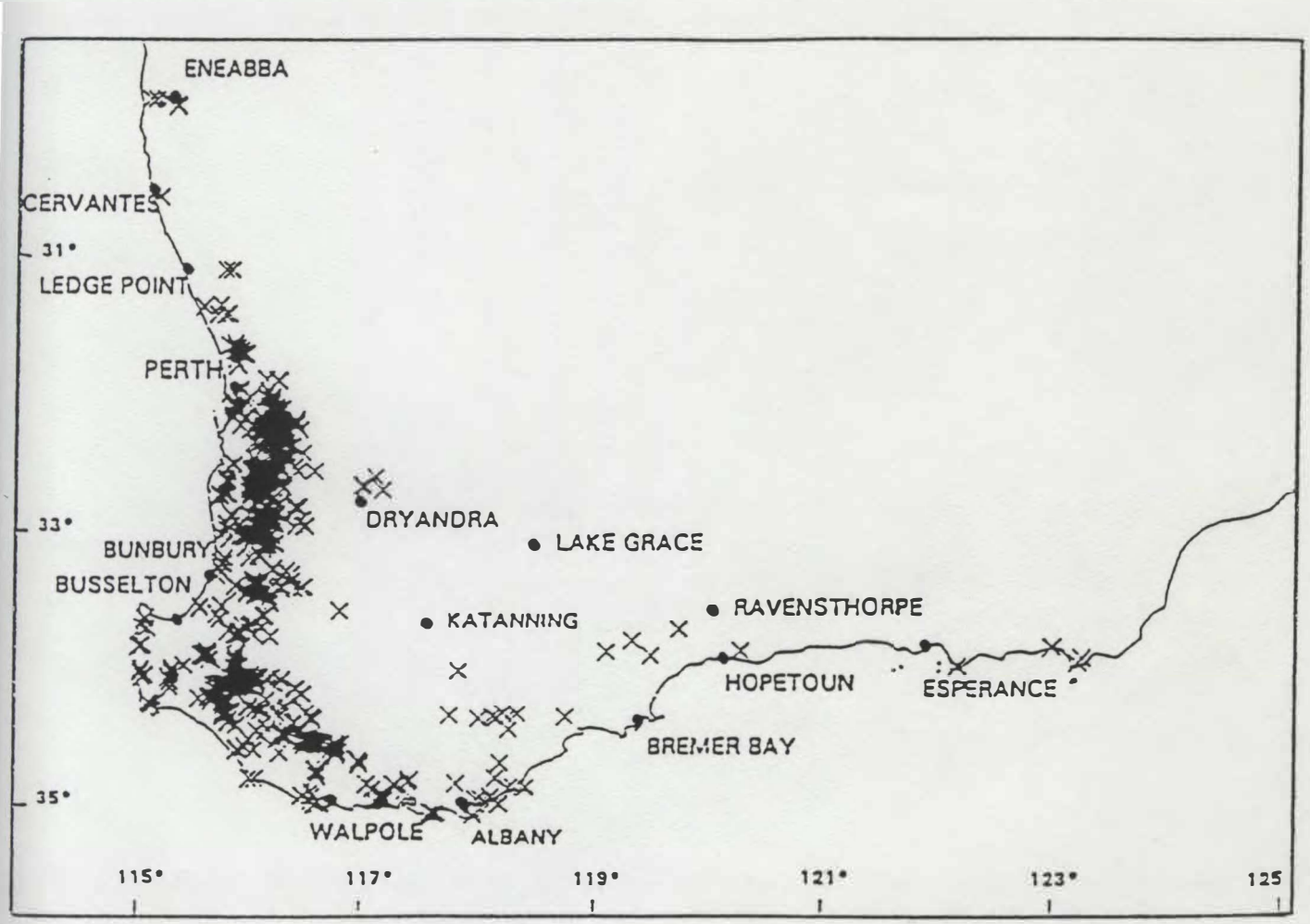

Figure 1.2 Distribution of *P. cinnamomi* in south-western Australia (from Shearer, 1994). The greatest incidence of *P. cinnamomi* occurs in the northern and southern jarrah forest. Map compiled from aerial maps and supplemented by isolation records.

It is principally a pathogen of woody perennial plant species, and herbaceous perennials, annuals and geophytes appear to be largely unaffected by the pathogen (Zentemeyer, 1980; Wills, 1993; Wills and Keighery, 1994). In south-western Western Australia it is estimated that 2 000 species of the estimated 9 000 species of vascular plants may be susceptible to infection (Wills,1989). There are many species recorded as hosts for the fungus, however the pathogen is particularly devastating to species from the families Proteaceae, Epacridaceae, Myrtaceae and Xanthorrhoeaceae (Weste and Marks, 1987). These families comprise the bulk of species on which the wildflower export and tourism relies (Wills and Robinson, 1994). In field studies of southern plant communities, 92% of the family Proteaceae were rated as susceptible to *P. cinnamomi*, of which *Banksia* represent a large proportion (Wills and Robinson, 1994).

1.3.3 Method of Infection

The mobility of zoospores and specific movement to their hosts makes them extremely potent particles for the local spread of the disease (Irwin *et al.*, 1995). Flagella enable the zoospores to move actively in water for short distances before they encyst and germinate to initiate infection. *Phytophthora* species may exist as colonizers of root tissue in the form of mycelium producing sporangia, which can produce zoospores. They can also survive as oospores and chlamydospores (Zentemeyer, 1980). Environmental conditions conducive to infection include high soil moisture and temperature (20-30 °C) (Irwin *et al.*, 1995). Only periods in winter, spring and autumn are sufficiently moist in Australia to allow for growth and reproduction in the soil, unless there are periods of high summer rainfall.

P. cinnamomi, like several other *Phytophthora* species, targets roots for initial invasion, however, the fungus can also invade leaves and the trunk. Motile zoospores of *P. cinnamomi* in free water are chemically attracted (chemotaxis) to the roots (Zentemeyer, 1960) and encyst on the root surface. After encystment, a germ tube arises which penetrates the epidermal cell layer producing vegetative hyphae which colonise the root tissues. Growth is initially intercellular while intracellular haustoria are formed in the cortical cell layer. This induces protoplast collapse and cell wall hydrolysis, resulting in brown lesions in several hosts. The pathogen then extends into the major roots and may extend along the root cortex and into the stele. This can kill the vascular cambium and phloem, and destroy the sieve tubes and phloem parenchyma of both primary and secondary roots (Shearer *et al.*, 1987). By infecting the root system in this way, there is a severe reduction in the amount of water and nutrient movement throughout the plant, thus leading to deterioration and death if left untreated.

1.3.4 Management of *P. cinnamomi*

1.3.4.1 Hygiene

Phytophthora species are highly adapted to their soil environments and once established are extremely difficult to eradicate. The most effective strategy for their control is to maintain a high level of hygiene. Research has shown that the introduction, spread or intensification of the pathogen in uninfested, natural communities can be reduced by strict hygiene measures (Shearer and Tippett,

1989). The Department of Conservation and Land Management (CALM) of Western Australia has developed comprehensive hygiene protocols to quarantine the disease. Some of the procedures adopted for management of a diseased forest include; the quarantine of different forest areas, the prevention of all forest activities in areas until it is mapped for disease; the use of aerial photography and computerized mapping for accurate disease detection and location; the adoption of measures for those areas with disease to prevent its spread, improvement of drainage where feasible and the location of roads in valleys rather than ridges. More recently, the fungicide phosphorous acid, is also used in controlling *P. cinnamomi* and is proving to be effective (Armstrong, 1991).

1.3.4.2 Biological Control

The definition given for biological control by Cook and Baker (1983) is "the reduction of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man". Biological methods are seen as an environmentally safe form of disease control and are perceived to be a good alternative to the use of fungicides (Irwin *et al.*, 1995). However, the current rate of progress with biological methods of *Phytophthora* control is slow and will probably not be an option until well into the next century (Irwin *et al.*, 1995).

One method of biological control in use in the jarrah forest is to change the understorey composition from *B. grandis*, which is highly susceptible, to encouraging the growth of *Acacia pulchella* R. Br. as a means of reducing the inoculum potential of *P. cinnamomi* (Tippett and Malajczuk, 1979). In contrast to

B. grandis, the legume *A. pulchella* has been shown to reduce sporulation of *P. cinnamomi* (Shea *et al.*, 1978). *A. pulchella* roots contain extractable volatiles that inhibit mycelial growth, sporangium production and zoospore germination (Whitfield *et al.*, 1981). Malajczuk *et al.* (1984) showed that a *Rhizobium* species isolated from roots of *A. pulchella* is strongly antagonistic to *P. cinnamomi.*

Suppressive soils have been defined by Baker and Cook (1974) as those in which disease development is suppressed, even though the pathogen is introduced and susceptible hosts are present. Potential antagonists of *P. cinnamomi* have been isolated from avocado feeder roots growing in soil suppressive to *P. cinnamomi.* From the many fungi and bacteria screened by Gees and Coffey (1989) from these soils, a particular strain of *Myrothecium roridum* Tode ex Fr. was the most active in suppressing *P. cinnamomi.* Another antagonistic microorganism tested for its effectiveness on *P. cinnamomi* is *Pseudomonas cepacia* Burkn. It was added to the soil and the response of eight proteaceous species to inoculation with *P. cinnamomi* was tested (Turnbull *et al.*, 1989). The bacterium was found to have an antagonistic effect on growth of the fungus in the infected plants.

1.3.4.3 Chemical Control: Phosphonates

Chemical control of *Phytophthora* infection of woody plants can be separated into two main methods: fumigation of the soil, and the use of systemic fungicides (Marks and Smith, 1991; Zentemeyer, 1980). Fumigation of the soil with chemicals is often used to eliminate weeds and soilborne pathogens (Ribeiro and Linderman, 1991). Fumigation with chemicals, such as methyl bromide, is seen

to be an extreme measure since it affects virtually all microflora. If plants introduced into these treated soils are infected by or contaminated with *Phytophthora* species, the lack of competition from soil antagonists can lead to a very rapid increase and spread of *Phytophthora* populations (Coffey, 1991). Also trees planted in fumigated soils may be stunted for the first year or two; presumbly due to a lack of beneficial mycorrhizae and microbial activity (Linderman *et al.*, 1983). Most importantly, fumigation of the soil is often ineffective because the fumigant does not penetrate deep enough to reach *Phytophthora* propagules that exist at greater soil depths.

The systemic fungicides, based on phosphorous acid and metalaxyl, and other organic chemical fungicides, which include captafol, copper sulfate, ethazole (terrazole 35WP), maneb and benomyl, have been used in the past to control *P. cinnamomi* (Coffey, 1991). Within the last decade there have been several important studies on phosphonates as a chemical control option with promising results. Phosphonate is a derivative of neutralized phosphorous acid and has been used widely to control pre- and post-harvest plant diseases caused by Oomycetes.

The phosphonates are truly systemic fungicides which after application are rapidly absorbed and translocated in the xylem and the phloem. Its distribution is then subject to a normal source-sink relationship in the plant. The fungicide is known to mediate its activity through the host by interfering with the pathogens phosphate metabolism (Barchietto *et al.*, 1990; Smillie *et al.*, 1990; Niere *et al.*,

1990). One consequence of this disturbance is that pathogen virulence mechanisms are affected, leading to the activation of host defence mechanisms (Guest and Grant, 1991). The phosphonate ion increases considerably the secretion of elicitors (Perez *et al.*, 1995). The enhanced elicitor production, caused by phosphonate, would contribute in activating the plants defences against fungal attack. This may result in direct inhibition of infection and also modify the interaction between host and pathogen.

