Microbial Ecology of *Phytophthora cinnamomi* Suppressive Soils:

A Study of Biological Suppression of *P. cinnamomi* in Sub-Tropical Avocado Orchards on the East Coast of Australia

by

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Statement of Authentication

The work presented in this thesis, to the best of my knowledge and belief, is an original account, except where acknowledged in the text, of my own research endeavour. I hereby declare that I have not submitted work presented in this thesis, either in full or in part, for an award at this or any other institution.

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Abstract

This study focuses on the soil- and water-borne plant pathogen *Phytophthora cinnamomi* Rands and the phenomenon of *P. cinnamomi* suppressive soil. In particular, this thesis reports on the outcome of field surveys and glasshouse assays undertaken to locate *P. cinnamomi* suppressive soils and to confirm the involvement of biological processes in suppression. The potential role of cellulase and laminarinase in suppression was investigated and a molecular technique known as length heterogeneity PCR (LH-PCR) was used to analyse the structure and diversity of bacterial and fungal communities in avocado orchard soils that were suppressive and conducive to *P. cinnamomi*.

Four avocado orchards with *P. cinnamomi* suppressive soils were identified and soils were γ-irradiated to destroy their suppressive capacity, thus confirming biological suppression. Suppression was also partially transferred to γ-irradiated and conducive soils by mixing with 10% suppressive avocado soils.

Cellulase and laminarinase activities measured in avocado orchard soils inoculated with *P. cinnamomi* were not associated with disease severity in lupin seedlings during glasshouse assays involving the same soil samples. In addition, reduced disease severity in avocado seedlings grown in sterile and conducive soils mixed with 10% suppressive soils and inoculated with *P. cinnamomi* was not associated with cellulase or laminarinase activities in these treatments. When cellulase and laminarinase were added to sterile sand inoculated with *P. cinnamomi*, disease severity in lupins did not decrease, and the enzyme solutions appeared to have a phytotoxic effect on the plants.

Neither suppressive nor conducive soils had characteristic bacterial or fungal community LH-PCR profiles. In addition, LH-PCR indicated that bacterial and fungal community diversity was moderate to high in both suppressive and conducive soils (bacterial Shannon index $H' = 2.99 – 3.01$; fungal Shannon index $H' = 3.5 – 3.7$). However, minor shifts in bacterial and fungal community structure were observed in response to mixing conducive and irradiated soils with suppressive soils. This was associated with decreased disease severity in avocado seedlings in these treatments.
The shift in bacterial community structure was partially determined by the appearance and increased abundance of several bacterial 16S rDNA sequences, which were unique to the suppressive soils, in the mixed soil treatments. At least one fungal ITS2 sequence was abundant in the suppressive soils, less abundant in the conducive soil and increased in the conducive and irradiated soils after they were mixed with suppressive soils. It is suggested that the bacteria and fungi from which these sequences originated may be involved in suppression and further work should be undertaken to determine their identity and confirm their potential role in the development and maintenance of *P. cinnamomi* suppressive soils.
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## Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ARISA</td>
<td>Automated ribosomal intergenic spacer analysis</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>IGS</td>
<td>Intergenic spacer region</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribed spacer region</td>
</tr>
<tr>
<td>LH-PCR</td>
<td>Length Heterogeneity PCR</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales</td>
</tr>
<tr>
<td>NSW DPI</td>
<td>NSW Department of Primary Industries</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCA</td>
<td>Potato-carrot agar</td>
</tr>
<tr>
<td>PSA</td>
<td>Phytophthora selective agar</td>
</tr>
<tr>
<td>Qld</td>
<td>Queensland</td>
</tr>
<tr>
<td>TGGE</td>
<td>Temperature gradient gel electrophoresis</td>
</tr>
<tr>
<td>TRFLP</td>
<td>Terminal restriction fragment length polymorphism</td>
</tr>
<tr>
<td>VIC</td>
<td>Victoria</td>
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Chapter 1

Introduction

1.1 The Phytophthora cinnamomi problem

*Phytophthora cinnamomi* Rands is a widely distributed soil- and water-borne Oomycete. The genus name is derived from the Greek language and means plant (*phyto*) destroyer (*ptthora*) (Erwin and Ribeiro 1996). *Phytophthora* is a large genus containing many species that are important plant pathogens. Possibly the best known of these is *P. infestans* (Mont.) de Bary, the cause of late blight of potatoes, which became infamous after the Irish potato famine in the 1840s (Zentmyer 1983; Erwin and Ribeiro 1996; Brown 1997a).

*P. cinnamomi* was described by Rands in 1922 (cited in Erwin and Ribeiro 1996) as the causal agent of stripe canker in *Cinnamomum burmannii* (Nees) Blume (cinnamon tree) in Burma. Speculation and debate concerning the origins of *P. cinnamomi* have been ongoing for several decades (Newhook and Podger 1972; Pratt et al. 1973; Broadbent and Baker 1974a; Newhook 1978; Zentmyer 1983; Erwin and Ribeiro 1996; Linde et al. 1997, 1999). Pratt et al. (1973) and Broadbent and Baker (1974a) postulated that *P. cinnamomi* was indigenous to the east coast of Australia. However, this hypothesis was challenged by several authors (Newhook and Podger 1972; Newhook 1978) and recent DNA polymorphism studies indicate that *P. cinnamomi* populations in Australia and South Africa are introduced (Linde et al. 1997, 1999). *P. cinnamomi* is now believed to have originated from across a wide area within New Guinea-Malaysia-Celebes and been introduced to the other parts of the world where it has been recorded (Linde et al. 1999).

*P. cinnamomi* is renowned as one of the most ubiquitous and destructive plant pathogens. Within the genus *Phytophthora*, *P. cinnamomi* is the species with the largest host range (over 1000 host plants) and it has been recorded in over 70 countries (Zentmyer 1983). To provide an example of the destructive potential of *P. cinnamomi*, an estimated 202,500 ha of the jarrah forests (*Eucalyptus marginata*
Donn ex Sm.) in Western Australia were destroyed by *P. cinnamomi* between 1927 and 1986 (Jones and Elliot 1986). *P. cinnamomi* is responsible for widespread dieback epidemics in many other forest areas within Australia, including the Brisbane Ranges and the Grampians to the west of Melbourne, the coastal forests of East Gippsland, Victoria (Vic) (Gregory 1983) and many forest areas within Queensland (Qld) and New South Wales (NSW) (Weste 1983). One of the most recent reports of a *P. cinnamomi* infestation comes from a 5 ha area of sub-alpine vegetation within the Barrington Tops National Park, NSW (McDougall et al. 2003).

The spread of *P. cinnamomi* through the Australian landscape has the potential to cause the extinction of many vulnerable Australian native plants (Reiter et al. 2004). *P. cinnamomi* also poses a threat to economically important crops such as almond, avocado, blueberry, cherry, chestnut, peach, pineapple, plum and several forestry timbers (Erwin and Ribeiro 1996). The pathogen is of particular importance to the avocado (*Persea americana* Mill.) industry because it has the potential for destroying avocado orchards in a short time frame (McKenzie et al. 1983). Phytophthora root rot of avocado is a major problem in nearly all areas of the world in which avocados are grown (Zentmyer 1980).

The pathogen mainly infects the roots of its host causing root rot, and without treatment the host usually dies. In Australian native plant communities there are few options for managing *P. cinnamomi*, except limiting human traffic, machinery and livestock in vulnerable areas and undertaking management activities that encourage an understorey of tolerant / resistant plants (e.g. *Acacia* spp.) to reduce *P. cinnamomi* inoculum (Podger and Keane 2000; D'Souza et al. 2004). In conventional avocado production systems, control is achieved through an integrated approach, involving cultural practices that minimise conditions conducive to *P. cinnamomi*, planting *P. cinnamomi* tolerant root stocks (Ploetz et al. 2001; Ploetz et al. 2002), and chemical control using metaxyl or, more commonly, phosphorus acid and phosphonate (Pegg et al. 1987, 1988, 1992). Certified organic avocado production systems depend on cultural practices and *P. cinnamomi* tolerant cultivars to manage *P. cinnamomi* root rot. Several potential microbial biocontrol agents have been identified. However, application of these in the field has met with minimal success (Erwin and Ribeiro...
Some cultural practices (e.g. application of organic mulches) aim to achieve biological control by inducing conditions that suppress *P. cinnamomi*.

The phenomenon known as *P. cinnamomi* suppressive soil was first reported (and studied extensively) by Broadbent and Baker in an avocado orchard on Tamborine Mountain on the east coast of Australia in the early 1970s (Broadbent et al. 1971; Broadbent and Baker 1974a, 1974b; Cook and Baker 1983). Since then, *P. cinnamomi* suppressive soils have been reported in several other parts of the world and there have been significant efforts to elucidate and exploit the mechanisms that result in suppression. Research to date has provided a substantial body of evidence supporting the hypothesis that *P. cinnamomi* suppression is mostly mediated by biological processes (Section 1.7). However, the specific mechanisms of biological suppression require further investigation and there is a need for further research to determine the importance of microbial community structure and diversity in *P. cinnamomi* suppressive soil (Section 1.9). This study was initiated to investigate: i) biochemical mechanisms involved in microbial suppression of *P. cinnamomi* and ii) whether microbial community structure and diversity influence suppression of *P. cinnamomi* in soil. The following literature review examines research findings from published studies with relevance to these objectives.

The review is presented in 8 sections covering:

1. *Pathology of* *P. cinnamomi* (Section 1.2). This section provides an overview of the biology and pathology of *P. cinnamomi*. The process of host infection and the development of diseases caused by *P. cinnamomi* are described with an emphasis on avocado plants.

2. *Definitions for ‘suppressive’ and ‘conducive’ soils* (Section 1.3). Several authors have proposed definitions for ‘suppressive’ and ‘conducive’ soils. In this section definitions are refined for this study.

3. *Conditions conducive to *P. cinnamomi* survival and pathogenicity* (Section 1.4). Factors associated with the soil environment and processes within the host that contribute to the survival and pathological success of *P. cinnamomi* are discussed.
4. **Abiotic characteristics of P. cinnamomi suppressive soils** (Section 1.5). There have been a number of studies carried out with the intention of determining the physicochemical characteristics of *P. cinnamomi* suppressive soils and whether manipulation of these has practical application for *P. cinnamomi* control. These studies are reviewed with the aim of identifying whether there are abiotic factors potentially involved in *P. cinnamomi* suppression. Topics covered include: soil type, structure and drainage; soil pH; soil nitrogen; calcium and other nutritional factors; soil organic matter.

5. **Association between soil organic matter and P. cinnamomi suppressive soils** (Section 1.6). A number of studies have indicated that high organic matter may be important in the development and maintenance of *P. cinnamomi* suppressive soils. This section examines the role of soil organic matter in the development of conditions that result in *P. cinnamomi* suppression.

6. **Biological agents implicated in P. cinnamomi suppressive soils** (Section 1.7). A number of studies have demonstrated that *P. cinnamomi* suppression is principally mediated by biological processes. This section reviews findings from these studies, many of which have investigated the role of specific antagonistic soil microfauna and microflora in *P. cinnamomi* suppression.

7. **Involvement of microbial metabolites in P. cinnamomi suppression** (Section 1.8). This section reviews evidence for the involvement of microbial metabolites in disease suppressive soils with a focus on the possible involvement of soil cellulase and laminarinase activities in *P. cinnamomi* suppression. Methods for estimating cellulase and laminarinase activities in soil are also considered.

8. **Microbial community structure and diversity in P. cinnamomi suppressive soils** (Section 1.9). The concepts of microbial community structure and diversity are defined and several DNA-based methods for investigating these are outlined. The section ends with a discussion on the potential importance of microbial community structure and diversity in the development and maintenance of soils that suppress soil-borne phytopathogens including *P. cinnamomi*. 
Key findings and gaps in knowledge identified in the literature review are summarised in Section 1.10. The specific aims for this study and an outline of subsequent thesis chapters are presented in Section 1.11.

1.2 Pathology of *P. cinnamomi*

1.2.1 Process of infection

*P. cinnamomi* is a member of the Oomycete group within the Pythiaceae family, which are commonly referred to as “water moulds”. The Oomycota are fungi like in morphology (i.e. filamentous hyphae forming a mycelium) but unlike fungi, which have cell walls principally composed of chitin, the cell walls of Oomycetes are composed of cellulosic and non-cellulosic beta-linked glucan polymers (Bartnicki-Garcia and Wang 1983). Like other Oomycetes, *P. cinnamomi* reproduces asexually by producing non-motile chlamydospores and motile zoospores (Ribeiro 1983) and reproduces sexually by heterogametangial contact to produce thick walled, non-motile, oospores (Elliot 1983). Zoospores act as the principal structures involved in the infection process (Erwin and Ribeiro 1996). Upon release from the sporangium, zoospores remain motile for at least several minutes, typically hours, and in some cases several days. As zoospores swim they change direction frequently, travelling at a rate of \(~6\mathrm{cm}\ 10\mathrm{h}^{-1}\). However, the rate of travel is highly variable and, in soil, is probably more dependent on the velocity and duration of water movement (Carlile 1983). Zoospores tend to swim upwards (geotaxis) and against the directional flow of water. It is thought that this occurs so that zoospores remain near the surface of soils where host rootlets are more abundant. By swimming against the directional flow of water, they avoid being carried downward through the soil profile (Carlile 1983; Gisi 1983; Weste 1983).

Zoospores also have a tendency to swim toward plant roots when they are in close proximity. Accumulation of swarming zoospores around plant roots commonly occurs quickly enough to indicate that they are attracted to plant roots and not merely immobilised on arriving at the root surface by random movement (Carlile 1983). This attraction appears to be a chemotactic response stimulated by plant root exudates, mainly amino acids, sugars, growth factors and ethanol (Carlile 1983;
Weste 1983; Erwin and Ribeiro 1996). In addition, zoospores are aerobic and are possibly attracted to the air-water interface on the root surface where oxygen concentrations are highest (Guest and Brown 1997). Electrotaxis may also affect the direction in which zoospores swim (Carlile 1983). Plant roots emit weak electric currents and it has been demonstrated that zoospores will swim toward, accumulate at, and encyst at weakly charged anodes (+) (Carlile 1983; Gisi 1983). *Phytophthora* zoospores have negatively charged anterior flagella and it is believed that this facilitates their attraction to positively charged surfaces at the zone of plant root tip elongation (Guest and Brown 1997).

After a zoospore reaches a solid object (such as a plant root or organic and inorganic particles) it develops a cell wall and encysts (Erwin and Ribeiro 1996). An abundance of solid obstacles can hasten the rate of encystment, thus reducing the number of zoospores that reach the plant root (Costa et al. 1996). Zoospores that encyst but fail to reach a solid surface are, under favourable conditions, free to be moved to other sites carried by water flow or other vectors (Carlile 1983; Erwin and Ribeiro 1996). During the early stages of encystment, zoospores produce an adhesive substance which allows them to attach firmly to solid surfaces. Within 1 to 6 h of encystment, a germ tube is produced that is chemotropically oriented toward the zone of root tip elongation (Carlile 1983). *Phytophthora* spp. are loosely defined as necrotrophs, meaning that they require the death of host cells in advance of invasion. As the germ tube penetrates the root cell wall and the hypha grows into the host tissues, the pathogen produces toxins that kill the host cell, allowing invasion to occur (Carlile 1983; Weste 1983; Guest and Brown 1997). If the germ tube fails to penetrate the root cell wall the cyst forms a microsporangium, from which secondary zoospores emerge (Weste 1983).

1.2.2 Development of disease in the host plant

*P. cinnamomi* causes several plant diseases including heart rot, collar rot, trunk cankers and root rot on a large variety of hosts (Erwin and Ribeiro 1996). Collar rot affects several plant species including *Actinidia chinensis* Planch (Kiwi Fruit). Collar rot is characterised by lesions that form on the lower stem or just below soil level following infection of the root system. As the bark sloughs away from around
the lesion, browned to blackened vascular tissues of the host plant are exposed (Erwin and Ribeiro 1996). Trunk cankers, which affect plant species such as *Macadamia integrifolia* Maiden and Betche (Macadamia), appear as roughened, sunken, deep furrowed lesions that may extend more than 2 m up the trunk, eventually girdling the trunk and lateral branches of the host. Trunk cankers develop from *P. cinnamomi* entering root and trunk wounds. The severity of the disease is increased by prolonged heavy rain (Erwin and Ribeiro 1996). *P. cinnamomi* can also cause collar rot and trunk cankers in avocado trees. However, in avocados *P. cinnamomi* primarily infects the root system causing root rot. Low hanging foliage and avocado fruit may also become infected if in contact with the soil surface, or if spores are splashed from the soil onto the fruit. Root infection generally leads to a slow decline eventually causing death and, in some cases, especially in younger plants, disease can spread rapidly causing sudden wilt (Erwin and Ribeiro 1996).

Plants that have been infected by *Phytophthora* spp. generally show disease symptoms that are similar to diseases caused by other facultative plant pathogens (Keen and Yoshikawa 1983). During the process of disease development, the fine feeder roots of infected avocado plants show a brown to brownish-black firm rot and become shrivelled, brittle and necrotic. The destruction of the fine feeder roots reduces the host’s ability to absorb water and nutrients (Broadley 1992; Erwin and Ribeiro 1996). A shortage of water to the above-ground parts of the plant may indirectly be the cause of metabolic alterations that occur throughout the rest of the plant (e.g. respiratory increases, increased auxin levels, increases in polyphenol oxidase and other enzymes, and altered accumulation of photosynthates). However, phytotoxic metabolites produced by *P. cinnamomi*, especially carbohydrate polymers, may also be involved in water shortage and wilting. In addition, as the mycelium grows into and through intercellular spaces within the host root tissues, it physically blocks the relocation of water, nutrients and plant metabolites (Keen and Yoshikawa 1983).

The progression of *Phytophthora* root rot in avocados is typified by stunted growth, leaves becoming necrotic and as the disease advances wilting occurs, followed by heavy leaf fall and dieback of aerial parts of the plant (Plate 1.1) (Broadley 1992; Erwin and Ribeiro 1996). Growth flushes on diseased plants are weak or absent. The
canopy becomes very sparse and may be reduced to a skeleton of main branches, which are susceptible to sunburn (Broadley 1992). A declining avocado tree may flower heavily, setting a large crop of undersized fruit. This response is thought to be caused by an accumulation of carbohydrates in the aerial parts of the plant resulting from the loss of roots. Water stress, caused by the destruction of feeder roots, can also reduce fruit set and contribute to early fruit drop during the fruit-filling stage (Broadley 1992).

Plate 1.1 Avocado tree affected by root rot caused by *P. cinnamomi*.

### 1.3 Definitions for disease ‘suppressive’ and ‘conducive’ soils

Over the past century there have been many reports of disease suppressive soils in field situations, where the incidence and severity of disease is influenced by soil characteristics (Alabouvette et al. 1996). The concept of a ‘suppressive soil’ was introduced by Menzies (1959), who applied the term to describe the phenomenon of soils that suppressed *Streptomyces* potato scab in California. By the 1970s the terms ‘disease suppressive soil’ and its antonym ‘disease conducive soil’ were widely adopted (Hornby 1983). While a conceptual basis for a suppressive soil was alluded
to by Smith and Snyder (1972), it appears that the first sound definition for a disease suppressive soil was proposed by Baker and Cook (1974). Baker and Cook (1974) defined a disease suppressive soil as a soil in which either the pathogen cannot establish, becomes established but fails to produce disease, or becomes established and causes disease at first but diminishes with continued cultivation of the crop.

Alabouvette et al. (1996) argued that the terms ‘disease suppression’ and ‘disease conduciveness’ fail to account for the fact that, in every natural soil, expression of disease occurs at (or is limited to) various degrees of incidence and severity. Suppression occurs along a continuum from ‘highly suppressive’ to ‘highly conducive’ rather than simply being suppressive or conducive. If the concept of Alabouvette et al. (1996) is applied, a suppressive soil can therefore be defined as a soil in which disease incidence and severity remain low, despite the presence or introduction of a pathogen, a susceptible host plant, and environmental conditions favourable for development of disease. Accordingly, the term ‘conducive soil’ can be defined as a soil in which disease incidence and severity are moderate to high in the presence of the pathogen, a susceptible host, and environmental conditions favourable for development of disease.

There are generally 2 modes of disease suppression - ‘specific’ and ‘general’. Specific suppression occurs when there are exclusive interactions involving one agent or several specific agents that suppress a soil-borne plant pathogen. An example of specific suppression is the well known phenomenon of take-all decline (Hornby 1979; Stirling and Stirling 1997; van Bruggen and Semonov 2000). Take-all is a widespread disease of cereals that is caused by the soil-borne fungus Gaeumannomyces graminis (Sacc.) von Arx and Oliver (Hornby 1979). Specific suppression of the take-all fungus has been attributed to antagonistic bacteria such as fluorescent Pseudomonas spp. (Cook and Baker 1983; Weller et al. 1988) and fungi such as Trichoderma spp. (Simon and Sivasithamparam 1989). In soils that are generally suppressive, suppression results from the cumulative effects of complex interactions between the pathogen and a multiplicity of biotic and / or abiotic factors within the soil environment (Stirling and Stirling 1997, van Bruggen and Semonov 2000). General suppression has been reported to occur in both natural ecosystems and in agroecosystems (van Bruggen and Semonov 2000). A recent example of
general disease suppression is provided by van Os et al. (2001), who found an association between suppression of *Pythium* root rot in bulbous *Iris* and increases in soil microbial biomass and activity.

1.4 Conditions conducive to *P. cinnamomi* survival and pathogenicity

As for most soil-borne plant diseases, the severity of disease caused by *P. cinnamomi* depends on the inoculum density of the pathogen in the soil. When *P. cinnamomi* populations are low this relationship is linear, and when the numbers of propagules are high this relationship is sigmoidal (Weste 1983). Disease potential may also fluctuate independently of the pathogen population in relation to changes in the environment or in the host (Duniway 1983; Schmitthenner and Canaday 1983; Weste 1983; Erwin and Ribeiro 1996).

There are a number of factors that may contribute to host susceptibility to infection by *P. cinnamoni*. Firstly, the walls of the host root cells form a physical barrier, but there is little evidence that this is a mechanism of resistance against *Phytophthora* spp. (Keen and Yoshilawa 1983). However, it is reasonable to suggest that it would be easier for a *Phytophthora* germ tube to penetrate a newly formed root cap or a freshly wounded root or trunk than a highly lignified cell wall. Preformed chemical inhibitors in plant tissues may also be important in general resistance to *P. cinnamomi*. For example, borbonols-alkyl-substituted lactones, present in certain cultivars of *Persea* spp., may function as resistance factors against *P. cinnamomi* (Keen and Yoshikawa 1983). Other plant metabolites associated with increased resistance to *Phytophthora* spp. include simple phenolic compounds, several enzymes, and phytoalexins (Keen and Yoshikawa 1983). Plants that do not produce such chemical inhibitors may be more susceptible to infection by the pathogen.

Differences in host susceptibility to *P. cinnamomi* can also be related to the age or life stage of the plant. Some plants may be more susceptible to diseases caused by *P. cinnamomi* at the seedling stage but more resistant during later stages of development and *vice versa*. Physiological changes in the host related to seasonal and environmental variables may also predispose the host to infection (Shea and
Pegg (1977a) noted that avocado seedlings growing in the shade beneath mature avocado trees were destroyed by *P. cinnamomi*, whereas those growing in direct sunlight within inter-row spaces were barely affected. To provide another example, intermittent waterlogging favours sporangial production, and subsequent zoospore dispersal and host infection by *P. cinnamomi*. Waterlogging may also predispose the host to infection by altering the metabolism of the host (e.g. affecting production of lignin, phenolics and phytoalexins; Cahill and McComb 1992), or by changing the soil and rhizosphere microbial community so that it is no longer antagonistic or no longer confers resistance to the host (Shea and Broadbent 1983).

Soil temperature and soil moisture are probably the most critical environmental factors determining the activity of *P. cinnamomi*. *P. cinnamomi* becomes inactive almost instantly in soils when the temperature drops below 0°C. At temperatures between 6°C and 7°C, the fungus becomes inactive within 2 days, whereas at 14°C inactivation takes around 16 days (Erwin and Ribeiro 1996). Oospores are the most resistant survival unit of *Phytophthora* spp.; under laboratory conditions they can withstand freezing at -20°C (Weste 1983; Erwin and Ribeiro 1996). *P. cinnamomi* is rarely found in soils where temperatures are consistently below 10°C (Weste 1983). The optimum range for pathogenic activity of *P. cinnamomi* is between 15°C and 27°C. Pathogenic activity becomes significantly reduced as temperatures approach 33°C, at which point the pathogenic activity of *P. cinnamomi* ceases (Zentmyer 1981).

Temperature does not act as an isolated variable in the population dynamics of *P. cinnamomi*. Within the temperature range optimal for disease expression (15°C to 27°C) the number of pathogen propagules is also dependent on the soil matric water potential ($\psi_m$) (Duniway 1983; Weste 1983). This is because the population density of *P. cinnamomi* in soil is largely due to the production of sporangia and the dispersal of zoospores, which are dependent on the presence of free water (Duniway 1983). However, at $\psi_m$ values greater than -0.1 MPa, the population of *P. cinnamomi* is primarily regulated by temperature (Weste 1983). Soil moisture requirements and limitations for the various growth stages of *P. cinnamomi* are shown in Table 1.1.
Table 1.1 Soil moisture requirements for mycelial growth, production of various propagules and dispersal of *P. cinnamomi* (data from Duniway [1983] and Weste [1983]). $\Psi_m =$ soil matric water potential.

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Soil $\Psi_m$ requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelium</td>
<td>-2.5 to -3.5 MPa</td>
</tr>
<tr>
<td>Chlamydospores</td>
<td>-0.003 to -10 MPa</td>
</tr>
<tr>
<td>Sporangia</td>
<td>-0.001 to -0.25 MPa</td>
</tr>
<tr>
<td>Dispersal and movement of zoospores</td>
<td>-0.001 to -1 MPa (varies depending on soil texture)</td>
</tr>
</tbody>
</table>

The movement of water in soil is a critical factor in the dispersal of zoospores and therefore the spread of disease. Over 50 years ago, Zentmyer and Richards (1952) demonstrated that phytophthora root rot was spread through the translocation of zoospores via irrigation channels. The progress of disease attributed to water dispersal has been measured at 400 m year$^{-1}$ on a downhill slope and at 6.6 m year$^{-1}$ on an uphill slope of 4°, suggesting that mycelial growth can also contribute to the spread of the pathogen (Weste 1983). Soil texture and soil structure affect soil drainage and therefore the ability of zoospores to move through the soil. In soils with larger pore sizes, such as gravel or sandy soils, the movement of zoospores is less inhibited than in clay soils. A compacted, fine textured, silty soil, however, completely restricts the movement of zoospores except at the surface (Marks and Smith 1981; Duniway 1983; Weste 1983; Erwin and Ribeiro 1996). Shallow soil may be underlaid by a hardpan, which is an impervious subsoil layer or rock base that impedes drainage (Weste 1983; Erwin and Ribeiro 1996). Any low lying land, poorly drained soil or soil lying over a hardpan may easily become and remain saturated for an extended period and, therefore, provide conditions conducive to the production and dispersal of *P. cinnamomi* zoospores (Weste 1983; Erwin and Ribeiro 1996). These soils become colonised more quickly, which results in the development of a large pathogen population (Weste 1983).

The production of sporangia (and subsequently zoospores) also requires external microbial factors that may be absent or suppressed in some soils (Baker 1978; Malajczuk 1983). Bacteria such as *Pseudomonas* and *Chromobacterium* have been implicated in the production of compounds that stimulate the formation of sporangia (Ribeiro 1983). Mycoparasitic *Trichoderma* spp. have been associated with the
initiation of sexual reproduction in *P. cinnamomi* (Elliot 1983). According to Shea and Broadbent (1983), the microbial factors responsible for sporangial formation appear to be ubiquitous and non-specific. While it may appear that these reproductive responses in *P. cinnamomi* result from commensal interactions, it is more likely that these responses are a defensive survival strategy (Weste 1983). Nonetheless, soils with populations of bacteria that produce compounds that stimulate sporangial formation are likely to contribute toward the pathogenic activity of *P. cinnamomi*.

The effect of soil chemistry on populations of *P. cinnamomi* and on the development of disease is complex, and the response of different *Phytophthora* spp. is highly variable (Schmitthenner and Canaday 1983). *P. cinnamomi* tolerates a relatively wide pH range; however, the optimal soil pH for sporangial production by *P. cinnamomi* is pH 6 to 6.5 (Zentmyer 1980). In some instances the pathogenic activity of *P. cinnamomi* has been favoured by high nutrient content in the soil. Marks et al. (1972) reported an increase in dieback of eucalyptus trees treated with a 17:9:7 mix of N:P:K fertiliser. However, applying nutrients and increasing mineral salts generally reduces disease or has no effect on *P. cinnamomi* (Schmitthenner and Canaday 1983; Weste 1983).

*P. cinnamomi* is protected by continued occupancy of the colonised host tissue, providing that sufficient moisture is maintained (Weste 1983). Evidence for this was provided by Shea et al. (1980), who recovered *P. cinnamomi* from the dead tissue of a *Banksia* sp. 2 years after the plant had died. In the absence of a living host, the chances of survival are increased by the capacity for independent saprophytic activity by *P. cinnamomi*. Growth and reproduction can also occur at the saprophytic stage. Saprophytic survival may occur in the form of mycelium, chlamydospores or oospores contained within organic materials such as dead plant roots (Weste 1983; Downer et al. 2001a). Therefore, under favourable environmental conditions, it would be expected that certain components of soil organic matter might contribute to maintaining higher densities of *P. cinnamomi* propagules. However, the greatest hazards to survival of *P. cinnamomi* are desiccation and high populations of antagonistic or competitive soil microorganisms (Weste 1983). Higher soil organic matter content supports a larger, more active and diverse microbial community, of which many members will be antagonistic, competitive or predatory toward *P.
In addition, the highest levels of disease incidence and severity occur in soils with depleted levels of organic matter, low levels of microbial activity and low numbers of actinomycetes and aerobic bacteria (Broadbent and Baker 1974a; Malajczuk 1983; Shea and Broadbent 1983; Weste 1983).

In summary, the soils that are most conducive to the survival of *P. cinnamomi*, and the expression of the plant diseases that it causes, are likely to be those: i) that have poor drainage and / or are consistently moist; ii) with soil temperatures consistently ranging between 15°C and 27°C; iii) that possess microbial populations that produce compounds that are stimulatory to sporangia formation; iv) that have a moderate pH range; and v) that are low in organic matter and do not support a large, active and diverse antagonistic soil microbial community.

### 1.5 Abiotic characteristics of *P. cinnamomi* suppressive soils

A number of soil abiotic factors have been investigated for their potential role in *P. cinnamomi* suppressive soils. Results from these investigations have indicated that *P. cinnamomi* suppressive soils may possess attributes that include: i) being well-drained with good structure; ii) pH 5.5 to 7.0; iii) high levels of ammonium and nitrate nitrogen (N); iv) high calcium levels; and v) high organic matter content (Erwin and Ribeiro 1996). Several workers have attempted to induce *P. cinnamomi* suppression by manipulating these and other abiotic factors with varying and often contradictory results (Schmitthenner and Canaday 1983; Shea and Broadbent 1983; Erwin and Ribeiro 1996). The following subsections outline key findings from these and other studies that have investigated the role of soil physicochemical characteristics in *P. cinnamomi* suppressive soils.

#### 1.5.1 Soil type, structure and drainage

Soils first associated with the phenomenon of *P. cinnamomi* suppression in Australia were red ferrosol soils (previously krasnozem; Morand 1994; Isbell 2002; McKenzie et al. 2004) of basaltic origin (Morand 1994; Isbell 2002; McKenzie et al. 2004). Red ferrosols were also associated with *P. cinnamomi* suppression in eucalypt forests located at the foothills of Mt Dandenong, Vic (Marks and Smith 1981). Red ferrosol
soils are typically well drained, strongly structured light to moderate clays to clay-loams (Isbell 2002; McKenzie et al. 2004). In suppressive avocado soils located in south-eastern Qld, the clay content of the soil consists of illite, kaolinite and montmorillonite (Broadbent and Baker 1974). While montmorillonitic clay minerals have been implicated in suppression of *Fusarium* wilt (Amir and Alabouvette 1993; Alabouvette et al. 1996), there is no evidence of a relationship between any specific type of clay mineral and suppression of *Phytophthora* root rot (Broadbent and Baker 1974a). However, clay minerals play an important role in soil aggregation (Alabouvette et al. 1996; Höper and Alabouvette 1996) and there is some evidence that destruction of soil aggregates may diminish the suppressive capacity of the soil. This is thought to be related to disturbances to soil microbial communities associated with the aggregates (Marks and Smith 1981).

As discussed in Section 1.4, persistent or frequent waterlogging provides conditions conducive to *P. cinnamomi* survival and infection and therefore maintaining good soil drainage is an important first line of defence against *P. cinnamomi*. Soil structure influences the flow rate and directional movement of water through the soil and over the soil surface. The strongly structured aggregates typical of ferrosol soils facilitate their capacity for good drainage (Morand 1994; Isbell 2002; McKenzie et al. 2004). However, even in these and other free draining soil types, such as the sandy and loam soils under the jarrah forests of Western Australia, frequent, prolonged or heavy rainfall provides conditions adequate for the development of diseases caused by *P. cinnamomi* (Shea et al. 1978; 1980; Hardy et al. 1996). Therefore, while management of soil moisture may play a role in minimising conditions that are conducive to *P. cinnamomi*, the capacity of the soil to drain free water is unlikely to be a primary factor determining the soil’s suppressive capacity.

1.5.2 Soil pH

Broadbent and Baker (1974a) noted that the *P. cinnamomi* suppressive soils they studied had a pH range between pH 5.5 and 7.0. A study of soils located in Taiwan suppressive to 3 species of *Phytophthora* (*P. parasitica* Dastur, *P. palmivora* Bulte and *P. capsici* Leonian) found no correlations between suppression of any *Phytophthora* species and soil texture, soil colour or the type of surface vegetation.
However, soils with pH 5.0 or lower were generally more suppressive than those with a higher pH (Ann 1994). A study of soils from 155 locations spread across the island of Hawaii found that soils $\leq$ pH 4.0 or $\geq$ pH 8.0 tended to be more suppressive toward *P. cinnamomi* than soils with $\sim$pH 6 (Ko and Shiroma 1989). A quadratic regression analysis of data from this study showed that the relationship between soil pH and *P. cinnamomi* suppression was significantly, but only weakly, correlated (Ko and Shiroma 1989). The results from these and other studies (Weste 1983) suggest that *P. cinnamomi* tolerates acid and alkaline conditions ranging from pH 4.0 to pH 8.0.

Addition of elemental sulphur to soil to lower the soil pH to 3.8 has been used successfully to control *P. cinnamomi* in pineapple crops in Australia (Pegg 1977b). However, reducing soil pH to effectively control *P. cinnamomi* only has practical applications for crops that are highly acid-tolerant, such as pineapples (Pegg 1977b; Weste 1983). Reducing soil pH is not suitable for avocados, which have an optimum pH range of 5.0 to 5.5 (pH 6.0 to 6.5 in soils high in manganese), and have a limited ability to tolerate conditions below pH 5.0 (Broadley 1992). The ideal pH range for avocados (and the pH range of suppressive soils studied by Broadbent and Baker [1974a]), is also within the range suitable for *P. cinnamomi* reproduction and pathogenic activity (Section 1.4). Therefore, it appears that soil pH is not a causal factor in *P. cinnamomi* suppressive soils where suppression occurs within a pH range suited to (or tolerated by) *P. cinnamomi*.

1.5.3 Soil nitrogen

Broadbent and Baker (1974a) identified high ammonium (NH$_4$) and nitrate (NO$_3$) content as attributes of the *P. cinnamomi* suppressive soils investigated during their study. However, they also noted that increases in sporangium formation were accompanied by increases in NH$_4$, which was attributed to waterlogging. Broadbent and Baker (1974a) noted that total N measured in 2 suppressive soils was consistently higher than total N measured in 2 conducive soils. Rather than being linked to suppression, however, the higher levels of total N in the suppressive soils were more likely to be related to the associated cultural practices which involved N amendments.
Other researchers have had variable results using N fertilisers to suppress *Phytophthora* diseases (Schmittener and Canaday 1983; Erwin and Ribeiro 1996). Increasing soil NO$_3$ was shown to reduce avocado root rot in one study, but the authors concluded that the effects were probably due to high salinity rather than a direct interaction between NO$_3$ and *P. cinnamomi* (Bingham et al. 1958). Limited success was achieved by increasing NO$_2$ in soil (Zentmeyer and Bingham 1956). Addition of N fertilisers to soil was shown to increase little leaf disease of pine caused by *P. cinnamomi*, and fertilisation of *Eucalyptus* spp. with N, P and K either increased disease or had no effect (Marks et al. 1972). Studies that have had more success with N amendments have associated HNO$_2$ and NH$_3$ (Tsao and Zentmyer 1979) and high-N organic amendments such as alfalfa meal with decreased disease incidence and severity (Schmittener and Canaday 1983). However, even during these studies, N amendments did not markedly suppress *P. cinnamomi* (Erwin and Ribeiro 1996). Therefore, the role (if any) of soil N in *P. cinnamomi* suppression is probably indirect.

1.5.4 Calcium and other soil nutritional factors

Both the suppressive rainforest soils and the suppressive avocado soils studied by Broadbent and Baker (1979a) were high in exchangeable cations; calcium (Ca) in particular. Similarly, high exchangeable cations, including Ca, occur in the *P. cinnamomi* suppressive loam soils supporting jarrah forests in Western Australia (Malajczuk 1979a). There has been some success achieved by adding Ca to soils to suppress *Phytophthora* diseases including *P. cinnamomi*. In one study, the addition of calcium carbonate (CaCO$_3$) to soil reduced infection of *E. marginata* seedlings by *P. cinnamomi* (Boughton et al. 1978). However, there are also instances where application of Ca to soils has increased disease incidence and severity. For example, Halsall (1980) found that additions of CaCO$_3$ to soil increased infection and disease severity in *Eucalyptus* spp. normally tolerant to *P. cinnamomi*. In other instances, root rot of avocado has been reduced by applications of calcium nitrate (Ca(NO$_3$)$_2$.4H$_2$O) (with ammonium sulphate [(NH$_4$)$_2$SO$_4$]) to soils naturally infested with *P. cinnamomi* (Lee and Zentmyer 1982), and Ca amendments have been found to interfere with zoospores from *P. parasitica* (Vonbroembsen and Deacon 1997).
The strongest evidence suggesting a possible link between Ca levels in soil and *P. cinnamomi* suppression was provided by Messenger et al. (2000a, 2000b). In a pot experiment, Messenger et al. (2000a, 2000b) found that the addition of gypsum (calcium sulphate = CaSO$_4$) to soil reduced *P. cinnamomi* infection rates in avocado seedlings by up to 71%. Sporangial formation in these same treatments was reduced by 74%, and zoospore production and colony forming units of *P. cinnamomi* were also reduced in soil amended with Ca(NO$_3$)$_2$.4H$_2$O or CaCO$_3$. Sporangial production was reduced by soil extracts from gypsum-amended soil and irrigation of soil with gypsum solutions reduced sporangial formation *in vivo*. Messenger et al. (2000a, 2000b) also presented evidence to suggest that reductions in infection of avocado seedlings grown in gypsum amended soil was not due to avocado growth response, increased root resistance or reduced root membrane permeability. In their conclusions, Messenger et al. (2000a, 2000b) alluded to a direct interaction between gypsum and *P. cinnamomi*. However, exactly how Ca in the form of gypsum (CaSO$_4$) affects *P. cinnamomi* is not known.

Other soil nutrients such as P, K and several minor plant nutrients, have also been considered as possible mechanisms to control disease caused by *P. cinnamomi* (Schmitthener and Canaday 1983; Shea and Broadbent 1983; Erwin and Ribeiro 1996). However, while there has been some success in applying these to reduce *Phytophthora* inoculum and reduce the severity of *Phytophthora* infections, these elements have not been identified as factors associated with soils that suppress *P. cinnamomi* or other *Phytophthora* species (Erwin and Ribeiro 1996).

### 1.6 Association between soil organic matter and *P. cinnamomi* suppressive soils

High soil organic matter is a factor commonly implicated in *P. cinnamomi* suppression. High organic matter was a key attribute of the *P. cinnamomi* suppressive soils studied by Broadbent and Baker (1974a). In California (USA), soils suppressive to avocado root rot caused by *P. cinnamomi* typically have high organic matter content (Casale 1990). There are numerous examples where organic matter has been applied to soils in the form of various materials (including: green manures [Baker 1978]; alfalfa meal [Zentmyer 1963]; chicken manure [Broadbent et al. 1989]; sewage sludge [Tsao 1977]; bioenhanced mulches [Costa et al. 1996];
eucalyptus trimmings [Downer et al. 2001a]; and yard trimmings [Downer et al. 1999]) with the intention of controlling *P. cinnamomi*, or as a first line of defence to minimise impacts caused by *P. cinnamomi* infestations. In many instances these organic amendments have induced *P. cinnamomi* suppressive conditions within the soil. There have also been a number of studies that have shown that *Phytophthora* diseases can be controlled in pots by regulating the quantity and composition of the organic matter in potting media (Gerrettson-Cornell et al. 1976; Hoitink et al. 1977, 1986, 1991; Shea and Broadbent 1983; Aryantha et al. 2000). However, not all soils with high organic matter are suppressive (Casale 1990) and some *P. cinnamomi* suppressive soils are actually low in organic matter content (Halsall 1982a, 1982b). An example of suppressive soil with low organic matter content was found in the Tallaganda State Forest in NSW (Halsall 1982a, 1982b).

Malajczuk (1983) considers that the main role of organic matter is related to microbial antagonism of the pathogen in both the bulk soil and in the rhizosphere. However, there are probably several ways in which organic matter contributes to the suppression of *P. cinnamomi* in soils. Firstly, the quality of the organic amendments has a strong influence on whether or not the pathogen is suppressed (Malajczuk 1983). Humic and fulvic substances that remain at the end of the decomposition process elicit a variety of responses from plants and soil microbes (Linderman 1989). It is possible that these substances have a direct or indirect effect on *P. cinnamomi* (Ribeiro and Linderman 1991). Secondly, Costa et al. (1996) found that organic matter levels, increased by adding mulch to the soil, were positively correlated with a reduction in the incidence of disease in avocado seedlings. They also found that the number of zoospores trapped on organic particles increased with increasing levels of organic matter. This might explain why Broadbent and Baker (1974a) found that, in their suppressive soils, *P. cinnamomi* sporangia only form in niches of organic matter. However, Costa et al. (1996) state that the specific mechanisms involved in the trapping of zoospores on organic particles is poorly understood and it is not known if this phenomenon simply results from zoospores encysting after bumping into solid organic particles or if it results from saprophytic antagonism when zoospores come into contact with organic particles in the soil. Thirdly, microbial cell counts and microbial activity are positively correlated with increasing amounts of soil organic matter (Henis 1986; Costa et al. 1996; Yang et al. 2003; Yao et al.)
and soil microbial activity and microbial populations are both negatively correlated with disease incidence and severity (Chen et al. 1988; You and Sivasithamparan 1995; Costa et al. 1996; Downer et al. 2001a). Therefore, the most probable role of organic matter in soils that are suppressive to P. cinnamomi is that it provides substrates for large numbers of saprophytic organisms that are antagonistic and/or effectively out-compete P. cinnamomi which is known to have weak saprophytic abilities (Costa et al. 1996; Erwin and Ribeiro 1996). The interactions between P. cinnamomi and other soil organisms are discussed fully in Section 1.7 (below).

1.7 Biological agents implicated in P. cinnamomi suppressive soils

Phytophthora species are highly affected by other soil microorganisms, which can be either stimulatory or antagonistic (Malajczuk 1983). The principal cause of suppression of P. cinnamomi in soil is considered to be microbial (Erwin and Ribeiro 1996) and much evidence has been presented in support of this hypothesis. For example, during pot experiments in which Jacaranda acutifolia Humb. and Bonpl., E. marginata and Eucalyptus sieberi L. Johnson seedlings were used as test plants, soils sampled from south-eastern Qld retained their ability to suppress P. cinnamomi after they were treated with steam up to 60°C, but became conducive after they were treated at 100°C (Broadbent et al. 1971; Broadbent and Baker 1974a). Confirmation of the involvement of biological processes in P. cinnamomi suppressive soils has been demonstrated in other studies by terminating biological activity through autoclaving, fumigating or γ-irradiating the soil (Malajczuk 1977; Halsall 1978, 1982a; Marks and Smith 1981; Casale 1990; Duvenhage et al. 1991; Ann 1994). Soils are then tested for loss of suppression by comparing development of disease in susceptible plants grown in sterile soils and in non-sterile suppressive soils. The loss of suppression observed in response to each biocidal treatment indicates that P. cinnamomi suppression is mediated by biological processes.

To date, much of the work that has been undertaken in an attempt to understand the biological processes involved in P. cinnamomi suppressive soils has aimed to identify microbial agents involved in P. cinnamomi suppression. Harvesting and cultivating microbial antagonists that can be exploited for use in biological control
has been the main aim for these studies. Therefore, it appears that much of this work has been undertaken based on an assumption that the mode of suppression in these soils is specific (Section 1.3). Direct study of microorganisms in *P. cinnamomi* suppressive soils has reinforced assumptions regarding the importance of microbial factors in suppression and provided insight into the functions, composition and diversity of microbial communities associated with them. However, despite advances in our understanding, there is still much uncertainty regarding the specific biological agents and/or mechanisms involved in *P. cinnamomi* suppressive soils. This section reviews outcomes from research undertaken with the aim of identifying biological agents involved in *P. cinnamomi* suppression.

1.7.1 *Meso- and Microfauna*

The possible involvement of mycophagous soil fauna in suppressive soils has mostly been ignored by plant pathologists, however, soil microfauna may contribute toward reducing *P. cinnamomi* propagules (reviewed by Malajczuk 1983; Erwin and Ribeiro 1996). Protozoa, especially vampyrellid and testate amoebas, physically perforate fungal walls and chemically digest the cellular contents (Old 1978, 1979; Malajczuk 1979a; Chakraborty and Old 1982). Malajczuk (1979a) observed perforations typical of vampyrellid amoebas in the hyphae of *P. cinnamomi* in soils suppressive to the pathogen. Observations have also been made of vampyrellid amoebas attacking and lysing hyphae and chlamydospores of *P. cinnamomi* (Old 1978, 1979; Old and Oros 1980; Chakraborty and Old 1982) and of small naked amoebas, belonging to the Hartmannellidae, lysing and ingesting zoospores (Palzer 1976, cited in Malajczuk 1983). The potential role of mesofauna such as nematodes and mites in *P. cinnamomi* suppressive soils has received little attention. This is probably because the results presented so far indicate that nematodes and mites are often associated with increased disease severity (Malajczuk 1983).

Mycophagous microfauna are widespread and non-specific, grazing on a wide range of soil fungi and fungal propagules. However, high numbers of these organisms are common in *P. cinnamomi* suppressive soil and therefore soil microfauna may contribute to general suppression by reducing *P. cinnamomi* inoculum (Malajczuk 1983). Their general non-specific predatory behaviour limits their potential use for
biological control of *P. cinnamomi* (Old and Patrick 1979; Malajczuk 1983) and this probably explains why researchers have mostly continued to ignore the microfauna found in *P. cinnamomi* suppressive soils.

### 1.7.2 Bacteria

Broadbent and Baker (1974a) found that the suppressive avocado soils they studied had higher populations of bacteria (especially *Bacillus* spp.) and actinomycetes than in all but one of the conducive soils (Table 1.2). In other studies, lysis of *P. cinnamomi* hyphae in soil has been positively correlated with increases in microbial numbers, including bacteria (Malajczuk 1983; Erwin and Ribeiro 1996). Malajczuk (1979a, 1979b) isolated bacteria, actinomycetes and fungi from suppressive and conducive soil and rhizosphere samples associated with the susceptible *E. marginata* and the less susceptible *Eucalyptus calophylla* R. Br. (Table 1.3). They recorded the largest percentage of antagonistic bacteria and actinomycetes in non-rhizosphere suppressive loam soil and in rhizosphere soil from *E. marginata* seedlings grown in suppressive loam soil. Other workers have observed similar trends in avocado and forest soils (e.g. Weste and Vithanage 1978; Halsall 1982a, 1982b; Murray 1987; Mass and Kotze 1989; Dunvenhage et al. 1991; You and Sivasithamparam 1994, 1995; Costa et al. 1996; Downer et al. 2001a) and at least one study (Tippett 1978, PhD thesis cited in Erwin and Ribeiro 1996) demonstrated that adding soil with large numbers of bacteria to biologically deficient soil can result in a significant reduction in *P. cinnamomi* populations.

**Table 1.2** Total populations of bacteria and actinomycetes measured in suppressive and conducive avocado soils (adapted from Broadbent and Baker 1974a).

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Total Bacteria (cfu g⁻¹ soil)</th>
<th>Actinomycetes (cfu g⁻¹ soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Suppressive:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red basaltic avocado</td>
<td>6.1 x 10⁶</td>
<td>1.8 x 10⁵</td>
</tr>
<tr>
<td>Red basaltic avocado</td>
<td>9.7 x 10⁶</td>
<td>7.5 x 10⁵</td>
</tr>
<tr>
<td><strong>Conducive:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red basaltic avocado</td>
<td>1.0 x 10⁷</td>
<td>9.5 x 10⁵</td>
</tr>
<tr>
<td>Red basaltic avocado</td>
<td>3.0 x 10⁶</td>
<td>7.0 x 10⁴</td>
</tr>
<tr>
<td>Sand jarrah forest</td>
<td>1.9 x 10⁵</td>
<td>5.0 x 10⁴</td>
</tr>
</tbody>
</table>
Table 1.3 Total populations of bacteria and actinomycetes in the rhizospheres of *E. calophylla* and *E. marginata* grown in suppressive loam and conducive laterite soils (adapted from Malajczuk 1979a).

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Bulk Soil</th>
<th><em>E. calophylla</em> rhizosphere</th>
<th><em>E. marginata</em> rhizosphere</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacteria (cfu g(^{-1}) soil)</td>
<td>Actinomycetes (cfu g(^{-1}) soil)</td>
</tr>
<tr>
<td>Suppressive Loam:</td>
<td></td>
<td>3.3 x 10(^6)</td>
<td>1.1 x 10(^7)</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>1.9 x 10(^8)</td>
<td>2.2 x 10(^7)</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td></td>
<td>1.5 x 10(^7)</td>
<td>7.0 x 10(^7)</td>
</tr>
<tr>
<td>Antagonists (%)</td>
<td></td>
<td>3.7 x 10(^7)</td>
<td>3.7 x 10(^7)</td>
</tr>
<tr>
<td>Conducive Laterite:</td>
<td></td>
<td>5.2 x 10(^5)</td>
<td>1.4 x 10(^7)</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>7.3 x 10(^7)</td>
<td>3.2 x 10(^7)</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td></td>
<td>3.7 x 10(^7)</td>
<td>1.6 x 10(^7)</td>
</tr>
</tbody>
</table>

There are 2 ways by which soil bacteria may suppress *Phytophthora* root rot. Baker (1978) speculated that, in some suppressive soils, bacteria that stimulate the production of zoosporangia (Section 1.4) may be suppressed, or that the stimulatory compounds they produce might be destroyed by other soil microorganisms. A second process that may result in suppression involves direct bacterial antagonism of *P. cinnamomi* propagules. In the suppressive avocado soils studied by Broadbent and Baker (1974a, 1974b), *P. cinnamomi* was abundant but produced mostly abortive sporangia. Further examination revealed that bacteria were swarming around the sporangia walls. This intense colonisation by the bacteria may have been responsible for the abortive sporangia.

Other studies have shown that *P. cinnamomi* may produce prolific numbers of zoospores but that root infection is reduced by microorganisms that attack the zoospores and zoospore germ tubes as they emerge (Malajczuk et al. 1977b; Nesbitt et al. 1981a; Malajczuk 1988). Antagonistic bacteria may also operate to reduce *P. cinnamomi* inoculum in soil by attacking the mycelium, sporangia or possibly the more resistant chlamydospores and oospores (Sneh et al. 1977; Malajczuk 1983). Several workers have observed bacteria intensively colonising the hyphae of *Phytophthora* spp. (Broadbent and Baker 1974a, 1974b; Malajczuk et al. 1977b, 1984, 1984; Nesbitt et al. 1981b; Malajczuk 1988). This attraction appears to be a chemotactic response to metabolites, possibly phenylalanine and glucose, exuded from *Phytophthora* hyphae (Nesbitt et al. 1981c). If this is so then the attraction of these bacteria to *P. cinnamomi* propagules may be related to the bacteria feeding on the exudates rather than on the *P. cinnamomi* propagules. It is also possible that in
the process of feeding on exudates the bacteria may produce metabolites that degrade \textit{P. cinnamomi} cells.

The understoreys of eucalyptus forests in Australia are typically dominated by either grasses, proteaceous species (e.g. \textit{Banksia} spp.) or legumes (e.g. \textit{Acacia} spp.), depending on the fire regime (Shea and Malajczuk 1977; Shea et al. 1978, 1979; Murray 1987; D’Souza et al. 2004). \textit{P. cinnamomi} tends to be highly active in eucalyptus forests that have a proteaceous understorey (Shea et al. 1980; Murray 1987; Wilson et al. 2000; D’Souza et al. 2004). \textit{Acacia} dominated understoreys have been associated with \textit{P. cinnamomi} suppressive soils in the jarrah forests of Western Australia (Tippett and Malajczuk 1979; Malajczuk et al. 1984; D’Souza et al. 2004) and the root nodule bacteria (rhizobia) that form symbiotic relationships with these \textit{Acacia} spp. are capable of reducing zoospore survival (Malajczuk et al. 1984). Actinomycetes are frequently associated with suppressive forest soils in Australia (Table 1.4). Antagonistic \textit{Micromonospora carbonacea} \textbf{Luedemann} and \textbf{Brodsky} and \textit{Streptomyces violascens} have been isolated from the rhizosphere of \textit{Banksia grandis Willd.} (El Tarabily et al. 1996). Other \textit{Streptomyces} spp. have also been implicated in \textit{in vitro} antagonism (Rose et al. 1980) and in suppression of \textit{P. cinnamomi} in soil supporting Australian native vegetation (Halsall 1982a, 1982b). Several other studies have associated antagonistic bacteria and actinomycete isolates with \textit{P. cinnamomi} suppression in Australian forest soils (Table 1.4), but the identity of these isolates was not determined (Malacjczuk 1979a; Keast and Tonkin 1983; Murray 1987; Malacjzuk 1988).

Broadbent et al. (1971) screened 3500 bacteria and actinomycetes isolated from various soils for antagonism toward several soil-borne plant pathogens including \textit{P. cinnamomi}. Most of the antagonistic isolates were from the genera \textit{Bacillus}, \textit{Pseudomonas} and \textit{Streptomyces}. In subsequent work, Broadbent and Baker (1974a, 1974b) demonstrated that \textit{Pseudomonas putida} \textbf{Trevisan} and \textit{Pseudomonas fluorescens Migula}, isolated from suppressive avocado soils, caused massive lysis of \textit{P. cinnamomi} mycelium \textit{in vitro}. They also found that \textit{Bacillus subtilis} var. \textit{niger Smith}, isolated from the same soils was involved in sporangial breakdown
Table 1.4 Studies of antagonistic bacteria in *P. cinnamomi* suppressive soils.

<table>
<thead>
<tr>
<th>Citation</th>
<th>Context</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broadbent et al. (1971); Broadbent and Baker (1974a; 1974b)</td>
<td>Suppressive avocado soils – eastern Australia</td>
<td><em>Pseudomonas</em> spp. <em>Pseudomonas putida</em> <em>Pseudomonas fluorescens</em> <em>Chromobacterium</em> spp. <em>Bacillus subtilis</em> <em>Actinomyces</em></td>
</tr>
<tr>
<td>Malajczuk (1979a; 1988)</td>
<td>Rhizosphere of <em>E. marginata</em> and <em>E. sieberi</em> grown in suppressive forest soil Western Australia</td>
<td>Non-specific bacteria Non-specific actinomycetes</td>
</tr>
<tr>
<td>Halsall (1982a; 1982b)</td>
<td><em>Eucalyptus</em> spp. forest soils New South Wales, Australia</td>
<td><em>Streptomyces</em> spp.</td>
</tr>
<tr>
<td>Malajczuk et al. (1984)</td>
<td><em>In vitro</em> study of antagonistic <em>Rhizobium</em> isolated from native legumes Western Australia</td>
<td>15 <em>Rhizobium</em> spp. isolates</td>
</tr>
<tr>
<td>Murray (1987)</td>
<td>Eucalyptus forest, antagonism in rhizosphere Western Australia</td>
<td>Non-specific bacteria Non-specific actinomycetes</td>
</tr>
<tr>
<td>Duvenhage et al. (1990); Duvenhage and Kotze (1993)</td>
<td>Suppressive avocado soils South Africa</td>
<td><em>Bacillus azotoformans</em> <em>Pichinoty Bacillus megaterium de Bury</em> <em>Pseudomonas</em> spp.</td>
</tr>
<tr>
<td>Stirling et al. (1994)</td>
<td>Suppressive avocado soil Queensland, Australia</td>
<td>9 non-specific actinomycetes 3 fluorescent <em>Pseudomonas</em> spp. 3 <em>Bacillus</em> spp. <em>Serratia marcescens</em></td>
</tr>
<tr>
<td>El Tarabily et al. (1996)</td>
<td><em>Banksia grandis</em> rhizosphere Australia</td>
<td><em>Micromonospora carbonacea Streptomyces violascens (Preob. and Svesh.) Pridham</em></td>
</tr>
<tr>
<td>You et al. (1996)</td>
<td>Suppressive mulch applied to avocado trees Western Australia</td>
<td>1600 isolates inhibitory <em>in vitro</em>; in vivo some <em>Streptomyces</em>, <em>Agromyces</em>, <em>Micromonospora</em> and <em>Actinomadura</em> isolates were suppressive</td>
</tr>
<tr>
<td>Yang et al. (2001)</td>
<td>Healthy avocado root tips – bacterial community 16S rDNA DGGE profiles California, USA</td>
<td>Bacterial rDNA community profile obtained from suppressive soil. <em>Pseudomonas</em> sp. 2 uncultured soil bacteria Uncultured <em>Pseudomonas</em> sp. <em>Polyangium</em> sp. <em>Cytophaga</em> sp. Unidentified eubacterium</td>
</tr>
<tr>
<td>Yin et al. (2004)</td>
<td>Suppressive avocado soil. Bacteria identified by combining substrate utilisation assays with rDNA intergenic sequences California, USA</td>
<td><em>Bacillus mycoides Renibacterium salmoninarum Streptococcus pneumoniae</em></td>
</tr>
</tbody>
</table>

(Broadbent and Baker 1974b). *Bacillus* spp. and *Pseudomonas* spp. have also been implicated as antagonistic bacteria common in suppressive avocado soils in South Africa (Mass and Kotze 1989; Duvenhage et al. 1990) and near Maleny in Qld (Stirling et al. 1992). Of the 164 bacteria (including several actinomycetes) isolated from the suppressive avocado soils near Maleny, 9% were antagonistic toward *P.*
cinnamomi in vitro. These antagonistic isolates included 3 Bacillus, 3 fluorescent Pseudomonas, 9 unidentified actinomycete isolates and an isolate identified as Serratia marcescens Bizio. However, when individual isolates or a combination of isolates were added to non-sterile and sterile soil leachates from the suppressive soil, lysis of P. cinnamomi mycelium only occurred in leachate from the non-sterile suppressive soil (Stirling et al. 1992). This indicates that these bacterial isolates may not be actively antagonistic toward P. cinnamomi in vivo. This also suggests that there may have been other unidentified (and possibly unculturable) organisms within the non-sterile soil leachate that were responsible for the suppressive capacity of the soil.

Yang et al. (2001) used a DNA-based method to identify bacteria associated with healthy avocado roots. These included Pseudomonas sp., Polyangium sp., Cytophaga sp., an uncultured Pseudomonas sp., 2 uncultured soil bacteria and an unidentified eubacterium. Several of these bacteria were dominant on healthy roots and absent from diseased roots (Yang et al. 2001). Combining a culture-based method with DNA sequencing, Yin et al. (2004) took a novel approach to identifying bacteria that occupy the same resource niche as P. cinnamomi in avocado soils. Carbon substrates known to attract P. cinnamomi zoospores were used to bait and grow soil bacteria with the capacity to utilise the same substrates. Intergenic rDNA sequences were obtained from the bacteria that grew on the substrates. Sequences from 3 bacteria related to Bacillus mycoides Flügge, Renibacterium salmoninarum Sanders and Fryer and Streptococcus pneumoniae (Klein) Chester, were characteristic of a post-epidemic soil that had apparently become suppressive. Yin et al. (2004) hypothesised that, because these and other functionally similar bacteria respond to the same biochemical cues that attract zoospores, they may contribute to P. cinnamomi suppression by occupying and competing for the same root infection sites targeted by P. cinnamomi.

While P. cinnamomi exhibits saprophytic survival strategies (Section 1.4), it is a weak saprophytic competitor (Malajczuk 1983), faring poorly in surface organic layers where larger numbers of saprophytes dominate the microbial community (Downer et al. 2001a). Changes in microbial populations were monitored in suppressive organic mulches applied to avocado orchards during a study in Western
Australia (You and Sivasithamparam 1995). Results from this study indicated that populations of bacteria and actinomycetes increased sharply following infestation of the mulch by *P. cinnamomi*. As suggested by the authors of this study, this phenomenon might be explained by bacteria and actinomycetes rapidly multiplying in response to the introduction of a new substrate in the form of *P. cinnamomi* propagules (You and Sivasithamparam 1995). In a subsequent study, 1600 actinomycetes isolated from the suppressive mulch were tested for antagonism toward *P. cinnamomi* (You et al. 1996). All 1600 isolates inhibited growth of *P. cinnamomi in vitro*. Ten of these isolates varied in their ability to suppress the pathogen and root infection during a pot assay with a susceptible *Antirrhinum* sp. (snapdragon). Of these, only a few isolates from the genera *Streptomyces*, *Agromyces*, *Micromonospora* and *Actinomadura* showed potential for suppressing the pathogen and development of disease (You et al. 1996). These results highlight the disparity that often occurs between observations made *in vitro* and *in vivo*.

1.7.3 Fungi

*Phytophthora* spp. are antagonised in several ways by a variety of soil fungi. The most common forms of antagonism involve mycoparasitism and/or production of metabolites that inhibit growth or destroy *P. cinnamomi* propagules (Malajczuk 1983). Ectomycorrhizal fungi may also contribute to the protection of susceptible plant roots from *P. cinnamomi* by: (i) forming a mantle that provides a physical barrier to penetration; (ii) producing antibiotics that inhibit growth and reproduction; (iii) utilising surplus plant exudates that may act as biochemical signals to *P. cinnamomi* hyphae and zoospores; (iv) providing habitat for other antagonistic rhizosphere microorganisms; (v) improving plant vigour; and (vi) inducing the plant to produce compounds that protect it from infection (Shea and Broadbent 1983; Erwin and Ribeiro 1996; Borowicz 2001). The fungi that form ectomycorrhizal associations with plant roots are usually Basidiomycetes (e.g. genera include: *Amanita*, *Boletus*, *Lactarius*, *Pisolithus*, *Rhizopogon*, *Russula*, *Suillus* and *Thelephora*) and to a lesser extent Ascomycetes, Zygomycetes and anamorphic fungi (Ogle and Brown 1997).
A number of ectomycorrhizal fungi have been linked with *P. cinnamomi* disease suppression in plantation conifers and in eucalyptus forest species (e.g. Marx 1969, 1970, 1972, 1973a, 1973b; Pratt 1971; Malajczuk 1979a; Finlay and McCracken 1991). However, according to Coffey (1992), avocado trees do not develop ectomycorrhizal associations and therefore, it is highly unlikely that ectomycorrhizal fungi are involved in *P. cinnamomi* suppression in avocado soils. The arbuscular mycorrhizal fungi may have an indirect role in suppression of *Phytophthora* root rot in some plant species by contributing to the health of the plant through providing nutrients to the roots thus enabling the host to resist or tolerate infection (Malajczuk 1983; Borowicz 2001). The involvement of arbuscular mycorrhizae in the suppression of *Phytophthora* root rot of avocados has been investigated (Davis et al. 1978; Matare and Hattingh 1978; Malajczuk 1988), however, it appears that these fungi have little to no effect on *P. cinnamomi* infection (Malajczuk 1983).

Several antagonistic fungal species are frequently associated with *P. cinnamomi* suppression (Table 1.5). These include species in the genera *Penicillium*, *Trichoderma*, *Aspergillus* and, less frequently, *Myrothecium* and *Epicoccum*. Each of these produces metabolites that actively inhibit *P. cinnamomi* *vitro* and presumably *in vivo* (Malajczuk 1983; Brown et al. 1987; Finlay and McCracken 1991; Chambers and Scott 1995; Almassi et al. 1996; Downer et al. 2001b; Jang et al. 2001). In particular, *Trichoderma* spp., *Myrothecium* spp. and *Trichoderma virens* (Mill., Giddens and Foster) von Arx isolated from suppressive soil have shown extreme antagonism toward *P. cinnamomi* during the saprophytic stage via antibiosis and mycoparasitism (Malajczuk 1983; Munnecke 1984; Gees and Coffey 1989; Finlay and McCracken 1991; Chambers and Scott 1995). The attraction of mycoparasites to *P. cinnamomi* propagules is presumably mediated by metabolites exuded by *P. cinnamomi* (Malajczuk 1983). Generally, lysis of *P. cinnamomi* hyphae by mycoparasitic fungi is rapid and involves the parasite coiling around the *P. cinnamomi* hyphae and subsequently forming structures that penetrate the cell wall (Malajczuk 1983). There are also a number of fungi that are capable of parasitising the thick-walled oospores of *P. cinnamomi*. These include certain members of the oomycetes, hyphomycetes, and chytrids (Sneh et al. 1977; Erwin and Ribeiro 1996). Examples include *Humicola fascoatra* Traaen, *Anguillospora pseudolongissima* Ranzoni, *Hyphochytrium catenoides* Karling (Daft and Tsao 1983), *Dactylella*
<table>
<thead>
<tr>
<th>Citation</th>
<th>Context</th>
<th>Fungal antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malajczuk (1979b)</td>
<td><em>E. marginata</em> <em>E. sieberi</em> Western Australia</td>
<td>2 <em>Boletus</em> spp., <em>Russula</em> sp., <em>Lactarius</em> sp., <em>Ramaria</em> sp.</td>
</tr>
<tr>
<td>Murray (1987)</td>
<td>Eucalyptus forest Antagonism in rhizosphere Western Australia</td>
<td>Various fungi not specified</td>
</tr>
<tr>
<td>Gees and Coffey (1989)</td>
<td>Suppressive avocado soil California, USA</td>
<td><em>Myrothecium roridum</em></td>
</tr>
<tr>
<td>Casale (1990)</td>
<td>Suppressive avocado soil California, USA</td>
<td><em>Trichoderma</em> sp. and several unidentified fungal isolates</td>
</tr>
<tr>
<td>Finlay and McCracken (1991)</td>
<td>Biocontrol of <em>P. cinnamomi</em> infection of <em>Lupinus albus</em> and <em>Erica vagans</em></td>
<td><em>Epicoccum purpurascens</em></td>
</tr>
<tr>
<td>Duvenhage and Kotze (1993)</td>
<td>Suppressive avocado soil South Africa</td>
<td><em>Paecilomyces lilacinus</em>, <em>Aspergillus candidus</em>, <em>Trichoderma hamatum</em></td>
</tr>
<tr>
<td>Duvenhage and Kohne (1997)</td>
<td>Suppressive avocado soil South Africa</td>
<td><em>Trichoderma harzianum</em>, <em>Trichoderma hamatum</em></td>
</tr>
<tr>
<td>McLeod et al. (1995)</td>
<td>Healthy avocado roots South Africa</td>
<td><em>Trichoderma virens</em>, <em>Trichoderma hamatum</em>, <em>Trichoderma koningii</em> <em>Oudemans</em></td>
</tr>
<tr>
<td>Chambers and Scott (1995)</td>
<td>Suppressive chestnut soil South Australia</td>
<td><em>Trichoderma virens</em>, <em>Trichoderma hamatum</em>, <em>Trichoderma koningii</em> <em>Oudemans</em></td>
</tr>
<tr>
<td>Costa et al. (1996; 2000)</td>
<td>Suppressive mulch California, USA</td>
<td><em>Trichoderma virens</em>, <em>Trichoderma harzianum</em></td>
</tr>
<tr>
<td>Borneman and Hartin (2000)</td>
<td>Fungal community DNA study of suppressive avocado soil California, USA</td>
<td>Dominant fungal sequences isolated from soil DNA samples were related to: <em>Tritirachium</em>, <em>Aspergillus</em>, <em>Pleospora</em>, <em>Petriella</em>, <em>Monilinia</em>, <em>Exophiala</em></td>
</tr>
<tr>
<td>Downer et al. (2001a)</td>
<td>Suppressive eucalyptus wood chip mulch applied to avocado trees California, USA</td>
<td>Most frequently isolated genera: <em>Penicillium</em>, <em>Aspergillus</em>, <em>Trichoderma</em>, <em>Sporothrix</em>, <em>Basidiomycetes</em>, <em>Phanerochaete chrysochoriza</em> <em>Burdsall</em>, <em>Ceraceomyces tessulatus</em> (Cooke) <em>Jüllic</em></td>
</tr>
</tbody>
</table>
spermatophaga Drechsler (Sneh et al. 1977) and Catenaria anguillulae Sorokin (Daft and Tsao 1984). The succession of fungi that parasitise P. cinnamomi propagules is determined by the soil water status, with oomycetes and the chytrids being favoured under moist conditions (Malajczuk 1983).

A number of antagonistic fungi associated with P. cinnamomi suppressive soils (Table 1.5) have also been assessed in vivo for their potential use in biocontrol. Reductions in root infection in avocado seedlings during glasshouse experiments and then in field experiments were achieved by introducing a strain of Myrothecium roridum Tode:Fr. isolated from healthy avocado roots (Gees and Coffey 1989). Epicoccum purpurascens Ehrenberg successfully protected Lupinus albus L. and Erica vagans L. from P. cinnamomi infection during pot experiments (Finlay and McCracken 1991). Some success in reducing Phytophthora root rot of Rhododendron spp. and citrus grown under controlled conditions was achieved with a number of different Penicillium spp. isolates (Tsao et al. 1991; Ownley and Benson 1992; Fang and Tsao 1995). Organic mulches inoculated with Trichoderma virens and Trichoderma harzianum Rifai reduced root infection in avocado seedlings and reduced viability of sporangia (Costa et al. 1996). Variable success has been achieved in reducing Phytophthora root rot with Paecilomyces lilacinus (Thom.) Samson, Aspergillus candidus Link:Fries and Trichoderma hamatum (Bonorden) Bainier in avocado orchards in South Africa (Duvenhage and Kohne 1997). Despite the attention that Trichoderma species have received (e.g. Broadbent and Baker 1974a; Mass and Kotze 1989; Casale 1990; Duvenhage and Kotze 1993; Chambers and Scott 1995; McLeod et al. 1995; Costa et al. 1996, 2000), they have rarely been effective as long-term biological control agents against P. cinnamomi (Erwin and Ribeiro 1996). The reason for this is most probably that they perform poorly in wet soils which are more favourable to P. cinnamomi (Coffey 1992).

Avirulent, hypovirulent (pathogen with reduced pathogenicity) and non-pathogenic strains of soil-borne pathogens have been found to provide cross protection in several disease suppressive soils such as in those that suppress take-all disease of wheat (Wong 1980). Finlay and McCracken (1991) investigated the potential for controlling P. cinnamomi using avirulent isolates of other Phytophthora spp. Several isolates initially reduced the disease rating over 2 months. However, those plants that
appeared to be protected at the end of the second month showed severe wilting or death by the seventh month. Finlay and McCracken (1991) concluded that cross protection against *P. cinnamomi* using other *Phytophthora* spp. has no potential as a method of biological control.

1.8 Involvement of microbial metabolites in *P. cinnamomi* suppression

1.8.1 Non-enzymatic microbial metabolites

Inhibition and lysis of *P. cinnamomi* propagules caused by bacteria and fungi are frequently attributed to antibiosis. There is evidence from a number of *in vitro* studies of several known and unknown microbial metabolites that disrupt *Phytophthora* growth, reproduction and pathogenicity (Malajczuk 1983; Erwin and Ribeiro 1996). While inhibiting mycelial growth, some of these metabolites also stimulate asexual (i.e. sporangia) and homothallic (i.e. oospores produced by selfing) reproduction in *Phytophthora*. For example, metabolites produced by *Trichoderma* spp. stimulate homothallic sexual reproduction in *Phytophthora* spp. (Section 1.4; Brasier 1971, 1972, 1975; Pratt et al. 1972; Reeves and Jackson 1972; O’Brien 1991). This stimulatory effect is associated with volatile or soluble metabolites that also act as antagonistic mechanisms to vegetative growth (Brasier 1975).

A number of microbial metabolites that potentially have deleterious effects on *Phytophthora* inoculum densities in soil have been identified. *P. cinnamomi* mycelial growth was shown to be inhibited by terrecyclic acid A, produced by an antagonistic *Aspergillus terreus* Thom strain (Almassi et al. 2003), and by diacetylphloroglucinol produced by several *Pseudomonas* spp. (Bedini et al. 1999). Indole-3-ethanol from *Zygorrhynchus moelleri* Vuillemin (Brown and Hamilton 1992) and flavopins from the mycoparasitic *E. purpurascens* (Brown et al. 1987) are known to inhibit zoospore germination. Other fungal compounds that inhibit *P. cinnamomi* include 6-(pent-1-enyl)-alpha-pyrone from *Trichoderma viride* Persoon:Fries (Moss et al. 1975), 6-pentyl-alpha-pyrone from *T. koningii* (Benoni et al. 1998) and metabolites from 5 classes of volatile compounds (alcohols, esters, ketones, acids and lipids) produced by *Muscodor albus* Worapong, Strobel and Hess. *M. albus* is a recently described (Strobel et al. 2001; Worapong et al. 2001) endophytic fungus isolated from
Cinnamomum zeylanicum Blume (cinnamon tree). The effects of these metabolites on *P. cinnamomi* have mostly been determined during *in vitro* experiments. Observing the effects of these metabolites within the soil environment is more difficult and for this reason there is a lack of specific evidence confirming their potential role as mechanisms involved in *P. cinnamomi* suppressive soils.

1.8.2 Enzymes involved in suppression

Several enzymes produced by bacteria and fungi have been associated with suppression of various plant pathogens. Enzymes frequently implicated in antagonism of phytopathogenic fungi include chitinase and glucanases. This appears logically sound considering that the cell walls of most fungi are principally composed of chitin and glucans (Alabouvette et al. 1996). Certain isolates of *S. marcescens*, *Streptomyces viridodiasticus* (Baldacci) Pridham and *M. carbonacea* have been shown to suppress basal drop disease of lettuce caused by *Sclerotinia minor* Jagger (El Tarabily et al. 2000). When interactions between these soil bacteria (1 bacterium and 2 actinomycetes) and *S. minor* were studied *in vitro*, the 3 antagonists were found to produce high levels of chitinase and β-1,3-glucanase, and all 3 isolates caused extensive hyphal lysis when *S. minor* was presented as the sole carbon source (El Tarabily et al. 2000).