The water solubility and mobility of phosphonates has provided an effective and economical way of treating root diseases caused by *Phytophthora*. Trunk injection of phosphonate is now a widely-adopted treatment for *Phytophthora* diseases of tree crops, including temperate and tropical trees. Methods such as these were used by Pegg *et al.* (1987) to protect and cure, avocado trees from *P. cinnamomi*. Infected plants recover rapidly when given injections into the trunk of phosphonate and another fungicide, metalaxyl. The results showed greater yields than controls in the third season after starting treatment. Marks and Smith (1990; 1992) have tested phosphonate as prophylactic and curative agents against *P. cinnamomi* in *Leucadendron*, *Rhododendron* and *Eucalyptus*. Phosphonate was unable to eradicate *P. cinnamomi* within established infections (trunk) within the three genera, however, maximum efficacy was achieved with foliar sprays of phosphonate before inoculation.

Tests carried out by CALM researchers have shown phosphonates to provide effective protection against the fungus in jarrah and several threatened *Banksia*

species including *B. brownii* and *B. seminuda* (A.S. George) B.L. Rye (Guest, 1995). They have also aerially applied the fungicide to affected forest from aircraft. Aerial application of phosphonate permits treatment of most remote areas cost-effectively, without disturbance to the treated and neighbouring areas (Komorek and Shearer, 1995).

Extensive and regular treatments of soil with fungicides such as metalaxyl to control *P. cinnamomi* is expensive and potentially phytotoxic to members of the Proteaceae (Dixon *et al.*, 1990; Sivasithamparan, 1991). Phosphonates are cost-effective, non-toxic to animals and degrade rapidly to phosphate in the soil (Hollingsworth, 1993).

1.4 *Banksia* species and *P. cinnamomi*

1.4.1 Susceptibility to *P. cinnamomi*

The Proteaceae are a key element in Western Australian ecosystems with 618 species and subspecies, the greatest concentration in the world (Wills, 1993). In the southwest forests *P. cinnamomi* has been shown to be particularly pathogenic to proteaceous understorey species which include banksias as a major component. The high susceptibility of the genus can be attributed to several morphological characteristics documented by Shea *et al.* (1978). The large carbohydrate reserves available in these plants provide an important niche for survival of *P. cinnamomi* when conditions outside the roots are unsuitable for vegetative growth. This

allows the fungus to survive several seasons and enables a large inoculum potential to accumulate (Dell and Malajczuk, 1989).

Banksias also have specialized proteoid root mats which lie in the upper soil horizon in close proximity to jarrah and numerous other plants' feeder roots, thus leading to cross infection. Also the root clusters of many *Banksia* species occur in shallow depressions into which moisture is directed after rain. This results in local microenvironments which are favourable for sporulation.

Studies to determine the susceptibility of *Banksia* species to *P. cinnamomi* have been performed in Hawaii and Australia (Cho 1981; 1983; Dixon *et al.*, 1984; McCredie *et al.*, 1985b; Tynan, 1994). Horticulturally important Western Australian species such as *B. hookeriana* Meisn., *B. victoriae* Meisn., *B. baxteri* and *B. occidentalis* R. Br. have shown high susceptibility in plantations. These studies also indicated that resistant plants may occur within susceptible species, such as *B. coccinea* and *B. baxteri* (Tynan, 1994). This has not been confirmed by more detailed studies.

1.4.2 Symptoms of Disease

There is some confusion surrounding the Australian usage of the term dieback. It differs to the conventional definition of "progressive dying back from the tips of twigs, branches or tops" which is a general phenomenon of arborescent shrubs and trees whenever there are ecosystem stresses (Davison and Shearer, 1989). The disease which Australian foresters refer to as dieback results in spectacular

damage and often widespread mortality. This may vary from sudden and lethal wilt of apparently healthy trees to chronic dieback over many years prior to death. In most cases *P. cinnamomi* has been implicated as the causal organism of this disease.

General symptoms of the disease associated with the presence of *P. cinnamomi* include leaf yellowing, leaf shedding without replacement, microphylly, epicormic shooting, stunting, loss of fine branches - occurring first in the upper crown but later extending throughout the plant and to the larger branches (Dell and Malajczuk, 1989). The primary symptoms of infection are evident as advancing fronts of necrosis (lesions) in the inner bark of roots and stems. The fungus kills its host by destroying roots and girdling the base of the stem, depriving the plant of access to water and nutrients (Bridgewater and Edgar, 1994). By this action *P. cinnamomi* indirectly causes plant death by the rotting of the root system.

In *Banksia* species the disease is characterized by rapid wilting and death of new shoots and other unlignified tissues followed by marginal scorching of older leaves. Although these symptoms may be restricted to the top half of a mature plant, the disease eventually spreads, causing intense browning of vascular elements in the collar region of the stem, and finally, death of the plant (Cho, 1981; Dixon *et al.*, 1984; McCredie *et al.*, 1985c).

1.5 Mechanisms of Resistance to Fungal Pathogens

1.5.1 Host-Pathogen Interactions

The biochemistry of plant-parasite relationships and the involvement of phenolic compounds has been examined in detail (see Strobel, 1976; Friend, 1981; Jones, 1983; Hahlbrock and Scheel, 1989 for reviews). It has been assumed that the fundamental role of phenolic compounds in plants is to protect them against disease caused by fungi and other pathogens (Friend, 1981). This assumption is due to the accumulation of phenolic material in many plants after infection (Tarr, 1972).

Phenolic compounds consist of an aromatic ring with one or more hydroxyl groups (Mole and Waterman, 1994). They include such classes as lignins, anthocyanins, flavonoids, coumarin derivatives and quinone derivatives (Lea and Leegood, 1993). These compounds are derived from phenylalanine and their biosynthesis is commonly referred to as the phenol pathway (Lea and Leegood, 1993). The products of this pathway constitute the secondary metabolism which occurs in plants in response to infection. The substances referred to above are essentially the 'first line' of chemical defence. In live tissues one option of defence is to synthesize more of these substances. Alternatively, they may be released from a stored, inactive form.

The one system of chemical defence in plants thought to be specific to microbial infection is where substances, not found before infection, are synthesized as a result of infection. The problem in studying such compounds has always been the uncertainty that these metabolites are of defensive significance. Where a defensive significance is apparent, such compounds are called phytoalexins

(Fritig and Legrand, 1993). Both the speed of their induction and their toxicity are vital to their utility to the plant. The substances reported to be involved are mostly structurally complex and include isoflavonoids, stilbenes, benzofurans and the simple phenolic acid, benzoic acid (Daniel and Purkayastha, 1995).

1.5.2 Mechanisms of Resistance

1.5.2.1 Phenolics

Enzyme activity in the phenol pathway changes conspicuously following infection with fungal pathogens. This led to the conclusion that resistance might be correlated with changes in phenol metabolism of the host (Dickinson and Lucas, 1977). The enzyme that has been considered important in these responses is the key enzyme involved in phenolic biosynthesis, phenylalanine ammonia-lyase (PAL). The subcellular location of PAL is mainly cytoplasmic, although it may also be associated with some membranous organelles.

In dicotyledons (Magnolidae) PAL converts phenylalanine to *trans*-cinnamic acid. This is a point where amino acids are diverted from protein synthesis to phenylpropanoid metabolism, providing a skeleton which forms the basis for compounds such as lignins, coumarins, polyphenols and flavonoids.

There are three mechanisms proposed to function in the response of plants to infection. The first mechanism involves increases in pentose phosphate when tissues become infected, yielding erythrose phosphate and NADPH (Kosuge,

1969). An increase in these two substances directly affects the shikimic acid pathway, resulting in a net increase in phenolic compounds and is the basic mechanism of biochemical resistance in higher plants (Dickinson and Lucas, 1977).

Another mechanism is the feedback inhibition or activation mechanism. This works by inhibiting or activating an enzyme by-product in the pathway in which the enzyme is involved. An example of this is PAL, which is strongly inhibited by its product *trans*-cinnamic acid and also *para*-coumarate (Wheeler, 1975).

The third mechanism, repression or induction of enzyme synthesis, can also be illustrated using PAL. The *de novo* synthesis of PAL following injury can be suppressed by inhibitors of protein synthesis, for example puromycin and cycloheximide. This suggests that synthesis of PAL in healthy tissue is repressed and that derepression occurs following infection. Stimulation of PAL activity in plants increases rapidly after a lag of 3-6 hours following wounding and reaches a maximum between 24 and 48 hours (Friend *et al.*, 1973).

It appears that PAL activity is a highly sensitive indicator of disturbances in the normal state of the cell (Bailey, 1986). Its activity is induced in response to infection by fungi or by exposure to the polysaccharides derived from them. PAL activity has also been correlated with production of phytoalexins and increased lignification.

1.5.2.2 Lignin

Lignin is known to be one of the most abundant biopolymers on earth and is resistant to degradation by most microorganisms (Vance *et al.*, 1980). It is this fact which associates lignin with disease resistance. The deposition of phenolic-derived lignin material occurs in the cell walls or cell wall fractions of plants following infection with an incompatible fungus (Legrand *et al.*, 1975). Such a process is generally referred to as lignification.

The possible role of lignin formation in disease resistance is yet unresolved. Evidence for its formation as a resistance mechanism is limited to only a number of cases (Vance *et al.*, 1980). Induced lignification around infection sites is generally accompanied by increased activity of a number of enzymes, namely PAL (Sarkanen and Ludwig, 1971). For example, Friend (1979) recorded increases in the enzymes PAL, *o*-methyltransferase and peroxidase in resistant lignifying potato tissues. This and other studies suggest that there is a concerted increase in enzyme activity as lignin biosynthesis is induced (Sarkanen and Ludwig, 1971).

Increase in enzyme activity may reflect activation or induction of multienzyme complexes associated with lignin biosynthesis. It has also been suggested by Friend (1979), that the activation of enzymes and deposition of lignin-like material occurs more rapidly in resistant than susceptible tissues. This was shown by a more rapid increase in PAL production in resistant, rather than susceptible potato tubers inoculated with *P. infestans* (Mont.) de Bary.

In several recent studies an obvious association between the formation of lignin after infection and some degree of resistance to the pathogen was evident. These studies examined lignin deposition in almond, avocado, soybean, corn, pine and native species of *Acacia* and *Eucalyptus* (Bonhoff *et al.*, 1987; Cahill and Weste, 1983; Cahill *et al.*, 1989; Cahill and McComb, 1992; Friend *et al.*, 1973; Phillips *et al.*, 1987).

Ride (1978) suggested five ways in which lignification works as a mechanism of a plants' resistance. Firstly, lignification may make walls more resistant to mechanical penetration. Within cells lignin is laid down near the walls to form a network of hemicellulose fibres. In fully lignified tissues this matrix forms a physical barrier which is impenetrable to the polysaccharides of fungal pathogens. Lignified zones, however, are not always effective barriers against penetration (Vance *et al.*, 1980).

Secondly, lignification of the wall at the point of attack may render it resistant to dissolution by fungal enzymes. Lignin formation results in chemical alteration of the polymeric carbohydrates in the cell wall of the host, so they can no longer act as substrates for the cell wall degrading enzymes of the pathogen (Friend, 1981). An example of this is the effect of lignification in resistant tuber tissue of potato infected with *P. infestans* (Aveyard *et al.*, 1973). The mechanism of invasion of *P. infestans* involves hydrolysis by an extracellular fungal galactanase of the polymeric β-1, 4-galactan component of the pectic fraction of

the potato cell wall (Aveyard *et al.*, 1973). Although it is possible that a mechanical barrier could prevent the hydrolytic action of the galactonase by separating the enzyme and the substrate, it seems more probable that lignification would be acting as a chemical barrier to the fungus.

Thirdly, lignification of walls may restrict diffusion of enzymes and toxins from the fungus to the host, and of water and nutrients from the host to the fungus, which would starve the fungus. Fourthly, phytoalexins and free radicals produced during polymerisation, may inactivate fungal membranes, enzymes, toxins and elicitors as a result of their antimicrobial properties. Finally, the hyphal tips of the pathogen may become lignified and lose plasticity which is necessary for growth. This is due to the mycelium absorbing the lignin.

1.5.3 Phenolics as Biochemical Markers for Resistance

The mechanisms which underlie the resistance of a host to a particular pathogen usually have as their basis specific alterations in host biochemistry. Constituents of the phenylpropanoid pathway are used as biochemical markers of resistance of the host against infection. This has been attempted in studies on field resistant *E. calophylla* R. Br. and field susceptible, E. *marginata*. Lignin, total soluble phenolics and PAL concentrations were compared between the two species to identify possible resistant responses (Cahill and McComb, 1992).

Resistance and susceptibility require definition. Weste and Marks (1987) define resistance (to *P. cinnamomi*) as a response occurring after penetration and limited colonization. Field resistance is characterized by the formation of anatomical barriers to fungal growth, such as callose deposits that may enclose individual hyphae and the periderm that may seal off the lesions. No secondary symptoms occur in the shoots, and other physiological changes are absent. Nevertheless, the pathogen remains viable and may reproduce on the field-resistant host.

Anatomical changes which result in barriers to invasion within root tissues do not generally occur in susceptible individuals (Cahill *et al.*, 1993). However, it has been shown to occur in several avocado root stocks, mature secondary tissues of *E. marginata* and infected seedling roots of several *Banksia* species (Phillips *et al.*, 1987; Tippett and Hill; 1984).

Support for the concept of phenolic compounds and its derivitives as biochemical markers is demonstrated from several studies on *E. marginata* and *E. calophylla*. Comparisons in PAL alterations between species has been examined by Cahill and McComb (1992). *E. calophylla* and *E. marginata* were infected with *P. cinnamomi* and the temporal and spatial changes of PAL activity, lignin concentration and concentration of soluble phenolics in the roots was measured. There was found to be increased activity of PAL, lignin and phenolics within 48 hours after infection in *E. calophylla*. In contrast these changes were absent in the roots of the susceptible *E. marginata*.

Cahill *et al.* (1993) reported that lines of micropropagated *E. marginata*, selected on the basis of their resistance to *P. cinnamomi* increased their activity of PAL, lignin and phenolic synthesis. Higher production of these compounds and lesion restriction was associated with the resistance of selected clonal lines. The contribution to resistance which both lignin and phenolics may have was indicated by their relative increased levels compared to other seedlings.

1.5.4 Breeding for Resistance

Evidence for resistance to *P. cinnamomi* has been established in several tree species including eucalypts and pines (Tippett *et al.*, 1985; Stukely and Crane, 1994; Butcher *et al.*, 1983). Clonal propagation of such individuals is considered to be a useful way to fully utilise their genetic potential. Techniques are now available for cloning seedlings and mature trees of certain species (McComb *et al.*, 1996), as a result making it possible to propagate resistant biotypes. These can then be produced in large quantities and integrated into breeding programmes, for use in commercial plantations, and for forest and mining rehabilitation.

A similar variation in tolerance has been observed in *Banksia* species (Dixon *et al.*, 1984; McCredie *et al.*, 1985b; Cho, 1983; Tynan *et al.*, 1995). Clonal propagation of this group has proved difficult, however, grafting techniques from disease resistant root-stocks have been used to cultivate horticulturally

important species of *Banksia* (McCredie *et al.*, 1985a; Turnbull, 1991) and to date banksias have been micropropagated with only limited success (Tynan, 1994).

Techniques developed to identify factors that contribute to tolerance or resistance in other plant groups need to be adapted to develop a reliable method to screen for these factors in *Banksia*. A biochemical screening method for *Banksia* would result in a better understanding of how to combat *P. cinnamomi* infection in this susceptible genus.

1.6 Aims

1. To determine if biochemical analysis can indicate resistance in *Banksia* species both inter and intraspecifically.

2. To determine if compounds used in biochemical defence in *Banksia* species alter in concentration over time in response to infection by *P. cinnamomi*.

3. To determine how phosphonates may affect the early responses of *Banksia* species to infection by *P. cinnamomi*.

4. To determine necessary growth requirements for *Banksia* species maintained in an aeroponics system.

5. To produce a reliable method of inoculating *Banksia* roots in an aeroponics system.

1.7 Hypotheses

1. The level of phenolics produced in *Banksia* species after infection with *P. cinnamomi* is related to genetic differences between individuals/species and is correlated with resistance to infection

2. The level of lignin produced in *Banksia* species infected with *P. cinnamomi* is related to genetic differences of individuals/species and is correlated with resistance to infection

3. Phosphonates alter early responses of *Banksia* species infected with *P. cinnamomi*

2. GENERAL MATERIALS AND METHODS

2.1 Plant Material And Maintenance

Seedlings of *B. menziesii*, *B. attenuata* and *B. grandis* were either purchased from commercial nurseries or germinated from seed. Plants were grown in an aeroponics system (Figure 2.1; Figure 2.2) where they were contained in 50 mm bottomless pots which allowed roots to extend down into the airspace towards a film of water at the base of the tub.

Plants were watered twice daily and fertilised three times per week (1 g L^{-1}) with Thrive® (Yates and Co. Pty Ltd, NSW, Australia). An iron supplement, Librel Fe.Lo®, (Atlas Interlates, Lancshire, England) was also given to plants weekly (0.1 g L^{-1}). Shoots and dead or unhealthy roots were regularly trimmed and the aeroponics tubs cleaned with bleach and water. When plants were approximately 3-6 months of age root material was used for inoculation and subsequent biochemical analysis.

Perlite was used to cover the tops of the pots to retain moisture but was later removed due to prominent algal growth throughout the perlite. Perlite was also a hinderance when removing plants from the system by falling into the tub and blocking drainage holes. Tubs were drained frequently as those plants with longer roots which extended into the liquid at the base of the tubs began to rot.

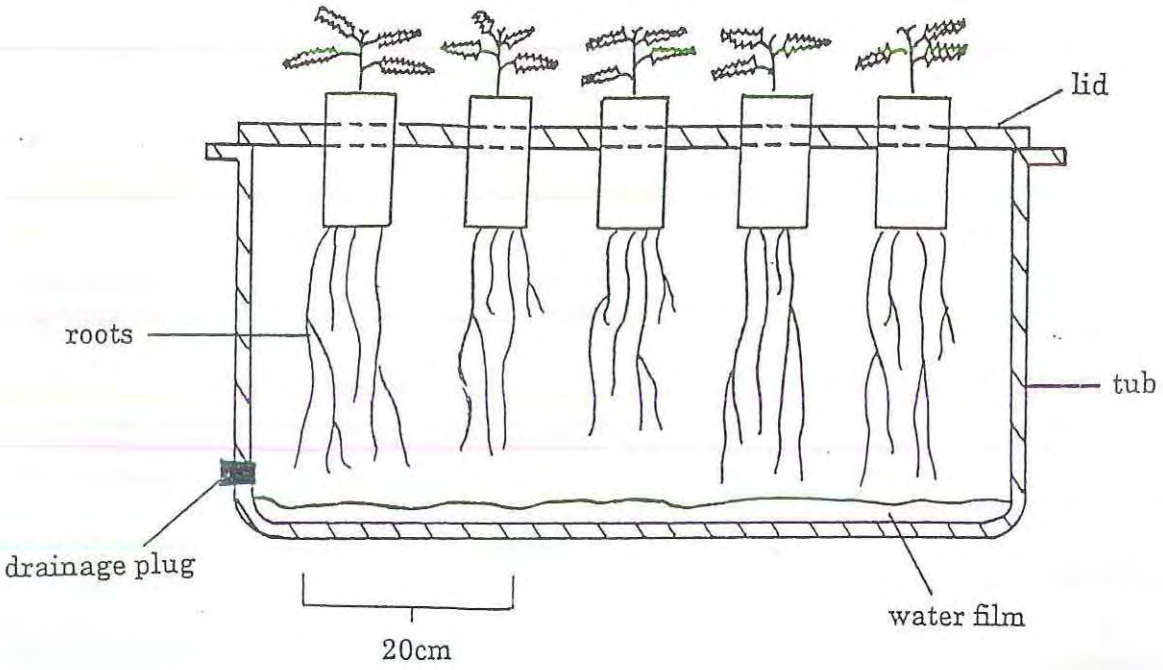

Figure 2.1 Aeroponics system. The roots extend down into the airspace and are watered frequentley to prevent desiccation. A thin film of water at the base of the tubs maintains the humidity and the water level is kept below the root tips by drainage plugs.

Figure 2.2 Aeroponics System. Root systems of healthy *B. menziesii* individuals growing down towards the water at the base of the tub. White bar represents 4 cm.

The first batch of seedlings used in the project were re-potted into bottomless pots to discourage seedlings from becoming root bound and to allow roots to grow down into the airspace. The base of the pots was covered with plastic netting to stop the seedling from falling through while still allowing root growth. However, due to small amounts of soil being lost from the pots when grown in this way the following batch of seedlings were left in the original pots which had gaps around the perimeter of the base only. Although this prevented the loss of soil and produced some good quality roots, the number of roots produced by an individual was limited. Six to eight roots per plant were needed for biochemical analysis and as this requirement was not met the base of all pots were removed and replaced with mesh to encourage root growth. In those plants with slow root growth, such as individuals of *B. grandis,* black plastic bags were wrapped around the plants individually to improve humidity and eliminate any light penetrating the system. Holes were punched in the bottom of the bags to allow drainage of water. This method increased the growth rate of roots compared to those individuals without bags (see section 3.3.5) and was therefore applied to all plants.

2.2 *P. cinnamomi* Maintenance and Zoospore Production

The *P. cinnamomi* isolate (MP27) used in this study was obtained from Murdoch University, courtesy of Dr Giles Hardy. It was maintained on 10% V8 agar (10% V8 juice [Campbell's Soups (Aust.) Pty Ltd, Lemnos, Australia], 0.002% β-sitosterol [Sigma Chemical Co., St Louis, MO], 0.01% $CaCO_3$ and 1.7% Bitek gar [Difco, Detroit, MI]) and subcultured regularly.

The method used for zoospore production was based on that of Gees and Coffey (1989). Eight to ten mycelial plugs (3 mm^2) were cut from the edge of a colony that had been growing on V8 agar for seven days. The plugs were placed in a 90 mm diameter Petri dish and covered with V8 broth (5% V8 juice, 0.01% $CaCO_3$ and 0.002% β-sitosterol) and incubated in full light at 25 °C for 24 hours. The plugs were then rinsed three times with sterile, deionised water (SDW), resuspended in 10 mL non-sterile soil extract (1% soil mixed with SDW, stirred overnight, filtered through a No.1 Watman filter paper) and replaced in the incubater until sporangia were visible (approximately two days). Cultures were placed at 4 °C for 30 minutes in darkness and then exposed to light (180 μmol m^{-2} s^{-1}) at 18 °C for one hour to stimulate the release of zoospores from the sporangia. The zoospore suspension was filtered through a sterile Kimwipe® [Kimberley-Clark (Aust.) Pty Ltd, NSW, Australia] before inoculation in Experiment 1. Zoospore concentration was determined by counting the number of zoospores with a haemacytometer and the suspensions contained between 1.5 to 3 x 10^4 zoospores per mL.