A novel protein showing β-1,3-glucanase activity was isolated from a *Bacillus amyloliquefaciens* (ex Fukumoto) Priest strain highly antagonistic toward *Colletotrichum lagenarium* (Pass.) Ellis and Halst. (Kim and Chung 2004). Observations made via confocal and electron scanning microscopy suggested that the enzyme acted on the cell wall of *C. lagenarium*. The enzyme was also stable at 80°C for 20 minutes and exhibited antifungal activity against several other plant pathogenic fungi (Kim and Chung 2004). Enzymes associated with antagonism of *Botrytis cinerea* Pers.: Fr. on *Phaseolus vulgaris* L. leaves by *B. subtilis* and *B. megaterium* include chitinase, β-1,3-glucanase, β-1,4 glucanase (cellulase), β-glucosidase, protease and lipase (Saad et al. 2005). However, the role of these enzymes in suppression of *B. cinerea* has not been confirmed.
Enzymes produced by *Pseudomonas* spp., (e.g. Fridlender et al. 1993, Sindhu and Dadarwal 2001) which, along with *Bacillus* spp. and various actinomycetes which were common antagonists in the *P. cinnamomi* suppressive soils studied by Broadbent and Baker (1974a), have been implicated in antagonism of various phytopathogens. Chitinase activity was not involved in biocontrol of *Rhizoctonia solani* Kühn, *Sclerotium rolfsii* Saccardo and *Pythium ultimum* Trow by *Pseudomonas cepacia* Palleroni and Holmes (Fridlender et al. 1993). However, a β-1,3-glucanase produced by *P. cepacia* caused significant damage to *R. solani* hyphae (Fridlender et al. 1993). In another study, several *Pseudomonas* strains isolated from the rhizosphere of chickpea inhibited growth of *Pythium aphanidermatum* (Edson) Fitzp. and *R. solani* *in vitro* (Sindhu and Dadarwal 2001). The *Pseudomonas* isolates were also found to produce large amounts of cellulase and chitinase in liquid culture. However, the extent of fungal growth inhibition was not related to the activities of these enzymes (Sindhu and Dadarwal 2001).

Evidence for the potential involvement of enzymes produced by fungi in suppression of various phytopathogenic fungi comes from direct study of (mainly *in vitro*) interactions between fungal antagonists and target fungal pathogens. Many of these fungi specialise as mycoparasites and the enzymes that they produce play an important role in their parasitic activity. For instance, proteases produced by *Verticillium biguttatum* Gams degrade the cell walls of *R. solani* enabling *V. biguttatum* to penetrate, dissolve and feed on *R. solani* propagules (McQuilken and Gemmell 2004). Proteases were also implicated as a mechanism involved in biocontrol of *B. cinerea* by *T. harzianum* (Elad and Kapat 1999). In this example, proteases produced by *T. harzianum* appeared to inactivate enzymes produced by *B. cinerea* and involved in host infection (Elad and Kapat 1999). In both these examples chitinase and β-1,3-glucanase were also investigated, but these enzymes did not appear to be important during the initial stages of parasitism. This indicates that some mycoparasites may produce enzymes that attack polymers that bind cell wall components rather than produce enzymes that target more abundant components such as chitin and glucans.

However, chitinase and glucanases are associated with in some instances of fungal antagonism and mycoparasitism. β-1,3-glucanase produced by *Penicillium*
purpurogenum Stoll was implicated in *in vitro* antagonism of *Monilinia laxa*
Aderhold et Ruhland (causal agent of peach twig blight) and *Fusarium oxysporum*
Schlechtendahl:Fries f. sp. lycopersici (Saccardo) Snyder and Hansen (causal
agent of tomato wilt), but chitinase was not involved (Larena and Melgarejo 1996).
An α-1,3-glucanase was implicated in the mycoparasitic activity of *T. harzianum*
(Sanz et al. 2005). The ability of various *Trichoderma* spp. to protect *P. vulgaris*
from damping off caused by a *Pythium* sp. and *R. solani* was not related to chitinase
or β-1,3-glucanase production *in vitro*. However, chitinase (but not β-1,3-glucanase)
activities were correlated with reduced incidence and severity of damping off when
*P. vulgaris* was grown in soil (Cotes et al. 1994). Low concentrations of β-1,3-
glucanase and cellulase from *T. harzianum* were shown to inhibit germination of
encysted zoospores and elongation of germ tubes from *P. ultimum* (Thrane et al.
1997).

Several microbial enzymes have also been shown to inhibit growth and lyse
*Phytophthora* propagules during *in vitro* studies. For example, Budi et al. (2000)
 hypothesised that the mechanisms by which a *Paenibacillus* sp. (isolated from the
mycorrhizosphere of *Sorghum bicolour* [L.] Moench) antagonised *P. parasitica*
included production of extracellular cellulolytic, proteolytic, chitinolytic and
pectinolytic enzymes. However, when Budi et al. (2000) treated *P. parasitica*
cultures with commercial enzyme preparations, only proteases inhibited mycelial
growth. In another study, proteases from several *Pseudomonas* spp. acted as a growth
inhibitor, but cellulase and collagenase also inhibited mycelial growth (Bedini et al.
1999). Several other studies have found inhibitory and degradative effects of
cellulases on *Phytophthora* propagules (Brown et al. 1987; Fridlender et al. 1993; El
Tarabily et al. 1996; Downer et al. 2001b) and cellulases are used in procedures to
isolate various *Phytophthora* components. For example, *Phytophthora cactorum*
(Lebert and Cohn) Schroeter oospores are separated from mycelial mats by first
degrading the mycelium with cellulase (Sneh 1972). In genetic studies, protoplasts
are isolated from *Phytophthora* cells by completely degrading the cell walls using
cellulase and laminarinase (Layton and Kuhn 1988; Erwin and Ribeiro 1996). The
degradative effect of these enzymes on *Phytophthora* propagules is not surprising
considering that, unlike true fungi, the cell walls of Oomycota are principally
composed of cellulosic β-1,4-glucans and non-cellulosic β-1,3- and β-1,6-glucans
As demonstrated by the examples presented above, studies that have aimed to understand the role of microbial enzymes in controlling fungal plant pathogens have mainly focused on specific microorganisms and their interactions with phytopathogens \textit{in vitro}. However, in addition to the involvement of lytic enzymes in mycoparasitism and antagonism through direct contact with fungal phytopathogens, their accumulation in soil may contribute to general suppression (Section 1.3) and the subsequent development and maintenance of disease suppressive soils. Surprisingly, relatively few studies have investigated this hypothesis.

Several studies have associated disease suppression with microbial activity in soil determined by measuring soil enzyme activities using broad spectrum enzyme assays such as fluorescein-diacetate hydrolysis (Boehm et al. 1997; Kim et al. 1997; Downer et al. 2001a). Suppression of blight and root rot diseases of cereals caused by \textit{Fusarium culmorum (Smith) Saccardo} has been correlated with high soil cellulolytic activity of β-glycosidase (Knudsen et al. 1999; Rasmussen et al. 2002), cellobiohydrolase and, to a lesser extent, endocellulases (Rasmussen et al. 2002). These and other enzymes are produced by the soil microbial community during degradation of organic residues (Kiss et al. 1978; Atlas and Bartha 1998; Rasmussen et al. 2002) and therefore provide an indication of saprophytic microbial activity in the soil. However, these enzymes are not known to specifically inhibit or lyse \textit{Fusarium} propagules. Therefore, correlations between an accumulation of these enzymes in soil (which provides an indication of microbial activity) and suppression of \textit{F. culmorum} indicates that suppression may be related to competition rather than direct interactions between these enzymes and the pathogen.

High microbial activity and large populations of antagonistic bacteria, actinomycetes and fungi are found in soils with high organic matter content, which is a characteristic of \textit{P. cinnamomi} suppressive soils (Section 1.6). Therefore, competitive processes within the soil microbial community may be involved in \textit{P. cinnamomi} suppression. Populations of cellulase and laminarinase producing bacteria such as \textit{Bacillus} spp., \textit{Pseudomonas} spp. and \textit{Streptomyces} spp. and fungi such as
*Trichoderma* spp., *Trichoderma* spp., *Penicillium* spp. also tend to be common residents in *P. cinnamomi* suppressive soils (Broadbent and Baker 1974a; Malajczuk 1983; Mass and Kotze 1989; Casale 1990; Costa et al. 2000). Where organic matter levels are adequate the activity of these and other cellulase and laminarinase producing microorganisms would lead to an accumulation of these enzymes in the soil. Considering the effects of these enzymes on *Phytophthora* propagules during *in vitro* studies, it would be expected that high levels of cellulase and laminarinase activity in soil would result in conditions less favourable to *P. cinnamomi*. Conversely, in soils with limited availability of organic substrates, populations of cellulolytic saprophytes are likely to be much lower and, therefore, *P. cinnamomi* degrading cellulase and laminarinase would be reduced. This would be expected to result in a soil environment that is more conducive to *P. cinnamomi* (Downer et al. 2001a).

The association between high organic matter content, large populations of antagonists, high microbial activity and suppression of *P. cinnamomi* was noted by Downer et al. (2001a, 2001b), who investigated the hypothesis that cellulase and laminarinase act as principal mechanisms of *P. cinnamomi* suppression. Their approach was unique in that enzymes known to inhibit and lyse a phytopathogen during *in vitro* studies were specifically assayed in field samples. Downer et al. (2001a) applied mulch consisting of eucalyptus trimmings to soils beneath avocado trees in California and compared these with unmulched trees. *P. cinnamomi* inoculum and root infection were reduced beneath the mulch. This reduction was associated with increased microbial activity as determined by non-specific soil enzyme hydrolysis of fluorescein-diacetate. Reductions in *P. cinnamomi* inoculum and root infection were also negatively correlated with cellulase and laminarinase activities and the activities of unknown enzymes active against *P. cinnamomi* cell walls from within the mulch and at the mulch-soil interface.

In a follow-up study, Downer et al. (2001b) undertook experiments to demonstrate the effects of cellulase and laminarinase at various concentrations on *P. cinnamomi* propagules. The destructive potential of both cellulase and laminarinase was greatest at mostly high concentrations. At concentrations >10 units mL$^{-1}$ cellulase prevented the development of chlamydospores, zoosporangia and zoospores. Cellulase also
caused severe damage to mycelium at concentrations >25 units mL\(^{-1}\). At concentrations up to 25 units mL\(^{-1}\) laminarinase had little effect on chlamydospore, zoosporangia or zoospore formation and mycelium. The most pronounced effect of laminarinase was on reductions in zoospore survival and encystment of excised roots at concentrations between 10 and 100 units mL\(^{-1}\). Germination of chlamydospores was stimulated by both enzymes at concentrations <10 units mL\(^{-1}\). Downer et al. (2001) also noted that chlamydospores that formed in dead root tissues were shielded from the destructive effects of cellulase, which suggests that \(P. cinnamomi\) may survive spikes in enzyme activity within field soils by the same means. However, Downer et al. (2001) hypothesised that if these protected chlamydospores germinated as sporangia and released zoospores, the zoospores would subsequently be destroyed by laminarinase (Downer et al. 2001).

While it is tempting to propose that the findings from the study of Downer et al. (2001a, 2001b) demonstrate that cellulase and laminarinase are principal mechanisms involved in suppression of \(P. cinnamomi\), their results do not provide sufficient evidence to draw such definitive conclusions. Their first study merely demonstrated an association between higher enzyme activities and reductions in \(P. cinnamomi\) inoculum and root infections. In their second study, cellulase and laminarinase clearly affected several life stages of \(P. cinnamomi\). However, the relationship between enzyme activities measured in the suppressive soils and the enzyme concentrations used in their laboratory experiments was not determined. Therefore, the potential role of cellulase and laminarinase in \(P. cinnamomi\) suppressive soils requires further confirmation.

1.8.3 Methods for estimating soil cellulase and laminarinase activities

The soil enzyme assay used by Downer et al. (2001a) was a modification of the method described by Alef and Nannipieri (1995). The method involves passive extraction of extracellular enzymes by suspending a measured amount of soil in buffered substrate (e.g. carboxymethylcellulose or laminarin) solutions and agitating for a specified time, usually 4 h (Schinner and von Mersi 1990; Deng and Tabatabai 1994a, 1994b; Downer et al. 2001a). Following the extraction-reaction step, enzyme activity is estimated by using a colorimetric method to determine reducing sugars.
released during the enzyme-substrate reaction. The colorimetric method used by Downer et al. (2001a) involves the use of potassium cyanide and ferric-ferrocyanide reagents (Schinner and von Mersi 1990; Deng and Tabatabai 1994). Other colorimetric methods for determining reducing sugars in solution include phenol-sulphuric acid, anthrone-sulphuric acid, dinitrosalicylic acid (DNS) and alkaline copper (Dygert et al. 1965; Schinner and von Mersi 1990; Deng and Tabatabai 1994; Gander et al. 1994) or a glucose oxidase:peroxidase system (Benefield 1971).

Several issues have been identified in relation to each of these methods (Gander et al. 1994; Deng and Tabatabai 1995) which indicate that there are opportunities to improve existing methods for measuring activity of soil glucanases. Firstly, enzymes are present in soil as free enzymes released from living cells, disintegrating cells and cell fragments. However, enzymes are also bound to cell constituents and organic compounds within the soil matrix and are present within both proliferating and non-proliferating cells (Tabatabai 1994). For this reason it is highly likely that passive extraction procedures (which involve incubation of soil suspended in a substrate solution) results in an underestimate of true enzyme activity potential within the soil. This issue might be improved by enzyme extraction procedures that involve chemical and/or mechanical disruption of the soil matrix.

The second issue relates to the use of colorimetric assays for determining reducing sugars released during the enzyme reaction. Each of the colorimetric methods for determining reducing sugars have been evaluated by a number of workers (Dygert et al. 1965; Schinner and von Mersi 1990; Deng and Tabatabai 1994, 1995; Gander et al. 1994). Each method varies with respect to sensitivity and reliability, and all are affected by interferences from within the soil-enzyme complex, including pigments, trace elements, and organic matter (Dygert et al. 1965; Deng and Tabatabai 1994, 1995; Gander et al. 1994). Another issue is related to limitations on the number of samples that can be handled during a single run. These limitations occur because: i) the size of containers required to hold the buffer and soil during the enzyme reaction step become bulky when handled in large numbers, which may restrict the number of samples that can be processed at one time; ii) the use of single celled or flow cell spectrophotometers to measure colour absorbance can be laborious and slow; iii) separate runs are required when determining the activities of more than one enzyme;
iv) the cost of substrates such as laminarin may be a limiting factor for use in enzyme reaction procedures that require substrate solution volumes of several mL.

Increasing the number of samples that can be handled and enzymes that can be assayed during a single run might be achieved by reducing the volume of substrate solution and soil used in the assay and by taking advantage of microplate technology. This was recognised by Wirth and Wolf (1992), who developed a microplate based multi-enzyme colorimetric assay, which included assays for both cellulases and laminarinases. Enzymes are passively extracted by agitating soil in a buffer solution. After 60 min, the soil suspension is centrifuged and the supernatant is transferred to a microplate well containing substrates labelled with a soluble remazol brillant blue (RBB) dye. The plates are incubated for the required period of time and then the reaction is terminated by adding hydrochloric acid, which results in precipitation of non-degraded polymeric substrate. The microplates are centrifuged and then the supernatant is transferred to a second microplate. Soluble RBB cleaved from the substrate during the enzyme reaction is then measured using a spectrophotometric plate reader. This innovation circumvents many of the handling issues stated above. However, the method still relies on passive extraction of free enzymes, which can lead to an underestimation of total soil enzyme activities. The method also relies on a colorimetric based assay which could be affected by interferences from within the soil matrix.

A possible solution to issues associated with colorimetric assays is the use of methods that employ fluorogenic technology. Substrates labelled with fluorophores such as 4-methylumbelliferone (4-MUF or 4-MUB) and 7-amino-4-methyl coumarin have been used effectively in microplate assays to measure soil enzyme activity and diversity (Miller et al. 1998; Marx et al. 2001). A soil suspension is prepared and aliquots from the soil suspension are placed directly into microplate wells containing substrate labelled with the fluorophore. The plates are incubated for a predetermined optimum period of time and then hydrolysed substrates are detected by measuring the fluorescence intensity in each microplate well using an automated microplate reader fitted with fluorescence emission filters. These emerging methods have tremendous potential for the study of soil enzymes. However, (at the time of writing) there are a limited number of fluorophore labelled substrates available commercially.
For example, there are no fluorophore labelled substrates available that can be used to directly measure cellulase or laminarinase activities. Therefore, these fluorogenic microplate methods will remain unsuitable for measuring the activities of these enzymes until labelled substrates are made available.

However, the fluorometric Amplex Red\textsuperscript{©} glucose assay developed by and supplied in kit form by Molecular Probes (USA) may be suitable for determination of reducing sugars (which mostly constitute D-glucose) following hydrolysis of carboxymethylcellullose and laminarin substrates. During the Amplex Red glucose assay, glucose oxidase reacts with D-glucose to form hydrogen peroxide ($\text{H}_2\text{O}_2$) which, in the presence of horseradish peroxidase, reacts with the Amplex red reagent to generate red-fluorescent resorufin. The quantity of resorufin produced during the reaction is determined using excitation wave lengths near 530nm and emission maxima of approximately 590nm. The entire assay can be carried out in 96 well microplates and can be completed within 1 hour (Molecular Probes 2001).

Traditional soil enzyme assays could be improved by developing an enzyme extraction procedure which involves mechanical disruption of the soil, microplate adaptations of the substrate-enzyme reaction and the use of the Amplex Red glucose assay to determine glucose released during the soil-enzyme reaction. The development of such a procedure has the potential to: i) improve the sensitivity of existing methods; ii) increase the number of samples that can be handled and handling efficiency; and iii) circumvent issues associated with colorimetric determinations of reducing sugars.

1.9 Microbial community structure and diversity in \textit{P. cinnamomi} suppressive soils

There is a substantial body of work that provides evidence for the possible involvement of specific bacterial and fungal isolates (Section 1.7), and microbial metabolites (Section 1.8), in antagonism and suppression of \textit{P. cinnamomi}. However, only a limited number of studies have investigated the importance of microbial community structure and diversity in the development and maintenance of \textit{P. cinnamomi} suppressive soils. The term “community structure” refers to the
taxonomic composition of an ecological community but may also be used with reference to “community organisation”, which relates to the distribution of individuals among species within a community (Dunbar et al. 2000). An ecological community may have major structural differences related to differences in the presence, absence or abundance of numerically dominant species, or minor structural differences related to the presence, absence or abundance of one or several less dominant species. Taxonomic diversity within ecological communities has 2 main components, “species richness” and “species evenness”. Species richness is simply defined as the total number of species within a community. Species evenness, on the other hand, refers to the distribution of individuals within taxonomic groups. The abundance distribution of individuals may be spread evenly among each species within the community, or may be concentrated within a single or several species that are numerically dominant within the community (Magurran 1991).

Over the past 2 decades our awareness of the importance of soil microbial communities in ecosystem functioning has increased substantially (Ovreas 2000). Soil microbial communities are crucial to nutrient cycling, detoxification of environmental contaminants and plant health (Atlas and Bartha 1998). As such, adverse changes to the structure and diversity within soil microbial communities may have long term implications for ecosystem stability (Ovreas 2000). In agroecosystems this translates to deterioration of crop productivity and decline in the sustainability of farming systems. For instance, changes within the soil microbial community that lead to imbalances in community structure and diversity may allow the development of deleterious populations within the soil (Alabouvette et al. 2004). For this reason, microbial community structure and diversity may also be important in the development and maintenance of biological processes that result in \textit{P. cinnamomi} suppressive soils and soils that suppress other soil-borne phytopathogens.

Studies that have provided insight into the phylogenetic structure and diversity of microbial communities in \textit{P. cinnamomi} soils have mostly been undertaken using culture-based isolation techniques (Section 1.7). However, comparisons between culture-based and direct, microscopic enumeration of microbial cells have indicated that <1% of microbial communities in environmental samples are culturable (Torsvik et al. 1990; Atlas and Bartha 1998; Head et al. 1998). In addition, DNA-based
studies have suggested that genetic diversity of bacteria in soil may be 200 times greater than the diversity of bacteria that can be cultured from the soil (Torsvik et al. 1990). This issue imposes serious limitations on the conclusions that can be drawn from culture-based and phenotypic assessments of microbial community structure and diversity. Consequently, there may be many more antagonistic bacteria and fungi involved in suppression than have been identified. Microbial community DNA profiling methods have the capacity to detect both culturable and non-culturable fractions within soil microbial communities (Head et al. 1998; Kitts 2001; Alabouvette et al. 2004; Mazzola 2004). Therefore, microbial community DNA profiling methods have the potential for identifying non-culturable constituents of microbial communities that may be unique to *P. cinnamomi* suppressive soil (Borneman and Hartin 2000; Yang et al. 2001). In this section common culture independent microbial community DNA profiling methods are outlined and evidence implicating microbial community structure and diversity in disease suppressive soils is examined.

1.9.1 *DNA-based methods for analysis of microbial community structure and diversity*

There is a substantial body of research that has demonstrated the use of microbial community DNA profiling methods to assess microbial community structure and diversity in a range of environments (e.g. Liu et al. 1997; Dunbar et al. 1999, 2000; Marsh 1999; Smit et al. 1999; Hedrick et al. 2000; Hill et al. 2000; Dees et al. 2001; Hagn et al. 2003; Kowalchuk et al. 2003). These methods have the capacity to provide descriptive and quantitative information and discriminate between soil microbial communities in comparative studies. Microbial community DNA profiling methods are frequently used to observe changes in microbial community structure and diversity in relation to agronomic management (e.g. Dunbar et al. 1999; Hagn et al. 2003), impacts due to contamination (e.g. Ibekwe et al. 1997; Trevors 1998; Lord et al. 2002) and other environmental perturbations (e.g. Liu et al. 2000; Norris et al. 2002; Fierer et al. 2003). However, DNA-based methods for assessing microbial community structure and diversity have only recently been applied to the study of disease suppressive soils (discussed in Section 1.9.2). Several microbial community DNA profiling methods are available but each has its advantages and disadvantages.
Clone libraries / direct sequencing

The most comprehensive approach to analysing microbial community composition involves the construction of clonal DNA libraries (Mazzola 2004). These are constructed by cloning amplicons (using appropriate vectors which are transformed into *Escherichia coli* cells) from PCR amplified community DNA extracted from environmental samples, isolating and purifying the resulting plasmid and sequencing the target gene. The sequences are analysed for matches to known sequences archived in nucleotide databases (e.g. NCBI Blast nucleotide database). Where sequences share high homology with archived sequences, inferences can be made with regard to the phylogenetic identity of the sequence. Matches may not be found for many sequences obtained from complex microbial communities (such as those found in soil) which indicates that these sequences are from unknown species or species which have not been sequenced.

A definitive inventory of the composition of a soil community requires sequencing of thousands of clones from a single soil sample which may contain as many as 4000 species g\(^{-1}\) soil (Torsvik et al. 1990). This makes the construction of clone libraries from complex microbial communities labour intensive and costly. For these reasons, this approach is not commonly used to examine microbial community structure and diversity, especially where a number of samples require analysis (Valinsky et al. 2002; Mazzola 2004). However, even sequencing of a small fraction of the microbial community may still yield information that cannot be acquired by other means (e.g. Borneman et al. 1996; Borneman and Triplett 1997). This was demonstrated in a study of bacterial diversity in pasture soils located in Wisconsin, USA (Borneman et al. 1996). In this study, 124 sequences were obtained which revealed that bacteria in the soils were from 3 major groups (Cytophaga-Flexibacter-Bacteroides, low-G+C-content gram positive group, members of the class Proteobacteria) and several unknown clades were also identified.

In many studies DNA-based microbial community profiling methods are used with the primary aim of determining whether there are differences between communities or whether changes occur in a soil community in response to specific events. The most suitable methods for this purpose are those that provide more rapid analysis of
multiple samples and less inventorial evaluations of microbial communities than clone libraries. There are several methods that meet these criteria, the more commonly used of these include denaturing gradient gel electrophoresis (DGGE; Muyzer 1999), temperature gradient gel electrophoresis (TGGE; Muyzer 1999) and terminal restriction fragment length polymorphism analysis (TRFLP; Liu et al. 1997; Marsh 1999; Mills et al. 2003).

**DGGE / TGGE**

DGGE and TGGE both operate on a similar principle. Mixed PCR amplified community DNA is separated by electrophoresis on a gel system using increasing gradients of denaturant (DGGE) or temperature (TGGE). DNA molecules move through the gel until they reach the denaturant or temperature gradient at which they melt. As the double-stranded DNA melts it branches which slows its migration through the gel (Muyzer 1999; Hill et al. 2000). The gels are stained using an intercalating dye to reveal individual bands which each correspond to different nucleotide sequences (Hill et al. 2000). In combination, these bands form a microbial community DNA profile (also referred to as a fingerprint).

After a profile is generated it can be analysed to determine whether there are differences between communities and to identify the bands that contribute to any differences. A major benefit of DGGE / TGGE is that amplicons of the same length but with different sequence information are separated and therefore each band in the profile should correspond to a unique sequence (Hill et al. 2000). Another major benefit is that bands can be excised from the gel and sequenced, which allows inferences to be made regarding their phylogenetic identity. Selecting and sequencing only bands of interest is far more efficient than constructing comprehensive clone libraries. However, DGGE / TGGE has a limited capacity to resolve bands when they are close together or when the profile contains a large number of bands (Muyzer et al. 2004).

**TRFLP**

In the TRFLP method fluorescently labelled oligonucleotide primers (5'-end labelled) are used to amplify community DNA during PCR. The PCR product is digested with restriction enzymes and then the digested amplicons are separated
using an automated DNA analyser (which may be an ultra-thin polyacrylamide or capillary gel based system) which uses a laser to detect the fluorescently labelled terminal restriction fragments (TRFs). Data is conveyed to software which generates an electropherogram and automatically digitises the data. TRF lengths are calculated against a DNA size standard. The fluorescence intensity of each TRF (represented as a peak in the electropherogram) is also determined to provide data regarding peak height and area which corresponds to the abundance of the TRF in the profile.

TRFLP has several advantages over DGGE. Firstly, TRFLP is more time efficient with the potential to produce hundreds of TRFLP patterns within a week (Kitts 2001). Software associated with the automated DNA analyser automatically and instantly digitises the electropherogram which circumvents the time consuming requirement to manually digitise gel images. The digitised data can be imported into spreadsheets for further processing or imported directly into statistical analysis software. The most important advantage of TRFLP is its precision and high definition which allows comparisons to be made between runs. However, TRFLP also has several disadvantages. Firstly, phylogenetically unrelated species may share TRFs of similar lengths and will therefore be represented by the same TRF in the profile (Horz et al. 2000; Mills et al. 2003). This is partially overcome for the purposes of analysis by referring to each peak in the profile as an operational taxonomic unit or OTU (Horz et al. 2000). Determining the identity of individual OTUs is made more difficult than in DGGE because DNA is destructively sampled and cannot be reclaimed. The matching of OTU sizes in silico is imprecise but this can be overcome by using multiple enzyme digests (Kitts 2001). However, the additional time required to complete the restriction enzyme digestion step and handle the additional data negates some of the efficiency gains. There are also problems associated with incomplete digestion of amplicons (Clement et al. 1998; Osborn et al. 2000) and the formation of single-stranded amplicon fragments during PCR which leads to secondary “Pseudo TRFs” (Egert and Friedrich 2003).

**LH-PCR**

A recently introduced method known as Length Heterogeneity PCR (LH-PCR; Suzuki et al. 1998; Ritchie 2000) shares the advantages of TRFLP but has the added advantage of having no enzyme digestion step. As with TRFLP, community DNA is
amplified using fluorescently labelled primers, amplicons are separated using an automated DNA analyser during which an electropherogram is generated and then the data is digitised. Unlike TRFLP, which identifies sequence length variations based on restriction site variability, LH-PCR uses natural length variations across specific hyper-variable regions of DNA (Ritchie 2000; Mills et al. 2003).

Mills et al. (2003) compared TRFLP and LH-PCR for use in monitoring bacterial community dynamics in response to nutrient amendments applied to petroleum-contaminated soils. They found that LH-PCR provided higher estimates of bacterial diversity than TRFLP. They also found that LH-PCR was more robust and technically simpler than the TRFLP method. LH-PCR was more reproducible than TRFLP, which was attributed to the loss of TRF data due to variability between digests and limitations associated with resolving small bp TRFs. In contrast, LH-PCR amplicons were more easily resolved, thus providing more data points to include in the analysis. LH-PCR also showed a greater ability to detect subtle changes in bacterial community structure (Mills et al. 2003).

In addition to the study by Mills et al. (2003), LH-PCR has also been used successfully to assess bacterial community dynamics and diversity in marine environments (Suzuki et al. 1998; Bernhard and Field 2000), during on-site treatment of pulp and paper mill waste waters (Tirrola et al. 2000) and in pasture soils (Ritchie et al. 2000). However, LH-PCR has never been used to analyse microbial communities in disease suppressive soils. In addition, the LH-PCR method has not been used to analyse soil fungal community structure and diversity. A method similar to LH-PCR, known as automated rRNA intergenic spacer analysis (ARISA; Fischer and Triplett 1999; Ranjard et al. 2000), has been used to analyse fungal community DNA (Ranjard et al. 2001; 2003). Both methods involve amplification of community DNA using fluorescently labelled primers, separation of amplicons using an automated DNA analyser and sequence length variations of different OTUs are measured and used for comparisons. However, ARISA specifically targets longer sequence domains across intergenic spacer regions (IGS) whereas, in LH-PCR, smaller subunits adjacent to or within IGS regions are targeted. Therefore, the LH-PCR method is distinctly different to ARISA.
Until recently (Martin and Rygeiwicz 2005), fungal specific primers suitable for use with LH-PCR analysis of fungal community DNA had not been evaluated. Martin and Rygeiwicz (2005) designed and evaluated primers that have been used for identification of Dikaryomycota (which include 2 major fungal clades Ascomycota and Basidiomycota) isolated from soil, and for analysis of Dikaryomycota DNA extracted from plant tissues. However, these primers are homologous to Dikaryomycota and therefore are unlikely to amplify fungal DNA from other clades. Therefore, the usefulness of these primers for assessing fungal community structure and diversity is limited.

Oligonucleotide primers selected for use in community DNA profiling methods need to be specific to, but universal within, the group being targeted (i.e. bacteria, fungi, etc.). For analysis of bacterial communities, primers that amplify DNA from the small subunit (SSU) 16S rDNA region are frequently used. The main reasons for this are that the 16S rDNA gene is found universally in bacteria and contains both highly conserved and variable sequence regions (Hill et al. 2000). Therefore, primers that target regions within the 16S rDNA gene are likely to amplify a broad range of bacteria. In fungi the 18S, 5.8S and 28S rDNA genes, which are traversed by the hyper-variable internal transcribed spacer ITS 1 and ITS 2 regions (Figure 1.1), have proven useful for the same reasons (Hill et al. 2000).

![Figure 1.1](image)

**Figure 1.1** Schematic representation of the 18S, 5.8S and 28S ribosomal genes and the hyper-variable ITS1 and ITS2 regions that traverse them.

A number of fungal specific and universal primers have been developed (e.g. White et al. 1990; Gardes and Bruns 1993; Smit et al. 1999; Borneman and Hartin 2000; Anderson et al. 2003) and used successfully to profile fungal communities in a range of environmental samples including soil (Smit et al. 1999; van Elsas et al. 2000; Hagn 2003; Nikocheva et al. 2003; Ranjard et al. 2001). Some of these primers may
be suitable for use in LH-PCR depending on the electrophoresis system used for separating amplicons. However, none of these primers is suitable for use in LH-PCR where an ABI Prism 3730 automated capillary DNA analyser (Applied Biosystems, USA) is used. The reason for this is that the ABI 3730 can only measure amplicons <500 bp (at the time of writing the largest size standard manufactured by ABI for use with the ABI 3730 was 500 bp) and each of these primers produce amplicons >500 bp. Turenne et al. (1999) designed the ITS86-F forward primer and coupled this with the ITS4 reverse primer (White et al. 1990) for use in screening human blood for medically important fungal pathogens. In their evaluation they demonstrated that this primer pair was fungal specific and universal. These primers also produced sequence lengths <500 bp, which indicates that they may be suitable for use in LH-PCR analysis of fungal community DNA from environmental samples. However, this primer pair will require further evaluation for its potential use in fungal LH-PCR.

As with all PCR based analyses of microbial community DNA, LH-PCR is limited by issues associated with extraction of nucleic acids, biases associated with PCR and methods used for amplicon separation and analysis (Suzuki et al. 1996; van Winzingerode et al. 1997; Head et al. 1998; Ritz 1998; Tein et al. 1999; Hill et al. 2000; Kitts 2001). As occurs with TRFLP and ARISA, several species may share the same sequence length and therefore be represented by a single OTU in the profile. The capacity to resolve OTUs in a profile often diminishes during electrophoresis of longer domains (Suzuki et al. 1998). An advantage that LH-PCR may have over ARISA is that shorter domains are targeted and, therefore, higher resolution of OTUs can be achieved by analysing shorter sequences (Suzuki et al. 1998). A disadvantage that LH-PCR shares with TRFLP and ARISA is that DNA is destructively sampled during electrophoresis and therefore OTUs of interest cannot be recovered and sequenced to reveal their identity. However, it is possible to make inferences with regard to the identity of OTUs appearing in LH-PCR profiles where 2 or more domains are amplified (Suzuki et al. 1998) or by cloning directly from the PCR product followed by sequencing inserts that match the length of selected OTUs.

In considering these issues, it appears that LH-PCR has a limited ability to obtain a total and true measure of microbial community structure and diversity. Despite these limitations (which are common to all community DNA profiling methods), LH-PCR
provides a powerful tool where the primary purpose is a rapid appraisal of microbial community structure and diversity for comparative purposes (Mills et al. 2003). Where patterns of interest are observed with LH-PCR, consideration can be given to whether the use of more resource intensive methods (such as constructing clone libraries) to identify constituents within the microbial community is justified.

1.9.2 Microbial community structure and diversity in P. cinnamomi suppressive soils

As discussed in Sections 1.7.2 and 1.7.3, a varied array of soil bacteria and fungi have been screened for in vitro antagonism toward P. cinnamomi and for their potential involvement in P. cinnamomi suppressive soils. Bacteria commonly isolated and implicated in suppression of P. cinnamomi in soils include those from the genera Bacillus and Pseudomonas, and from the Actinomycetes (Section 1.7.2; Table 1.4). Fungi associated with P. cinnamomi suppression belong to the genera Pencillium, Trichoderma, Aspergillus, Myrothecium and Epicoccum (Section 1.7.3; Table 1.5). Each of these groups, along with other antagonistic bacteria and fungi, are probably active in the soil simultaneously. This suggests that, rather than suppression resulting from a single or a few specific microbial agents, it may result from communities of microorganisms that contain a number of key phylogenetic groups. In this instance, the principal mode of suppression could be considered to lie somewhere between specific and general suppression (Section 1.3), because suppression is dependent on the presence and activity of key taxonomic groups in the soil.

Microbial community structure is thought to be influential in several incidences of soils that suppress soil-borne plant pathogens (van Elsas et al. 2002; Garbeva et al. 2006) other than P. cinnamomi. For example, culture-based studies have suggested that an abundance of non-pathogenic Fusarium spp. and fluorescent Pseudomonas spp. may be critical in the development of soils that are naturally suppressive to Fusarium wilt (Mazzola 2002). The presence of specific fluorescent Pseudomonas spp. in soil microbial communities has also been implicated in the suppression of take-all of wheat, caused by G. graminis var. tritici Walker (Mazzola 2002). Suppression of Rhizoctonia root rot of apple trees caused by R. solani is induced with the cultivation of wheat cultivars that stimulate populations of specific
fluorescent pseudomonad genotypes antagonistic toward *R. solani* (Mazzola 2002). Diminished capacity for soil microbial communities to suppress Rhizoctonia root rot of apple has also been correlated with increasing orchard age and declines in rhizosphere populations of *B. megaterium, Burkholderia cepacia* (Palleroni and Holmes) Yabuuchi and *P. putida* (Mazzola 1999).

Recent DNA based studies have further reinforced assumptions regarding the importance of microbial community structure in disease suppression. In one study survival of *F. oxysporum* f. sp. *lycopersici*, following wheat bran-amended solarization, was reduced by increased activity of facultative anaerobes (Momma 2005). Subsequent work involving DGGE analysis of bacterial community DNA revealed the emergence of dominant bands that were unique to the wheat bran-amended solarized soils. This implies that bran-amended solarization caused a shift in bacterial community structure and that the bacteria represented by these bands may have been responsible for reduced survival of the pathogen (Momma 2005). In another study, the structure of microbial communities involved in the suppression of *P. aphanidermatum* (which causes disease in cucumber [*Cucumis sativus* L.] plants) in rock wool slabs was investigated by cultivation on selective media and by DGGE analysis (Postma et al. 2006). Culture-based isolation indicated that suppression of *P. aphanidermatum* was correlated with an abundance of culturable filamentous actinomycetes and *Trichoderma* spp. Bacterial community profiles generated by DGGE showed a significant relationship between the composition of microbial communities and disease suppressiveness. In agreement with results from the culture-based component of the study, dominant bands excised from the DGGE gel and sequenced matched sequences from several actinomycetes including: *Streptomyces, Mycobacterium, Microbacterium, Rhodococcus, Curtobacterium*, and *Tsukamuraella* (Postma et al. 2006).