2.3 Phenolic Analysis

The assay for the determination of total phenolics was based on techniques of Swain and Hillis (1959). Three segments, each of 2 cm were cut from the roots beginning at the root tip. These were ground using a mortar and pestle and then extracted twice in 4 mL of methanol. Insoluble material was removed by centrifugation (5 000 g, 5 min.) and 1 mL of extract was added to 0.5 mL of 2 M Folin-Ciocalteau reagent [Sigma Chemical Co., St Louis, MO] then mixed thoroughly. After three minutes, 0.5 mL of 1 M Na_2CO_3 was added and left to react for 1 hour with the solution being mixed intermittently. Samples were centrifuged (5 000 g, 5 min.) and 1 mL of supernatant was then recentrifuged in 2 mL centrifuge tubes (5000 g, 10 min.). The absorbance of 1 mL of this supernatant was measured at 725 nm on the spectrophotometer.

The concentration of total phenolics in the extract was calculated from a standard curve using *para*-coumaric acid as the standard. The lower limit of detection of the assay is approximately 0.01 ug mL^{-1} of *para*-coumaric acid equivalents.

2.4 Lignin Analysis

This assay used methods modified from Cahill and McComb (1992), Doster and Bostock (1988) and Hammerschmidt (1984). Root segments were extracted in 4 mL of methanol over 24 hours and the solids then dried at 37 °C for 24 to 48 hours and their dry weights determined. The samples were then ground in 4 mL of 0.5 M NaOH and incubated for 24 hours at room temperature to hydrolyse

wall-bound phenolic acids (Cahill and McComb, 1992). The incubation mixture was neutralised with 0.5 mL of 2 M HCL and the residue collected by centrifugation at 500 g. The residue was washed with 4 mL of SDW and 5 mL of methanol was added and the solids recovered by centrifugation. The pellet was air-dried for 24 hours and resuspended in 5 mL of 2 M HCL to which 0.5 mL of thioglycolic acid (TGA) [Sigma Chemical Co., St Louis, MO] was added. The samples were capped and placed in an oven at 95 °C for 4 hours and then cooled on ice. The acid was drained off and the pellet rinsed with 5 mL of SDW and incubated for 18 hours with 5 mL of NaOH. The supernatant was then collected and the solids rinsed with 4 mL of SDW which was then added to the NaOH supernatant. This was acidified with 1 mL of 2 M HCL and placed at 4 °C for four hours to precipitate the lignothioglycolic acid (LTGA). The supernatant was centrifuged for 10 minutes at 850 g to collect the precipitate. The supernatant was drained off and 3 mL of 0.5 M NaOH was added and the supernatant collected by centrifugation. The absorbance of 1 mL of the supernatant of LTGA was determined at 280 nm (A280) with a spectrophotometer. The amount of LTGA in the sample was calculated as the absorbance at 280 nm^{-1} mL^{-1} g^{-1} dry weight.

TGA is toxic (corrosive) and all stages of the procedure involving this compound were carried out in a fume cupboard.

2.5 Plating Of Roots

Portions (5 mm) from the first three segments of a root were cut and placed sequentially onto Corn Meal Agar (CMA) [Unipath Ltd, Hampshire, England]) in 90 mm Petri dishes supplemented with the antibiotics and fungicides Hymexazol (50 mg L^{-1}) [Sankyo Co. Pty Ltd, Tokyo, Japan], Pimaricin (10 mg L^{-1}) [Sigma Chemical Co., St Louis, MO], Cefotaxime (100 µg L^{-1}) [Sigma Chemical Co.. St Louis, MO] and Rifampicin (50 mg L^{-1}) [Sigma Chemical Co., St Louis, MO] (medium abbreviated to HPCR). Plates were sealed and stored in the dark at 25 ^{0}C. Portions were excised from root segments used in phenolic and lignin analysis. The presence of *P. cinnamomi* was confirmed within two days.

P. cinnamomi was determined by its characteristic growth pattern. When uncertainties arose with fungi of similar growth patterns, stained slides of *P. cinnamomi* mycelium taken from a live culture were used to compare hyphal characteristics of the mycelium growing from the plated roots.

3. PRELIMINARY INOCULATION TESTS

3.1 Introduction

The most appropriate procedure of inoculation of plant roots is species, age and propagation dependant. Mature plants of tree or shrub size can be inoculated by adding infected plugs of agar to the soil of the potted plants as was done on studies of *Pinus radiata* (Butcher *et al.*, 1984). Mature *Banksia* species have been inoculated by placing infected millet seed below the surface of the soil surrounding the plant (Dixon *et al.*, 1990). Another method used by Shearer *et al.* (1987) to infect roots of *E. marginata* and *B. grandis* involves retrieving the roots, applying an agar disc cut from an active culture to the freshly sliced root tip and then incubating at 25 ^{0}C.

Methods of root inoculation used for young plants and seedlings grown and contained in soil have involved the application of inoculum as a homogenate to the soil (Cho, 1981; 1983). There have also been studies where seedlings have not been grown on a soil medium, such as Bonhoff *et al.* (1987), who grew soybean seedlings which under aseptic conditions on wet filter paper and Tippett *et al.* (1977) who inoculated roots of eucalypt which were grown aseptically, while being supported on filter paper bridges in test tubes. Both studies inoculate roots by submerging root tips in a zoospore suspension and their techniques have been termed 'dip inoculation' by Hahn *et al.* (1985).

The most commonly used technique for plants not grown in a soil medium is the drop inoculation method. Plant roots are exposed from their medium and inoculated with a drop of zoospore suspension at the root tip. Species that have been successfully infected by this method include avocado (Phillips *et al.*, 1987), *E. calophylla, E. marginata* (Cahill and McComb, 1992), *E. sieberi* and *E. maculata* (Dawson and Weste, 1984). Studies carried out by Cahill and Weste (1983), Cahill *et al.* (1989) and Tynan (1994) included inoculating members of the genera *Acacia, Banksia, Juncus, Xanthorrhoea,* and *Zea.*

Presently, there is no documented inoculation procedure for plants growing in an aeroponics system as described in section 2.1. Both the drop and dip inoculation methods are applicable to such a system. However, the drop inoculation technique has been most widely used in other exposed-root systems and thus was chosen as the method of inoculation in initial experiments (see section 4.2.4).

After difficulties were incurred recovering *P. cinnamomi* from infected roots individuals of both *B. menziesii* and *B. attenuata* were used in a series of experiments that investigated differing methods of inoculation. Several preliminary trials were performed to provide a reliable method of inoculation in the aeroponics system. This resulted in adjustments to previous methods (see section 4.2.3) used by Cahill and McComb (1992). The effect of specific antibiotics, surface sterilisation of roots and modification of the inoculation technique, on the rate of recovery of *P. cinnamomi* was examined.

3.2 Materials And Methods

3.2.1 Trial 1

Four seedlings of *B. menziesii* were inoculated to examine lesion developement caused by *P. cinnamomi* after four days. For inoculation of roots, plants were removed from the aeroponic tubs to the laboratory bench. All but the three healthiest roots chosen for inoculation were cut away and the three remaining were inoculated with a 10 µL drop of zoospore suspension (yields of 1-3 x 10^4 per mL) delivered with a micropipette. The plants were then left on the bench for 24 hours before being returned to the aeroponics system. Over the 24 hour period the plants were sprayed with SDW and covered with plastic to prevent desiccation. The roots were harvested after four days, cut sequentially and plated on CMA supplemented with Pimaricin (10 mg L^{-1}), Cefataxime (100 µg L^{-1}) and Rifampicin (50 mg L^{-1}) as in section 2.5.

3.2.2 Trial 2

In an initial experiment (Experiment 1) recovery of *P. cinnamomi* was low and contaminated with *Pythium* and other fungal species. Three month old seedlings of *B. menziesii* were inoculated and used to assess the effect of the fungicide Hymexazol added to the selective CMA. Three roots per plant were inoculated using the methods described in section 4.2.4. A separate set of *B. menziesii* seedlings were treated with distilled water in place of inoculum and served as controls in this trial. Plants were maintained under glasshouse conditions and roots were harvested after four days. After harvesting, roots were cut into 5 mm

segments, and plated sequentially onto CMA supplemented with Pimaricin, Cefataxime and Rifampicin at the same concentrations as Trial 1 with the addition of Hymexazol at 50 mg L^{-1}.

3.2.3 Trial 3

Twenty five individuals of *B. menziesii* and *B. attenuata* were used to test effectiveness of ethanol for surface sterilisation. Five plants per treatment were used for surface sterilisation which involved dipping roots into a beaker of 75% ethanol for 15 seconds followed by several rinses in sterile distilled water. Treatments included: control plants, where roots were uninoculated and unsterilised; inoculated but not sterilised; and inoculated and sterilised roots. In the last treatment half of the plants roots were sterilised before inoculation and the other half were sterilised after inoculation. After inoculation, plants were left covered with plastic on the bench for one hour, as opposed to 24 hours, to reduce desiccation.

3.2.4 Trial 4

In Trial 4 an additional method of inoculation was tested using *B. menziesii*. Ten individuals were used as a control and drop inoculated with water. Another set of seedlings were drop inoculated using the technique outlined in section 3.2.1. A further ten individuals were clamped upright on a retort stand with root tips submerged (up to 5 mm) in a zoospore suspension for 15 minutes and misted regularly to prevent desiccation. All plants were then returned to the aeroponics systems until they were harvested.

3.2.5 Trial 5

In Trials 1 to 3 inoculated plants were placed in newly set up aeroponics tubs which were cool and dry. The lack of humidity in these new tubs was thought to be the factor responsible for the dessicated roots. Therefore, Trial 3 was repeated using aeroponics tubs which had been previously set up in the glasshouse and left for a week.

3.2.6 Trial 6

In this trial ten individuals of *B. menziesii* were used as a control, and ten individuals were inoculated with unfiltered zoospore suspension (see section 2.2). The dip inoculation method was used in an effort to improve recovery of *P. cinnamomi*. Phenolic analysis was performed on roots from this trial (see section 4.2.4).

3.2.7 Trial 7

Initially plants produced adequate root growth, however, possibly due to seasonal variation, later root production decreased. Plant growth methods were then modified to increase root growth. Black plastic bags were attached around the seedling pots which prevented light penetration and increased the humidity around the roots. The effectiveness of applying these bags was monitored by comparing mean root increase in individuals with bags and without, using *B. attenuata, B. menziesii* and *B. grandis*.

3.3 Results

3.3.1 Trial 1

P. cinnamomi was recovered from three out of the four plants. Lesions extended from 0.5 cm to 4 cm from the root tip (Table 3.1).

3.3.2 Trials 2, 3 and 4

No *P. cinnamomi* was recovered from roots inoculated in these trials. Also, in all trials it was noted that roots had appeared to have dried out by the time they were harvested.

3.3.3 Trial 5

In this trial roots were returned to established systems and as a result, roots were moist and fleshy. Measurement of zoospore yields using a haemacytometer showed zoospore suspensions which were filtered through a sterile Kimwipe® had a decrease in the number of mobile zoospores compared with unfiltered suspensions. *P. cinnamomi* was recovered on one plate from an individual in the dip inoculation treatment.

Table 3.1 Lesion development in seedlings of *B. menziesii* after being inoculated with a drop of *P. cinnamomi* zoospore suspension (3 x 10^4 per mL). Means calculated from three replicates.

Plant number	Mean lesion length (mm) ± se
1	16 ± 0.9
2	26 ± 0.4
3	-
4	32 ± 0.4

Table 3.2 Lesion length from plated roots of *B. menziesii* following dip inoculation with *P. cinnamomi* zoospores (2.5 x 10^4 per mL). Means calculated from five plants.

Plant number	Mean lesion length (mm) ± se
1	20 ± 0
2	29 ± 0.6

3.3.4 Trial 6

Inoculation for Trial 6 used unfiltered zoospore suspension and *P. cinnamomi* was recovered from seven of the ten roots. The roots used in this trial were moist and the presence of *P. cinnamomi* up the root length ranged from 5 mm to 50 mm (Table 3.2; Table 3.3).

3.3.5 Trial 7

Individuals of all three species that had their roots placed in black plastic bags had a significantly greater increase in mean root length but this increase varied between the three species. The species that showed the greatest response to this treatment was *B. menziesii* (Figure 3.1) which by the end of week six had a mean increase of 120 mm in contrast with the control which had only 20 mm. *B. grandis* (Figure 3.3) had the lowest root increase of 15 mm over four weeks, only 5 mm more than the control. Those individuals with bags displayed an initial spurt of root growth in the first week which then, generally, leveled off.

Table 3.3 Summary of trial results, alterations and problems encounted while developing a technique for inoculation of *Banksia* roots in an aeroponics system.

	Trial					
Response	1	2	3	4	5	6
P. cinnamomi present	+	-	-	-	+	+
Recovery of *P. cinnamomi* (%)	75	-	-	-	10	60
root condition	moist	dry	dry	dry	moist	moist
variables added	Hymexazol	ethanol dip	dip inoculation			
variables altered		plants left on bench for 1 hour only		aeroponics tubs set up a week prior	used unfiltered zoospore suspension	
problems encountered	*fungal competition *root dessication when left out for 24 hours	*ethanol too harsh/drying *inoculation method too drying	*aeroponics tubs not humid	*filtering zoospore suspension reduced yield		

3.4 Discussion

These trials were required to identify the variables in the aeroponics system that caused difficulties in inoculating plant roots. Plants grown in this system were susceptible to desiccation and therefore a method that minimised root exposure needed to be identified. Inoculation of plants grown in this system were successful when alterations (see Table 3.3) were made to previous methods used to inoculate *Banksia* species (see section 3.2.1).

Failure to attain a high rate of recovery of *P. cinnamomi* was amended in several ways. Firstly, competition with *P. cinnamomi* by other fungal species on plates was reduced with the addition of the specific fungicide Hymexazol. The restriction of growth of other species also enabled easier identification of *P. cinnamomi* mycelium from plated roots. Surface sterilisation was another method used in Trial 3 to eliminate competition; treatment with ethanol, however, caused roots to dry out and subsequently the ethanol treatment was not continued.

Another factor thought to be responsible for low recovery of *P. cinnamomi* was the drying of roots. This was initially thought to be a result of being removed from the humid aeroponic system for 24 hours and this time was therefore reduced to 1 hour in Trial 3. In Trial 4 the dip inoculation method was used which again reduced the time required for roots to be out of the system to 15 minutes. This, however, did not improve the condition of the roots and it was then thought that returning plants to a newly set up system had an effect on root

moisture levels. Establishing the aeroponics systems a week prior to placing inoculated seedlings into the tubs ensured the humid conditions.

It is likely that all the above factors combined eventually resulted in high recovery of *P. cinnamomi* from infected plants. Adequate recovery of *P. cinnamomi* in Trial 6 enabled Experiments 2,3,4 and 5 in the following chapter to be performed knowing that inoculation was likely to be successful.

Decrease in root growth noted in all species in the glasshouse from May to October may be attributed to seasonal changes. Due to the the success in Trial 7, all other plants in aeroponics systems were grown with the plastic bags. As a result of this, and possibly due to increasing light periods, by November growth returned to rates similar to those present in initial experiments.

4. LIGNIN AND PHENOLIC ANALYSIS

4.1 Introduction

Compounds that have been used as a measure of resistance to *P. cinnamomi* in a range of species are lignin, total phenolics and phenylalanine ammonia-lyase. Biochemical assays for these compounds performed on eucalypts (Cahill and McComb, 1992) have determined PAL activity in extracts from roots by the conversion of *L*-phenylalanine to cinnamic acid, lignin as its thioglycolic acid derivative (section 2.4) and total soluble phenolics from methanolic extracts using the Folin-Ciocalteau reagent (section 2.3). Analysis of these compounds in response to infection by *P. cinnamomi*, to date, has not been investigated in *Banksia* species. Banksias are considered more susceptible than *Eucalyptus* species (Shearer and Tippett, 1989) and as a result may lack this induced phenolic synthesis.

Quantitative analysis of phenolic compounds in relation to *P. cinnamomi* infection has been carried out on many plants including almonds, wheat, potatoes, plums, soybean and eucalypts. On the basis of phenolic activity, these plants show varying susceptibility to *P. cinnamomi*. Studies on clonally propagated seedlings of *Eucalyptus* (Cahill *et al.*, 1992; 1993) have demonstrated genetically based resistance to *P. cinnamomi*, which has been correlated to increased activity of phenolic products in roots after infection. This has also been demonstrated in sedges and grasses (Cahill *et al.*, 1986; Phillips and Weste, 1984) and avocado rootstocks (Gabor and Coffey, 1991; Phillips *et al.*, 1987).

Therefore, one aim of this research was to determine if the response to infection with *P. cinnamomi* involves increased activity of the phenolic pathway and associated changes in both lignin and phenolic synthesis.

Phosphonates, used in chemical control of *P. cinnamomi*, have increased "resistance" in certain species by triggering host defence mechanisms (Smillie *et al.*, 1989). A study performed on *Banksia* and *Eucalyptus* species trunk-injected with phosphonate showed that treated plants produce cork cambium which contained lesions caused by *P. cinnamomi* (Shearer, pers. comm.). This study also set safe limits for phosphonate injection to avoid phytotoxicity.

Agricultural species, such as avocado are treated with phosphonate twice yearly at 40% concentration, however, Australian natives are highly sensitive to phosphonate application having evolved in low phosphate soils. Although *Banksia* species were shown to tolerate high concentrations of up to 10 to 20%, these levels were not recommended as phytotoxic reactions occurred. The most effective concentration that controlled *P. cinnamomi* with least side effect, was 1%. In these studies the above recommendations were used and plants were treated with 0.5 to 1% concentrations of Fos-Ject 200®.

Previous to this study it was not known if, and how, phosphonates alter resistance responses to *P. cinnamomi* in *Banksia*. This chapter examines the correlation between phenolic and lignin production and phosphonate application in *Banksia* species.

4.2 Material And Methods

4.2.1 Phosphonate Application

Two foliar applications, two weeks apart of Fos-Ject 200® Fungicide [UIM Agrochemicals (Aust.) Pty Ltd.] were applied to the treatments requiring phosphonate. Recommended phosphonate concentrations of 0.5 to 1% were trialed on both *B. menziesii* and *B. grandis* individuals. Those sprayed with 1% concentrations exhibited leaf burning, stunted growth and often subsequent death. Concentrations of 0.5% did not produce any phytotoxic responses, only minor leaf burns in some individuals of *B. menziesii*. Consequently, phosphonate was applied at 0.5% (to the point of run off or leaf wetness) for all subsequent experiments.

4.2.2 Design of Biochemical Analysis Experiments

Each experiment was performed on both *B. menziesii* and *B. grandis* sampling at four or both four and twelve days after inoculation. Four treatments were used: a control; phosphonate application; phosphonate application and inoculation with *P. cinnamomi*; and inoculation with *P. cinnamomi* only.

The roots of five randomly chosen individuals from each treatment were excised at four and twelve days after zoospore inoculation. Six roots were taken from the individuals chosen, three of these were allocated to phenolic analysis and the remainder to lignin analysis. As some studies have shown a difference in phenolic and lignin content (Cahill and McComb, 1992), the roots were divided

into three, 2 cm sections beginning at the root tip. The phenolic assay used the Folin-Ciocalteau Reagent as outlined in section 2.3. For lignin analysis, roots are extracted in 4 mL methanol for 24 hours. The samples were then dried in an oven and weighed. The concentration of lignin in the sample was determined as its TGA derivative (section 2.4). Portions of the roots used were plated out to recover *P. cinnamomi*, as outlined in section 2.5.

4.2.3 Experiment 1

The effect of *P. cinnamomi* infection on phenolic and lignin activity for treatment, time after inoculation and root segment was investigated in this initial experiment. Individuals of *B. menziesii* were inoculated using the drop inoculation method outlined in section 4.2.1. Roots were harvested and analysed for phenolic and lignin concentrations at four and twelve days after inoculation. At both intervals, root sections were plated out (see section 2.5) to confirm the presence of *P. cinnamomi*.

4.2.4 Experiment 2

Plants used in Trial 6 inoculation test were analysed for phenolic concentration (see section 3.2.6). A lignin assay was not performed and roots were harvested at day four only. The methods used for total phenolic analysis were followed from section 2.3.

4.2.5 Experiments 3, 4 and 5

The inoculation technique outlined in section 3.2.4 was used in these experiments. Design of the experiments is outlined in section 4.2.2. Experiments 3 and 4 harvested root material of *B. menziesii* and *B. grandis*, respectively four days after inoculation. Experiment 5 used *B. menziesii*; root material at four and twelve days after inoculation was analysed for phenolics and lignin.

4.2.6 Statistical Analysis

Treatment, time and root segment means were compared using either a two way or three way analysis of variance (ANOVA). Where a significant difference was found at the 95% confidence level, a two way or one way ANOVA respectively, was performed on the factor/s that were significant. Where appropriate Scheffe's post-hoc multiple range test was performed to determine which treatment means differed significantly ($p<0.05$) from each other.