In some instances of disease suppressive soils the structural organisation of microbial communities may not be as important as other factors. For example, in one study DGGE profiles generated for soil bacteria and fungal communities were found to be poor indicators of *Pythium* suppression (Kowalchuk et al. 2003). In addition, DGGE microbial community profiles were also limited in detecting changes within communities after rigorous soil treatments such as fumigation and flooding. While
suppression was observed in both untreated naturally suppressive soils and sterilised soils amended with compost, the DGGE profiles from these soils indicated that the composition of bacteria and fungal communities were different. This indicates that suppression of *Pythium* root rot can be influenced by different communities (Kowalchuk et al. 2003).

Microbial diversity is considered to be an important factor in the maintenance of soil health and quality which incorporates the concept of disease suppression (Garbeva et al. 2006). During 2 complementary studies involving culture-based and culture-independent DGGE comparisons between permanent grassland, grassland planted to maize, long-term arable land and arable land turned into grassland, suppression of *R. solani* was correlated with increasing levels of microbial diversity (van Elsas et al. 2002; Garbeva et al. 2006). Results from the 2nd study (Garbeva et al. 2006) indicated that treatments with the highest above ground plant diversity (grassland and grassland planted to maize) also yielded the highest levels of microbial diversity. Species diversity within *Bacillus* and *Pseudomonas* communities were also positively correlated with suppression of *R. solani* (Garbeva et al. 2006).

While many culture-based studies have been undertaken in an attempt to identify microbial agents specifically involved in suppression of *P. cinnamomi* (Section 1.7), there are 2 studies which have used a culture independent DNA-based approach to investigate microbial community structure in *P. cinnamomi* suppressive soils. Yang et al. (2001) undertook a study of bacterial communities associated with healthy and diseased avocado roots grown in soil naturally infested with *P. cinnamomi*. They used DGGE to compare 16S rDNA profiles acquired from rhizosphere bacterial communities living on the tips of healthy and *P. cinnamomi* infected avocado roots. Yang et al. (2001) found that (based on DGGE profiles) the composition and structure of bacterial communities associated with healthy roots were consistently similar to each other, and were significantly different to bacterial communities associated with infected roots. The structure of bacterial communities on healthy root tips was also similar to that of healthy root tips taken from plots that had been continuously bioaugmented (via an irrigation system) with the biocontrol agent *P. fluorescens* strain 513. Ribosomal DNA bands with the highest intensity were excised from the DGGE gels, cloned and sequenced and compared against nucleotide
sequence databases. Several of these clonal sequences originated from previously uncultured bacteria. This result highlights the capacity for molecular approaches to generate information beyond that which can be obtained using traditional culture-based methods.

With the main purpose of designing and testing fungal specific primer pairs for amplifying fungal community DNA from environmental samples, Borneman and Hartin (2000) compared a \textit{P. cinnamomi} suppressive avocado soil with soil from a diseased avocado orchard. They used one of their primer pairs to amplify fungal community DNA from each soil and then cloned and sequenced the amplicons. The identities of the clonal sequences were determined by comparing them with published sequences. Borneman and Hartin (2000) found notable differences in the phylogenetic composition of the suppressive and conducive soils. The 62 clones that Borneman and Hartin (2000) sequenced belonged to 10 genera, 4 of which were exclusive to the suppressive soil. In the suppressive avocado soil the dominant genera were \textit{Tritirachium}, \textit{Aspergillus}, \textit{Pleospora}, \textit{Petriella}, \textit{Monilinia} and \textit{Exophiala}. Results from a culture-based study (Downer et al. 2001a) of the same soil suggested that the dominant genera were different to those identified using the molecular approach. The culture-based approach suggested that the dominant fungal genera were \textit{Aspergillus}, \textit{Penicillium}, \textit{Sporothrix}, \textit{Phoma}, \textit{Trichoderma} and \textit{Fusarium} (Borneman and Hartin 2000; Downer et al. 2001a).

Borneman and Hartin (2000) and Yang et al. (2001) are the only studies that have used a culture independent molecular approach in attempts to characterise microbial community structure in \textit{P. cinnamomi} suppressive soils. Both these studies were carried out on soils supporting avocados in California (USA) and the role of microbial community structure in \textit{P. cinnamomi} suppressive soils within the Australian context is yet to be investigated by means of a culture independent approach. In addition, Yang et al. (2001) investigated bacterial community structure associated with healthy and diseased avocado root tips, but the role of community structure in the universal soil matrix has not been investigated. Borneman and Hartin (2000) only compared soils from one suppressive orchard and one diseased orchard and therefore more locations and more soil types need to be examined to determine if fungal community structure is important in other \textit{P. cinnamomi} suppressive soils.
The limitations of culture-based studies and the outcomes from the Yang et al. (2001) and Borneman and Hartin (2000) studies indicate a need for further research into the importance of microbial community structure in *P. cinnamomi* suppression using DNA-based community profiling methods. Further, while it has been suggested that high microbial diversity may be an important factor in disease suppressive soils (Alabouvette et al. 2004), at the time of writing no studies had been undertaken with the specific aim of examining microbial diversity in *P. cinnamomi* suppressive soils.

1.10 Summary and outcomes

Interest in *P. cinnamomi* suppressive soils has prevailed over several decades since Broadbent et al. (1971) and Broadbent and Baker (1974a, 1974b) first identified the phenomenon in eastern Australia. However, land uses and/or management practices at the original avocado orchards where *P. cinnamomi* suppressive soils were identified by Broadbent and Baker (1974a) in the early 1970s have since changed (ascertained during preliminary investigations for this study). While work was undertaken on *P. cinnamomi* suppressive mulches applied to avocado orchards in Western Australia in the mid-1990s (You and Sivasithamparam 1994; 1995; You et al. 1996), *P. cinnamomi* suppressive avocado soils have not been reported on the east coast of Australia since 1992 (Stirling et al. 1992). Consequently, no locations on the east coast of Australia suitable for studying *P. cinnamomi* suppression were known when this review was written. Therefore, before investigations into factors involved in *P. cinnamomi* suppressive avocado soils can be undertaken, suitable study locations will need to be identified.

Many physicochemical soil variables have been investigated for their possible involvement in *P. cinnamomi* suppression. Soil drainage and soil moisture are critical issues in determining a soil’s conduciveness to *P. cinnamomi* survival and pathogenicity. Calcium, particularly when in the form of CaSO$_4$, also appears to be an important abiotic factor in *P. cinnamomi* suppressive soils, but its specific mode of action is not fully understood. Another factor frequently associated with *P. cinnamomi* suppressive soils is high soil organic matter content. However, the role of soil organic matter appears to be indirect, mainly as a substrate supporting microbial activity. A significant number of studies have demonstrated that the removal of the
microbial community by pasteurising or sterilising suppressive soils results in loss of suppression. This provides a very strong indication that *P. cinnamomi* suppression is mediated by biological factors. However, while there have been few reports implicating naturally occurring abiotic processes in *P. cinnamomi* suppression (Section 1.5), where soils are believed to be suppressive, bioassays involving a susceptible host and biocidal treatment of the soil to confirm biological suppression are necessary. The reasons for this are that: i) biological suppression cannot simply be assumed; there is always some possibility that abiotic processes may be involved; ii) in conventional systems chemical fungicides (e.g. copper based fungicides) may be responsible for reductions in *P. cinnamomi* inoculum and disease incidence and severity.

Some suppressive soils have higher numbers of bacteria and fungi than conducive soils and most have a higher ratio of antagonistic microorganisms. A number of studies have associated a diversity of different genera and specific species of bacteria and fungi with *P. cinnamomi* suppressive soils (Section 1.7). *In vitro* studies have shown that many of these produce various metabolites that inhibit or destroy *P. cinnamomi* propagules. Among these metabolites are the enzymes $\beta$-1,3 glucanase (laminarinase) and $\beta$-1,4 glucanase (cellulase). Downer et al. (2001a) associated the activity of these enzymes with disease suppression under *P. cinnamomi* suppressive mulch. They also demonstrated the destructive effects of these enzymes on *P. cinnamomi* during a laboratory study (Downer et al. 2001b). However, the relationship between enzyme activity measured in the soil and in the mulch, and the enzyme concentrations used in their laboratory study, was not determined and therefore further evidence is required to confirm the role of cellulase and laminarinase in *P. cinnamomi* suppressive soils. In addition, existing methods used for determining soil cellulase and laminarinase activities are affected by several issues associated with enzyme extraction procedures and colorimetric methods used for determining the quantity of reducing sugars released during enzyme-substrate reactions (Section 1.8.3). This indicates that there are opportunities to improve these methods.

While a large number of bacteria and fungi are antagonistic toward *P. cinnamomi* when grown in culture, their application *in vivo* rarely results in effective biological
control. In addition, observations from the relevant literature suggest that no single species or taxonomic group of bacteria or fungi is solely responsible for the occurrence of *P. cinnamomi* suppressive soils. Until recently, most of the information on microbial community structure and diversity has been founded on culture-based studies. Culture-independent molecular DNA analysis methods have the potential to identify a much larger proportion of the microbial community than can be achieved using culture-based techniques. Several microbial community DNA profiling methods are available. A recently introduced method known as length heterogeneity PCR (LH-PCR) has shown potential for overcoming some of the disadvantages of other community DNA profiling methods. However, to date LH-PCR has only been used for analysis of bacterial communities and primers suitable for examining fungal community DNA using the LH-PCR method need to be identified and evaluated.

A number of studies have demonstrated the potential importance of microbial community structure and diversity in the development and maintenance of soils that have the capacity to suppress several different soil-borne phytopathogens. At least 2 culture-independent microbial community DNA studies have provided evidence to suggest that microbial community structure may play a role in suppression of *P. cinnamomi*. Observations by Yang et al. (2001) suggested that bacterial communities associated with healthy avocado root tips have a distinctively different structure than diseased roots. Borneman and Hartin (2000) noted differences in fungal community structure between 2 avocado soils with different abilities to suppress *P. cinnamomi* root rot. The limitations of culture-based studies and the outcomes from these studies indicate a need for further investigation of microbial community structure and microbial diversity in *P. cinnamomi* suppressive soils and their roles in *P. cinnamomi* suppression.
1.11 Thesis aims and thesis chapter contents

1.11.1 Aims

The broad aims for this thesis are to investigate the role of cellulase and laminarinase in *P. cinnamomi* suppression and to examine microbial community structure and diversity in *P. cinnamomi* suppressive soils. The specific aims for the studies in this thesis are presented below.

1. Locate *P. cinnamomi* suppressive soils suitable for further study and, where suppressive soils are identified, determine whether suppression is biological.

2. Develop an improved assay for estimating soil cellulase and laminarinase activities and compare cellulase and laminarinase activities in soils with varying abilities to suppress *P. cinnamomi*.

3. Determine whether cellulase and laminarinase have the capacity to reduce *P. cinnamomi* infection in test plants.

4. Examine bacterial community structure and diversity in *P. cinnamomi* suppressive soils using LH-PCR.


6. Examine fungal community structure and diversity in *P. cinnamomi* suppressive soils using LH-PCR.
1.11.2 Chapter contents

Chapter 2 – Materials and Methods. This chapter covers methods which were common to the experimental work reported in each chapter including methods for sterilising materials, preparation of culture media, isolation of *P. cinnamomi* from plant roots, confirmation of *P. cinnamomi* isolate identity and pathogenicity, maintenance of *P. cinnamomi* cultures, preparation of inoculum for pot experiments, germination of lupin seeds, disease severity assessments for lupin seeds and sterilisation of soil using γ-irradiation.

Chapter 3 - Identification of *Phytophthora cinnamomi* Suppressive Soils. Ten avocado orchards and 2 rainforest remnants located in north-eastern NSW and south-east Qld were selected for a suppressive soil screening survey. Soils were sampled from each location and the ability of the soil to suppress the development of root rot in inoculated lupin seedlings was determined in a glasshouse assay. A subsequent assay was carried out to confirm the suppressiveness of selected soils and to confirm that suppression was biologically mediated in these soils. A third glasshouse assay was also carried out to confirm that the selected soils could suppress the development of root rot in avocado seedlings and to determine whether suppression can be transferred to a conducive soil or restored to a soil in which suppression has been destroyed through γ-irradiation.

Chapter 4 - Cellulase, Laminarinase and *P. cinnamomi* Suppression. A microplate enzyme assay developed for use in this study (Appendix 1) was applied to measure cellulase and laminarinase activity in soils with differing ability to suppress *P. cinnamomi*. Soil cellulase and laminarinase activities were also determined at 3 sampling times during the third glasshouse experiment presented in Chapter 3. This chapter also reports on the results from a glasshouse experiment that aimed to determine the effects of a range of enzyme concentrations on *P. cinnamomi* infection of lupin seedlings.
Chapter 5 – Structural Characteristics of Bacterial Communities Associated with P. cinnamomi Suppressive Soils Determined by 16S rDNA Length Heterogeneity PCR. Microbial community DNA was extracted from soil samples collected for the suppressive soil screening survey reported in Chapter 3. A molecular community DNA profiling method, known as length heterogeneity PCR (LH-PCR), was used to analyse the structure and diversity of the bacterial communities in these samples. Changes in bacterial community structure and diversity were also monitored in relation to transferral and restoration of suppression during the third glasshouse experiment reported in Chapter 3.

Chapter 6 - Structural Characteristics of Fungal Communities Associated with P. cinnamomi Suppressive Soils Determined by ITS Length Heterogeneity PCR. Due to a lack of primers available for use in fungal LH-PCR, 3 primer pairs were assessed for their suitability. One of these primer pairs was selected for use in LH-PCR analysis of the fungal community DNA from soil samples collected for the initial suppressive soil screening survey (Chapter 3). This chapter also reports on changes in fungal community structure and diversity in relation to transferred and restored suppression during the third glasshouse experiment reported in Chapter 3.

Chapter 7 – General Discussion. Key outcomes from the study of microbial ecology of P. cinnamomi suppressive soils are summarised and discussed within the context of previous research findings from published studies. The implications of the outcomes from this study for managing P. cinnamomi suppressive soils and for future research are discussed.
Chapter 2

Materials and methods

The materials and methods described in this chapter are those that are common to more than one experimental chapter. Each of the following experimental chapters contains materials and methods sections that are specific to the work reported in that chapter.

2.1 Autoclave sterilisation and culture disposal

Unless stated otherwise, autoclave sterilisation was carried out at 121°C and 105 kPa. Media and equipment were routinely autoclaved for 20 min and discarded cultures were autoclaved for 45 min prior to disposal.

2.2 Culture media

Potato-carrot agar (van der Plaats-Niterink 1981) with β-sitosterol (PCA-β) was used for routine growth and maintenance of pure *P. cinnamomi* cultures. β-sitosterol was added to the agar to increase the production of asexual spores (Englander and Turbitt 1979; Englander and Roth, 1980; Ribeiro 1983). PCA-β was prepared by combining 20 g potato and 20 g carrot (both scrubbed and diced) with 500 mL deionised water in a screw-cap reagent bottle. In a second reagent bottle, 15 g agar (Amyl Media, Australia) was added to 500 mL deionised water. Both bottles were steamed in an autoclave at atmospheric pressure for 10 min. After steaming, the molten agar was cooled to 50°C in a water bath. The potato-carrot broth was transferred to 50 mL plastic centrifuge tubes (Sarstedt, Australia) and centrifuged (Model GT-70, Spintron, Australia) at 3000 revs min⁻¹ for 10 min. The supernatant (cleared potato-carrot broth) was combined with the molten agar. A 1 mL aliquot of 2% β-sitosterol in chloroform (ICN Biomedical, USA) was added to the potato-carrot agar. Deionised water was added to bring the final volume to 1 L and then the solution was mixed and autoclaved. The molten PCA-β was cooled in a water bath to 50°C before
pouring approximately 15 mL into 9 cm disposable γ-irradiated petri-dishes (Sarstedt, Australia). After the agar had solidified the petri-dishes were sealed in sterile polyethylene plastic bags and stored at 4°C for up to 2 months.

A Phytophthora selective agar (PSA) (Massago et al. 1977; Tsao and Guy 1977) was used to isolate and reisolate *P. cinnamomi* from soil and plant material and to purify *P. cinnamomi* cultures. To make the PSA, the PCA-β basal media was prepared as described above and then the following antibiotics were added to agar cooled to 50°C before pouring (final concentrations given below):

- 10 µg mL⁻¹ Pimaricin (Sigma, USA) (stock solution in sterile deionised water)
- 50 µg mL⁻¹ Rifampicin (ICN Biomedical, USA) (stock solution in methanol)
- 50 µg mL⁻¹ Tachigaren - 99.5% Hymexazol - (Sankyo, Japan) (stock solution in sterile deionised water)

### 2.3 Source of *P. cinnamomi* isolate

The *P. cinnamomi* isolate used in all experiments was baited from soil (Section 2.4) collected in May 2002 from an avocado orchard located at Alstonvale, NSW (description and map co-ordinates provided in Section 3.2.1). Several areas within this orchard had a history of *P. cinnamomi* infection (Mr Alan Campbell, pers. comm. 2002; Mr John Dirou, pers. comm. 2002). Soil was sampled from beneath an avocado tree that exhibited symptoms typical of *P. cinnamomi* infection (Section 1.2.2).

### 2.4 Isolation of *P. cinnamomi* from soil

The lupin baiting method used to isolate *P. cinnamomi* from soil was adapted from Pratt and Heather (1972). In a clear 225 mL plastic cup, 20 g soil was suspended in 200 mL sterile distilled water. A fitted plastic lid with 5 holes was placed on top of the cup. Radicles emerging from 5 germinated *Lupinus angustifolius* L. (narrow leaf lupin) seeds (Section 2.10) were inserted through the holes in the lid until they were
partially submerged in the soil suspension. The lupins were incubated using a 12 h day / night cycle of 25°C / 18°C in a glasshouse until necrotic lesions appeared on the radicles (3 - 4 d). The lupins were removed from the pots and *P. cinnamomi* was isolated from necrotic tissues on the radicles as described in Section 2.5.

### 2.5 Isolation of *P. cinnamomi* from plant roots

Plant roots with necrotic lesions were excised from the plant, surface disinfected (1 min in 70% ethanol), washed in sterile distilled water, blotted dry on sterile filter paper (Whatman No. 1), cut into 0.5 cm sections and plated onto PSA. The plates were incubated in the dark at 24°C. Within 2 – 3 d, distinctive coralloid hyphae and abundant chlamydospores characteristic of *P. cinnamomi* (Erwin and Ribeiro 1996) were observed growing on the agar (Plate 2.1). The images shown in Plate 2.1 were obtained using an Olympus DP 70 digital camera mounted on an Olympus BX51 microscope (Olympus, Australia).

![Plate 2.1](image)

(i) Coralloid hyphae (x200) and (ii) chlamydospores (x400) distinctive of *Phytophthora cinnamomi*.

Small sections of colonies suspected of being *P. cinnamomi* were transferred to PSA and incubated in the dark at 24°C. After 4 d the plates were inspected for any contaminating material. Colonies suspected of being contaminated, or representing species other than *P. cinnamomi*, were discarded. Pure cultures on the remaining plates were subcultured on PCA-β.
2.6 Confirmation of isolates as *P. cinnamomi*

Isolates were confirmed as *P. cinnamomi* by stimulating production of sporangia in non-sterile soil extract (Pratt and Heather 1972; Pratt et al. 1973). To produce the soil extract, soil samples were mixed with sterile deionised water in a conical flask and placed on an orbital shaker (Bioline, Edwards Instruments, Australia) for 1 h at 180 revs min\(^{-1}\). The soil suspension was transferred to a 50 mL plastic centrifuge tube (Sarstedt, Australia) and centrifuged (Model GT-70, Spintron, Australia) at 4000 revs min\(^{-1}\) for 10 min. The supernatant was transferred to a sterile 9 cm plastic petri-dish, and ten 8 mm diameter agar discs taken from the periphery of a 4 d old pure *P. cinnamomi* colony were submerged in the soil extract. The plates were incubated at ambient temperature on a window sill with regular day and night cycles over 3 d. The mycelial mats were then removed and inspected (at x200 and x400 magnification) for non-papillate sporangia (Plate 2.2), typical of *P. cinnamomi* (Erwin and Ribeiro 1996). The image shown in Plate 2.2 was obtained using an Olympus DP 70 digital camera mounted on an Olympus BX51 microscope (Olympus, Australia).

![Plate 2.2 Non-papillate sporangia of *P. cinnamomi* (x400).](image)

The pure *P. cinnamomi* cultures were examined by Dr Gordon Stovold (Plant Pathologist, NSW DPI, Alstonville) and tested for pathogenicity (Section 2.7) to provide further confirmation that the isolates were *P. cinnamomi*. 
2.7 Testing P. cinnamomi cultures for pathogenicity

Pure isolates were tested for pathogenicity by passing through lupin (L. angustifolius cv. New Zealand blue) seedlings. To achieve this, γ-irradiation sterilised soil (Section 2.12) was inoculated with P. cinnamomi mycelial mats grown on cellophane discs (Section 2.9). Germinated lupin seeds were prepared as described in Section 2.10 and 5 were transplanted into each inoculated pot. Uninoculated control pots were also planted with lupins to provide a means for detecting entry of contaminating material (i.e. cross contamination of P. cinnamomi from a source other than the inoculum from the mycelial mats).

To provide ideal conditions for the development of disease (Erwin and Ribero 1996; Drenth and Sendall 2001), all pots were maintained in a temperature controlled glasshouse on a 12 h day / night cycle of 25°C / 18°C and were initially flooded with sterile distilled water for 3 d followed by 3 d drying. After 12 d, the lupins were recovered from the soil, washed in sterile distilled water to remove adhering soil and rated for disease severity (Section 2.11). All isolates tested positive for pathogenicity. The most virulent isolate (i.e. the isolate yielding the highest disease severity rating) was selected for use in all experiments involving P. cinnamomi.

2.8 Maintenance of P. cinnamomi cultures

For routine maintenance, P. cinnamomi cultures were grown on PCA-β (Section 2.2) in sterile 9 cm plastic petri-dishes. Stock cultures were also maintained on PCA-β squares in 10 mL sterile deionised water in 25 mL McCartney bottles. Stock cultures were stored in the dark at 24°C. To avoid virulence decline, as typically occurs in P. cinnamomi cultures stored over 6 - 12 months (Pietkiewicz 1978; Erwin and Ribeiro 1996; Zarzycka 1996), stock cultures were passed through lupins (Section 2.7) and reisolated from lupin roots (Section 2.5) at 3 - 4 monthly intervals.

2.9 Preparation of P. cinnamomi cellophane disc inoculum

Mycelial mats of P. cinnamomi grown on cellophane discs (Hwang et al. 1975; Malajczuk et al. 1977b) were used to inoculate soil in all pot experiments reported in
this thesis. Clear cellophane film was cut into 50 mm discs, washed twice in distilled water, wrapped in aluminium foil, sterilised by autoclaving, left to stand for 24 h to allow any thermophilic spores to germinate, re-autoclaved and then dried in an oven at 45°C. Sterile cellophane discs were transferred aseptically to the surface of PCA-β agar plates. The cellophane was allowed to moisten over several hours and then smoothed to ensure even contact with the agar surface. Using a sterile 8 mm diameter stainless steel corer, agar discs were cut from the periphery of a 5 d old *P. cinnamomi* culture. Each agar disc was transferred to the centre of a cellophane disc. Inoculated discs were incubated at 24°C in the dark for up to 7 d before being used to inoculate soil.

### 2.10 Lupin seed germination

*L. angustifolius* (cv. New Zealand blue) seed was germinated prior to being used in baiting assays and in pot experiments. The lupin seed was sourced from Rockfield Pty Ltd, Sassafras, Tasmania, Australia. Lupin seed was germinated on a bed of sterile vermiculite. The vermiculite was washed in distilled water, drained and dried in an oven at 50°C. Distilled water was added to the vermiculite at a ratio of 1: 3.5 (v/v). The moistened vermiculite was placed in a deep tray, covered with aluminium foil, autoclaved for 30 min, left to stand for 24 h and then re-autoclaved for 30 min. The lupin seed was prepared for germination by surface disinfecting in 70% ethanol for 2 min. The seed was then drained immediately, washed in sterile distilled water and soaked in sterile distilled water overnight. The following day seed was drained and spread across the surface of the sterile vermiculite. The vermiculite tray was covered with the aluminium foil and incubated at 25°C for 2 - 3 d. Germinated seed with 2 – 3 cm radicles was selected for use in all experiments.

### 2.11 Disease severity assessments for lupin seedlings

Disease severity in lupin seedlings was assessed using an arbitrary disease severity rating (DSR) system adapted from Greenhalgh and Lucas (1984). The 0 - 5 scale used by Greenhalgh and Lucas (1984) was divided to form a scale of 0 - 10. Table 2.1 outlines the criteria used for rating root rot severity based on this scale and Plate
2.3 shows examples of how the scale was applied to attain average DSRs for lupin seedlings harvested from individual pots.

Table 2.1 Criteria used for determining disease severity of lupin seedlings grown in soil or other media inoculated with *P. cinnamomi*.

<table>
<thead>
<tr>
<th>Disease severity rating (DSR)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Healthy roots with no visible lesions</td>
</tr>
<tr>
<td>1</td>
<td>Minor lateral root rot with less than 10% of root tips necrotic or minor rot on tap root tips or both</td>
</tr>
<tr>
<td>2</td>
<td>Minor to slight lateral root rot with up to 20% of root tips necrotic or minor to slight rot evident on tap root tips or both</td>
</tr>
<tr>
<td>3</td>
<td>Slight lateral root rot with up to 30% of root tips necrotic or minor to slight rot evident on tap root tips or both</td>
</tr>
<tr>
<td>4</td>
<td>Slight to moderate rot with up to 40% of the lateral root area necrotic or slight to moderate rot evident on tap root or both</td>
</tr>
<tr>
<td>5</td>
<td>Moderate lateral root rot with up to 50% of root area necrotic or moderate rot evident on tap root or both</td>
</tr>
<tr>
<td>6</td>
<td>Moderate to severe lateral root rot with up to 60% of root area necrotic and moderate rot evident on tap root</td>
</tr>
<tr>
<td>7</td>
<td>Severe root rot of both lateral roots and tap roots with some development of lateral roots</td>
</tr>
<tr>
<td>8</td>
<td>Severe rot with tap root completely necrotic and some development of lateral roots but lateral roots completely necrotic</td>
</tr>
<tr>
<td>9</td>
<td>Severe rot with tap root completely necrotic and no development of lateral roots but plant still living</td>
</tr>
<tr>
<td>10</td>
<td>No development of lateral roots, tap root completely rotted and plant dead</td>
</tr>
</tbody>
</table>
Plate 2.3 Lupin seedlings with varying root rot severity assessed according to a disease severity rating scale from 0 to 10. No seedlings scored a DSR of 10 and therefore the scale shown in this photograph ends at 9.

2.12 Sterilisation of soil by γ-irradiation

Soil was sterilised using γ-irradiation to efficiently destroy fungi and bacteria (Jackson et al. 1967; McLaren 1969; Powlson and Jenkinson 1976) with minimal disturbance to soil chemical and physical properties (Trevors 1996). Soils were sealed in 10 L plastic drums and sent to Steritech Pty Ltd (Wentworth Park, Sydney) for γ-irradiation from a Cobalt 64 source at a dose of 2.5 Mrad (2.0 Mrad h⁻¹). After irradiation, sealed containers were opened in a biological safety cabinet and a subsample was removed aseptically to check for sterility. A 10-fold dilution series was made by adding 1 g of soil to 10 mL of sterile saline solution (0.75% NaCl) and then performing a further two 10-fold dilutions. To check for contamination, 100 µL of each dilution level was spread on PCA-β agar plates. Plates were incubated at 25°C for up to 4 weeks and checked for microbial growth daily for the first 7 d and then every 2 - 3 d thereafter. No fungal or bacterial colonies appeared on any agar plate on any occasion, therefore confirming the sterility of the irradiated soils.
Identification of *Phytophthora cinnamomi* suppressive soils

3.1 Introduction

Virtually all soil ecosystems possess some biological capacity to limit the activity of certain soil-borne plant pathogens (Alabouvette et al. 1996; Alabouvette 1999; Mazzola 2004). This is evidenced by many studies which have demonstrated the complete loss of the soil’s capacity to restrict the development of disease in a host plant after microbial activity has been removed by biocidal treatments, pasteurising or sterilising (Hornby 1983; Alabouvette et al. 1996; Erwin and Ribeiro 1996; Mazzola 2004). Soils with the capacity to prevent or impose significant limitations on the development of disease in a suitable host under conditions otherwise favourable for disease expression are termed “disease suppressive soils” (Menzies 1959; Baker and Cook 1974; Hornby 1983; Alabouvette et al. 1996). The antonym of this term is “disease conducive soils”, which is used to describe soils in which the pathogen expresses disease within the host with little or no hindrance from sources within the soil environment.

The causal agents of disease suppressive soils are not exclusively biological in origin. Abiotic processes within the soil may have a direct or indirect effect on the suppressive capacity of a soil. For instance, suppression of Fusarium wilt (Panama disease) of banana plantlets has been associated with montmorillonitic clay minerals (Stotzky et al. 1961; Stotzky and Martin 1963; Alabouvette 1999). Another example is the association between vermiculitic clay minerals and suppression of black root rot of tobacco, caused by *Thielaviopsis basicola* (Berkeley and Broome) Ferraris (Stutz et al. 1989). In this instance, however, a strain of *P. fluorescens* highly antagonistic toward *T. basicola* was also associated with the presence of vermiculitic clay minerals in the soil (Stutz et al. 1989). Other physicochemical factors such as soil pH and soil macro and micro nutrients have been investigated for their potential role in disease suppressive soils (Alabouvette et al. 1996; Höper and Alabouvette
Where associations between abiotic factors and suppressive soils have been determined, they are also frequently associated with biotic factors that are more directly responsible for disease suppression (Alabouvette et al. 1996; Höper and Alabouvette 1996; Alabouvette 1999; van Bruggen and Semonov 2000; Mazzola 2004).

*P. cinnamomi* suppressive soils have previously been characterised as being well drained, having a pH between 5.5 and 7.0 and possessing high levels of NH$_4$, NO$_3$, Ca cations, cation exchange capacity and organic matter content (Broadbent and Baker 1974a). However, mixed results from experimental manipulations of these parameters have suggested that these factors may have an indirect role in *P. cinnamomi* suppressive soils (Broadbent and Baker 1974a; Schmitthenner and Canaday 1983; Shea and Broadbent 1983; Broadbent et al. 1989; Erwin and Ribeiro 1996). Rather, it appears that the physicochemical characteristics of the soil may provide conditions that encourage and maintain a microbial community antagonistic toward *P. cinnamomi*. Certainly, the majority of studies undertaken with the aim of determining the mode of *P. cinnamomi* suppression have presented cumulative and compelling evidence that *P. cinnamomi* suppression is predominantly mediated by biological processes (Broadbent and Baker 1974a; Malajczuk et al. 1977a, 1977b; Halsall 1978; Malajczuk 1979c; Marks and Smith 1981; Halsall 1982a; Malajczuk 1983; Shea and Broadbent 1983; Ko and Shiroma 1989; Casale 1990; Duvenhage et al. 1991; Stirling et al. 1992; Ann 1994; Erwin and Ribeiro 1996).

Broadbent et al. (1971) and Broadbent and Baker (1974a) presented the earliest reports of *P. cinnamomi* suppressive soils from avocado orchards and rainforest soils located on the east coast of Australia. Treating these soils with steam at 100°C resulted in the soil losing its suppressive capacity. A number of other studies also found that *P. cinnamomi* suppressive soils lost their suppressiveness after they were steamed (Halsall 1978; Marks and Smith 1981; Halsall 1982a; Ko and Shiroma 1989; Ann 1994), dry heated to 200°C (Halsall 1982a), or sterilised using γ-irradiation (Malajczuk 1977a; Duvenhage et al. 1991). Each of these treatments removes biological activity from the soil (Trevors 1996) and the authors of these studies concluded that *P. cinnamomi* suppression was mostly mediated by biological processes. However, using heat to pasteurise or sterilise a soil can also affect its
chemical and physical attributes (Trevors 1996). Therefore, the loss of suppression observed in response to heating the soil may have resulted from thermo-induced changes to the chemical and physical attributes of the soil. This possibility can be disregarded, however, based on similar results being obtained using \( \gamma \)-irradiation (at dosages of around 2.5 Mrad; Jackson et al. 1967; McLaren 1969; Alef and Nannipieri 1995; Trevors 1996) to sterilise the soil, which has minimal impact on soil physical and chemical attributes (Trevors 1996).

*P. cinnamomi* suppressive soils have previously been identified and investigated in a number of ecosystems including subtropical rainforests along the east coast of Australia (Broadbent and Baker 1974a), the jarrah forests in Western Australia (Malajczuk 1975, 1977a, 1979a, 1979b; Malajczuk and McComb 1979; Shea et al. 1983), cool temperate forest surrounding the foothills of Mount Dandenong in Vic (Marks and Smith 1981) and the sclerophyll forests in the Tallaganda State Forest in NSW (Halsall 1982a, 1982b). Phytophthora suppressive soils were also identified in a range of soil types supporting various plant communities across the island of Hawaii, USA (Ko and Shiroma 1989). However, much of the research associated with the phenomenon of *P. cinnamomi* suppressive soils has been undertaken in avocado orchard soils located in California (Casale 1990; Menge et al. 1994; Costa et al. 1996; Costa et al. 2000; Downer et al. 2001a), South Africa (Mass and Kotze 1989; Duvenhage et al. 1991) and Australia (Broadbent and Baker 1974a; Stirling et al. 1992). Within the Australian context, *P. cinnamomi* suppressive avocado soils were identified in the early 1970s at Tamborine Mountain (Tamborine Mt), south-eastern Qld, and at a few locations in north-eastern NSW (Broadbent et al. 1971; Broadbent and Baker 1974a). The most recently reported observation of *P. cinnamomi* suppressive avocado soils was in 1992 from an orchard located near Maleny, north of Brisbane, Qld (Stirling et al. 1992). Since then there have been no published reports of *P. cinnamomi* suppressive avocado soils in Australia. Therefore, to investigate the processes and mechanisms associated with *P. cinnamomi* suppressive avocado orchard soils located in Australia, suitable study sites need to be located.
With over 1000 avocado orchards scattered across Australia (Horticulture Australia Limited 2004), selection criteria needed to be developed during the present study to identify suitable locations to test for *P. cinnamomi* suppressive soils. The primary criteria used included:

1) Selection of locations where growers reported minimal problems with *P. cinnamomi* root rot in their orchard. It should be noted that minimal occurrence or absence of *P. cinnamomi* root rot may result from the use of prophylactic chemical treatments (e.g. metaxyl, fosetyl-Al and phosphonate based fungicides), which is a common practice in conventional orchards (Pegg 1992; Kaiser et al. 1997). In organically managed orchards, control of Phytophthora root rot is more reliant on inducing biological processes believed to be responsible for *P. cinnamomi* suppression. Therefore, organically managed orchards with minimal *P. cinnamomi* root rot were targeted to yield *P. cinnamomi* suppressive soils.

2) Exclusion of orchards or sections of orchards prone to frequent saturation or waterlogging. Frequent waterlogging is not conducive to the development of *P. cinnamomi* suppressive soils (Erwin and Ribeiro 1996).

3) Selection of rainforest sites and conventionally and organically managed orchards in which organic matter is actively maintained (by regular additions of organic materials via mulching, cover cropping or by natural leaf / debris fall). The reasons for this are that: i) suppressive avocado orchard soils have been characterised as having high levels of organic matter (Broadbent and Baker 1974a; Nesbitt et al. 1979; Malajczuk 1983; Shea and Broadbent 1983; Menge et al. 1994; Casale et al. 1995; Costa et al. 1996) and ii) maintaining a high level of organic matter assists in sustaining highly active, diverse and potentially antagonistic soil microbial communities (Cook and Baker 1983; You and Sivasithamparam 1994, 1995; Downer et al. 2001a).

When sampling soil from beneath avocado trees to assess suppressive capacity, the soil was taken from beneath healthy plants no deeper than the top few centimetres. The assumptions behind this sampling strategy were that: i) the feeder root zone of avocado trees generally occurs within the top few centimetres of the soil profile,
especially where a mulch layer is present (Downer et al. 2001a), ii) *P. cinnamomi* mainly attacks the feeder roots of avocado trees (Coffey et al. 1988; Broadley 1992) and therefore an abundance of healthy feeder roots may indicate suppression and iii) the highest levels of microbial activity are found in the surface organic layers of the soil (Burns 1983; Atlas and Bartha 1998; Downer et al. 2001a; Tiquia 2002), and high microbial activity has been associated with *P. cinnamomi* suppressive soils (Malajczuk 1983; Erwin and Ribeiro 1996; Downer et al. 2001a). Another factor to consider when sampling soils for assessing suppressiveness is associated with adverse or variable temperatures during transport and storage. Firstly, soil samples should be assessed for their suppressive capacity as soon as possible. Secondly, storing soil at the commonly advised temperature of 4°C initiates biological dormancy (Alef and Nannipieri 1995) and therefore, due to the apparent importance of biological activity in *P. cinnamomi* suppression, this should be avoided and the soils should instead be kept at close to, or slightly cooler than, ambient temperature.

Screening soil for suppressiveness can be undertaken by examining the effects of the soil directly on the pathogen, or by examining the effects of the soil on the development of disease in a suitable host. One approach involves counting cultured *P. cinnamomi* chlamydospores (under magnification) that germinate or fail to germinate when incubated with the soil (Ko and Shiroma 1989; Ann 1994). Where a large volume of soil samples are to be tested this approach provides a simple method for screening soils for their suppressive potential. However, in the absence of a suitable host, this assay does not confirm the capacity of the soil to suppress the development of disease.

Testing soil for disease suppression can also be undertaken using bioassays involving inoculation of potted soil with the pathogen, followed by sowing susceptible host plants into the infected soil (Broadbent and Baker 1974a; Malajczuk 1977a; Wildermuth et al. 1979; Duvenhage et al. 1991). The suppressive capacity of various soils has also been demonstrated by mixing a small amount (as little as 1%) of the suppressive soil with a conducive soil, which causes the conducive soil to become suppressive (Malajczuk et al. 1977a; Alabouvette et al. 1979; Wildermuth et al. 1979; Cook and Baker 1983). Disease severity at the end of the assay is usually assessed visually, which may simply involve examining plants for disease symptoms.
such as poor growth (e.g. Broadbent et al. 1971) or a numerical value is generated to represent the extent of disease incidence and severity. The latter is more useful for statistical analyses. Methods for generating numerical values include counting infected plants (which determines disease incidence), measuring plant growth, yield or weight (Duvenhage et al. 1991), estimating the percentage root area affected by disease (Malajczuk 1977a; Duvenhage et al. 1991) or by estimating the extent of disease severity using an index based on a graduated scale that uses specific criteria (e.g. Greenhalgh and Lucas 1984). Plant growth and biomass can be affected by variables other than root disease and, therefore, while containing an element of subjectivity, assessments based on estimating disease severity provide a more robust indicator of disease expression.

Plant species used in bioassays for assessing *P. cinnamomi* suppression have included *E. marginata* and *E. calophylla* (Malajczuk 1977a), *J. acutifolia* (Broadbent and Baker 1974a), susceptible *P. americana* (avocado) varieties (e.g. Edranol and Topa Topa; Kellam and Coffey 1985; Gabor et al. 1990; Weste and Hinch 1991; Rahimian and Casale 1992; Costa et al. 2000; Messenger et al. 2000b) and *L. angustifolius* (narrow leaf lupin; Broadbent and Baker 1974a; Duvenhage et al. 1990). Lupins germinate easily, grow quickly and seedlings are highly susceptible to *P. cinnamomi*. Therefore, they make an ideal test plant for baiting *P. cinnamomi* from soil (Pratt and Heather 1972) and for *P. cinnamomi* suppressive soil screening assays. Confirmation that the soils have the capacity to suppress the development of disease in the host plant(s) associated with the system under study should also be considered an important part of demonstrating disease suppression.

In the following study, avocado growers located in south-eastern Qld and north-eastern NSW were interviewed to identify orchards that were likely to yield *P. cinnamomi* suppressive soil. These regions were chosen as a starting point for this study because they were the same regions from which *P. cinnamomi* suppressive soils were first reported (Broadbent and Baker 1974a). Soils from 10 avocado orchards were sampled for an initial suppressive soil screening bioassay in which lupin seedlings were used as the test plant. Soil samples from 2 rainforest remnants were included in the assay for comparative purposes. Four avocado orchards that possessed suppressive soils during the first screening assay were sampled and
assayed a second time. However, on this occasion each soil sample was γ-irradiated to remove biological activity from the soil. Using the lupin *P. cinnamomi* suppressive soil assay, irradiated soils were compared with the non-irradiated suppressive soils with the aim of confirming that suppression was biologically mediated. In a third glasshouse experiment the suppressive capacity of soils from the 4 orchards was further demonstrated using avocado seedlings as the test plant and by mixing suppressive soils with conducive soil.

### 3.2 Materials and methods

#### 3.2.1 Study locations

To identify potential *P. cinnamomi* suppressive soils, informal telephone interviews were conducted in early 2002. Fifty-eight avocado growers were contacted from north-eastern NSW and south-eastern Qld. During discussions with the orchard managers, information was extracted (notes were recorded during and after each interview) to provide answers for each of the following questions:

1. Are there major problems with *P. cinnamomi* root rot in the orchard?

2. Are there blocks within the orchard where *P. cinnamomi* does not appear to be a significant problem?

3. Are there areas within the orchard where *P. cinnamomi* is a greater problem than in other areas? Are these problem areas subjected to frequent waterlogging / saturation?

4. What is the estimated ratio of healthy : diseased plants in the orchard?

5. How is *P. cinnamomi* managed? Is phosphonate used to treat diseased trees or is it used regularly as a preventive measure? Does the orchard manager place emphasis on cultural practices for the management of Phytophthora root rot? Do these practices include inputs of organic matter in the form of organic mulches, cover cropping or natural leaf and debris fall from older trees?
6. Does the orchard manager believe that the soil within the orchard has the ability to suppress *P. cinnamomi*?

7. Is there rainforest vegetation nearby that may be suitable for comparison?

8. Is the orchard manager willing to allow access to the orchard and are there practical/logistical issues related to sampling soil from the orchard?

Information provided by interviewees was used to compile a short-list of orchards on the basis of their potential for yielding *P. cinnamomi* suppressive or *P. cinnamomi* conducive soils (Section 1.3). Orchards were short-listed where: i) the entire orchard or large sections of the orchard showed minimal *P. cinnamomi* infection; ii) the orchard had a high percentage of healthy trees (>80%); iii) waterlogging was minimal or absent; iv) emphasis was placed on cultural practices to manage *P. cinnamomi* (especially maintenance of soil organic matter).

The short-listed orchards included 3 organically managed avocado orchards and 5 conventionally managed orchards. Soils from an organically managed orchard and a conventionally managed orchard potentially possessing conducive soil (due to the high proportion of diseased trees reported by orchard managers) were also included. Comparisons were also made with soil sampled from 2 subtropical rainforest remnants that were adjacent to the orchards located at Tamborine Mt and Pretty Gully. Finally, a dairy pasture soil found to be conducive to the development of *P. cinnamomi* root rot in lupin seedlings during preliminary experiments was included to provide a benchmark for conduciveness. Table 3.1 details the abbreviated codes used for each location throughout the presentation of results in this and subsequent chapters; nearest town or locality and global position coordinates; management regime; and suspected status of the soil in terms of suppressiveness. Figure 3.1 shows the study area within Australia and the towns or localities nearest to each study location.
Table 3.1 Study locations, codes used for each study location, management regime and suspected suppressiveness / conduciveness status for each location.

<table>
<thead>
<tr>
<th>Code</th>
<th>Locality</th>
<th>Latitude Longitude</th>
<th>Management</th>
<th>Suspected status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta1</td>
<td>Tamborine Mountain, Qld</td>
<td>27° 56’ S 153° 10’ E</td>
<td>Conventional</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Ta2</td>
<td>Tamborine Mountain, Qld</td>
<td>27° 56’ S 153° 11’ E</td>
<td>Conventional</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Trf</td>
<td>Tamborine Mountain, Qld</td>
<td>27° 56’ S 153° 11’ E</td>
<td>Rainforest</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Pr1</td>
<td>Pretty Gully, NSW</td>
<td>28° 36’ S 152° 37’ E</td>
<td>Organic</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Pr2</td>
<td>Pretty Gully, NSW</td>
<td>28° 35’ S 152° 38’ E</td>
<td>Conventional</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Prf</td>
<td>Pretty Gully, NSW</td>
<td>28° 36’ S 152° 37’ E</td>
<td>Rainforest</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Cud</td>
<td>Cudgen, NSW</td>
<td>28° 06’ S 153° 33’ E</td>
<td>Conventional</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Eur</td>
<td>Eureka, NSW</td>
<td>28° 31’ S 153° 26’ E</td>
<td>Conventional</td>
<td>Conducive</td>
</tr>
<tr>
<td>Tu1</td>
<td>Tuckombil, NSW</td>
<td>28° 49’ S 153° 28’ E</td>
<td>Organic</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Tu2</td>
<td>Tuckombil, NSW</td>
<td>28° 49’ S 153° 28’ E</td>
<td>Conventional</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Als</td>
<td>Alstonvale, NSW</td>
<td>28° 38’ S 153° 26’ E</td>
<td>Organic</td>
<td>Suppressive</td>
</tr>
<tr>
<td>Ura</td>
<td>Uralba, NSW</td>
<td>28° 54’ S 153° 25’ E</td>
<td>Organic</td>
<td>Suppressive</td>
</tr>
<tr>
<td>C</td>
<td>Wollongbar, NSW</td>
<td>28° 50’ S 153° 25’ E</td>
<td>Dairy Pasture (control soil)</td>
<td>Conducive (in preliminary trial)</td>
</tr>
</tbody>
</table>

Figure 3.1 Map showing study area and localities
© copyright Commonwealth of Australia (Geoscience Australia 2006)
3.2.2 Soil sampling

There were 3 soil sampling events associated with the experimental work in this Chapter. Soil was sampled from each of the 10 avocado orchards and the 2 rainforest remnant locations (Section 3.2.1; Table 3.1) during the first sampling event (Section 3.2.5), and from 4 selected avocado orchards during subsequent sampling events (Sections 3.2.6 and 3.2.8). The criteria for selecting the 4 orchards were based on evidence of suppression presented in Section 3.3.2 (also refer to Section 3.3.3).

At each of the 10 avocado orchards, 4 healthy avocado trees were selected at random and soil was taken from beneath each tree. During subsequent sampling events (at each of the 4 selected orchards), soil was sampled from the same 4 trees. Soil was sampled from the base of each tree at 4 evenly spaced points in a 1 - 2 m radius from the trunk. These 4 samples were combined to form a single composite sample representative of the soil from beneath that particular tree. Within the 2 rainforest locations, 4 individual soil samples were collected at 5 m intervals along a 20 m transect. The dairy paddock control soil was taken from a single sampling point, near the centre of the paddock, on each sampling occasion (Sections 3.2.5, 3.2.6 and 3.2.8).

Sampling involved the removal of any surface litter to expose the soil and, when sampling from beneath an avocado tree, to expose avocado feeder roots. Soil was sampled at a depth of 0 cm - 5 cm. This sampling strategy was based on an assumption that the soil within the feeder root zone of healthy avocado trees would provide the best chance for detecting *P. cinnamomi* suppressive soil (Section 3.1). To avoid cross contamination between samples, all equipment used for soil sampling was washed thoroughly and surface disinfected with 70% ethanol between sampling points. During the first sampling event, all 13 locations (Section 3.2.1 and Table 3.1) were sampled over 5 d. During subsequent sampling events, the 4 selected locations were sampled over 4 d. Soil samples were contained in polyethylene bags and kept cool in an insulated box during transportation back to the research facility for processing at the end of each day.
3.2.3 Handling soil samples

To avoid microbial contamination, samples were homogenised while still contained in their 90 cm x 60 cm polyethylene bags. This was carried out by manually dividing and then remixing the soil sample several times while still in the bag as well as mimicking the mechanical action of a tumbler for 5 min. After mixing, each soil sample was divided into subsamples for the experiments and analyses listed below. When taking subsamples, plastic bags were turned inside out and used to hand grab the required amount of soil. This technique prevented cross contamination between samples by avoiding direct contact with the soil sample and by avoiding reuse of utensils.

Subsamples were collected and stored appropriately for the following experimental work:

- DNA extractions (Chapters 5 and 6). Stored at -20°C immediately.
- Chemical analyses (Section 3.2.4). Air-dried at 40°C and then sealed and stored at ambient temperature.
- Cellulase and laminarinase assays (Chapter 4). Stored at 4°C for no more than 3-4 d before being assayed (second and third sampling events only).
- γ-irradiation sterilisation. Transported to irradiation facility within 1 - 2 d after sampling (Section 2.12).
- Soil moisture determinations (Section 3.2.4). Conducted immediately.

Soil remaining in the original polyethylene bags was used for glasshouse assays. For the irradiated treatments, soils were transported at ambient temperature to the irradiation facility and returned within 14 d (Section 2.12). Soils for non-irradiated treatments were stored in a temperature controlled room at 20 - 25°C until the irradiated treatments were returned.
3.2.4 Soil nutrient analyses

Soil moisture content at the time of sampling was determined according to method 2B1 described by Rayment and Higginson (1992). Soil chemical analyses were carried out on composite samples representing each location. Composite samples were obtained by combining an equivalent amount (250 g) of soil from each of the 4 samples taken from each location. Soil chemical analyses were carried out by Nutrient Advantage Laboratory Services (Incitec Pivot Pty Ltd) Werribee, Victoria, Australia. Soils were tested for: pH (1:5 CaCl₂); electrical conductivity (1:5); total N, Colwell P; oxidisable organic carbon; cation exchange capacity (CEC) - Ca⁺⁺, Mg⁺⁺, Na⁺ and K⁺. Nutrient Advantage Laboratory Services Pty Ltd conducted all soil chemical testing according to methods recommended by Rayment and Higginson (1992).

3.2.5 Experiment 1: P. cinnamomi suppressive soil screening survey

Soils sampled from each of the 10 avocado orchards and 2 rainforest locations described in Section 3.2.1 were screened for P. cinnamomi suppression. Four samples were collected from each orchard and rainforest location (48 soil samples in total). Soil was also sampled from the Wollongbar dairy pasture (Section 3.2.1). The pasture soil sample was γ-irradiation sterilised (Section 2.12) and used for positive (inoculated with P. cinnamomi) and negative (not inoculated with P. cinnamomi) control treatments. All soil samples were assessed for their suppressive potential using the bioassay described in Section 3.2.7.

3.2.6 Experiment 2: Confirmation of biological suppression

Based on the results from the suppressive soil screening survey (Section 3.3.2), avocado orchards were selected at 4 locations for further study. The main purpose of this experiment was to confirm observations made during the suppressive soil survey (Experiment 1) and to determine whether suppression was mediated by biological and/or abiotic processes.
The 4 avocado orchards selected were:

1. Tamborine Mt 1, Qld (Ta1)
2. Pretty Gully 1, NSW (Pr1)
3. Tuckombil 1, NSW (Tu1)
4. Tuckombil 2, NSW (Tu2)

At each orchard, 4 soil samples were collected from beneath the same 4 avocado trees that were sampled during Experiment 1 (Section 3.2.5). Each composite field soil sample was halved. One half was stored at 20°C (Section 3.2.3), while the remaining portion was sent for γ-irradiation treatment (Section 2.12). The irradiated portion was included in the assay to test for abiotic suppression of *P. cinnamomi* in the sampled soils. The dairy pasture soil was sampled (Section 3.2.2), γ-irradiation sterilised (Section 2.12) and used for both positive and negative control treatments (Section 3.2.5). Procedures for the glasshouse assay component of this experiment are described in Section 3.2.7.

3.2.7 *P. cinnamomi* suppressive soil bioassay using lupins

Suppressive soil bioassays were undertaken to assess soils for their potential to suppress *P. cinnamomi*. The suppressive soil screening assay described here is similar in principle to that used by Duvenhage et al. (1991). Each soil sample was distributed between 4 replicate 500 mL square pots. To avoid cross contamination, soil samples were transferred to pots by pouring the soil directly from the plastic sample bag. All pots were surface disinfected in 2% sodium hypochlorite before use. Latex gloves were worn during pot preparation and either cleaned with 70% ethanol or replaced between samples. A 1 cm layer of soil was placed in the bottom of each pot. A mycelial mat of *P. cinnamomi* grown over cellophane film (Section 2.9) was transferred aseptically to a central position over the soil layer and then the pot was filled with the remaining soil. Before transferring, the mycelial mat was trimmed to the size of the cellophane disc. One half of the γ-irradiated dairy pasture soil was treated in the same manner to show that *P. cinnamomi* caused root rot in lupins in the assay system (i.e. a positive control for disease expression). The remaining γ-irradiated soil was treated similarly, but the *P. cinnamomi* mycelial mat was replaced
with a sterile cellophane disc. This treatment served as a negative control (i.e. no *P. cinnamomi*) to check for the absence of disease expression in uninoculated lupins.

The pots were placed into large (550 mL) round polypropylene containers (Channol, Australia). These containers acted as flood chambers during the experiment. Initially, the soil was watered to container capacity and incubated in a temperature controlled glasshouse on a 12 h 26°C day / 18°C night cycle. After 3 d, germinated lupin seeds (Section 2.10) were transplanted into each pot. To avoid damaging lupin radicles, dibble holes were made aseptically in the soil before transplanting using a stainless steel skewer. The pots were laid out in a randomised complete block design in the glasshouse (Plate 3.1). The floor of the glasshouse was treated with copper sulphate prior to and after the screening assay.

![Plate 3.1 Suppressive soil screening assay. Photo shows lupin seedlings 3 d after transplanting (plants on sill are not part of the experiment).](image)

Each pot received 100 mL distilled water and was incubated for a further 3 d (environmental conditions were as described above) to allow the lupin seedlings to establish. Once established, 5 of the most evenly growing lupins in each pot were selected and the remaining 5 were removed. The soil in each pot was then flooded by filling the flood chambers to maximum capacity. The excess water was allowed to evaporate from the pots over 4 d and then this cycle was repeated. Early in the assay, lupin seedlings in the positive control pots (irradiated pasture soil inoculated *P.*
cinnamomi) exhibited symptoms of root rot. The assay was terminated (usually within 15 d) when there was an obvious difference in vigour between lupins growing in the positive control and those growing in the negative control (irradiated pasture soil without P. cinnamomi). At the end of the experiment, the lupins in each pot were harvested, washed with distilled water, gently blotted dry using absorbent paper and then rated for disease severity (Section 2.11). To confirm that root rot symptoms had been caused by P. cinnamomi, roots were excised from the lupins and plated onto PSA as described in Section 2.5.

3.2.8 Experiment 3: Transfer of suppression

The main aim for this experiment was to determine if suppression could be restored to a sterile soil and transferred to a conducive soil. The same 4 orchards that were selected for Experiment 2 (Section 3.2.6) were sampled as described in Section 3.2.2. However, on this occasion, the 4 soil samples from the 4 trees in each orchard were combined and homogenised to give one composite sample for each orchard. A portion of each of the 4 avocado orchard soil samples was stored at 20°C while the remaining portion was γ-irradiation sterilised (Section 2.12). The conducive dairy pasture control soil (Section 3.2.1) was also sampled and treated in the same manner. Avocado seedlings were used as the test plant to confirm that the observations made during Experiments 1 and 2 were relevant to P. americana.

Avocado seed germination

The P. americana var. Topa Topa (Topa Topa) fruit collected to provide the seed for this experiment were harvested from a single tree in an orchard located on the Cudgen Plateau near Murwillumbah, NSW. Both the fruit and the labour to harvest them were generously donated by Mr Graham Anderson. Topa Topa avocados were selected because this variety is highly susceptible to P. cinnamomi (Erwin and Ribeiro 1996) and is commonly used for susceptible control treatments in pot and field experiments involving P. cinnamomi (Kellam and Coffey 1985; Gabor et al. 1990; Weste and Hinch 1991; Rahimian and Casale 1992; Costa et al. 2000; Messenger et al. 2000). Care was taken while harvesting the Topa Topa avocados to ensure that the fruit did not come into contact with material that could potentially cause contamination with Phytophthora propagules (e.g. ground, soil, rotting fruit).
After harvesting, the fruit was surface disinfected in a large vat containing a strong (concentration not known) chlorine solution.

The experiment was established 8 weeks after the fruit was harvested, during which time the fruit was stored in a cool room at 4°C. Before germination, the Topa Topa seeds were removed from the fruit and the shoot end of each seed was trimmed to improve germination (Sauls and Campbell 1980; Bergh 1988). All fruit was washed in 2% sodium hypochlorite for 5 min before the seed was removed. The seeds were also washed in 2% sodium hypochlorite for 1 min and rinsed once in sterile distilled water before being placed in deep plastic trays of sterile coarse river sand with the radicle end facing down and the sand just covering the shoot end of the seed. To prepare the germination trays, the river sand was washed twice with distilled water, drained and dried in an oven at 50°C. Distilled water was added to the sand in a deep plastic tray at a ratio of 1:2 (v/v). The tray was covered with aluminium foil and autoclaved for 40 min. The tray was left to stand for 24 h and then autoclaved again for 40 min.

The seed trays were covered with aluminium foil and incubated in a temperature controlled glasshouse at 25°C (+/- 2°C). Some seeds germinated very early (~ 2 weeks), but the majority produced a radicle within 5 weeks, with 90% of all seeds successfully germinating. The seeds that germinated early were removed and only seeds that produced a 2 - 4 cm radicle within 1 week of each other were selected for use in experiments.

Soil treatments
Following γ-irradiation of soils (Section 2.12), treatments were established as shown in Table 3.2. For mixed soil treatments, a 90% volume of either irradiated or conducive (pasture) soil was mixed with a 10% volume of non-irradiated suppressive soil from each of the 4 selected locations. The irradiated soil treatments from each location were mixed with their respective non-irradiated soil (i.e. 90% Tamborine Mt irradiated soil + 10% Tamborine Mt non-irradiated soil). Each soil treatment was prepared in a 20 L plastic drum (a separate drum was used for each soil treatment and each drum was washed in 2% sodium hypochlorite and rinsed with distilled water before use). The soil in each treatment was mixed by hand to break up clods.
(latex gloves were worn and these were replaced with fresh gloves between samples), sealed in the drum with a fitted lid and then tumbled for 10 min.

**Table 3.2** Treatments established for the transfer of suppression experiment (Experiment 3).

<table>
<thead>
<tr>
<th>Location</th>
<th>Non-irradiated</th>
<th>Irradiated</th>
<th>Irradiated + 10% Non-irradiated</th>
<th>Conducive (pasture) + 10% Non-irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamborine Mt 1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Pretty Gully 1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Tuckombil 1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Tuckombil 2</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Conducive (pasture)</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After mixing, portions of each soil treatment were subsampled for laboratory analyses. A portion of the subsample was stored at -20°C for DNA extractions (Chapters 5 and 6) and a portion was stored at 4°C prior to carrying out enzyme assays (Chapter 4) (week 0 samples).

**Glasshouse experiment**

Each soil treatment was distributed between 5 replicate pots. The pots used for this experiment were square 1 L black UV stabilised polypropylene pots (150 mm height x 90 mm width at top x 65 mm width at bottom). Each pot was rinsed in 2% hypochlorite before use. Each pot was inoculated with a *P. cinnamomi* mycelial mat (Section 2.9) as described in Section 3.2.7. The soil in each pot was moistened to maximum water holding capacity and then the pot was placed in a 550 mL polypropylene container (Channol, Australia) for flooding. The pots were transferred to a temperature controlled glasshouse and placed in a randomised complete block design (Plate 3.2). The glasshouse was maintained on a 12 h 26°C day / 18°C night cycle throughout the experiment.
The pots were incubated for 14 d before germinated Topa Topa avocado seeds were transplanted into each pot (1 seed per pot). Germinated seeds were transplanted by first removing approximately 100 mL soil from the centre of the pot and then using a stainless steel skewer (dipped in 100% ethanol and flamed between each pot) to form a hole in which to insert the radicle. The 100 mL volumes of soil removed to make way for the avocado seeds were retained for enzyme activity analyses (Chapter 4) and microbial community DNA analyses (Chapters 5 and 6) (week 2 samples). Avocado seedlings were left to establish over a 6 week period, during which the soil was kept moist by adding 100 mL distilled water to the pots every 2 - 3 d. At the end of this 6 week period, the avocado seedlings were well established and the first flood event was initiated by filling the flood chamber to maximum capacity with distilled water. The excess moisture was allowed to evaporate over 4 days and then the soils were kept moist by adding 100 mL distilled water to the pots every 2 d for a further 10 d. This cycle was repeated 3 times and the experiment was terminated 12 weeks after transplanting the avocado seeds (14 weeks after inoculation).
**Seedling harvest and disease severity assessment**

Avocado seedlings were harvested by placing the pot on its side on a plastic sheet (new plastic sheet for each treatment), loosening the soil and root ball from the edge of the pot and gently removing and shaking the avocado seedling to remove adhering soil from the roots. The soil was then broken up and mixed with gloved hands (latex gloves changed between each treatment), divided, and mixed again for 5 min. Subsamples of soil from each pot were taken for enzyme activity analyses (Chapter 4) and microbial community DNA analyses (Chapters 5 and 6) (week 14 samples).

Remaining soil adhering to the avocado seedling root was gently removed by washing in distilled water. Excess moisture was removed from the root using absorbent paper and then the root was rated using the 0 - 5 disease severity rating (DSR) scale of Greenhalgh and Lucas (1984) (Table 3.3). Roots with necrotic lesions were excised from avocado seedlings and plated onto PSA (as described in Section 2.5) to confirm *P. cinnamomi* infection.

Table 3.3 Disease severity rating system used to assess *P. cinnamomi* root rot in avocado seedlings (after Greenhalgh and Lucas 1984).

<table>
<thead>
<tr>
<th>Disease severity rating (DSR)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Healthy roots with no visible lesions</td>
</tr>
<tr>
<td>1</td>
<td>Minor lateral root rot with less than 10% of lateral root tips necrotic</td>
</tr>
<tr>
<td>2</td>
<td>Moderate lateral root rot with 10-50% of lateral root tips necrotic or tip of tap root slightly rotten or both</td>
</tr>
<tr>
<td>3</td>
<td>Severe lateral root rot with greater than 50% of lateral root tips necrotic or 5-30% of the tap root rotten from tip or both</td>
</tr>
<tr>
<td>4</td>
<td>Severe tap root rot with greater than 30% of the tap root necrotic but with some healthy lateral roots above lesions</td>
</tr>
<tr>
<td>5</td>
<td>Tap and lateral roots completely rotten or plant dead</td>
</tr>
</tbody>
</table>

3.2.9 *Data analysis*

Disease severity ratings from Experiment 1 were analysed by fitting a mixed linear model with locations specified as fixed effects, while glasshouse replicates and soil
samples within each location determined the random components. Ninety-five percent confidence intervals were calculated for predicted mean DSRs (determined by fitting the mixed linear model) for each location. Linear regressions were also conducted to assess relationships between mean DSRs and soil chemical parameters measured for each location. The DSR data for Experiment 2 were analysed as described for Experiment 1, with the exception that linear regressions were not carried out.

Analysis of variance (ANOVA) was used to test for differences between locations and the various soil treatments within Experiment 3. The ANOVA was structured to accommodate tests of impacts due to location and treatment and their interaction. Where F values were significant (p<0.05), mean DSRs were separated using least significant difference (LSD).

All model fitting was carried out with the assistance of Mr Stephen Morris (Biometrician, NSW Department of Primary Industries) using the Genstat software package Version 5 (Genstat Committee 1997). All graphs were prepared using Origin Version 5.0 (Microcal Software 1997).

3.3 Results

3.3.1 Soil abiotic properties

Soils at each of the sampling locations were derived from weathered tertiary basalt originating from volcanic activity (in north-eastern NSW) 20 - 24 million years ago (Morand 1994; Graham 2001). These soils are classified as red ferrosols under the Australian soil classification system (previously red Krasnozem soil; Morand 1994; Isbell 2002; McKenzie et al. 2004). The red ferrosol soils within the study area typically lack texture contrast between A and B horizons, are high in free iron oxide and are dark to reddish brown, deep, well drained, self-mulching, moderate to strongly structured light to medium clays or clay loams (Morand 1994; Isbell 2002; McKenzie et al. 2004). Chemical properties determined for soil samples from each study location are described in Table 3.4. The moisture content of soil samples at the time of sampling for each occasion is presented in Table 3.5.
Table 3.4 Soil chemical characteristics for each study location. Units for P are mg kg$^{-1}$ dry soil and units for cations and CEC are milli-equivalents 100 g$^{-1}$ dry soil. OC = organic carbon.

<table>
<thead>
<tr>
<th>Location</th>
<th>pH</th>
<th>CaCl$_2$ EC (1:5) dS m$^{-1}$</th>
<th>Total N %</th>
<th>P (Colwell)</th>
<th>OC %</th>
<th>Ca$^{2+}$</th>
<th>Mg$^{2+}$</th>
<th>Na$^+$</th>
<th>K$^+$</th>
<th>CEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta1</td>
<td>5.7</td>
<td>1.15</td>
<td>0.76</td>
<td>560</td>
<td>8.5</td>
<td>1.2</td>
<td>0.26</td>
<td>0.96</td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>Ta2</td>
<td>5.4</td>
<td>0.38</td>
<td>0.76</td>
<td>540</td>
<td>8.0</td>
<td>0.0</td>
<td>0.19</td>
<td>0.99</td>
<td>27.7</td>
<td></td>
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<tr>
<td>Trf</td>
<td>5.4</td>
<td>0.30</td>
<td>0.64</td>
<td>36</td>
<td>7.2</td>
<td>0.2</td>
<td>0.24</td>
<td>1.30</td>
<td>22.4</td>
<td></td>
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<tr>
<td>Pr1</td>
<td>6.5</td>
<td>0.65</td>
<td>0.74</td>
<td>1600</td>
<td>7.7</td>
<td>1.9</td>
<td>0.43</td>
<td>3.00</td>
<td>36.1</td>
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</tr>
<tr>
<td>Pr2</td>
<td>6.2</td>
<td>0.19</td>
<td>0.31</td>
<td>100</td>
<td>3.6</td>
<td>0.7</td>
<td>0.07</td>
<td>2.20</td>
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</tr>
<tr>
<td>Prf</td>
<td>6.6</td>
<td>0.29</td>
<td>0.59</td>
<td>41</td>
<td>5.8</td>
<td>0.2</td>
<td>0.10</td>
<td>1.90</td>
<td>24.1</td>
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</tr>
<tr>
<td>Cud</td>
<td>5.5</td>
<td>0.35</td>
<td>0.59</td>
<td>510</td>
<td>6.0</td>
<td>1.2</td>
<td>0.14</td>
<td>1.60</td>
<td>24.1</td>
<td></td>
</tr>
<tr>
<td>Eur</td>
<td>5.4</td>
<td>0.18</td>
<td>0.48</td>
<td>46</td>
<td>4.1</td>
<td>0.6</td>
<td>0.22</td>
<td>0.38</td>
<td>14.3</td>
<td></td>
</tr>
<tr>
<td>Tu1</td>
<td>6.3</td>
<td>0.27</td>
<td>0.64</td>
<td>220</td>
<td>6.2</td>
<td>1.0</td>
<td>0.20</td>
<td>1.30</td>
<td>27.5</td>
<td></td>
</tr>
<tr>
<td>Tu2</td>
<td>6.6</td>
<td>0.34</td>
<td>0.57</td>
<td>170</td>
<td>5.8</td>
<td>1.3</td>
<td>0.24</td>
<td>1.70</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td>Als</td>
<td>6.9</td>
<td>0.28</td>
<td>0.53</td>
<td>130</td>
<td>5.1</td>
<td>0.5</td>
<td>0.24</td>
<td>1.10</td>
<td>30.8</td>
<td></td>
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<tr>
<td>Ura</td>
<td>5.8</td>
<td>0.39</td>
<td>0.77</td>
<td>500</td>
<td>8.5</td>
<td>2.0</td>
<td>0.48</td>
<td>1.30</td>
<td>28.3</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5.4</td>
<td>0.35</td>
<td>0.58</td>
<td>310</td>
<td>5.3</td>
<td>1.5</td>
<td>0.77</td>
<td>0.078</td>
<td>17.2</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5 Soil moisture (%) at time of sampling for each experiment. Values represent means of 4 samples from each location with duplicate soil moisture measurements taken for each sample.

<table>
<thead>
<tr>
<th>Location</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta1</td>
<td>42.0</td>
<td>36.7</td>
<td>34.3</td>
</tr>
<tr>
<td>Ta2</td>
<td>32.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trf</td>
<td>27.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pr1</td>
<td>33.3</td>
<td>27.2</td>
<td>23.7</td>
</tr>
<tr>
<td>Pr2</td>
<td>15.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prf</td>
<td>26.1</td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
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<td>28.6</td>
<td>33.3</td>
</tr>
</tbody>
</table>

87
3.3.2 Experiment 1: Suppressive soil screening survey

Evidence of root rot was observed on lupin roots in all soil treatments, with the exception of the non-inoculated negative control treatment (DSR = 0). The highest DSRs were recorded for lupin seedlings in the positive control (C) soil, followed by soils from 2 organic orchards at Uralba (Ura) and Alstonvale (Als), and from 2 conventional orchards at Cudgen (Cud) and Tamborine Mt 2 (Ta2) (Figure 3.2). The mean DSR for the control soil (C) was significantly higher than mean DSRs observed for all other locations, as evidenced by 95% confidence intervals (represented by error bars in Figure 3.2). Locations with soils that most consistently yielded the lowest DSRs (<3) included Tamborine Mt 1 (Ta1-conventional), Tuckombil 2 (Tu2-organic), Pretty Gully 1 (Pr1-organic), Pretty Gully 2 (Pr2-conventional) and Tuckombil 1 (Tu1-conventional). The predicted mean DSRs for these 5 locations were not significantly different according to 95% confidence intervals (Figure 3.2). The 2 rainforest locations (Trf and Prf) scored a moderate disease severity rating (3 - 4).

![Figure 3.2](image-url)

**Figure 3.2** Predicted mean disease severity ratings (n=16) for lupins grown in soil samples collected from 10 avocado orchards, 2 rainforest remnants and 1 dairy pasture. Error bars represent 95% confidence intervals. Refer to text and Table 3.1 for details of location codes.
Regression analyses (data not shown) did not indicate any relationships between mean DSRs and soil chemical parameters (Table 3.4) measured for each location ($r^2$ for each soil nutrient parameter ranged between 0 and -0.15). There was no relationship between soil moisture at time of sampling (Table 3.5) and DSRs ($r^2 = 0$).

### 3.3.3 Experiment 2: Confirmation of biological suppression

In this experiment, non-irradiated soil samples from Tamborine Mt 1 (Ta1) and Pretty Gully 1 (Pr1) consistently yielded the lowest DSRs (<2.1; Figure 3.3). Minor infections were evident on lupins grown in soil samples from Tuckombil 1 (Tu1) and Tuckombil 2 (Tu2), with Tuckombil 1 (Tu1) and Tuckombil 2 (Tu2) being significantly more diseased than Tamaborine 1 (Ta1) according to 95% confidence intervals. Tuckombil 1 (Tu1) was also significantly more diseased than Pretty Gully 1 (Pr1). An increase in DSR in response to irradiation sterilisation of the soils was most noticeable in soil samples from Tamborine Mt 1 (Ta1) and Pretty Gully 1 (Pr1) (Figure 3.3). There was also an increase in DSR in response to irradiation sterilisation in soil samples from Tuckombil 1 (Tu1) and Tuckombil 2 (Tu2).

![Figure 3.3](image-url) Predicted mean disease severity ratings ($n=16$) for lupins grown in non-irradiated field soils from 4 avocado orchards (x axis) and in the same soils following $\gamma$-irradiation sterilisation (y axis). Error bars represent 95% confidence intervals. Refer to text and Table 3.1 for details of location codes.
3.3.4 Experiment 3: Transfer of suppression

As occurred with lupins in Experiments 1 and 2 (Sections 3.3.2 and 3.3.3), non-irradiated soil (N) from Tamborine Mt 1 (Ta1), Pretty Gully 1 (Pr1), Tuckombil 1 (Tu1) and Tuckombil 2 (Tu2) yielded low DSRs on avocado seedlings (Figure 3.4). ANOVA showed that there were significant differences between treatments (p<0.01) and between treatments and the non-irradiated (N) conducive soil (C) (p<0.05). While there were no significant differences between any of the non-irradiated orchard soils (N; Ta1, Pr1, Tu1 and Tu2), root rot severity in avocado seedlings increased significantly (p<0.05) in response to irradiation sterilisation (i) of soil from each location (Figure 3.4). However, the increased mean DSR associated with the irradiated soils (i) was significantly decreased (p<0.05) by mixing the irradiated soils with non-irradiated suppressive soils (iN) from each location. For the Tuckombil 1 (Tu1) and Tuckombil 2 (Tu2) irradiated soils, addition of 10% non-irradiated suppressive soil (iN) reduced disease severity to levels that were not significantly different from the non-irradiated soils (N) from the same locations.

![Figure 3.4](image-url)

**Figure 3.4** Mean disease severity ratings (n=5) for avocados grown in non-irradiated avocado orchard soils and in soils subjected to various treatments (refer to text for details of treatments). Bars associated with means represent LSDs. Significant differences (p<0.05) occur where the distance between means is greater than the LSD. The non-irradiated conducive soil (C) was mixed with 10% (v/v) of non-irradiated suppressive soil from each of the 4 avocado orchards (refer to text for details of treatment codes).
The non-irradiated (N) conducive dairy pasture soil (C) yielded a significantly higher mean DSR than any of the other non-irradiated (N) avocado soils (Figure 3.4). There were no significant differences (p<0.05) between the non-irradiated (N) conducive soil (C) and 3 of the 4 (Pr1, Tu1 and Tu2) irradiated soils (i). The exception was the irradiated (i) Tamborine Mt treatment (Ta1), which produced significantly more disease on avocado seedlings than the non-irradiated (N) conducive pasture soil (C) (p<0.05). Addition of 10% suppressive soil from the 4 orchards (Ta1, Pr1, Tu1 and Tu2) to the conducive pasture soil (C) resulted in a significant decrease (p<0.05) in mean DSR in all cases. For the conducive soil (C) + Tuckombil 1 (Tu1) and the conducive soil + Tuckombil 2 (Tu2) treatments, mean DSR values were similar to those for the non-irradiated (N) Tuckombil 1 (Tu1) and Tuckombil 2 (Tu2) soils.

3.4 Discussion

The main aim for the series of experiments reported in this chapter was to identify P. cinnamomi suppressive soils for further study and, where these soils were identified, to determine whether suppression was influenced by biological or abiotic processes. During each of the 3 experiments, none of the soils completely suppressed the development of P. cinnamomi root rot in either lupin seedlings or avocado seedlings. However, if disease suppression is considered to occur along a continuum (Alabouvette et al. 1996), then any soil, in which no disease or minor to moderate disease severity occurs, can be defined as highly to slightly suppressive. This was evident in the series of experiments presented in this study, where the level of suppressiveness varied between soils in which a high level of active inoculum was supplied in conjunction with moisture and temperature conditions favouring disease establishment.