4.3 Results

4.3.1 Experiment 1

Concentrations of phenolics from *B. menziesii* ranged from 0.01 to 0.07 $\mu g\ g^{-1}$ of fresh weight (Figure 4.1a). There was no significant difference found over time, between root segments or between treatments. Mean concentrations of total

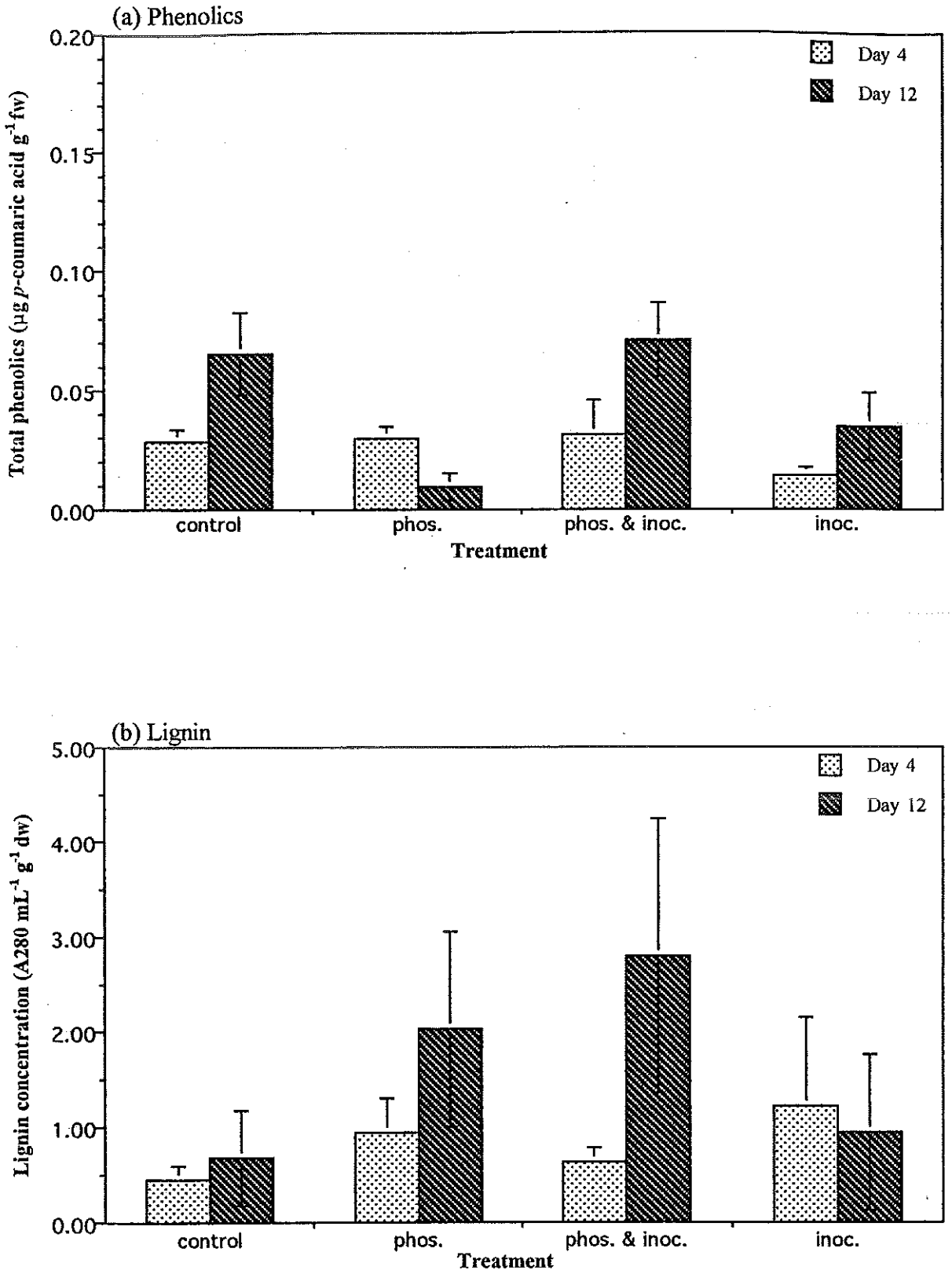

Figure 4.1 Mean concentrations of (a) total soluble phenolics; (b) lignin in *B. menziesii* for treatments at days four and twelve for Experiment 1. Values are means for five replicates. Vertical bars are standard errors.

phenolics and lignin were similar for day four, however, at day twelve there was a general increase for all treatments for lignin and in the control and inoculated treatments for phenolics (Figure 4.1). The highest concentrations of total phenolics and lignin were produced in the phosphonated and inoculated treatment combined at day twelve. No *P. cinnamomi* was recovered from root segments analysed in this experiment.

4.3.2 Experiment 2

In this experiment there was no significant difference between root segments, however, there was a difference ($p<0.05$) between phenolic levels in inoculated and uninoculated treatments of *B. menziesii* (Figure 4.2). Phenolic concentrations in uninoculated plant roots were clearly higher than in inoculated roots. The mean phenolic concentration for control and inoculated treatments was 0.01 and 0.005 $\mu g\ g^{-1}$ of fresh weight. *P. cinnamomi* was recovered from 60% of plated root segments.

4.3.3 Experiment 3

Phenolic levels in *B. menziesii* roots were higher in the control and phosphonate treatments (Figure 4.3a). The control had significantly higher phenolic levels than the phosphonate treatment.

There was no significant difference in the levels of lignin, however, the inoculated treatments generally had higher levels.

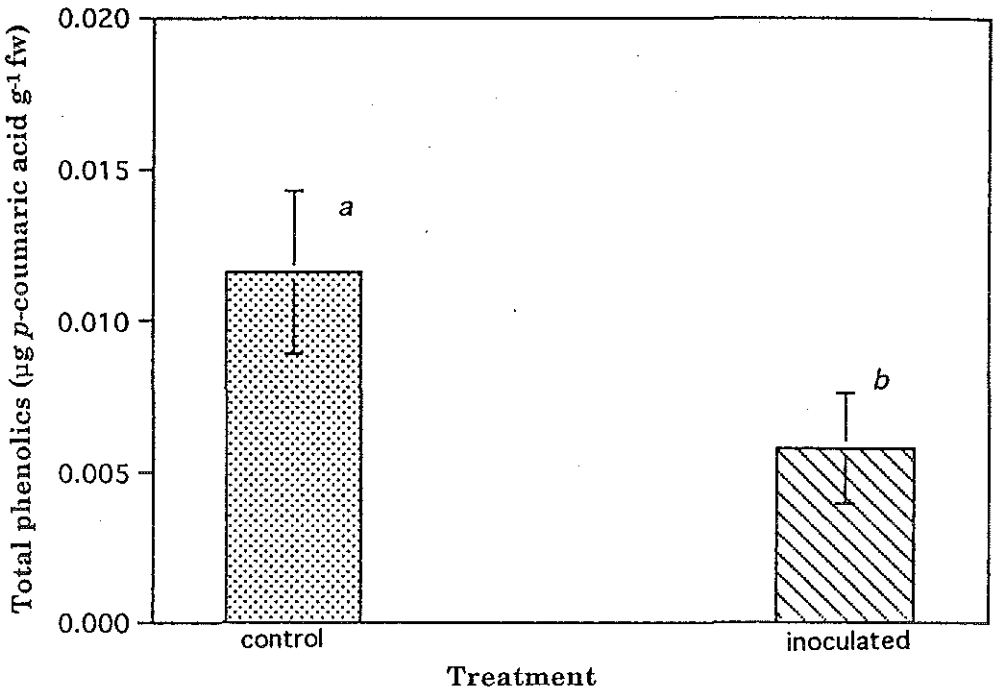

Figure 4.2 Mean concentration of total soluble phenolics in *B. menziesii* for treatments at day four for Experiment 2. Values are means for ten replicates. Vertical bars are standard errors. Letters represent significantly different treatments.

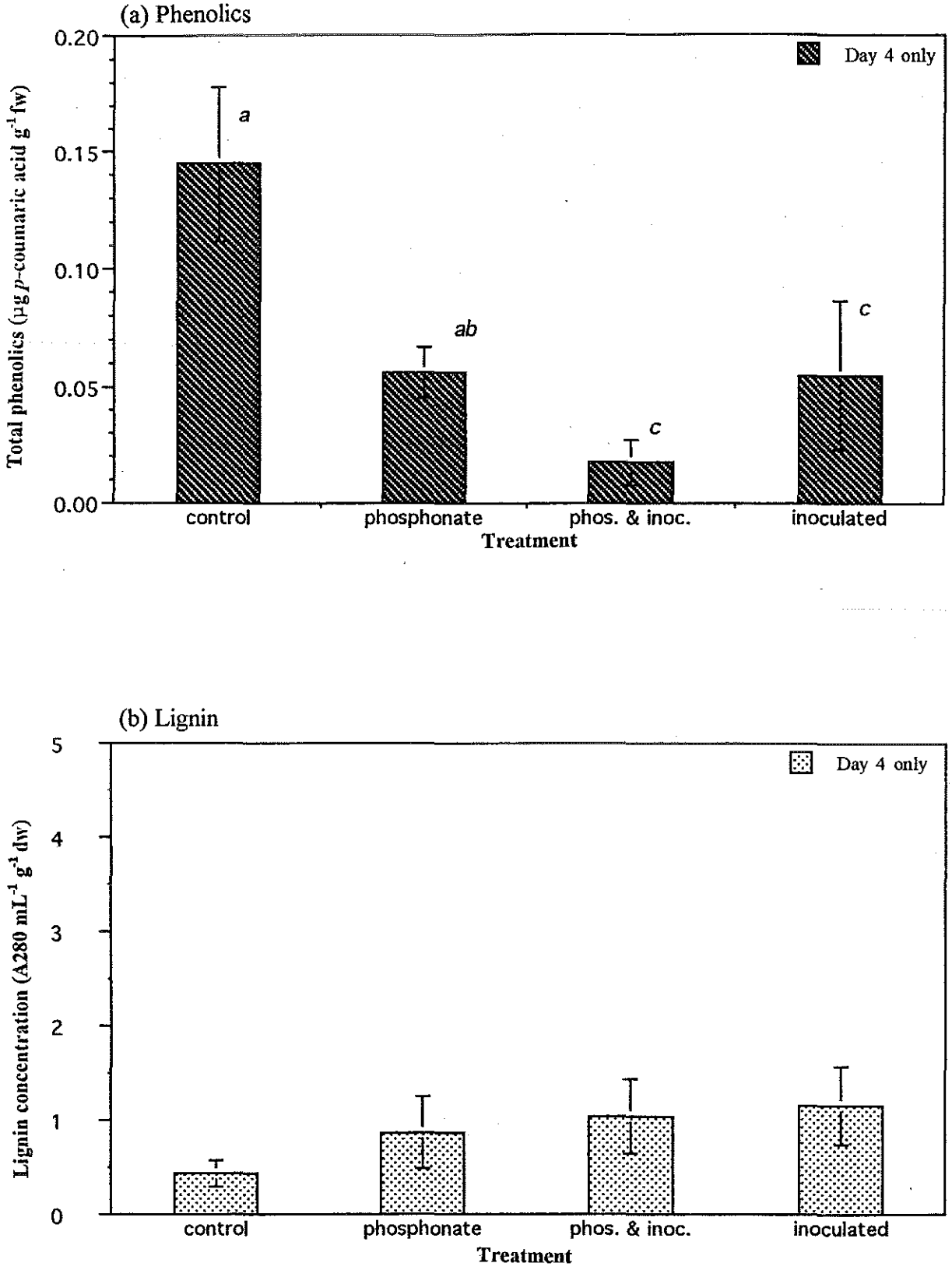

Figure 4.3 Mean concentrations of (a) total soluble phenolics; (b) lignin in *B. menziesii* for treatments at day four for Experiment 3. Values are means for five replicates. Vertical bars are standard errors. Letters represent significantly different treatments.