The DSR data presented for Experiment 1 (Section 3.3.2) suggested that avocado soils sampled from Tamborine Mt 1 (Ta1; conventional), Pretty Gully 1 (Pr1; organic), Pretty Gully 2 (Pr2; conventional), Tuckombil 1 (Tu1; conventional) and Tuckombil 2 (Tu2; organic) had the capacity to reduce the development of P. cinnamomi root rot in lupins (Figure 3.2). Some samples collected from other locations also reduced the severity of P. cinnamomi root rot in test plants (data not
shown). However, most of the soils from Eureka (Eur; conventional), Tamborine Mt 2 (Ta2; conventional), Alstonvale (Als; organic) and Uralba (Ura; organic) were slightly to moderately conducive to *P. cinnamomi* root rot. Prior to this study comparisons had not been made between conventional and organic management systems in relation to their performance in terms of *P. cinnamomi* suppression. The results presented here suggest that neither conventional nor organic avocado orchard management systems consistently favour the development of *P. cinnamomi* suppressive soils.

*P. cinnamomi* suppressive avocado soils have previously been identified as possessing soil pH levels between 5.5 and 7.0 and higher levels (than in conducive soils) of NH$_4$, N0$_3$, exchangeable Ca$^{++}$, CEC and organic matter content (Broadbent and Baker 1974a; Schmitthenner and Canaday 1983; Shea and Broadbent 1983; Erwin and Ribeiro 1996). In the present study, linear regressions were used to test for relationships between a number of soil properties measured for each location (Table 3.4) and DSR data recorded for lupin seedlings during Experiment 1. Results from these regressions suggested that none of the soil abiotic properties included in the analysis were associated with the ability of the soil to suppress the development of disease.

Newhook and Podger (1972) noted that few rainforest areas within Qld were affected by *P. cinnamomi* despite the presence of vegetation susceptible to the pathogen. Broadbent and Baker (1974a) also found that soils collected from several rainforest areas in Qld were highly suppressive toward *P. cinnamomi*. For this reason, the average DSRs recorded for soil samples from the Tamborine Mt and the Pretty Gully rainforest remnants that were higher than some avocado orchards in the present study were unexpected. The results suggested that these soils may be slightly conducive to *P. cinnamomi*. Another possibility is that the mechanisms responsible for suppression may not have been active at the time of sampling. Parameters that might affect these mechanisms include soil moisture, which is well recognised as a factor limiting microbial activity in soil (van Elsas et al. 1997). The time of sampling (late 2002) coincided with the peak of an extended drought which may have impacted on the rainforest areas more than the avocado orchards which (with the exception of Pretty Gully 2 and Eureka) were irrigated. It is possible that moistening and then
incubating the soils for 3 d may have been insufficient to completely activate biological processes assumed to be responsible for suppressing *P. cinnamomi*. However, there was no evidence of this from the regression analysis of the soil moisture (Section 3.3.1) and DSR data for Experiment 1 (Section 3.3.2). In addition, while soil moisture in the rainforest soils was lower than most of the orchard soils, their moisture content was between 26% and 27%, which was similar to the suppressive Tuckombil 2 orchard soil (28% soil moisture content).

Biological processes involved in suppression appeared to be active in soils sampled from the 4 selected orchards (Tamborine Mt 1, Pretty Gully 1, Tuckombil 1, Tuckombil 2) during Experiment 2 (Section 3.3.3) and Experiment 3 (Section 3.3.4). Soils from these locations consistently yielded low DSRs across all 3 experiments in both lupins and Topa Topa avocado seedlings and, for most of the soils sampled from these 4 locations, DSRs increased significantly in response to removal of microbial activity by $\gamma$-irradiation (Sections 3.3.3 and 3.3.4). These results are in accordance with Broadbent and Baker (1974a) and others (Malajczuk 1977a; Halsall 1978; Malajczuk 1979c; Marks and Smith 1981; Halsall 1982a; Ko and Shiroma 1989; Casale 1990; Duvenhage et al. 1991; Stirling et al. 1992; Ann 1994) who have demonstrated the biological nature of *P. cinnamomi* suppression. When demonstrating biological suppression of *P. cinnamomi* most workers pasteurised their soils, which resulted in a significant increase in root rot incidence and severity. Heating soils is known to alter physicochemical attributes of the soil (Trevors 1996) and therefore there could be some uncertainty when interpreting results obtained by heating soils. However, the loss of suppression in response to $\gamma$-irradiation sterilisation (which has minimal impact on the physicochemical attributes of soil; Trevors 1996), as demonstrated by Malajczuk (1977), Duvenhage et al. (1991) and in the present study, solidly supports the contention that *P. cinnamomi* suppression is biologically mediated.

Malajczuk et al. (1977a) restored suppression to sterile loam forest soil by adding a small amount (6.4%) of non-sterile suppressive soil. In the present study, suppression was at least partially restored to sterile soil (previously suppressive prior to sterilisation) by adding 10% non-sterile suppressive soil. Disease severity was also reduced in a conducive soil by adding 10% non-sterile suppressive soil. This
suggests that either biochemical mechanism(s) involved in suppression (Section 1.8) continued to function after the suppressive soils were diluted 1 in 10, or that the biological agents responsible for suppression (Sections 1.7 and 1.9) were able to quickly establish in the conducive and sterile soils and assert their antagonistic influence on *P. cinnamomi*. This is the first time that these possibilities have been investigated in relation to *P. cinnamomi* suppression when transferred to a conducive soil.

In the subsequent chapters of this thesis, comparisons of the activities of 2 soil enzymes (cellulase and laminarinase) and microbial community structure and diversity (determined using microbial community DNA profiling methods) are made between field samples with the aim of identifying biochemical or microbial community attributes that may be unique to either the suppressive or conducive soils identified during this study. Shifts in soil enzyme activity and microbial community structure and diversity during Experiment 3 were also monitored with the aim of detecting changes that may have been associated with treatments in which *P. cinnamomi* suppression was transferred.
Chapter 4

Cellulase, laminarinase and P. cinnamomi suppression

4.1 Introduction

Studies on P. cinnamomi suppressive soils have provided a body of evidence indicating that suppression is biologically mediated (e.g. Broadbent and Baker 1974a; Malajczuk 1979; Marks and Smith 1981; Halsall 1982a, 1982b; Marks and Smith 1983; Casale 1990; Duvenhage and Mass 1990; Ann 1994). In Chapter 3, the loss of suppression in soils after γ-irradiation, and the ability to suppress P. cinnamomi in γ-irradiated and conducive soils through inoculation with a 10% volume of suppressive soil, show that suppression in the avocado orchard soils used in this study is also biologically mediated. Specific biological agents (Pratt 1971; Broadbent and Baker 1974a, 1974b; Malajczuk et al. 1984; Murray 1987; Stirling et al. 1992; You et al. 1996) and mechanisms (Malajczuk 1979; Nesbitt 1979; Costa et al. 1996; Downer et al. 2001a, 2001b) potentially responsible for P. cinnamomi suppression in soil have been investigated. However, despite much effort to elucidate the mechanisms of P. cinnamomi suppression, the problem remains mostly unresolved.

A number of workers have reported lysis of P. cinnamomi propagules as an outcome of microbial antagonism and as a process implicated in P. cinnamomi suppression (Cook and Baker 1983; Malajczuk 1983; Erwin and Ribeiro 1996). Inhibition and lysis of P. cinnamomi propagules have been observed in soil, compost and soil extracts (Broadbent and Baker 1974a, 1974b; Hoitink et al. 1977; Malajczuk 1979; Nesbitt et al. 1979, 1981a; Halsall 1982a, 1982b; Stirling et al. 1992; Costa et al. 1996; Aryantha et al. 2000). Soils suppressive to P. cinnamomi typically contain large populations of bacteria, and antibiotic producing bacteria such as pseudomonads, Bacillus spp. and actinomycetes have been isolated from degraded P. cinnamomi propagules (Broadbent and Baker 1974a, 1974b; Nesbitt et al. 1985). Lysis of P. cinnamomi mycelium has also been observed following mycoparasitism by cellulolytic fungi such as Trichoderma spp. (Chambers and Scott 1995).
Antibiosis is frequently cited as a mechanism by which *P. cinnamomi* propagules are inhibited or lysed *in vitro* (Pratt 1971; Brown et al. 1987; Erwin and Ribeiro 1996). However, the role of specific microbial metabolites in suppression of *P. cinnamomi* in soil is poorly understood. As a member of the Oomycota, the cell walls of *Phytophthora* species are principally composed (80 to 90%) of cellulosic β-1,4-glucans and non-cellulosic β-1,3- and β-1,6-linked glucans (Bartnicki and Wang 1983). In the process of breaking down organic matter, a suite of soil microorganisms produce β-1,3- and β-1,4-glucanases (laminarinase and cellulase) (Tabatabai 1994). There is evidence from a number of *in vitro* studies that Phytophthora propagules are degraded by laminarinases and cellulases. Sneh (1972) developed a method to separate *P. cactorum* oospores from mycelial mats by first degrading the mycelium with cellulase. In genetic studies, protoplasts were isolated from *Phytophthora* cells by completely degrading the cell walls using cellulase and laminarinase (Erwin and Ribeiro 1996). Both enzymes have been implicated as mechanisms involved in *in vitro* microbial antagonism toward *Phytophthora* spp. (Brown et al. 1987; Fridlender et al. 1993; El Tarabily et al. 1996). Such mechanisms are in accordance with the characteristically high organic matter and high microbial activity measured in *P. cinnamomi* suppressive avocado soils (Broadbent and Baker 1974a; Shea and Broadbent 1983; Erwin and Ribeiro 1996). An accumulation of cellulases and laminarinases in soil may result in suppression of *P. cinnamomi* (Downer et al. 2001a, 2001b).

More specific evidence of the potential role of cellulase and laminarinase in degradation and suppression of *P. cinnamomi* in soil comes from 2 studies by Downer et al. (2001a, 2001b). In the first of these studies, both enzymes were associated with reduced *P. cinnamomi* inoculum in Californian avocado soils mulched with freshly chipped eucalyptus trimmings. In a second study in the laboratory, Downer et al. (2001b) demonstrated that various *P. cinnamomi* propagules were inhibited or degraded at moderate to high concentrations of cellulase and laminarinase. The relationship between *P. cinnamomi* suppression and these enzymes in Australian soils, and the effect of these enzymes on plant growth and development of Phytophthora root rot in plants, have not been investigated.
In the following study, cellulase and laminarinase activities were measured in field soils shown to suppress \textit{P. cinnamomi} in the suppressive soil screening assays reported in Chapter 3. Measurement of enzyme activities was achieved using a new method developed to overcome limitations identified in published methods (Section 1.8.3; Appendix 1). Enzyme activities were also measured at 3 stages during a glasshouse experiment in which suppressive soils were added to $\gamma$-irradiated soils and a conducive soil (Section 3.2.8). Soil enzyme activities were measured to: i) determine whether high cellulase and laminarinase activities were associated with \textit{P. cinnamomi} suppressive soils in subtropical Australia and ii) determine whether \textit{P. cinnamomi} suppression transferred to a conducive soil was accompanied by changes in cellulase and laminarinase activities. The effect of cellulase and laminarinase on plant growth and the development of \textit{P. cinnamomi} root rot in lupin seedlings were also investigated.

\section*{4.2 Materials and methods}

\subsection*{4.2.1 Field locations and soil sampling}

Soil was collected from 4 avocado orchards (Tamborine Mt., Pretty Gully, Tuckombil 1 and Tuckombil 2) on 2 occasions which coincided with early spring (September, 2003) and late summer (February, 2004). Details for field locations and procedures for sampling, handling and storage of soil samples were described in Sections 3.3.1 - 3.2.3.

\subsection*{4.2.2 Soil sampled during Experiment 3}

During Experiment 3 (refer to Chapter 3), soil was sampled from pots at 0, 2 and 14 weeks as described in Section 3.2.8. Techniques used to avoid cross contamination and conditions for handling and storing soil samples prior to analysis were described in Sections 3.2.2, 3.2.3 and 3.2.8.
4.2.3 Enzyme assays

Soil cellulase and laminarinase assays were carried out using the method developed by Keen and Vancov (Appendix 1). Enzymes were extracted from soil using 6 bead-beating cycles and the enzyme – substrate reactions were run over 4 h. Each soil sample was extracted and assayed in triplicate.

4.2.4 Effects of enzymes on plant growth and disease suppression

Clean, coarse river sand was mixed with vermiculite at a ratio of 1:1 (v/v; pH 4.9). The sand mix was washed twice in tap water, drained, autoclaved twice and dried at 105°C. After cooling, 10 mL of the sand mix was placed into 225 mL pots (plastic drinking cups with drainage holes; Castaway, Australia). *P. cinnamomi* mycelial mats grown over 20 mm diameter cellophane discs (Section 2.9) were transferred aseptically to the surface of the sand mix layer and then a further 190 mL sterile sand mix was added to each pot. The 225 mL pots were placed in broader 180 mL plastic drinking cups which acted as flood chambers. Industrial grade enzyme preparations (Novozymes, Australia) of cellulase (Cellusoft®; produced by a *Trichoderma* sp.; pH 5.5) and laminarinase (Cereflo®; also contains cellulase; produced by *Bacillus amyloliquefaciens* [ex. Fukumoto] Priest; pH 7.0) were diluted to give a range of concentrations in sterile deionised water, and 80 mL of each dilution was added to pots to give 1, 5, 10, 15, 20, 25 and 35 U enzyme mL⁻¹ sand mix. One enzyme unit is the amount of enzyme required to degrade an appropriate substrate to reducing carbohydrates with a reducing rate corresponding to 1 µmol glucose h⁻¹. Addition of 80 mL of enzyme solution to 200 mL of sand mix resulted in an initial moisture content equivalent to 59% of water holding capacity (predetermined as 49% moisture content of the sand mix). Each treatment was replicated 4 times and 6 control treatments were established as follows:
1. Deionised water
2. Deionised water + *P. cinnamomi*
3. Inactivated cellulase (Cellusoft®) 5 U mL⁻¹ sand mix
4. Inactivated cellulase (Cellusoft®) 5 U mL⁻¹ sand mix + *P. cinnamomi*
5. Inactivated laminarinase (Cereflo®) 5 U mL⁻¹ sand mix
6. Inactivated laminarinase (Cereflo®) 5 U mL⁻¹ sand mix + *P. cinnamomi*

Enzymes were inactivated before addition to the sand mix by autoclaving for 20 min.

The pots were incubated in a glasshouse (12 h cycle 25°C day / 18°C night) for 3 d and then 5 lupin seedlings (Section 2.10) were transplanted into each pot. Procedures used for transplanting lupin seedlings were described in Section 3.2.7. After transplanting the lupin seedlings, distilled water was added to each pot until the base stood in 5 mm of water (indicator mark placed on cup). For the next 2 d moisture was maintained to the indicator mark (topped up daily) and on the third day each pot was flooded to just below the brim of the flood chamber. The cups were incubated for a further 6 d during which the moisture was maintained as described above (water topped up daily to the 5 mm indicator mark). On the 6th day the 5 lupin seedlings in each pot were harvested, washed gently, blotted with absorbent paper to remove excess moisture and weighed. In addition, a ~1 cm section of each tap root tip (20 in total) was plated onto PSA (Section 2.2 and Section 2.5) and the number of roots from which *P. cinnamomi* grew were counted.

4.2.5 Analysis

Differences between cellulase and laminarinase activity measured for field soil samples were assessed using ANOVA. Enzyme activity results for the 4 field locations in spring and summer were then pooled, and means were calculated and analysed for correlations with the pooled means for disease severity ratings recorded for soils from the same locations in Experiments 1 (Section 3.3.2) and 2 (Section 3.3.3). Differences between locations and the various soil treatments within Experiment 3 (Section 3.3.4) were also tested using ANOVA. The ANOVA was structured to accommodate tests of impacts due to location and treatment and their
interaction. Where F values were significant (p<0.05), mean enzyme activities were separated using least significant difference (LSD).

Statistical analyses were carried out with the assistance of Mr Stephen Morris (Biometrician, NSW Department of Primary Industries) using the Genstat software package Version 5 (Genstat Committee 1997). All graphs were prepared using Origin Version 5 (Microcal Software 1997).

4.3 Results

4.3.1 Cellulase and laminarinase activity in freshly sampled field soils

Cellulase and laminarinase activities measured in *P. cinnamomi* suppressive soils collected from 4 avocado orchards (Ta1 = Tamborine Mt. 1; Pr1 = Pretty Gully 1; Tu1 = Tuckombil 1; Tu 2 = Tuckombil 2) ranged from 5.4 to 35.0 µg glucose produced g\(^{-1}\) dry soil h\(^{-1}\) and 16.3 to 80.9 µg glucose produced g\(^{-1}\) dry soil h\(^{-1}\), respectively (Table 4.1). With the exception of cellulase and laminarinase activities at Ta1 and laminarinase activity at Tu1, the activities of both enzymes measured in summer were 37% - 64% lower than in spring. Activities of both enzymes at Ta1 and laminarinase activity at Tu1 did not change between spring and summer. Differences in enzyme activities observed between the 4 locations were significant on both occasions (spring: cellulase p<0.01, laminarinase p<0.05; summer: cellulase p<0.05, laminarinase p<0.01). Comparison of means by LSD confirmed that cellulase activity in soil samples collected from Ta1 and Tu1 in spring were significantly lower (p<0.05) than in soil from the other 2 locations. Among the soil samples collected in late summer, cellulase activity at Pr1 was similar to Tu2 but only Pr1 had significantly higher (p<0.05) cellulase activity than Ta1 and Tu1. In both spring and summer, laminarinase activities measured in soil from Pr1 were significantly higher (p<0.05) than for soil from the other 3 locations.
Table 4.1 Cellulase and laminarinase activity measured at 4 avocado orchards on 2 occasions.

<table>
<thead>
<tr>
<th>Location</th>
<th>Cellulase activity*</th>
<th>Laminarinase activity*</th>
</tr>
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<tr>
<td></td>
<td>Spring</td>
<td>Summer</td>
</tr>
<tr>
<td>Ta1 - Tamborine Mt. 1</td>
<td>6.4</td>
<td>6.6</td>
</tr>
<tr>
<td>Pr1 - Pretty Gully 1</td>
<td>35.0</td>
<td>14.1</td>
</tr>
<tr>
<td>Tu1 - Tuckombil 1</td>
<td>9.2</td>
<td>5.4</td>
</tr>
<tr>
<td>Tu2 - Tuckombil 2</td>
<td>26.9</td>
<td>10.1</td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td>17.2</td>
<td>5.9</td>
</tr>
</tbody>
</table>

* Enzyme activity is expressed as µg glucose produced g⁻¹ dry soil h⁻¹. Values are means for 4 samples (3 replicate assays for each sample; n=12) collected from 4 locations.

A positive correlation ($r^2 = 0.96$) occurred between cellulase and laminarinase activities measured in field soils (Figure 4.1). However, there were no relationships between cellulase ($r^2 = 0.17$) or laminarinase ($r^2 = 0.15$) activities and disease severity ratings recorded for soils from the same locations during Experiments 1 (Section 3.3.2) and 2 (Section 3.3.3).

![Figure 4.1](image_url) **Figure 4.1** Correlation between cellulase and laminarinase activities in avocado orchard soils. Data points represent pooled means for enzyme activities measured in spring and summer ($n = 6$ for each data point).
4.3.2 Cellulase activity during Experiment 3

Over the duration of the 14 week transfer of suppression experiment (Experiment 3, Sections 3.2.8 and 3.3.4) cellulase activity decreased in the non-irradiated suppressive orchard soils (N) (Figure 4.2), with loss of cellulase activity occurring within the first 2 weeks (Figure 4.2). Despite this decrease, at each sampling time (weeks 0, 2 and 14) cellulase activity in the suppressive orchard soils (N) remained significantly higher (p<0.05) than in the: irradiated soils (i); conducive pasture soil (C); and the irradiated + 10% suppressive soil treatments (iN) (Figure 4.2). Cellulase activity in the suppressive orchard soils (N) was also significantly higher (p<0.05) than cellulase activity in the conducive pasture + 10% suppressive soil treatments (CN) at weeks 0 and 2, but not at week 14.

![Cellulase activity](image)

**Figure 4.2** Cellulase activities of *P. cinnamomi* suppressive avocado orchard soils (with and without γ-irradiation) and a *P. cinnamomi* conducive pasture soil in a glasshouse experiment (Experiment 3, Chapter 3). Columns represent pooled means for cellulase activity at 0, 2 and 14 weeks after soil was inoculated with *P. cinnamomi* (n=12; conducive n=3). Bars associated with means represent LSDs (p<0.05) for different treatments for each time point. Refer to text for details of treatment codes.
Irradiating the suppressive orchard soils (N) resulted in a 10 fold decrease in cellulase activity (Figure 4.2) measured 14 d post-irradiation (0 weeks). With the exception of the irradiation sterilised Ta1 soil (0.09 µg glucose g⁻¹ dry soil h⁻¹), cellulase activity in the irradiated treatments (i) fell below detection limits by the end of the second week (4 weeks post-irradiation) but recovered by week 14 (Figure 4.2). At week 0, pooled means for cellulase activity increased slightly but not significantly in response to mixing the irradiated soil samples with their respective non-irradiated suppressive orchard soils (IN). Cellulase activity in the irradiated + 10% suppressive soil treatments (IN) was similar at weeks 0 and 2 but increased by week 14 (Figure 4.2). A comparison of separated means (for treatment effects at each location) suggested that this increase was mainly due to a significant increase (p<0.05) in cellulase activity in response to adding the suppressive Pr1 soil to the same soil after it was irradiation sterilised (data not shown).

Initially (week 0), there were no changes in cellulase activity in response to mixing the conducive pasture soil (C) with the suppressive soils (CN; Figure 4.2). Cellulase activity declined in the conducive soil (C) and the conducive + 10% suppressive soil treatments (CN) over the first 2 weeks. At weeks 2 and 14, cellulase activity was higher in conducive + 10% suppressive soil treatments (CN) than in the conducive soil (C; Figure 4.2) but these differences were not significant. At week 2, cellulase activities were significantly (p<0.05) higher in the conducive (C) and conducive + 10% suppressive soil treatments (CN) than in the irradiated (i) and irradiated + 10% suppressive soil treatments (IN; Figure 4.2).

Comparisons of disease severity rating (DSR) data from Experiment 3 (Chapter 3) and cellulase activity measurements for the same experiment suggested that there was an association between low DSR and higher cellulase activity in the non-irradiated suppressive orchard soils (N) at 0, 2 and 14 weeks (Figure 4.3). There was also an association between high DSR and low enzyme activity in the irradiated soil treatments. However, this pattern was not consistent across the other soil treatments at any of the 3 sampling times (Figure 4.3).
Figure 4.3 Relationship between disease severity rating and cellulase activity in *P. cinnamomi* suppressive avocado orchard soils (with and without γ-irradiation) and a *P. cinnamomi* conducive pasture soil (Experiment 3, Chapter 3). Columns represent pooled mean disease severity ratings ordered from highest to lowest (*n* = 20; conducive *n* = 5). Disease severity was assessed once at week 14. Disease severity data shown at 0, 2 and 14 weeks are therefore the same. Solid circles represent pooled mean cellulase activity measured in soils sampled from each treatment at 0, 2 and 14 weeks (*n* = 12 conducive *n* = 3). Refer to text for details of treatment codes.

4.3.3 Laminarinase activity during Experiment 3

Laminarinase activity declined in the non-irradiated suppressive avocado orchard soils (N) over the 14 week transfer of suppression experiment (Figure 4.4). At each sampling occasion (0, 2 and 14 weeks), laminarinase activity in the suppressive orchard soils remained significantly higher (p<0.05) than in the: irradiated soils (i); conducive pasture soil (C); and the irradiated + 10% suppressive soil treatments (iN). At 0 and 2 weeks, laminarinase activity in the suppressive orchard soil (N) was also significantly (p<0.05) higher than in the conducive + 10% suppressive soil treatments (CN). However, no significant differences occurred between these 2 treatments at 14 weeks (Figure 4.4).
There was a large and significant (p<0.05) fall in laminarinase activity in the irradiated orchard soils (i) 2 weeks post-irradiation (Figure 4.4; 0 weeks). In the irradiated soils (i) laminarinase activity was similar at week 0 (5.2 µg glucose g⁻¹ dry soil h⁻¹) and week 2 (4 weeks post-irradiation; 5.0 µg glucose g⁻¹ dry soil h⁻¹), but increased significantly (p<0.05; 8.6 µg glucose g⁻¹ dry soil h⁻¹) at week 14 (Figure 4.4). Between week 0 and week 2, laminarinase activity increased significantly (p<0.05) in response to mixing the irradiated soils with their respective suppressive orchard soil (iN). However, by week 14 there was no significant difference between the pooled means for these 2 treatments.

Overall, laminarinase activity in the conducive pasture soil (C) declined by approximately 30% over the 14 week experiment (Figure 4.4). Laminarinase activity measured in the conducive soil was similar to that measured in the conducive + 10%
suppressive orchard soil treatments at each time point. However, separation of the pooled means (data not shown) suggested that laminarinase activity in the conducive soil was significantly (p<0.05) lower than in the conducive + 10% suppressive Pr1 soil treatment, but significantly higher than in the conducive + 10% suppressive Tu1 soil treatment. At week 2, laminarinase activity was significantly (p<0.05) higher in the conducive soil and conducive + 10% suppressive soil treatments than in the irradiated and irradiated + 10% suppressive soil treatments.

Comparisons between laminarinase activity and disease severity rating (DSR) data for Experiment 3 (Chapter 3) suggested that there was an association between low DSR and high laminarinase activity in the non-irradiated suppressive orchard soils (N) at each time point (Figure 4.5). Higher DSRs also appeared to be associated with lower laminarinase activity in the irradiated soil treatments (i). However, disease severity did not appear to be associated with laminarinase activity in any of the other treatments.
4.3.4 Effect of cellulase and laminarinase added to plant growth media

Lupin seedlings grew well in the water only control treatments (mean fresh weight 10.2 g per 5 seedlings) but grew poorly (mean fresh weight 2.1 g per 5 seedlings), and were highly affected by root rot (*P. cinnamomi* recovered from 80% of root tips) in the water inoculated with *P. cinnamomi* controls (Table 4.2). In the cellulase (Cellusoft©) treatments, lupin seedlings survived but grew poorly (compared to the water only controls) in concentrations of 20 U cellulase mL⁻¹ sand mix or less (Table 4.2) and died (failed to establish) in 25 and 35 U cellulase mL⁻¹ sand mix within 4 d of being transplanted. In treatments where lupins established there was a general trend of declining fresh weight with increasing cellulase concentration. The average lupin fresh weights recorded for the 1 and 5 U cellulase mL⁻¹ sand mix treatments...
were similar to the inactivated non-inoculated control treatments, which were approximately half the weight of lupins grown in the water only controls. *P. cinnamomi* was recovered from 80% of the 20 lupin root tips from both the inoculated water control and the 1 U cellulase mL\(^{-1}\) sand mix treatments. There was a general decline in the percentage of infected roots up to 15 U cellulase mL\(^{-1}\) sand mix but in the 20 U cellulase mL\(^{-1}\) sand mix treatments *P. cinnamomi* was recovered from 40% of root tips. *P. cinnamomi* was not recovered from the remains of lupins transplanted in the 25 and 35 U cellulase mL\(^{-1}\) sand mix treatments.

Table 4.2 Effect of enzymes on lupin growth and disease development.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cellulase (Cellusoft(^{\text{™}}))</th>
<th>Laminarinase (Cereflo(^{\text{®}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh weight (g) Infected root tips (%)</td>
<td>Fresh weight (g) Infected root tips (%)</td>
</tr>
<tr>
<td>Water only</td>
<td>10.2 0</td>
<td>10.2 0</td>
</tr>
<tr>
<td>†Water inoculated</td>
<td>2.1 80</td>
<td>2.1 80</td>
</tr>
<tr>
<td>Inactivated</td>
<td>5.2 0</td>
<td>ND 0</td>
</tr>
<tr>
<td>†Inactivated-inoculated</td>
<td>3.3 55</td>
<td>ND 40</td>
</tr>
<tr>
<td>†1 U mL(^{-1})</td>
<td>5.5 80</td>
<td>1.9 65</td>
</tr>
<tr>
<td>†5 U mL(^{-1})</td>
<td>5.1 50</td>
<td>ND 0</td>
</tr>
<tr>
<td>†10 U mL(^{-1})</td>
<td>3.1 10</td>
<td>ND 20</td>
</tr>
<tr>
<td>†15 U mL(^{-1})</td>
<td>3.1 15</td>
<td>ND 0</td>
</tr>
<tr>
<td>†20 U mL(^{-1})</td>
<td>3.0 40</td>
<td>ND 0</td>
</tr>
<tr>
<td>†25 U mL(^{-1})</td>
<td>ND 0</td>
<td>- -</td>
</tr>
<tr>
<td>†30 U mL(^{-1})</td>
<td>ND 0</td>
<td>- -</td>
</tr>
</tbody>
</table>

† Inoculated with *Phytophthora cinnamomi*. ND indicates no data due to failure of lupins to establish. Values for fresh weights are means of 4 replicate pots with 5 lupin seedlings in each pot. Values for infected root tips are expressed as the percentage of 20 root tips from which *P. cinnamomi* was recovered on Phytophthora selective agar (PSA, Section 2.2).

With the exception of the 1 U laminarinase mL\(^{-1}\) sand mix (Cereflo\(^{®}\)) treatments, lupin seedlings died within 3 d after being transplanted in all pots treated with Cereflo\(^{®}\) (Table 4.2). Lupin seedlings also died in both inactivated laminarinase control treatments (inoculated and non-inoculated). The roots of many of the lupin seedlings transplanted in 10 U laminarinase mL\(^{-1}\) sand mix, and in higher enzyme concentrations, had an appearance and texture consistent with partial digestion (i.e. moist, brown to black, limp and soft). For this reason, fresh weights were only recorded for the 1 U laminarinase mL\(^{-1}\) sand mix treatments. In 1 U mL\(^{-1}\) sand mix
laminarinase treatments, lupin seedlings grew poorly (1.9 g fresh weight) and *P. cinnamomi* was recovered from 65% of the seedling root tips. *P. cinnamomi* was also recovered from 40% of the seedlings from the inactivated and inoculated treatments and from 20% of the seedling remains from the 10 U laminarinase mL\(^{-1}\) sand mix treatments. *P. cinnamomi* was not recovered from the remains of lupins transplanted in the other laminarinase treatments.

### 4.4 Discussion

Temporal fluctuations in soil enzyme activities have been observed in a number of studies (Bandick and Dick 1999; Debosz et al. 1999; Aon and Colaneri 2001; Aon et al. 2001). In this study, soil cellulase and laminarinase activities were mostly higher in spring than in summer, and, in both seasons, the activities of both enzymes were higher in soils under organic management (Pretty Gully 1 and Tuckombil 2) than in soils under conventional management (Tamborine Mt. 1 and Tuckombil 1). Several researchers have found that organically managed soils are more biologically active than soils under conventional management, often attributing this to an absence of synthetic pesticides in the organic systems (Jaffe et al. 1998; Albiach 1999; Condron et al. 2000; Bettiol et al. 2002; Bullock et al. 2002; Oehl et al. 2004).

Production of enzymes by soil microorganisms is also affected by substrate availability in the form of organic matter (i.e. plant and animal residues) (Bandick and Dick 1999; Debosz et al. 1999; Zhang and Zhang 2000; Acosta-Martinez and Tabatabai 2001; Acosta-Martinez et al. 2003a, 2003b). The organically managed Pretty Gully 1 avocado orchard (Pr1) and both conventional orchards were actively mulched with organic residues (e.g. wood chips; chicken manure), whereas the mulch layer in the organically managed Tuckombil 2 orchard (Tu2) mostly consisted of naturally deposited plant debris. While organic inputs at each of the 4 orchards varied, the major difference was the use of fungicidal canopy sprays containing Cu in the conventional orchards (Tamborine Mt. 1, Mr James McCulloch pers. comm. 2003; Tuckombil 1, Mrs Bonnie Walker pers. comm. 2003). In previous studies of soils at the Tuckombil 1 avocado orchard, Merrington et al. (2001) and van Zwieten et al. (2004) found that an accumulation of Cu in the soil (from Cu-based fungicides)
caused a reduction in microbial biomass and activity and earthworm populations. Therefore, in the present study, differences in enzyme activities between the organically and conventionally managed orchards may have been related to the long term use of Cu fungicides. However, a more in-depth study of the effects of Cu on cellulase and laminarinase activities in avocado orchard soils needs to be undertaken to test this association.

Downer et al. (2001a) applied freshly chipped eucalyptus trimmings to soil beneath young avocado trees (2 years old) and compared these with unmulched trees within the same field location. The results from their study suggested an association between higher cellulase and laminarinase activity in mulched avocado plots and reduced *P. cinnamomi* inoculum potential when compared with unmulched soils. In the present study, cellulase and laminarinase activity measured in field samples were strongly correlated, but there were no relationships between enzyme activity and disease severity. In soil sampled from pots during Experiment 3 (Chapter 3), the highest levels of enzyme activities were recorded for the non-irradiated suppressive soils, which also had the lowest disease severity ratings. The lowest levels of enzyme activity were recorded for the irradiated soils, which produced the highest disease severity ratings. However, despite partial restoration of suppression in the irradiated + suppressive soil treatments and reductions in disease severity in the conducive pasture + suppressive soil treatments (compared to the conducive pasture soil), there were no associations between cellulase or laminarinase activity and disease severity in avocado seedlings.

In a second study, Downer et al. (2001b) demonstrated the effects of cellulase and laminarinase on different *P. cinnamomi* propagules. After adding enzymes to soil extracts they found that at low concentrations (1 U enzyme mL$^{-1}$ soil extract) cellulase stimulated formation of zoosporangia, zoospores, and chlamydospores. In contrast, concentrations >10 U cellulase mL$^{-1}$ soil extract consistently prevented the formation of these structures. At concentrations >25 U cellulase mL$^{-1}$ soil extract the mycelium was significantly degraded, and at 100 U cellulase mL$^{-1}$ soil extract hyphae and spores were completely destroyed. Interestingly, zoospore survival was unaffected at the higher cellulase levels. Laminarinase had minimal effect on zoosporangia, zoospores, and chlamydospore formation and germination of
Chlamydospores preformed in excised roots was enhanced at concentrations of 10 U mL\(^{-1}\) soil extract. The main impact of laminarinase on \textit{P. cinnamomi} in the Downer et al. (2001b) experiments was reduced zoospore encystment on excised roots at low concentrations and reduced zoospore survival at concentrations of >10 U mL\(^{-1}\) soil extract.

In the present study, lupin seedlings grown in sterile sand mix inoculated with \textit{P. cinnamomi} and treated with cellulase concentrations ≥25 U mL\(^{-1}\) sand mix and laminarinase concentrations of ≥5 U mL\(^{-1}\) sand mix, died within 3 - 4 d after planting and appeared to have been partially digested within several days. From 1 to 25 U cellulase mL\(^{-1}\) sand mix, plant growth declined with increasing cellulase concentrations. These results suggest that the enzyme concentrations required to disrupt \textit{P. cinnamomi} also affected plant growth and may have degraded plant cells. However, it should be noted that the enzyme preparations used by Downer et al. (2001b) were purified lyophilised powders (Sigma-Aldrich, USA and Calbiochem, USA), whereas the enzyme preparations used in this study (donated by Novozymes, Australia) were partially purified industrial grade aqueous solutions containing residual microbial cells and other unknown metabolites and compounds. Poor growth of lupin seedlings in inactivated cellulase controls and zero survival of lupins in the inactivated laminarinase controls, suggests that the enzyme preparations may have had a phytotoxic, as well as a possible enzymatic (in active enzyme treatments) effect on the seedlings. Despite these effects, \textit{P. cinnamomi} was recovered from lupin root tips grown in concentrations up to 20 U cellulase mL\(^{-1}\) sand mix and 1 U laminarinase mL\(^{-1}\) sand mix. In contrast to the findings of Downer et al. (2001b), this result suggests that \textit{P. cinnamomi} can survive and infect a host plant at moderate to high levels of cellulase activity.

The relationship between cellulase and laminarinase activities measured in avocado soils by Downer et al. (2001a) and the enzyme concentrations used in laboratory experiments with \textit{P. cinnamomi} propagules by Downer et al. (2001b) are not clear. In the present study, it was also difficult to infer relationships between soil enzyme activity measured in the field and glasshouse (Experiment 3, Chapter 3) samples and the concentrations of commercial enzyme preparations used in the pot experiment. This difficulty arises due to the difference between the relative units used to express...
enzyme activity in soil (µg reducing sugar glucose equivalents [or glucose] liberated from a substrate normalised on a g⁻¹ dry soil h⁻¹ basis) and the units used to express the specific catalytic potential of commercial enzyme preparations (U of enzyme activity normalised on a mg⁻¹ enzyme or mg⁻¹ total protein basis, where 1 U is the amount required to liberate 1 µmol reducing sugar glucose equivalents from a substrate min⁻¹ or h⁻¹). The issue is confounded further by manufacturers using different methods for determining reducing sugars which vary in their accuracy and sensitivity (e.g. Novozymes use the Somogyi-Nelson method, Nelson [1944]; Somogyi [1952]). To have greater meaning in relation to enzyme activities expected in field soil, the laboratory experiments carried out by Downer et al. (2001b) and the pot trial reported here should be repeated. However, before these experiments are repeated, calibration of the enzyme concentrations used in experiments with enzyme activity measured in field samples would be required. This could be achieved by adding the commercial enzyme preparations to an autoclave-sterilised soil (or a soil with low enzyme activity) in a series of dilutions and then using the same enzyme assay to measure enzyme activity in field samples and in the amended soils. Enzyme dilutions compatible with enzyme measurements in the field samples could then be used in the laboratory and glasshouse experiments.

There is evidence from in vitro studies of several known and unknown metabolites produced by soil microorganisms (other than cellulase and laminarinase) that disrupt P. cinnamomi and other Phytophthora spp. For example, Budi et al. (2000) isolated a Paenibacillus sp. (from the mycorrhizosphere of Sorghum bicolor) that was antagonistic toward P. parasitica and F. oxysporum. Budi et al. (2000) hypothesised that this antagonism resulted from extracellular cellulolytic, proteolytic, chitinolytic and pectinolytic enzymes produced by the Paenibacillus sp. isolate. When P. parasitica was treated with commercial enzyme preparations (including cellulase), only proteases inhibited mycelial growth. In another study, Brown et al. (1987) found that pigmented antibiotics, epicorazine and flavopin, produced by the mycoparasite E. purpurascens inhibited and disrupted mycelial growth and spore germination in Pythium spp. and Phytophthora spp. In addition to cellulase and laminarinase, these metabolites are potentially involved in P. cinnamomi suppressive soils but further work is required to determine the extent of their involvement.
The results presented in this chapter do not support the hypothesis that cellulase and laminarinase are principal mechanisms for \textit{P. cinnamomi} suppression in soil. Rather than having exclusive involvement, these enzymes may be part of a complex consisting of a number of mechanisms that result in \textit{P. cinnamomi} suppression. Further studies are required to confirm whether cellulase and laminarinase are involved in suppression and to determine the role of other metabolites in \textit{P. cinnamomi} suppressive soil.
Chapter 5

Structural properties of bacterial communities associated with *P. cinnamomi* suppressive soils determined by 16S rDNA Length Heterogeneity PCR

5.1 Introduction

There are 2 principal modes of biological suppression of soil-borne plant pathogens, i.e., “specific” and “general” (Section 1.3). Specific suppression occurs when there is an explicit interaction between one or several antagonists and the pathogen. General suppression results from the cumulative effects of multiple interactions involving the soil biological community and one or several soil-borne plant pathogens (Stirling and Stirling 1997; van Bruggen and Semonov 2000). The work reported in Chapter 4 aimed to determine whether the production of cellulase and laminarinase by microbial communities in soil is an important process involved in *P. cinnamomi* suppression. Involvement of these enzymes in *P. cinnamomi* suppression would suggest general suppression, whereby the functional attributes of the soil microbial community holds greater importance than its overall taxonomic structure or the presence of a specific antagonist in the soil. However, Chapter 4 provided little evidence to support this hypothesis. In the present chapter, a molecular technique was applied with the aim of determining whether bacterial communities within *P. cinnamomi* suppressive soils possess unique structural features, which might include specific groups of bacteria.

The term “community structure” may be used with reference to the taxonomic composition of an ecological community based on the presence or absence of various species associated with that community. Community structure may also be defined within a context that incorporates community composition with community organisation (Section 1.9) determined by the distribution of individuals among species within the community (Dunbar et al. 2000). However, the presence or abundance of a specific taxon (which may be critically important in terms of community function, e.g. disease suppression) may be the only variable that separates 2 or more ecological communities. Despite a high level of structural
similarity between the ecological communities, the presence (or differences in abundance) of this taxon contributes to minor differences in community structure. This example implies that, on its own, the term “community structure” fails to provide a means to communicate the extent of differences in the structural characteristics of ecological communities. Therefore, in the following study the term “gross community structure” is used to differentiate between large and minor differences in species composition and/or abundance within the soil microbial communities under investigation.

There have been a number of studies undertaken in an attempt to determine whether there are unique structural features of microbial communities associated with P. cinnamomi suppressive soils. Most of these studies have been carried out in the pursuit of specific microbial agents suitable for use in biological control. Broadbent and Baker (1974a) found that microbial communities associated with P. cinnamomi suppressive soils under avocado had higher numbers of bacteria and actinomycetes, particularly Bacillus spp. and Pseudomonas spp. In a follow-up study, Broadbent and Baker (1974b) isolated B. subtilis var. niger, a Flavobacterium sp. and a Pseudomonas sp. from degraded P. cinnamomi propagules. The Flavobacterium sp. and the Pseudomonas sp. caused lysis of hyphae and B. subtilis var. niger appeared to be the main agent involved in sporangial breakdown.

Other researchers have also observed higher numbers of bacteria in P. cinnamomi suppressive soils when compared with conducive soils (Malajczuk and McComb 1979; Halsall 1982b; Malajczuk 1983; Duvenhage and Mass 1990; Duvenhage et al. 1990). Contradictory data come from South Africa, where Mass and Kotze (1989) found larger numbers of bacteria (dominated by Pseudomonas spp.) in disease affected soils. However, in agreement with the findings of Malajczuk and McComb (1979), Mass and Kotze (1989) found that soils supporting healthy avocado trees had a higher ratio of antagonistic bacteria (including actinomycetes). Bacterial assemblages implicated in P. cinnamomi suppressive soils include several fluorescent Pseudomonas spp. (Broadbent and Baker 1974a; Stirling et al. 1994), Bacillus spp. (Broadbent and Baker 1974a and 1974b; Stirling et al. 1992), Rhizobium spp. (Malajczuk et al. 1984); Serratia spp. (Stirling et al. 1992) and a number of actinomycete isolates, mostly Streptomyces spp. (Broadbent and Baker
Malajczuk and McComb 1979; Duvenhage et al. 1990; Stirling et al. 1992; You and Sivasithamparam 1995; You et al. 1996). The succession of culture-based studies has provided sufficient evidence to suggest that the structural characteristics of a soil microbial community may be a factor in *P. cinnamomi* suppression.

Soil management practices (e.g. frequent inputs of organic matter) that aim to encourage high microbial activity are commonly recommended for achieving biological suppression of *P. cinnamomi* (Shea and Broadbent 1983; Turney and Menge 1994; Downer et al. 1999). However, assumptions of the importance of soil microbial community structure and diversity in *P. cinnamomi* suppression have mostly been made using information from culture-based studies. Classical culture-based methods are widely recognised as having limited scope for assessing gross community structure and diversity in most environments (it is estimated that <1% of soil microorganisms are culturable; Torsvik et al. 1990; Atlas and Bartha 1998). Analysis of whole community DNA using culture-independent molecular approaches permits analysis of both culturable and non-culturable constituents of microbial communities (Torsvik et al. 1990; Kitts 2001).

Yang et al. (2001) used denaturing gradient gel electrophoresis (DGGE) to generate bacterial 16S rDNA community profiles from healthy and *P. cinnamomi* infected avocado roots, and to track changes in bacterial community structure in the rhizosphere of avocado trees during repeated bioaugmentation with an antagonistic strain of *P. fluorescens*. They found that rhizosphere bacterial communities associated with healthy roots were structurally similar to those associated with healthy roots from bioaugmented avocado trees. However, rhizosphere communities associated with healthy and bioaugmented roots were distinctly different to communities associated with diseased roots. In addition, bacterial diversity appeared to be less important than gross community structure, with diseased roots producing more complex and variable DNA banding profiles than healthy and bioaugmented roots, which were consistently represented by simple profiles dominated by a few bands. Yang et al. (2001) cut and sequenced DNA bands unique to healthy root profiles from the DGGE gels and found that these were affiliated with published sequences from 3 uncultured soil bacteria, including an unidentified *Pseudomonas* sp., a *Polyangium* sp. and a *Cytophaga* sp. The results of Yang et al. (2001) highlight
the capacity for molecular methods to detect non-culturable soil microorganisms and discriminate between microbial communities.

However, using DGGE to characterise bacterial communities is not without its disadvantages. Preparing DGGE gels can be laborious, making DGGE less suitable for high-throughput analysis. DGGE also provides poor resolution when there are a large number of bands in the profile or when there are bands that are close to each other (Muyzer et al. 2004). Other microbial community DNA fingerprinting methods include the frequently cited Terminal Restriction Fragment Length Polymorphism method (TRFLP; Liu et al. 1997; Mills et al. 2003) and an emerging method known as Length Heterogeneity PCR (LH-PCR; Suzuki et al. 1998; Tiirola et al. 2003). Both methods provide rapid, high-throughput analysis of microbial community DNA and, due to their high resolution power, are suitable for analysing highly complex bacterial community DNA profiles. Both methods are based on the use of fluorescently labelled primers to amplify bacterial community 16S rDNA followed by amplicon separation using an automated DNA analyser. The main difference between the 2 methods is that TRFLP identifies sequence length variations based on restriction site variability, whereas LH-PCR is based on natural variations in sequence lengths across specific hyper-variable regions of DNA (Ritchie 2000; Mills et al. 2003).

TRFLP has several disadvantages including the additional time required to complete the restriction enzyme digestion step, problems associated with incomplete digestion of amplicons (Clement et al. 1998; Osborn et al. 2000) and the formation of single-stranded amplicon fragments during PCR which leads to secondary “Pseudo TRFs” (Egert 2003). The primary purpose of microbial community DNA fingerprinting techniques is to provide a rapid assessment of microbial community structure and diversity (Mills et al. 2003). As in both DGGE and TRFLP, a disadvantage of LH-PCR is that multiple, phylogenetically unrelated organisms may share the same sequence length across particular regions of DNA, and will therefore be represented by the same DNA fragment (Mills et al. 2003). For the purpose of analysis this issue is resolved by referring to each band or peak in a profile as an operational taxonomic unit or OTU (Horz et al. 2000).
Multivariate statistical methods known as principal components analysis (PCA; Clement et al. 1998; Klamer et al. 2002; Fierer et al. 2003) and cluster analysis (Liu et al. 1997; Dunbar et al. 2000; Hiraishi et al. 2000; Urakawa et al. 2000; Sessitsch et al. 2002; Sait et al. 2003) are commonly used in quantitative analyses of microbial community DNA fingerprint data. However, interpretation of cluster analysis is often constrained, especially where there are a large number of comparisons to be made (Rees et al. 2004). Issues arise with PCA if the data is not normally distributed and PCA is known to obscure underlying patterns in the data where the analysis is performed on non-linear data along large gradients (Rees et al. 2004). Rees et al. (2004) proposed an alternative approach with the intention of standardising the analysis of microbial community DNA profiles using protocols that are simple to perform while maintaining statistical rigour. The approach recommended by Rees et al. (2004) is primarily based on the tools already used by animal and plant ecologists interested in community dynamics at the macro-scale. The recommended approach involves the use of the Bray-Curtis similarity algorithm to determine the percentage similarity between community DNA profiles. A similarity matrix is constructed which is then visually interpreted by the use of multi-dimensional scaling (MDS). Where patterns appear in the MDS plot that suggest separation of groups, an analysis of similarity (ANOSIM) is performed which allows significance testing of the data groups. An exploratory method such as similarity percentage analysis (SIMPER) is then used to identify those OTUs that contribute to the differences between data groups (Rees et al. 2004).

Assemblage data from community DNA profiling methods can also be used to assess species (OTU) diversity. OTU richness can simply be determined as the total number of OTUs in a community, or community diversity indices can be used to assess the components of diversity. A large number of diversity indices have been proposed (reviewed in detail by Magurran 1991) and each emphasises the species richness or equitability (i.e. dominance or species abundance distribution) components of diversity to varying degrees. Traditional diversity indices widely adopted in macro-ecology include the Shannon (or Shannon-Wiener) diversity index and the Simpson index, which emphasises species abundance distribution (Clarke and Warwick 2001). These diversity indices have also been used by microbial ecologists to assess OTU diversity and evenness in microbial communities based on the TRFLP method.
(Dunbar et al. 2000) and to assess microbial community physiological diversity with data obtained using the BIOLOG system (Zak et al. 1994; Sharma et al. 1997).

In the following study, total microbial community DNA was extracted from soils collected for the suppressive soil screening glasshouse assay (Experiment 1 reported in Chapter 3) and during the transfer of suppression experiment (Experiment 3 reported in Chapter 3). These community DNA samples were analysed using LH-PCR with the aim of determining whether bacterial diversity and gross community structure were factors associated with *P. cinnamomi* suppression observed during the 2 glasshouse experiments. Data produced by LH-PCR were also analysed to determine whether there were individual bacterial OTUs that were specific to *P. cinnamomi* suppressive soils. Data produced by LH-PCR were analysed using the statistical procedures recommended by Rees et al. (2004) and OTU diversity and evenness of OTU abundance distribution were assessed using the Shannon diversity index and the Simpson index.

### 5.2 Materials and methods

#### 5.2.1 Field locations and soil sampling

Soil was collected from 10 avocado orchards and 2 rainforest remnants in September 2002. These samples were assayed for their ability to suppress *P. cinnamomi* root rot in lupin seedlings during Experiment 1 as detailed in Chapter 3. Details for field locations and procedures for sampling, handling and storage of soil samples were described in Sections 3.2.1 - 3.2.3.

#### 5.2.2 Soil sampled during Experiment 3

During Experiment 3, soil was sampled from pots at 0, 2 and 14 weeks after inoculating the pots with *P. cinnamomi* (Section 3.2.8). Techniques used to avoid cross contamination and conditions for handling and storing soil samples prior to DNA extraction were described in Sections 3.2.2, 3.2.3 and 3.2.8.
5.2.3 Disease severity rating data

The disease severity rating (DSR) data used during comparisons between 16S rDNA LH-PCR profiles in this chapter were the same as those reported in Chapter 3 for Experiments 1 and 3.

5.2.4 Soil DNA extraction

Microbial community genomic DNA was isolated from soil using a mechanical extraction method developed by Dr Tony Vancov and Mrs Karen Jury-Vancov (unpublished). For each soil sample, approximately 200 mg soil was transferred (using a stainless steel spatula, washed in 100% ethanol between each sample) to autoclave-sterile 2 mL plastic screw cap tubes (Scientific Specialists Incorporated, USA) containing 800 mg of 1 mm acid-washed ceramic beads (Saint Gobain, USA) and 300 mg acid-washed 0.1 mm glass beads (Sigma-Aldrich, USA). The soil was washed to remove gross contaminants by adding 500 µL autoclave-sterile phosphate buffered saline (0.1 mol L$^{-1}$, pH 7.0; Sambrook et al. 1989) to the tubes, vortexing briefly to mix and then centrifuging (Eppendorf 5810R model, Germany) at 13,000 revs min$^{-1}$ for 2 min. The supernatant was discarded and 520 µL sodium phosphate buffer (0.1 mol L$^{-1}$, pH 7.0; Sambrook et al. 1989) and 81 µL MT lysis buffer (1% SDS, 0.5% Extran, 1% polyvinylpyrrolidone 40, 50 mmol L$^{-1}$ EDTA, 100 mmol L$^{-1}$ Tris) were added to the soil pellet. Cells contained within the soil pellet were disrupted by bead-beating for 30 s at 5.5 m s$^{-1}$ using a FP120 FastPrep® Instrument (Bio101-Savant Instruments, USA). Tubes were centrifuged at 13,000 revs min$^{-1}$ for 5 min at 4°C. The supernatant was transferred to a sterile 1.5 mL micro-centrifuge tube (Astral Scientific, Australia). To remove humic acids, tannins and proteins (Thoss et al. 2002), 250 µL 7.5 mol L$^{-1}$ ammonium acetate and 6 µL 100 mg mL$^{-1}$ bovine serum albumin (BSA; Invitrogen, USA) was mixed with the extract and tubes were incubated on ice for 15 min. Tubes were centrifuged at 13,000 rpm for 5 min at 4°C and the supernatant was transferred to a sterile 1.5 mL micro-centrifuge tube. A 600 µL volume of 100% isopropanol (molecular biology grade; ICN Chemicals, USA) was added to each tube and the DNA suspension was precipitated at ambient temperature for 15 min. Tubes were centrifuged for 15 min at 13,000 revs min$^{-1}$, the supernatant discarded and the DNA pellet washed with 70% ethanol (in autoclave-
sterile deionised water). The ethanol was removed and the pellet dried in a vacuum chamber (John Morris Scientific, Australia) for 5 min at ambient temperature. The DNA pellet was resuspended overnight at 4°C in 60 µL distilled DNase- and RNase-free (ultrapure) water (Gibco BRL, USA). The following day, a final clean-up step was performed by passing the DNA sample through a polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich, USA) spin column (Berthelet et al. 1996). The column was prepared by transferring 600 µL PVPP slurry (PVPP in 20 mmol L⁻¹ potassium phosphate buffer, pH 7.4 [Sambrook et al. 1989], autoclaved for 20 min) to separation column tubes (Axygen Scientific, USA) and centrifuging for 5 min at 13,000 revs min⁻¹ to remove excess buffer. The column was transferred to a sterile catch tube and the DNA sample was passed through the column by centrifuging for 5 min at 13,000 revs min⁻¹. The purified DNA sample was stored in 10 µL aliquots in sterile micro-centrifuge tubes at -20°C until required (up to 18 months for Experiment 1 samples).

DNA extractions were carried out in duplicate for each soil sample collected for Experiment 1 (96 extractions in total) and single extractions were carried out for each sample collected from pots during Experiment 3 (3 replicate pots sampled for each treatment, 153 extractions in total).

5.2.5 Quantification of DNA

The quality of the sample DNA was verified by agarose gel electrophoresis. Sample DNA was quantified using the PICO green dsDNA quantification kit (Molecular Probes, USA). The assay was carried out according to the manufacturer’s instructions in microplates using 1 µL sample DNA. Fluorescence readings were carried out using an automated microplate reader fitted with 480 nm excitation and 520 nm emission filters (Fluorostar Galaxy, BMG Technologies, Germany). DNA samples used for PCR were diluted to 10 ng µL⁻¹ in ultrapure water.

5.2.6 PCR amplification

Bacterial 16S rDNA genes were amplified using a 5-carboxyfluoroscein labelled forward primer, 63F-FAM (5′-FAM-CAGGCCTAACACATGCAAGTC-3′) (Mills
et al. 2003), paired with a 355R (5'-GCTGCCTCCCGTAGGAGT-3') reverse primer (Suzuki et al. 1998) (both primers were manufactured by Invitrogen, USA). PCR were carried out in 50 µL volumes containing: 1.25 U Hot-master™ Taq polymerase (Eppendorf, Germany), 5 µL Hot-master™ Taq Buffer (Eppendorf, Germany), 200 µmol of each dNTP (Astral Scientific, USA), 20 pmol of each primer, 10 ng DNA template, 1 µL 10 mg mL⁻¹ ultrapure DNase- and Rnase-free BSA (Amersham Biosciences, USA) made up to volume with ultrapure water. PCR were carried out in a GeneAmp 9700 PCR thermocycler (Perkin Elmer-ABI, USA) under the following conditions: Hot-master™ Taq initialisation at 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 45 s, primer annealing at 55°C for 45 s and chain extension at 72°C for 2 min. A final extension cycle was performed at 72°C for 7 min before samples were held at 4°C.

Excess PCR reagents were removed from the PCR samples by PEG precipitation (Paithankar and Prasad 1991). PCR samples (50 µL) were transferred to sterile 1.5 mL micro-centrifuge tubes to which 50 µL PEG buffer (20% polyethylene glycol 8000 in 2.5 M NaCl) was added. The tubes were touch spun to mix and then incubated at 37°C for 60 min. Tubes were then centrifuged at 13,000 revs min⁻¹ at 4°C for 60 min. The supernatant was discarded and the pellet was washed with 400 µL 70% ethanol (in autoclave-sterile deionised water), centrifuged for 2 min, the ethanol was discarded and the DNA pellet was dried in a vacuum chamber for 5 min. The resulting amplicon DNA pellet was resuspended over 48 h (at 4°C and protected from light) in 40 µL ultrapure water.

5.2.7 Quantification of PCR products

The fluorescence intensity of PCR samples used for analysis was standardised using a method developed by Dr Tony Vancov (unpublished). A standard curve was constructed using the fluorescently labelled forward primer (63F-FAM) in concentrations ranging from 0-400 fmol DNA µL⁻¹ in 1 x TE buffer (10 mmol L⁻¹ Tris, 1 mmol L⁻¹ EDTA in sterile deionised water, pH 7.5). An aliquot (2 µL) from each PCR sample was diluted 100-fold in sterile TE buffer. For both samples and standards, 200 µL of diluted PCR product was transferred to a black 96 well microplate (Greiner Bio101, Germany). The fluorescence intensity of the PCR
sample was measured against the standard curve using an automated fluorescence microplate reader (Fluorostar Galaxy, BMG Lab Technologies, Germany) fitted with 485 nm excitation and 520 nm emission filters. The estimated concentration of the PCR sample was reported as fmol primer equivalent DNA µL$^{-1}$ (quantity of DNA with a fluorescence intensity equivalent to the fluorescence intensity of $x$ fmol labelled primer DNA µL$^{-1}$). Prior to amplicon separation (Section 5.2.8) all PCR samples were diluted so that their total fluorescence intensity was equivalent to the fluorescence intensity of 25 fmol primer DNA µL$^{-1}$.

5.2.8 Amplicon separation

A 1 µL volume of each diluted PCR product (i.e. equivalent to the fluorescence intensity of 25 fmol 63F-FAM primer DNA) was transferred to a 96 well ABI 3730 micro-titre tray (ABI, USA) or to sterile 1.5 mL micro-centrifuge tubes. Amplicon separation was performed by staff at the University of New South Wales Automated DNA Sequencing Facility (Kensington, NSW, Australia) using an ABI 3730 DNA analyser (ABI, USA). Samples were resuspended overnight in 15 µL Hi Di Formamide containing 5 µL mL$^{-1}$ LIZ500 size standard (ABI, USA). Samples were briefly centrifuged, heated to 95°C for 5 min, cooled on ice for 2 min and then run on the ABI 3730 DNA analyser. A 15 sec injection time was used and the average run time was approximately 50 min. Sample data were captured using Genemapper Software version 3.5 (ABI, USA).

5.2.9 Data handling and analysis

Sample data files produced by Genemapper were analysed using public domain software STRand Version 2.2.241 (Veterinary Genetics Laboratory, University of California, Davis) which is available from http://www.vgl.ucdavis.edu/STRand/. For each sample file, STRand generates an electropherogram and automatically digitises the data from the electrophoretic trace to report peak positions (OTU fragment sizes) measured in bp against the internal size standard (LIZ500; Section 5.2.8) and the height of each peak measured in fluorescence units (Figure 5.1). Following automatic selection of peaks (peak sensitivity for automatic selection was set to 100), visual
inspections of the electropherogram for each sample were carried out and peaks not detected by the software were manually selected for inclusion in the raw data set.

Figure 5.1 STRand interface showing electropherogram and data table.

Genemapper reads and displays OTU fragment sizes to 2 decimal places (e.g. 279.51 bp). However, DNA sequences occur as integer values (e.g. 279 or 280 bp) and, for this reason, it is necessary to include a procedure for OTU size calling and alignment in the analysis (Rees et al. 2004). Initially, the Treeflap Microsoft Excel macro (developed by Dr Chris Walsh, Monash University, Australia: www.wsc.monash.edu.au/~c-walsh/treeflap.xls), which rounds peak sizes to the nearest integer value and automatically aligns peaks against the rounded sizes (Rees et al. 2004), was applied to the raw data. However, the macro was found to inaccurately align replicate profiles and therefore OTU size calling and alignment was carried out manually.

Raw data sets for each sample were transferred to a Microsoft Excel Version 2002 (Microsoft, USA) spreadsheet for manual processing. Replicate profiles (generated
from replicate DNA extractions) were compared and aligned against each other. Rather than applying rigorous rounding rules (e.g. an OTU displayed as 279.50 is always rounded up to 280 bp and an OTU displayed as 279.49 bp is always rounded down to 279 bp), as applied in some TRFLP studies (Kitts 2001), size calling was based on alignment of profiles from replicate DNA extractions (e.g. where rep 1 has an OTU at 279.62 bp, rep 2 has an OTU at 278.86 bp and rep 3 has an OTU 279.23, all OTUs are rounded to 279 bp). During manual size calling the presence and size of an OTU appearing in the raw data was always confirmed by checking the electropherogram. After aligning replicate sample profiles, the data for each replicate were normalised by calculating the fluorescence intensity of each OTU as a percentage of the total fluorescence intensity of all OTUs in the profile. The data was normalised further by reassigning OTUs that accounted for 1% or less of the total fluorescence intensity to zero and recalculating the percentage fluorescence of each remaining OTU (Rees et al. 2004). The data for each replicate profile were then pooled (2 replicate profiles for each Experiment 1 soil sample and 3 replicate profiles for each treatment at each sampling time during Experiment 3) to form a single profile representing the soil sample or treatment by averaging the percentage fluorescence for each OTU across each replicate profile.

Normalised data sets for each sample were imported into the multivariate statistical software package, Primer Version 5 (Primer-E Ltd, Plymouth, UK). The Primer software was used to: i) generate a similarity matrix calculated using the Bray-Curtis similarity coefficient, ii) display the similarity results in multi-dimensional scaling (MDS) ordination plots and single linkage cluster analysis dendrograms, iii) carry out one-way analysis of similarity (ANOSIM) to determine whether significant differences occurred between treatments or groups of treatments (global R statistic reported) and, if so, to perform pairwise comparisons between treatments or paired groups of treatments (R statistic reported) and iv) similarity percentage analysis (SIMPER) to identify OTUs unique to specified groups of samples. Procedures for carrying out these analyses were as described by Rees et al. (2004) except that the data were not binary transformed (presence / absence of OTUs) for any of the analyses reported in this chapter. LH-PCR data acquired from Experiment 1 soil samples (Section 5.2.1) was explored by manipulating factors displayed as different symbols in the MDS plot to search for patterns potentially associated with the
following variables: suppressive soil vs. conducive soil (i.e. DSR <3 vs DSR >3); sampling location (i.e. individual orchard or forest sampling locations, i.e. Ta1 - Tamborine Mt 1, Prf - Pretty Gully rainforest); geographic location (sampling locations located within close proximity to one another, i.e. data from Ta1 + Ta2 + Trf = Tamborine Mountain); soil management regime (i.e. organic vs conventional vs rainforest); and incremental DSR values (i.e. DSR <4 vs DSR >4; DSR <5 vs DSR >5; etc.).

Species richness ($S$), the Shannon-Weiner diversity index ($H'$) and the Simpson evenness index ($1-\lambda$) were also calculated within the Primer software package. These diversity indices were used to compare OTU richness and evenness in 16S rDNA LH-PCR profiles from soils that yielded low and high DSRs during Experiment 1 (Section 5.2.1). These diversity measures were also used to analyse changes in OTU richness and evenness in groups of treatments during Experiment 3 (Section 5.2.2). Comparisons of means for OTU richness and diversity index values were carried out by calculating 95% confidence intervals using Origin Version 5.0 (Microcal Software, 1997).

5.2.10 Verification of bacterial community DNA LH-PCR data

Negative DNA template controls (ultrapure water) were included in each PCR run to check PCR reagents for contamination. Positive DNA template controls were also included in each bacterial community DNA LH-PCR run (5 runs in total and therefore 5 replicate positive controls). The positive control template DNA was extracted from sample #38 (a suppressive avocado soil collected from Tuckombil 2) and stored in single use aliquots to avoid repetitive freeze-thaw cycles and possible degradation of DNA. The positive controls were used to monitor reproducibility of the bacterial community LH-PCR profile data. Limits for interpreting differences between samples were determined by using the results from the positive control PCR samples to assess variability between runs using the Bray-Curtis similarity algorithm (Section 5.2.9). The contribution of the ABI 3730 DNA capillary analyser to variability in the bacterial community LH-PCR data was also assessed by loading 6 replicate aliquots from one PCR sample (obtained from sample #38) onto the ABI 3730 during a single run.
5.3 Results

5.3.1 Verification of 16S rDNA LH-PCR data

No contamination was detected in any of the negative water controls. Bray-Curtis similarity values between 16S rDNA LH-PCR profiles for positive controls (after normalising the data) included in each PCR / ABI 3730 analysis run was 83% to 93%. In a separate experiment, 6 replicate aliquots from a single PCR sample were run simultaneously on the ABI 3730 to determine the extent of variability contributed by the machine. Prior to normalising the data (by calculating relative ratios) for each profile, the level of variability contributed by the ABI 3730 appeared to be excessive with a coefficient of variation of 70% for the mean \( (n = 6) \) accumulated peak height. However, after normalising the data and removing OTUs that contributed <1% to the accumulated peak height, the level of similarity between replicate profiles was >96%. These results suggest that during a single ABI 3730 run, normalised 16S rDNA LH-PCR profiles from a single sample may differ by up to 4% (based on Bray-Curtis similarity). After normalising the data, a further 12% (based on Bray-Curtis similarity) dissimilarity between replicate 16S rDNA LH-PCR profiles may accumulate during separate PCR, dilution and handling, and separate runs on the ABI 3730. Therefore, in subsequent analyses differences between samples were determined where ITS2 LH-PCR profiles shared <83% Bray-Curtis similarity (determined from the lowest similarity value for the positive controls).

5.3.2 Bacterial LH-PCR profiles from Experiment 1 soil samples

Bacterial community 16S rDNA LH-PCR profiles were obtained from soil samples collected from 10 avocado orchards and 2 rainforest remnants for Experiment 1 (Chapter 3). Bacterial LH-PCR profiles from soil samples that yielded DSRs of less than 3 (low DSR - suppressive) were not exclusively dissimilar to those obtained from soil samples that yielded DSRs greater than 3 (high DSR - conducive) (Figure 5.2). ANOSIM confirmed this observation by returning a global R statistic of 0.094 \( (p<0.05) \), which indicates that the 2 groups were very similar to each other and also that there were minor and significant differences (Global R = ~0 indicates that groups are similar to each other, Global R = 1 indicates that groups are dissimilar to
each other; Clarke and Warwick 2001). In addition, the MDS stress value of 0.23 (Figure 5.2) indicates that the placement of data points in the plot was close to random (according to Clark and Warwick [2001] stress values >0.3 indicate arbitrary placement of data points, and MDS plots showing values of stress between 0.2 and 0.3 should be interpreted with caution). Manipulations of factorial groupings in the MDS plot did not provide any indication that 16S rDNA LH-PCR profiles were associated with any of the variables that were considered (Section 5.2.9). Overall, bacterial LH-PCR profiles were relatively similar between sampling locations (Figure 5.3) with 10 out of 12 locations showing Bray-Curtis similarity values close to 80% or greater, and all locations showing similarity values greater than 70%. According to SIMPER analysis there were no OTUs unique to low DSR yielding soil samples or to high DSR samples.

![Stress: 0.23](image)

**Figure 5.2** Multidimensional scaling plot based on Bray-Curtis similarities of 16S rDNA LH-PCR profiles for soil samples with different DSRs collected for Experiment 1.

○ = DSR <3
● = DSR >3
(DSR = disease severity rating)
Mean species richness ($S$) for both low DSR and high DSR soil samples was 24 OTUs. According to ANOVA, there were no significant differences between Shannon diversity index or Simpson index values for the 2 groups either. The Shannon index values suggested that marginally high levels of OTU diversity were present with a mean of $H' = 2.99$ for low DSR samples and a mean of $H' = 3.01$ for high DSR samples. The Simpson index suggested even abundance distribution of OTUs, with both groups having a mean index value of 0.94.

5.3.3 Bacterial LH-PCR profiles from Experiment 3 soil samples

Week 0
An MDS ordination based on Bray-Curtis similarity analysis of 16S rDNA LH-PCR profiles obtained from samples taken from pots at the start of Experiment 3 (week 0) suggested that the suppressive avocado orchard soils were similar to each other but were distinctly dissimilar to the conducive dairy pasture soil (Figure 5.4). The original structure of bacterial LH-PCR profiles in the suppressive orchard soils
appeared to be distorted following γ-irradiation treatment, which resulted in considerable variability occurring among these soil samples (Figure 5.4). The irradiated + suppressive soil and the conducive pasture + suppressive soil treatments formed 2 loosely associated clusters. The conducive pasture + suppressive soil treatments were separated from, but remained close to, the bacterial LH-PCR profiles from the conducive pasture soil (Figure 5.4). The stress value for the MDS analysis was 0.09 (Figure 5.4) which suggests that the observed patterns were real. However, the robustness of interpretations from the MDS plot can be improved by carrying out significance testing. ANOSIM returned a Global R statistic of 0.646 at a significance level of p<0.01, which indicated moderate and significant differences between groups. ANOSIM pairwise comparisons showed that there were no significant (R statistic = 0.083) differences between the suppressive soil and the irradiated soils. However, there was a significant (p<0.05 in each case) and high level of separation (R statistic ranged between 0.865 and 0.948) between the suppressive soils and each of the other soil treatment groups. There were no significant differences between the irradiated soils and the irradiated + suppressive soil treatments. Differences between the conducive pasture soil and the conducive pasture + suppressive soil treatments were moderate (R statistic = 0.563) and significant (p<0.05).
Figure 5.4 Multidimensional scaling plot based on Bray-Curtis similarities of 16S rDNA LH-PCR profiles for suppressive avocado orchard, irradiated orchard, irradiated + suppressive, conducive pasture and conducive pasture + suppressive soil treatments collected from pots at week 0 during Experiment 3.

▲ Suppressive orchard
● Irradiated orchard
▼ Irradiated + suppressive
■ Conducive pasture
♦ Conducive pasture + suppressive

Week 2
After 2 weeks incubation, MDS analysis showed that variability among the irradiated soil treatments had decreased and that they were now clearly separated from the suppressive orchard soils (Figure 5.5). Bacterial LH-PCR profiles from the conducive pasture soil grouped discretely from both the irradiated and suppressive orchard soils. Some scatter occurred in the data from the conducive pasture + suppressive soil and irradiated + suppressive soil treatments, which resulted in less well defined groupings for these treatments. The irradiated + suppressive soil treatments formed a loose cluster that appeared to be associated most closely with the irradiated treatments. Similarly, the conducive pasture + suppressive soil treatments formed a loose cluster that was most closely associated with the conducive pasture soil. The MDS stress value of 0.09 corresponds to a good ordination with little chance of error being made in interpreting patterns shown in the MDS plot (Figure
5.5). However, to support the observations from the MDS plot ANOSIM was carried out. ANOSIM confirmed that overall there were moderately high levels of separation between groups (Global $R$ statistic $= 0.77; p<0.01$). Pairwise comparisons showed that differences between the conducive pasture soil and the conducive pasture + suppressive soil treatments were moderately low ($R$ statistic $= 0.438; p<0.05$). The highest levels of separation occurred between the conducive pasture soil and each of the other treatment groups (in each case $R$ statistic $= 1$ and $p<0.05$). There were no significant differences between the irradiated soils and the irradiated soil + suppressive soil treatments. Differences between the irradiated soil + suppressive soil treatments and the conducive pasture + suppressive soil treatments were not significant.

**Figure 5.5** Multidimensional scaling plot based on Bray-Curtis similarities of 16S rDNA LH-PCR profiles for suppressive avocado orchard, irradiated orchard, irradiated + suppressive, conducive pasture and conducive pasture + suppressive soil treatments collected from pots at week 2 during Experiment 3.

- ▲ Suppressive orchard
- ● Irradiated orchard
- ▼ Irradiated + suppressive
- ■ Conducive pasture
- ♦ Conducive pasture + suppressive
At week 14, 16S rDNA LH-PCR profiles clustered more tightly into the 5 treatment groups than at weeks 0 and 2 (Figure 5.6). The MDS ordination showed a clear separation between the suppressive orchard soils, irradiated orchard soils and the conducive pasture samples (stress value = 0.1). The irradiated + suppressive soil and the pasture + suppressive soil treatments also formed clusters separated from the other treatment groups (Figure 5.6).

The Global R statistic from ANOSIM of bacterial LH-PCR data from week 14 was 0.832 (p<0.05), which confirmed that the overall differences observed between the 5 treatment groups were large and statistically significant. Pairwise ANOSIM comparisons demonstrated large (R statistic values greater than 0.7) and significant
(p<0.05 in all cases) differences between each of the treatment groups (Table 5.1).
There was a high level of separation between the suppressive orchard soils and the conducive pasture soil (R statistic = 1.000; p<0.05) and also between the suppressive orchard soils and the irradiated soils (R statistic = 0.990; p<0.05). The level of separation was slightly lower but remained significant (p<0.05) between the suppressive orchard soils and the conducive pasture + suppressive soil (R statistic = 0.969) and irradiated + suppressive soil (R statistic = 0.771) treatments (Table 5.1). There were also significant differences (p<0.05) between the conducive pasture soil and the conducive pasture + suppressive soil treatments (R statistic = 0.708), and the suppressive orchard soil and the irradiated soil + suppressive soil treatments (R statistic = 0.771).

Table 5.1 ANOSIM results for 16S rDNA LH-PCR profiles obtained from pots sampled during Experiment 3 at week 14. Similarity was calculated based on 4 replicate treatments within each treatment category. Significance level for pairwise comparisons was p<0.05 in each case.

<table>
<thead>
<tr>
<th>Pairwise treatment comparisons</th>
<th>R statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressive orchard v irradiated orchard</td>
<td>0.990</td>
</tr>
<tr>
<td>Suppressive orchard v irradiated + suppressive</td>
<td>0.771</td>
</tr>
<tr>
<td>Suppressive orchard v conducive pasture</td>
<td>1.000</td>
</tr>
<tr>
<td>Suppressive orchard v conducive pasture + suppressive</td>
<td>0.969</td>
</tr>
<tr>
<td>Irradiated orchard v irradiated + suppressive</td>
<td>0.708</td>
</tr>
<tr>
<td>Irradiated orchard v conducive pasture</td>
<td>1.000</td>
</tr>
<tr>
<td>Irradiated orchard v conducive pasture + suppressive</td>
<td>1.000</td>
</tr>
<tr>
<td>Conducive pasture v irradiated + suppressive</td>
<td>1.000</td>
</tr>
<tr>
<td>Conducive pasture v conducive pasture + suppressive</td>
<td>0.708</td>
</tr>
<tr>
<td>Conducive pasture + suppressive v irradiated + suppressive</td>
<td>0.927</td>
</tr>
</tbody>
</table>
Results from SIMPER analysis at week 14

Similarity percentage analysis (SIMPER) was carried out on data from week 14 (Table 5.2). SIMPER showed that pairwise similarities between treatments were generally moderate (ranging from 45% mean similarity between the suppressive orchard soils and the irradiated soils, to 71% mean similarity between the conducive pasture soil and conducive pasture + suppressive soil treatments). The mean similarity value between the suppressive orchard soils and the conducive pasture soil was 46% (Table 5.2). In contrast, the mean similarity between the suppressive soils and the conducive pasture + suppressive soil treatments was 61%. The suppressive orchard soil and the irradiated orchard soil treatments showed 45% similarity. Mixing the irradiated soils with a 10% volume of their respective suppressive soils resulted in 60% mean similarity between these and the suppressive soil treatments.