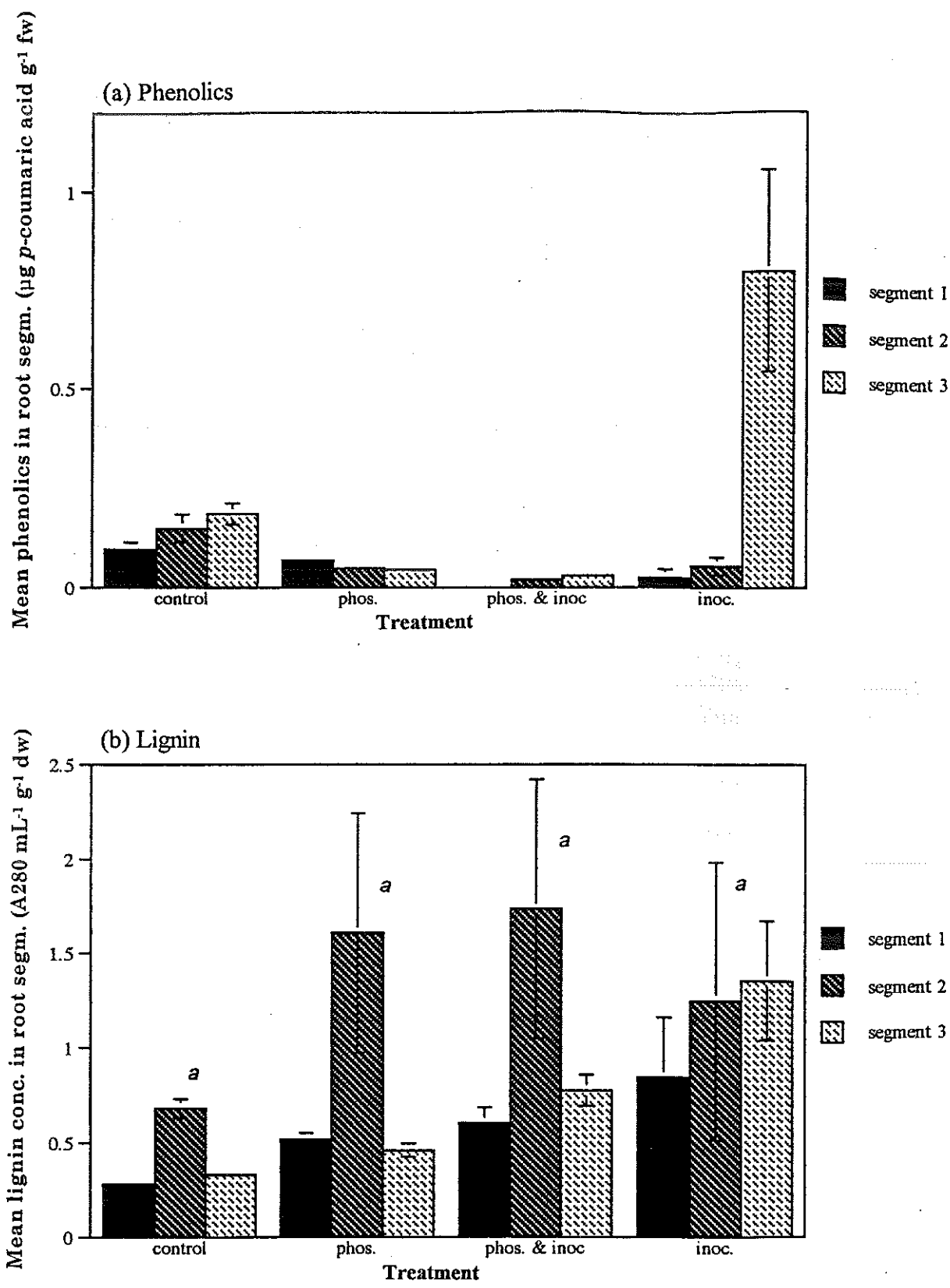

Figure 4.4 Mean concentrations of (a) total soluble phenolics; (b) lignin for treatments in *B. menziesii* root segments in Experiment 3. Values are means for five replicates. Vertical bars are standard errors. Letters represent root segments with significantly different concentrations.

In this experiment there was a significant difference in lignin concentrations between the root segments. Increased lignification was found in segment two (Figure 4.4b), absorbance doubling that of other segments.

P. cinnamomi was present on 90% of plates.

4.3.4 Experiment 4

Phenolic concentrations differed between treatments in this experiment. Phosphonated treatments had the highest phenolic activity (0.13 μg mg^{-1} fresh weight), particularly the inoculated treatment (Figure 4.5a). The inoculated treatment, with no phosphonate, was substantially lower than control levels. This suggests that phosphonate, and not infection with *P. cinnamomi*, is responsible for increased lignin levels in *B. grandis*.

There was no effect of treatment or root section on lignin concentration. However, both inoculated treatments had greater lignin content (0.23 mL^{-1} g^{-1} dry weight) than uninoculated treatments (0.14 mL^{-1} g^{-1} dry weight), with the phosphonate-inoculated treatment having the highest levels of lignin (see Figure 4.5b).

P. cinnamomi was recovered from 90% of plated *B. grandis* roots.

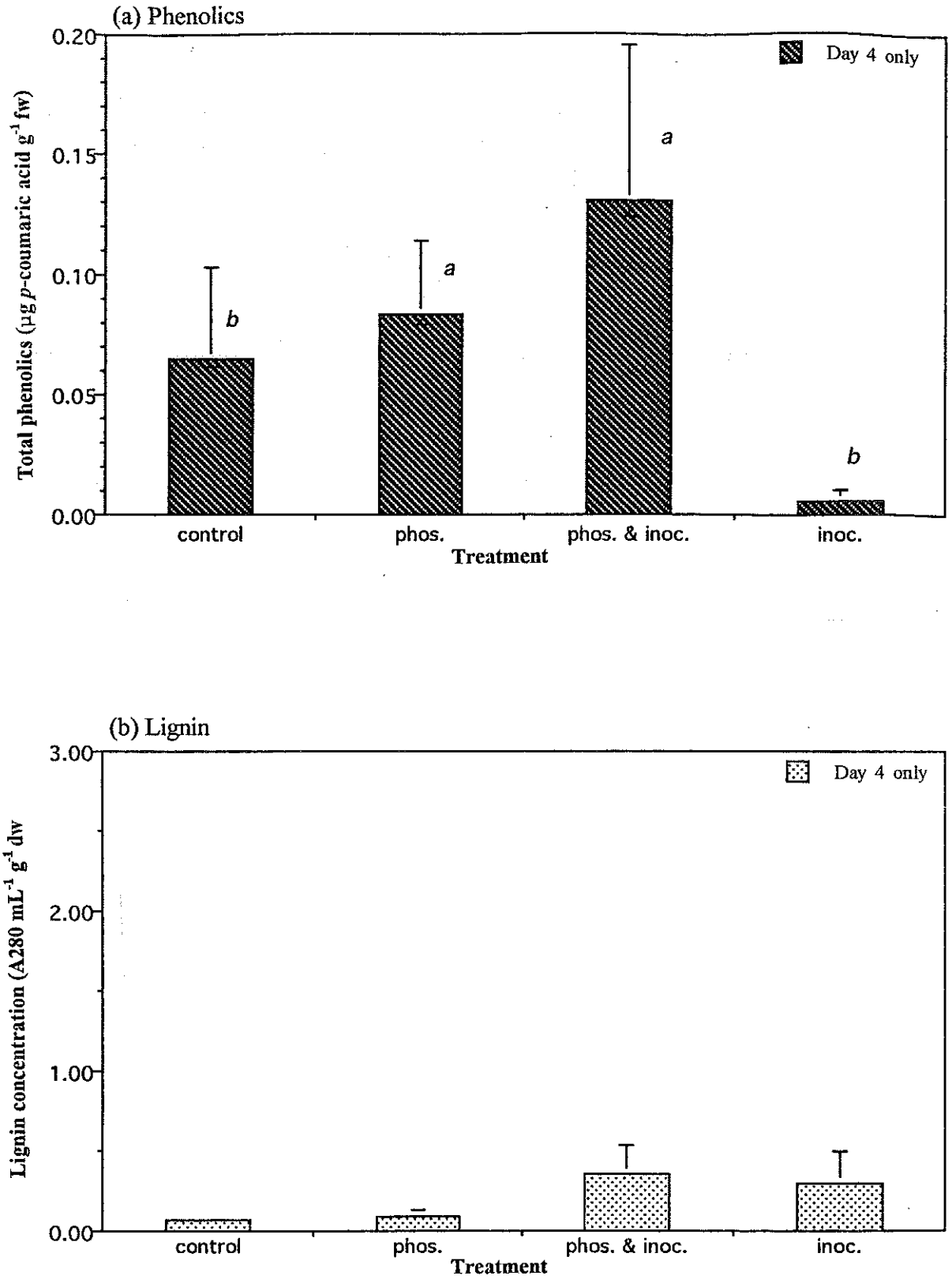

Figure 4.5 Mean concentrations of (a) total soluble phenolics; (b) lignin in *B. grandis* for treatments at day four for Experiment 4. Values are means for five replicates. Vertical bars are standard errors. Letters represent significantly different treatments.

4.3.5 Experiment 5

There was no effect of treatment or root section for phenolic concentration in *B. menziesii* at day four, but phenolic concentrations were higher in the uninoculated treatments (Figure 4.6a). At twelve days there was a significant effect due to treatments, both the control and phosphonate treatments had significantly higher levels of total phenolics than the inoculated treatments (Figure 4.6a).

There was an effect of time on total phenolics levels which were marginally higher in uninoculated treatments on day four and substantially higher on day twelve. After inoculation phenolic activity decreased progressively from days four to twelve (see Figure 4.6a).

Lignin levels increased significantly throughout the duration of the experiment and maximum levels were recorded in inoculated treatments for both day four and day twelve. There was no significant difference in lignin content in root segments for days four and twelve.

P. cinnamomi was recovered from 90% of *B. menziesii* roots.

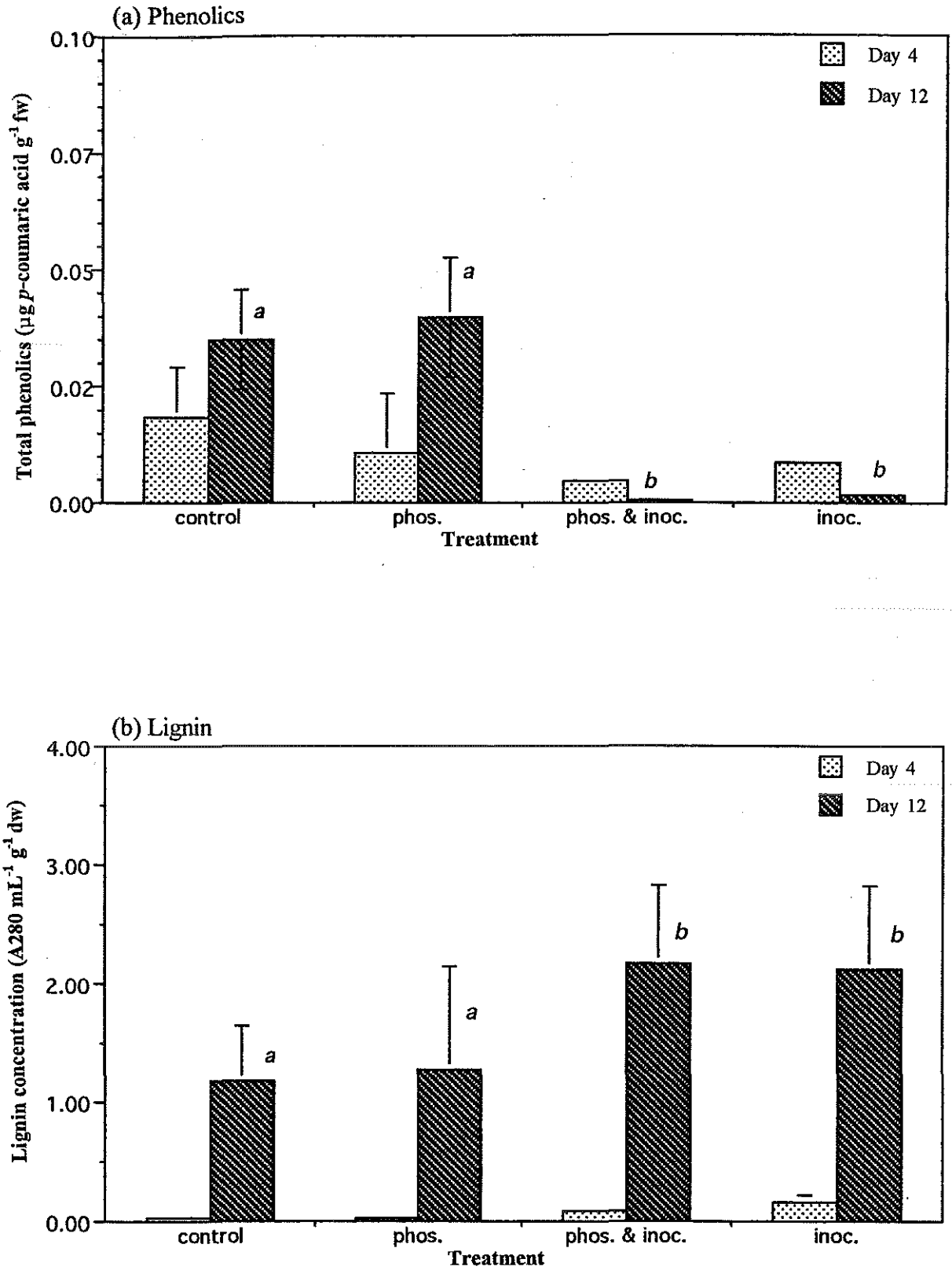

Figure 4.6 Mean concentrations of (a) total soluble phenolics; (b) lignin in *B. menziesii* for treatments at days four and twelve for Experiment 5. Values are means for five replicates. Vertical bars are standard errors. Letters represent significantly different treatments.

4.4 Discussion

4.4.1 Phenolics

Infection of *B. menziesii* and *B. grandis* root systems with *P. cinnamomi* resulted in differences in phenolic concentrations between treatments, and temporal changes within the root. In experiments where *P. cinnamomi* was recovered (2, 3, 4 and 5) inoculated treatments had significantly lower soluble phenolic concentrations. Experiment 5, day four, although not significant at the 95% confidence level, was significant at the 90% confidence level, indicating the same general trend.

Between species this response is consistent. In Experiment 4 (*B. grandis)* phenolic levels in the inoculated treatment were significantly lower than those in the control and phosphonated treatments. The phenolic concentration in the phosphonate-inoculated treatment, however, was high in this experiment. This appears to be an effect of phosphonate application rather than inoculation, as the phosphonated treatments are higher than control and inoculated treatments.

These lower phenolic concentrations in inoculated treatments may indicate that phenolic activity ceases soon after *P. cinnamomi* establishes itself in the host tissue. This is best illustrated in Experiment 5 (Figure 4.6a), where time was a significant factor and phenolic levels were very low twelve days after inoculation. Also, it may be possible that by four days after inoculation the phenolic pathway has already been activated, phenolic compounds have been synthesised in the

roots but as they are susceptible these haven't been effective in combating the fungus. As a result the root systems may already be dead by day four. Therefore in futher research phenolic levels in roots could be monitored at twelve to twenty four hour periods after infection. This was not included in this study as the response time of these compounds in banksias was unknown. It would also require a considerable increase in the number of plants and samples to be processed.

Spatial changes in concentrations of total soluble phenolics in *B. menziesii* and *B. grandis* were not significant. In roots of field resistant *E. calophylla* the highest increase in total phenolics above control levels were recorded in segments one and two (91-97% above control levels) while increases in segment three were 31% above control levels after inoculation. In root segments of *E. marginata* (susceptible) there were relatively small increases in the concentration of total phenolics after inoculation (20% of control values). As spatial changes within *B. menziesii* and *B. grandis* roots were not distinguishable and lower than those of susceptible *E. marginata*, this may indicate the even lower tolerance that *Banksia* species have to *P. cinnamomi*.

4.4.2 Lignin

Lignin produced in inoculated roots, unlike total phenolics, was significantly greater than in uninoculated plants. The higher lignin content in inoculated plants may be due to rapid death of the roots which first dry out producing lignins in the cell walls (Cahill *et al.*, 1989).

An effect of time existed for lignin in Experiment 5 (Figure4.6b). The significant increase in lignin concentration in inoculated plants over time may be a result of the *P. cinnamomi* lesion end point extending into healthy uninfected tissue resulting in heavily necrotic, infected tissue by day twelve (Phillips *et al.*, 1987).

Lignin concentrations in *B. grandis* were congruent with those found in *B. menziesii* where inoculated plants had higher lignin concentrations, with the highest levels present in phosphonate-inoculated plants.

In Experiment 3 the lignin concentrations between root segments were significantly different. The effect observed with regard to root segment for lignin and not phenolics is probably because lignin is produced and deposited at the site of infection and acts as a morphological barrier (Phillips *et al.*, 1987). Segment two was statistically different from one and three and contained higher concentrations of lignin. Although segment one was expected to produce the greatest amount of lignin after infection (Cahill and McComb, 1992), this first segment from inoculated roots may have dried out and reduced in weight by harvesting at day four. Segment two of infected roots were still fleshy but *P. cinnamomi* had extended into this segment and lignin had been deposited as a result. Segment three did not produce as much lignin as this segment may not have been invaded by the pathogen when harvested at day four. This is consistent with the lesion extensions that were obtained (Table 3.1; Table 3.2). In a study performed on *E. calophylla* roots the greatest increase in lignification

was found between 20-40 mm from the root tip which corresponds to root segment two in this study (Cahill and McComb, 1992).

4.4.3 Effect of Phosphonate on Phenolic and Lignin Levels

The increase in phenolic production in *B. grandis* in Experiment 4 may be attributed to differences in species and their response to the concentration of phosphonate application. *B. grandis* exhibited no side effects during preliminary phosphonate trials (see section 4.2.1) where as *B. menziesii* exhibited leaf burning. Uptake and possible translocation of phosphonates may be more efficient in *B. grandis* and this species may be more capable of tolerating the chosen concentration. Shearer (pers. comm.) found that *B. grandis* as well as *E. marginata*, produced dynamic defence mechanisms that were triggered by phosphonate application. Therefore, *B. grandis* may take up and utilise phosphonates more readily and consequently increase phenolic production.

An additional study on *B. grandis*, extended to include analysis of root material at day twelve would be appropriate to see the effects of time on phosphonate activity. Unfortunately, due to limited time and availability of plant material it was not possible for this experiment to be repeated and extended to twelve days.

5. GENERAL DISCUSSION

In this study biochemical responses of *Banksia* species to infection with *P. cinnamomi* were investigated. Measurement of these biochemical responses were used to assess their validity as indicators of resistance to *P. cinnamomi* infection. The reduced synthesis of phenolic substances within *Banksia* roots may be a result of rapid breakdown of metabolic activities (Cahill and McComb, 1992) such as electrolyte leakage, alterations in respiratory pathways, mineral content, concentrations of phytohormones and water relations causing desiccation and subsequent death. This suggests that unlike other native species, such as eucalypts, biochemical defence responses in the *Banksia* genus are not very effective. If so, this would explain the susceptibiltiy and rapid mortality in banksias, and perhaps in other proteaceous species, in comparison to other natives of the Swan Coastal Plain and southern jarrah forest of Western Australia.

Total soluble phenolics and lignin analysis would be useful to indicate resistance intraspecifically, as considerable differences in these compounds occurred between control and inoculated plants. Therefore these assays have the potential to identify individuals which have unusually high total soluble phenolic concentrations after inoculation or even greater concentrations of lignin deposited in inoculated treatments (above approximately 3.5 mL^{-1} g^{-1} dry weight). An application of such a response would be to identify individuals which exhibited significant increases in lignin and phenolic synthesis to use in

micropropagation to preserve and study their genetic potential. If successful, clonal propagation of tolerant *Banksia* individuals may be involved in future projects to rehabilitate mined and diseased areas (Podger *et al.*, 1996). Utilisation of genetically resistant individuals may be an avenue of biological control to complement other approaches.

Phenolic and lignin analysis could also be applied to investigate biochemical correlations with current ranking of *Banksia* species for susceptibility or tolerance. The species used in this study have been described as having high (*B. grandis*) to moderately-high (*B. menziesii*) susceptility by other researchers (Cho, 1983; Tynan *et al.*, 1995). However, in this study it was not possible to compare differences in phenolic levels between species, as difficulties arose in synchronising growth of two species. Furthermore, the logistics of running two experiments concurrently would make the processing of the samples very difficult. However, this might be achievable by optimising the treatment/s and time/s that exhibited the greatest differences and using only these to measure differences between a number of species. Further investigations of total soluble phenolics and lignin concentrations as indicators of resistance levels may be extended to species of other proteaceous genera or agriculturally important plants susceptible to *P. cinnamomi*. Improvement of the phenolic and lignin assays may result in an additional technique available for screening *Banksia* resistance, other methods from past research include the excised root assay (Tynan, 1994), histological tests and field resistance counts (Cho, 1983; McCredie

et al., 1985b). The phenolic and lignin assays appear to be the only biochemical tests available for *Banksia*.

Temporal changes in phenolic and lignin concentrations were only obvious from Experiment 5 as *P. cinnamomi* was not recovered from Experiment 1. This does not confirm that infection did not take place, but this is implicated in the results as no significant effect existed for treament, time or root segment. Results of Experiment 5 suggest that compounds synthesised in defence to *P. cinnamomi* infection in *B. menziesii* alter in concentration over time. Phenolics progressively decreased from day four to twelve and lignin increased. Increase in lignin by day twelve is in accordance with previous findings (Cahill and McComb, 1992).

In uninoculated treatments there was also an apparent difference between four and twelve days in phenolic and lignin concentrations. One reason for this may be that moving the plants to another glasshouse, that differs slightly in temperature, light intensity and airflow, could have stressed the plants causing phenolic compounds to be released. Harvesting of roots after four days and then again at twelve days may have also stressed the plants. These two factors could account for increased phenolic levels in uninoculated plants overtime.

An unexpected amount of time of this study was devoted to developing a technique that allowed easy access to roots. Many alterations were made to produce healthy seedlings in such a system. A reliable method of inoculating *Banksia* roots in such a system was also developed after no *P. cinnamomi* was

recovered from Experiment 1. As a result a new technique is available with possibilities for further research in root infecting pathogens.

In reference to the hypotheses proposed, it was not possible to use the level of total soluble phenolics and lignin produced in *Banksia* species after infection with *P. cinnamomi* to distinguish intraspecific variation. Such an investigation would require greater root production from individuals. Also *B. menziesii* and *B. grandis* were the only species used and are similar in susceptibility ranking. Perhaps inclusion of more species with a range of tolerances would depict this. *B. attenuata* could not be used as a third species in this project as it grew poorly in this system. However, as these two species have medium to low resistance this may be the response of this resistance level.

Response to phosphonate application by the parameters measured was not significant. Phosphonates appeared to have little effect in altering response to infection. This may be true for *B. menziesii* only as *B. grandis* showed significant increases in phenolic concentrations in plants treated with phosphonate. This may be related to differences between species in their tolerance to phosphonate. *B. menziesii* may require more frequent, less concentrated applications to induce resistance responses.

There are many avenues that could be pursued for further study. These include the use of these assays to indicate possible resistance between *Banksia* species, other genera of the Proteaceae family or other species of *Phytophthora.*

Foundations for further research have been set by availability of a system in which other species and root pathogens can be investigated and for which an appropriate inoculation technique exists.

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