Further analysis with SIMPER showed that 10 OTUs accounted for 50% of the dissimilarity observed between the suppressive orchard soils and the conducive pasture soil. Of these, OTUs with lengths of 283, 305, 313 and 318 bp were present in the suppressive orchard soils but were not detected in the conducive pasture soil at each of the 3 sampling times. These 4 OTUs accounted for 12% of the dissimilarity observed between the 2 treatments. OTUs with lengths of 283, 313 and 318 bp were detected in the conducive pasture + suppressive soil treatments but the 305 bp OTU remained absent. A further 6% of the dissimilarity between the conducive pasture soil and the suppressive orchard soils was explained by OTUs of 280, 296 and 332 bp, which were present in the pasture soil but were absent from the orchard soils. OTUs that were 283, 301, 313, 315, 324 and 382 bp in length were present in the suppressive orchard soils but were not detected in the same soils after they were irradiated. These OTUs accounted for 12% of the dissimilarity between the suppressive orchard soils and the irradiated soil treatments. By week 14, OTUs of 283, 313, 315 and 324 bp in length had been restored after mixing the irradiated soils with their respective suppressive orchard soil.
Table 5.2 Results from SIMPER pairwise similarity comparisons between treatments. Values represent % similarity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Suppressive</th>
<th>Irradiated</th>
<th>Irradiated + suppressive</th>
<th>Conducive pasture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiated</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irradiated + suppressive</td>
<td>60</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conducive pasture</td>
<td>46</td>
<td>50</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Conducive pasture + suppressive</td>
<td>61</td>
<td>47</td>
<td>54</td>
<td>71</td>
</tr>
</tbody>
</table>

Results from diversity analyses at week 14

OTU richness ($S = \text{total number of OTUs}$) and values for Shannon and Simpson diversity indices were higher for the conducive pasture soil ($S = 28 \text{ OTUs}; H' = 3.06; 1-\lambda = 0.94$) than for the suppressive orchard soils ($S = 23 \text{ OTUs}; H' = 2.85; 1-\lambda = 0.93$). However, based on 95% confidence intervals, differences between pooled means for the 2 treatment groups were not significant (Figure 5.7). All 3 diversity measures were reduced in the irradiated soils, but the greatest effect was related to a shift in OTU dominance as indicated by the Shannon and Simpson indices (Figure 5.7). Neither OTU richness ($S$) nor values for Shannon or Simpson diversity indices increased significantly in response to mixing 10% volumes of the suppressive soils with the conducive pasture soil (Figure 5.7).
5.4 Discussion

This is the first time that the culture independent 16S rDNA LH-PCR method has been used to analyse bacterial community structure and diversity in disease suppressive and conducive soils. The method was also used to monitor changes in irradiated soils and in a conducive pasture soil in response to mixing with *P. cinnamomi* suppressive soils. Previously, only one other study (Yang et al. 2001) had attempted to assess bacterial community structure, in relation to *P. cinnamomi* suppression, using a community DNA profiling method (DGGE). Prior to this and the present study, assumptions regarding the role of bacterial community structure and diversity were founded on culture-based studies. These culture-based studies typically provided an estimate of the total number of culturable bacteria g⁻¹ *P. cinnamomi* suppressive soil (Broadbent and Baker 1974a; Malajczuk et al. 1979; Murray 1987). A few studies also provided information regarding the total number of individual isolates harvested from the soil and tested for antagonism, including

![Graph showing mean OTU richness, Shannon diversity index and Simpson index for Experiment 3, week 14 data. Data points represent pooled means for each diversity measure. Error bars represent 95% confidence intervals for each mean.](image-url)
details for the ratio of antagonists within the culturable population (Malajczuk and McComb 1979; Mass and Kotze 1989; Duvenhage et al. 1990; Stirling et al. 1992). Only isolates of interest due to their antagonistic performance were identified to genus or species level, leaving the taxonomic identity and the differentiation between the remaining isolates unknown. Therefore, culture-based studies carried out to date have not provided a true appraisal of bacterial community structure or diversity within *P. cinnamomi* suppressive and conducive soils. As demonstrated in the present study, an advantage of microbial community DNA profiling techniques is that they provide information concerning a much larger proportion of the microbial community and are therefore useful for analysing microbial community structure and diversity.

In recognition of the limitations of culture-based techniques, Yang et al. (2001) applied DGGE to characterise bacterial communities in the rhizosphere of healthy and diseased avocado roots. Their results suggested that bacterial species richness (as represented by total number of OTUs) within the rhizosphere of healthy avocado root tips was slightly lower than rhizosphere communities associated with diseased roots. The results of Yang et al. (2001) also showed that the rhizosphere of healthy avocado roots was consistently colonised by a bacterial community structurally distinct from communities associated with *P. cinnamomi* infected roots. These differences were mainly due to several dominant OTUs that were unique to the healthy root tips. In the present study, LH-PCR did not detect differences between the gross structure of free-living (non-rhizosphere) bacterial communities in soil samples that yielded low DSRs and samples that yielded high DSRs during Experiment 1. OTU richness, diversity and evenness were similar in low and high DSR yielding soil samples and there were no OTUs unique to either group. These results suggest that bacterial diversity and the gross taxonomic structure of bacterial communities were not factors involved in determining disease severity in lupin seedlings during Experiment 1 (Section 5.2.1). Based on these results it appears that the gross structure of free-living bacterial communities within the bulk surface mineral layer of soil surrounding avocado trees may not be an important factor in *P. cinnamomi* suppression. As Yang et al. (2001) alluded to, suppression of *P. cinnamomi* by bacteria may primarily occur within the rhizosphere. This may explain why in some *P. cinnamomi* suppressive avocado soils the pathogen persists but the orchard
remains mostly healthy (Broadbent and Baker 1974a; Erwin and Ribeiro 1996). It may prove fruitful for future *P. cinnamomi* suppressive soil studies to compare bacterial communities in root-free soil with those of rhizosphere soil from the host plant.

A number of studies have shown that soil microbial communities are typically heterogeneous at both macro- and micro-scales (Atlas and Bartha 1998). It was surprising then that LH-PCR profiles were relatively similar (samples from 7 out of 10 locations were >80% similar and all locations were >70% similar) for soils sampled at locations spatially separated by distances of over 100 km (Section 3.2.1). Previous applications of LH-PCR have demonstrated that the method is reproducible and has a high level of discriminatory power (Ritchie 2000; Mills et al. 2003; Tiirola et al. 2003). Work undertaken for a separate project at the NSW DPI Wollongbar laboratories showed that LH-PCR was able to clearly discriminate between bacterial communities in different soil types and between bacterial communities in soils under different management regimes (unpublished data; Dr Tony Vancov, pers. comm. 2005). In addition, LH-PCR was able to detect large and significant differences between avocado soils and the dairy pasture soil samples taken during Experiment 3 (Section 5.3.3). These differences occurred despite all sampling locations (including the pasture) being on the same primary soil type (Section 3.2.1). The combination of these factors strongly suggests that the level of similarity observed between most samples was not an artefact of any discriminatory limitations imposed by 16S rDNA LH-PCR or of soil type. Therefore, based on the data obtained from 16S rDNA LH-PCR it appears that the gross structure of bacterial communities in the suppressive and conducive avocado orchard and rainforest soils were quite similar.

The high level of similarity observed between bacterial LH-PCR profiles from avocado orchard soils may indicate that orchard practices, or possibly the avocado trees themselves, encourage many taxonomic assemblages that dominate bacterial communities beneath avocado trees. There was also a high level of similarity between avocado soils and rainforest soils, which indicates that there may be factors common to both systems that determine the structure of bacterial communities within them. Broadbent and Baker (1974a) first observed *P. cinnamomi* suppression in an avocado orchard where the soil was managed under the “Ashburner system” (Erwin
and Ribeiro 1996). The Ashburner system aims to emulate conditions in rainforest soils, mainly through inputs of organic matter. In the current study, the soils beneath each orchard previously supported sub-tropical rainforest communities and inputs of organic material were actively practiced, or were at least maintained as natural leaf fall, in each orchard. Microbial communities in the organic debris and humus layers beneath avocado trees would be expected to mostly function as a saprophytic detrital system as occurs on the forest floor (Amaranthus et al. 1999). Perhaps, as Broadbent and Baker (1974a) predicted, inputs of organic matter beneath avocado trees encourages a micro-flora similar in function and composition to that found in rainforest soils. Certainly, the bacterial LH-PCR profiles obtained during this study suggest that this is possible. It is worth noting, however, that all sampling locations were on red ferrosol soils derived from Tertiary basalts of similar age (Section 3.2.1). Evidence from other studies indicates that microbial community structure and diversity are influenced by soil type (Garbeva et al. 2004). Therefore, had the sampling locations been on a number of different soil types there may have been greater discrimination between 16S rDNA LH-PCR profiles from each location.

During Experiment 3 (Chapter 3), suppressive orchard soils were mixed with the same orchard soils after γ-irradiation, and also with a conducive dairy pasture soil. By week 14 of the trial there was a 12-15% increase in similarity between LH-PCR profiles from mixed soil treatments and the orchard soils. This minor shift in community structure was not accompanied by significant increases in OTU richness ($S$), diversity (Shannon index) or OTU abundance distribution (the Simpson index). However, the shift in community structure coincided with a significant decrease in disease severity in both the conducive + suppressive soil treatments and the irradiated + suppressive soil treatments (Section 3.3.4). These results suggest that the gross taxonomic structure and diversity of bacterial communities were not factors involved in an increase in the suppressive capacity of the irradiated and pasture soils in response to mixing with suppressive soils. The minor shift in community structure does suggest, however, that there may have been some bacterial assemblages that were associated with an increase in suppressiveness in these soil treatments. Within the conducive pasture + suppressive soil treatments this minor change in community structure was partially explained by the establishment of OTUs with lengths of 283, 313 and 318 bp. These OTUs were common to the suppressive orchard soils but were
absent in the conducive soil throughout the trial. OTUs with lengths of 283 and 313 bp were also among a number of OTUs that vanished from the orchard soils after they were irradiated. These OTUs were also among 4 OTUs restored in the irradiated soils after they were mixed with the suppressive orchard soils. These results indicate that there were specific bacterial assemblages (represented by OTUs) that established in the mixed soil treatments and were incidentally associated with decreased disease severity in avocado seedlings. However, further study is required to determine whether these OTUs were responsible for this decrease in disease severity. To determine the identities of the OTUs, amplicons within the original LH-PCR sample need to be cloned, sequenced and analysed in silico for homology with published sequences in public-domain databases (e.g. NCBI Blast database [http://www.ncbi.nlm.nih.gov/BLAST/]). The length of the sequences can then be matched with the LH-PCR profiles to determine which OTUs represented which phylogenies. The additional work required to achieve this was beyond the scope of this study.

In conclusion, the results presented in this chapter provided little evidence to support the hypothesis that the gross structure of free-living soil bacterial communities is an important factor involved in *P. cinnamomi* suppression. However, there was some evidence to suggest that there may have been specific bacteria (represented by OTUs) that were involved in decreased disease severity in avocado seedlings during Experiment 3. The involvement of these OTUs in suppression requires confirmation through further study. Bacterial OTU richness and evenness did not appear to be an important factor in determining suppressiveness. However, it is likely that very low bacterial diversity would result in a lower number of antagonists in the soil, which would improve conditions for *P. cinnamomi* survival and pathogenicity. The constituency of soil microbial communities also includes a number of eukaryotic organisms that have been shown to antagonise or feed directly on *P. cinnamomi* propagules (Malajczuk 1983; Erwin and Ribeiro 1996). The following chapter (Chapter 6) reports on an original adaptation of the LH-PCR method applied to examine the structure and diversity of fungal communities in the same soils analysed in this chapter.
Chapter 6

Structural properties of fungal communities associated with *P. cinnamomi* suppressive soils determined by ITS2 Length Heterogeneity PCR

6.1 Introduction

Fungi play an important role in natural and managed terrestrial ecosystems. For example, a large number of fungi are plant pathogens, including *P. cinnamomi*. Another of their primary roles is as saprophytes involved in the decomposition of organic matter, subsequently supplying nutrients to organisms in other trophic levels, including plants. By forming mycorrhizal associations with plants, fungi may also transport nutrients directly to plant roots in return for substrates excreted by the plant (Atlas and Bartha 1997; Miller 2000).

Mycorrhizal fungi also protect plant roots from disease (Whipps 2001). For those plant species that have formed symbiotic relationships with ectomycorrhizal fungi, protection is provided by a physical and microbial barrier in the form of hyphal tissue on the root surface, termed the mantle (Malajczuk 1979a; Curl 1988). Among early reports of plant root protection from *P. cinnamomi* infection by fungi, Marx and Davey (1969) and Marx (1970; 1972) demonstrated the role of ectomycorrhizal fungi in protecting forest pines. Malajczuk (1979a) also demonstrated that, under certain conditions, ectomycorrhizae have the ability to protect *E. calophylla* and *E. marginata* from *P. cinnamomi*. Exploiting ectomycorrhizae to protect avocado roots would be a convenient strategy for biological control of root pathogens, however, *Persea* spp. do not form such symbiotic relationships (Coffey 1992). Therefore, where biological suppression of Phytophthora root rot in avocado trees occurs, it must result from other interactions.

The 2 basic modes of specific and general disease suppression (Section 1.3) that apply to soil bacteria, as discussed in Chapter 5, also apply to suppression influenced by soil fungi. The antagonistic interactions that may result in suppression can be categorised under the terms amensalism (e.g. antibiosis), mycoparasitism and
competition. These interactions occur within the rhizosphere or within root-free soil, especially where there is increased saprophytic activity in the surface mineral and detritus layers or where organic substrates are concentrated (Atlas and Bartha 1996).

There have been relatively few studies that provide insight into the structure (taxonomic composition and organisation within communities; Sections 1.9 and 5.1) and diversity (species richness and evenness; Sections 1.9 and 5.1) of fungal communities in soils biologically suppressive to *P. cinnamomi*. However, several culture-based studies have implicated soil fungi in *P. cinnamomi* suppression. Downer et al. (2001a) isolated a greater diversity of fungal species, and recorded higher numbers of fungal propagules, in soils beneath avocado trees under a suppressive mulch when compared with bare soil treatments. The dominant fungal populations in the mulched soils studied by Downer et al. (2001a) were in the genera *Aspergillus, Penicillium, Sporothrix, Phoma, Saccharomyces* and *Trichoderma*. Malajczuk and McComb (1979) found that, when grown in a Western Australian lateritic soil (with low fertility and low organic matter content), the incidence of Phytophthora root rot in *E. marginata* seedlings was much higher than when seedlings were grown in a more fertile loam soil with higher organic matter. The total numbers of fungal propagules on roots grown in the lateritic soil were considerably lower than in the rhizosphere of healthy plants grown in the loam soil.

In contrast, South African researchers (Mass and Kotze 1989) reported higher numbers of fungal propagules in disease affected soils when compared with soils supporting healthy avocado trees. Mass and Kotze (1989) found that *Trichoderma* spp. were more dominant in disease affected soils, whereas *Penicillium* spp. were predominantly associated with soils supporting healthy avocado trees. Malajczuk and McComb (1979) also reported higher counts of *Trichoderma* propagules on diseased roots. However, in contrast to Mass and Kotze (1989), diseased roots also possessed higher numbers of *Penicillium* propagules. Malajczuk and McComb (1979) suggested that both *Trichoderma* spp. and *Penicillium* spp. were more abundant on diseased roots because they were feeding on decaying root tissue. While predominantly saprophytic, *Trichoderma* spp. are also mycoparasites known to parasitise *Phytophthora* propagules (Malajczuk 1983). You and Sivasithamparam (1995) noted that the number of *Trichoderma* propagules increased in response to
inoculating organic mulch with *P. cinnamomi*. Thus, an alternative explanation to that provided by Malajczuk and McComb (1979) could be that *Trichoderma* populations were more abundant in the diseased soils due to increased *P. cinnamomi* inoculum.

Other evidence implicating soil fungi in *P. cinnamomi* suppression mainly comes from culture-based studies undertaken with the aim of identifying fungal antagonists for use in biological control. Among the earliest of these, Pratt (1971) identified 14 (out of 33) basidiomycete isolates that antagonised *P. cinnamomi* *in vitro*. *Trichoderma* spp. (Kelley 1976; Chambers and Scott 1995, McLeod et al. 1995), *T. virens* (Chambers and Scott 1995; Costa et al. 2000), *Penicillium* spp. (Murray 1987; Fang and Tsao 1995), *E. purpurascens* (Brown et al. 1987) and *Myrothecium* spp. (Munnecke 1984; Gees and Coffey 1989) are among the more commonly studied fungi known to antagonise *P. cinnamomi* (Malajczuk 1983; Erwin and Ribeiro 1996). Historically, testing individual isolates for their efficacy in controlling *P. cinnamomi* has met with limited success (Malajczuk 1983; Erwin and Ribeiro 1996). A recent example is a study carried out with the aim of testing the ability of bioenhanced mulches, inoculated with *T. harzianum* and *T. virens*, to reduce Phytophthora root rot of avocado (Costa et al. 2000). Mulches inoculated with either organism failed to completely control the pathogen. However, both *T. harzianum* and *T. virens* inoculated mulches reduced infection rates of avocado roots by 22 - 25% and 31 - 37%, respectively. The limited success of biocontrol field programs is mainly attributed to a lack of understanding of the ecological requirements of the biocontrol candidates (Stirling and Stirling 1997). In many cases, the complexity of interactions in disease suppressive soils extends beyond one or a few specific organisms. Suppression may be a cumulative effect of the activities of a diverse and densely populated microbial community. Whether the gross phylogenetic structure and species diversity within soil fungal communities are important factors in suppression of *P. cinnamomi* is not clear.

As discussed in Sections 1.9 and 5.1, the limitations of culture-based studies for investigating structural organisation and diversity within soil microbial communities are well known, and molecular methods involving analysis of whole community DNA are seen as a way to overcome these limitations. At the time of writing, only
one study had applied a molecular method to analyse soil fungal communities within
the context of *P. cinnamomi* suppression (Borneman and Hartin 2000). Borneman
and Hartin (2000) designed and tested 2 primer pairs (nu-SSU-0817-5′ and nu-SSU-
1536-3′; nu-SSU-0817-5′ and nu-SSU-1196-3′) for their ability to specifically
amplify fungal community DNA from environmental samples. They applied one of
these primer pairs to analyse fungal communities in 2 avocado orchard soils with
contrasting abilities to inhibit Phytophthora root rot. Their approach to analysing
fungal community DNA was to clone and sequence DNA fragments amplified using
one of the primer pairs (nu-SSU-0817-5′ and nu-SSU-1536-3′) and then determine
the most probable identity of the cloned sequences by comparing them with
were able to demonstrate that fungal communities in the 2 soils exhibited structural
differences. Ten fungal genera were identified from a total of 62 clones, with 4 of
these being unique to the suppressive soil. The dominant genera in the suppressive
soil as identified by the molecular approach (*Tritirachium*, *Aspergillus*, *Pleospora*,
*Petriella*, *Monilinia* and *Exophiala*) were very different to the dominant genera
identified by a culture-based approach (*Aspergillus*, *Penicillium*, *Sporothrix*, *Phoma*,
*Trichoderma* and *Fusarium*) using the same soils (Borneman and Hartin 2000;
Downer et al. 2001a). The contrasting results acquired from the 2 approaches
highlights the potential for community DNA profiling methods to provide insight
into microbial communities beyond that achieved by traditional culture-based
approaches.

Universal fungal primers, other than those designed by Borneman and Hartin (2000),
that have been tested for amplifying fungal community DNA (Borneman and Hartin
2000; Anderson et al. 2003) include: EF4 and EF3 (Smit et al. 1999); EF4 and fung5
(Smit et al. 1999); ITS1-F (Gardes and Bruns 1993) and ITS4 (White 1990); and
2234C and 3126T (Sequerra et al. 1997). These, and the Borneman and Hartin
(2000) primers, are suitable for profiling fungal community DNA using methods
such as DGGE (van Elsas et al. 2000; Hagn et al. 2003), TRFLP (Nikolcheva et al.
2003) and ARISA (Ranjard et al. 2001). As discussed in Sections 1.9 and 5.1, these
methods have several disadvantages and an alternative method for profiling
microbial communities is Length Heterogeneity PCR (LH-PCR; Suzuki et al. 1998).
In May 2005, Martin and Rygeiwicz (2005) published fungal specific primer pairs
suitable for use in LH-PCR analysis of fungal community DNA from environmental samples. However, when the present study was undertaken, LH-PCR had not been used to analyse soil fungal community DNA and fungal specific primers suitable for use with LH-PCR analysis had not been identified.

In this study, 3 primer pairs were assessed for their suitability for fungal community LH-PCR analysis. One of these primer pairs consisted of a forward primer named ITS86-F coupled with the ITS4 reverse primer (White 1990). Turenne et al. (1999) designed the ITS86-F primer (and used it with the ITS4 reverse primer) for use in screening human blood for medically important fungal pathogens. One of the primary criteria for selecting primer pairs for use in fungal community LH-PCR, where fingerprint separation is carried out using an ABI Prism 3730 automated DNA analyser (Applied Biosystems, USA), is that the primers produce amplicons no greater than 500 bp. The reason for this is that, at the time of writing, the largest size standard available for use with the ABI Prism 3730 is 500 bp. Two of the primer pairs met this criterion and these were further assessed by comparing sequences obtained from their amplification products with ribosomal databases, with the primary purpose of confirming that they specifically amplified fungal DNA. The ITS86-F and ITS4 reverse primer pair was then chosen for use in LH-PCR analysis of fungal community DNA from soil samples collected for Experiment 1 (Chapter 3) and at 3 time points during Experiment 3 (Chapter 3). Fungal community LH-PCR data were explored using the same multivariate methods applied in Chapter 5 to determine whether the structural organisation and diversity within soil fungal communities are factors associated with suppression of *P. cinnamomi*.

### 6.2 Materials and methods

#### 6.2.1 Field locations and soil sampling

Soil was collected from 10 avocado orchards and 2 rainforest remnants during September 2002. These samples were assayed for their ability to suppress *P. cinnamomi* root rot in lupin seedlings during Experiment 1 as detailed in Chapter 3. Details for field locations and procedures for sampling, handling and storage of soil samples were described in Sections 3.2.1 - 3.2.3.
6.2.2 Soil sampled during Experiment 3

During Experiment 3, soil was sampled from pots at 0, 2 and 14 weeks after inoculating the pots with *P. cinnamomi* (Section 3.2.8). Techniques used to avoid cross contamination and conditions for handling and storing soil samples prior to DNA extraction were described in Sections 3.2.2, 3.2.3 and 3.2.8.

6.2.3 Disease severity rating data

The disease severity rating (DSR) data used during comparisons between ITS2 LH-PCR profiles in this chapter were the same as those reported in Chapter 3 for Experiments 1 and 3.

6.2.4 Soil DNA extraction and quantification

Soil DNA extractions were carried out as described in Section 5.2.4. The same DNA samples used for analyses reported in Chapter 5 were also used in this study. Sample DNA was quantified as described in Section 5.2.5.

6.2.5 PCR amplification

Three ITS primer pairs were assessed for their suitability in soil fungal community LH-PCR. PCR amplification was carried out using primer pairs (P) as follows: P1) ITS86-F (Turenne 1999) and ITS4-R (White et al. 1990); P2) ITS1-F (Gardes and Bruns 1993) and ITS86-R; P3) ITS1-F and ITS4-R (Table 6.1). The ITS86-R primer sequence is the reverse complement of the ITS86-F primer. P1 targets the ITS2 region between the 5.8S rDNA and the 28S rDNA genes (Figure 6.1). P2 targets the ITS1 region between the 18S rRNA gene and the 5.8S rRNA gene. The P3 primer pair amplifies DNA across the internal transcribed spacer (ITS) region which includes the ITS1, 5.8S rRNA gene and ITS2 regions (Figure 6.1). All primers were manufactured by Invitrogen, USA.
Table 6.1 Details for primers assessed for their suitability in fungal community LH-PCR analysis.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Target genomic region</th>
<th>Primer Sequence (5′—3′)</th>
<th>Approx. product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>ITS2</td>
<td>GTGAATCATCGAATCTTTGAA</td>
<td>400</td>
<td>Turenne et al. (1999)</td>
</tr>
<tr>
<td>P1</td>
<td>ITS2</td>
<td>TCCTCCGCTTATTGATATGC</td>
<td>400</td>
<td>White et al. (1990)</td>
</tr>
<tr>
<td>P1</td>
<td>ITS1</td>
<td>CTTGGTCATTTAGAGGAAGTA</td>
<td>350</td>
<td>Unpublished</td>
</tr>
<tr>
<td>P1</td>
<td>ITS1</td>
<td>TTCAAGATTCCGATGATTCAG</td>
<td>350</td>
<td>Gardes and Bruns (1993)</td>
</tr>
<tr>
<td>P3</td>
<td>ITS</td>
<td>CTTGGTCATTTAGAGGAAGTA</td>
<td>700</td>
<td>Gardes and Bruns (1993)</td>
</tr>
<tr>
<td>P3</td>
<td>ITS</td>
<td>TCCTCCGCTTATTGATATGC</td>
<td>700</td>
<td>White et al. (1990)</td>
</tr>
</tbody>
</table>

Figure 6.1 Schematic representation of fungal ribosomal genes bridged by hyper-variable ITS regions targeted by the 3 primer pairs that were assessed for their suitability in soil fungal community LH-PCR analysis.

PCR were carried out as described in Section 5.2.6 except that: (i) the forward primer was 5′ HEX labelled for fungal community LH-PCR; (ii) 20 ng DNA template was used; and (iii) 30 cycles of denaturation at 94°C for 45 s, primer annealing at 55°C for 45 s and chain extension at 72°C for 2 min were used instead of 25 cycles.

6.2.6 Cloning and sequencing

As part of the primer assessment process, 2 separate clone libraries were constructed from P1 and P2 PCR amplification products. PCR products were purified by PEG precipitation as described in Section 5.2.6 prior to setting up cloning reactions. Cloning reactions were carried out with the pCR®2.1 Topo® plasmid vector system (Topo® TA 2.1 Cloning Kit; Invitrogen, USA). Ligation reactions were set up as described in cloning kit instructions using 4 µL of PCR product, 2 µL pUC19 control DNA (supplied with cloning kit) and a 30 min reaction time. Vector constructs were transformed into One Shot® electrocompetent Escherichia coli Escherich cells by electroporation using a BioRad Micropulser Electroporation Apparatus (BioRad, USA). Immediately after electroporation, 1 mL of S.O.C. medium (supplied with kit).
was added to the electroporation cuvette. The cuvette was inverted 3 times to mix. The contents of the cuvette were transferred to a sterile 2 mL centrifuge tube, laid horizontally and incubated at 37°C for 1 h on a shaker to allow expression of the indicator antibiotic resistance gene in transformants (determined by detecting synthesis of β-Galactosidase). Five 100 µL aliquots from each tube were then spread on 5 pre-warmed (37°C) Luria-Bertani (LB) agar plates (prepared as per cloning kit instructions: 1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.0) containing 50 µg mL⁻¹ ampicillin (LB₅₀). Plates were pre-treated with 40 µL IPTG (100 mmol L⁻¹ in ultrapure water; Invitrogen, USA) and 40 µL X-Gal (40 mg mL⁻¹ dimethylformamide; Invitrogen, USA) by spreading and drying on the solidified agar surface. Plates were incubated at 37°C until discrete blue and white colonies (white colonies = positive clones synthesising β-Galactosidase and dark blue colonies = negative clones) formed on the plates (~24 h). Using autoclaved toothpicks, 130 white colonies were randomly harvested from each of the 2 clone libraries and patch-streaked onto LB₅₀ agar plates (without IPTG / X-Gal) in positions aligned against a numbered grid template (~40 patch-streaks per agar plate). Each selected colony was also patch-streaked onto a second IPTG / X-Gal treated LB₅₀ agar plate using a corresponding numbered grid pattern. These plates acted as master or reference plates to provide confirmation that the selected colonies were positive clones. Both sets of plates were incubated at 37°C for 24 h and then at 4°C overnight (to allow for better colour development without streaked colonies growing into each other).

A total of 96 white transformant colonies from each of the 2 clone libraries (P1 and P2) were screened for positive inserts by performing colony PCR with the M13 forward and M13 reverse vector primers (supplied with cloning kit; Invitrogen, USA). Positive transformant colonies were selected (from the non-IPTG / X-Gal-treated LB₅₀ plates) at random and an autoclaved toothpick was used to transfer the colonies to 100 µL ultra-pure water in 1.5 mL sterile centrifuge tubes. The tubes were vortexed briefly and 1 µL of the cell suspension was used to provide the DNA template in the PCR. The PCR cocktail contained the same combination of reagents as described in Section 6.2.5, except that the primers were replaced with 20 pmol each of the M13 forward and reverse primers. PCR amplification of cloned DNA fragments were performed in a thermocycler under the following conditions: cell disruption and Hot-master™ Taq initialisation at 94°C for 4 min, followed by 25
cycles involving denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s and chain extension at 72°C for 1 min 30 s. Chain extension on the final cycle was at 72°C for a further 7 min before holding at 4°C. PCR products were visualised by agarose gel electrophoresis and positive PCR products were subsequently purified by PEG precipitation as described in Section 5.2.6.

A total of 60 clones were selected from each clone library (P1 and P2) for inserts of varying fragment length. The clonal DNA was sequenced in both forward and reverse directions (120 sequencing reactions for each clone library) using the BigDye Terminator version 3.1 (BDT V 3.1) cycle sequencing kit (Applied Biosystems, USA). Sequencing reactions were carried out in 20 µL volumes containing: 1µL BDT V 3.1, 3.2 pmol of either P1 or P2 forward or reverse primer, 1.5 µL BDT 5 x reaction buffer and 37 ng DNA template (DNA concentration of PCR products determined using the PICO green DNA quantification kit as described in Section 5.2.5), made up to volume with ultra-pure water. Sequencing reactions were carried out using the following thermo-cycle sequencing conditions: 96°C 1 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and chain extension at 60°C for 4 min, with a final rapid ramp and hold at 4°C. Unincorporated dyes were removed from the sequencing PCR products by ethanol precipitation. The sequencing PCR samples were transferred to 1.5 mL micro-centrifuge tubes to which 16 µL sterile deionised water and 64 µL non-denatured 95% ethanol were added. The tubes were vortexed to mix, incubated at room temperature for 15 min and centrifuged (Eppendorf 5810R model, Germany) for 20 min at 14,000 rpm. The supernatant was discarded immediately after centrifuging. A volume of 250 µL freshly prepared 70% ethanol was added to the tubes which were vortexed to mix and then centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pellet dried under vacuum for 10 min.

The purified sequence samples were analysed by staff at the University of New South Wales, Automated DNA Analysis Facility (UNSW, Kensington, NSW, Australia) using an Applied Biosystems model 3730 (ABI 3730) automated capillary DNA sequencer (Applied Biosystems, USA). Upon arrival at UNSW, 20 µL Hi Di Formamide was added to each sequence sample and the samples were mixed on a shaker for 1 h. Samples were then centrifuged briefly to spin down contents, heated
for 2 min at 95°C, placed on ice for 2 min and then run on the ABI 3730 for approximately 2 h. Data generated by the ABI 3730 were captured using ABI DNA Sequencing Analysis Software version 5.1.1.

### 6.2.7 Sequence data analysis

DNA sequences were edited (primer sequences for P1 and P2 libraries were identified and then vector sequences were removed), and consensus sequences obtained, using the BioEdit Sequence Alignment Editor software package (Tom Hall, Ibis Therapeutics, USA). The BioEdit Sequence Alignment Editor software package is available free from [http://www.mbio.ncsu.edu/BioEdit/bioedit.html](http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequenced clones were analysed for the presence of chimeras using the CHIMERA CHECK facility available on the Ribosomal Database Project (RDP) website: [http://rdp.cme.msu.edu/html/](http://rdp.cme.msu.edu/html/). None of the sequences were chimeric. Cloned sequences were compared against published nucleotide sequence data from known and unidentified organisms using the NCBI Blast nucleotide database [http://www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/). The closest matching sequences were retrieved from the database, aligned with the relevant cloned sequence and percentage homology was determined using pairwise alignment functions in BioEdit.

### 6.2.8 Quantification of PCR products

HEX labelled PCR products were purified to remove excess PCR reagents (including excess labelled forward primer) by the PEG precipitation method as described in Section 5.2.6. The quality of the purified PCR products and the negative controls were checked using agarose gel electrophoresis. The fluorescence intensity of PCR samples was quantified using the method detailed in Section 5.2.7 with the exception that the HEX-ITS86-F primer was used to construct a standard curve and fluorescence readings were carried out using 520 nm excitation and 555 nm emission filters.
6.2.9 Amplicon separation

HEX labelled PCR samples were diluted so that their fluorescence intensity was equivalent to the fluorescence intensity of 25 fmol µL⁻¹ HEX-ITS86-F primer and a 1 µL aliquot from each PCR dilution was analysed by staff at the University of New South Wales Automated DNA Sequencing Facility (Kensington, NSW, Australia) using an ABI 3730 DNA analyser (ABI, USA) as outlined in Section 5.2.8.

6.2.10 Data handling and analysis

Data handling and analyses of sample data files produced by Genemapper for the fungal community ITS2 LH-PCR profiles were the same as detailed in Section 5.2.9. However, using a 1% relative ratio cut-off was found to remove an excessive number of OTUs from each ITS LH-PCR profile and therefore the relative ratio cut-off was reduced to 0.5% (i.e. OTUs accounting for 0.5% or less of the accumulated fluorescence intensity were reassigned to zero and the relative ratio of each remaining OTU was recalculated). As described in Chapter 5, normalised data sets were imported into the multivariate software package, Primer Version 5 (Primer-E Ltd, Plymouth, UK) for analysis. Bray-Curtis similarity data for Experiment 1 was explored by manipulating factors displayed as different symbols in the MDS plot to search for patterns potentially associated with the following variables: suppressive soil vs. conducive soil (i.e. soils that yielded DSR <3 vs soils that yielded DSR >3); sampling location; geographic location; soil management regime (organic vs conventional vs rainforest); and incremental DSR values (i.e. DSR <4 vs DSR >4; DSR <5 vs DSR >5; etc.). Species richness (S), the Shannon-Weiner diversity (H') and the Simpson evenness indices (1-λ) were also calculated within the Primer software package. These diversity indices were used to compare OTU richness and evenness in fungal ITS2 DNA LH-PCR profiles from soils that yielded low and high DSRs during Experiment 1 (Section 6.2.1). These diversity measures were also used to analyse changes in OTU richness and evenness in groups of treatments during Experiment 3 (Section 6.2.2). Comparison of OTU richness and diversity indices was carried out by calculating 95% confidence intervals using Origin Version 5.0 (Microcal Software, 1997).
6.2.11 Verification of fungal community DNA LH-PCR data

Verification of fungal community DNA LH-PCR data was carried out as described in Section 5.2.10, except that there were 6 replicate positive controls and that data used to assess similarity of positive controls between runs was obtained using methods detailed in Sections 6.2.5, 6.2.8 and 6.2.9.

6.3 Results

6.3.1 Amplification of DNA from soil and fungal specificity of primers

All 3 primer pairs successfully amplified soil DNA. For each primer pair, PCR products appeared as intense bands on agarose gels. Primer pairs produced bands concentrated at approximately 400 bp (P1), 350 bp (P2) and 700 bp (P3). The primary criterion for selecting primer pairs for fungal community DNA LH-PCR, where fingerprint separation is carried out using an ABI 3730 automated DNA analyser, is that the primers produce amplicons less than 500 bp (Section 6.1). Consequently, assessments for fungal DNA amplification specificity were carried out by cloning and sequencing PCR products produced by P1 and P2 primer pairs only.

When compared against nucleotide sequences in the NCBI BLAST database, sequences from the P1 clone library were most closely aligned with published sequences acquired from 3 major fungal clades (Table 6.2). None of the P1 clonal sequences aligned with prokaryotic DNA sequences or eukaryotic sequences from organisms other than fungi. Thirty-nine P1 sequences were most closely related to 22 fungal species within the Ascomycota clade. There were 14 repeat fragments with a sequence length of 396 bp that had 88% sequence similarity with a *Verticillium* sp. (NCBI Blast accession number: AY172097). The remaining clonal sequences were evenly distributed between the other 21 Ascomycete species. A total of 6 sequences aligned with 6 Basidiomycete species and 7 sequences aligned with 4 Zygomycete species.
Table 6.2 Closest matches in NCBI BLAST database to sequences from P1 (ITS2) clone library.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Number of clones</th>
<th>Sequence length (bp)</th>
<th>Closest match</th>
<th>Accession number</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Massarina corticola</td>
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<td></td>
<td>1</td>
<td>282</td>
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<td>AY533556</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>285</td>
<td>Eliurema sp.</td>
<td>AY148442.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>Lecythophora sp.</td>
<td>AY219880</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>286</td>
<td>Chrysosporium lobatum</td>
<td>AJ131688.1</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>287</td>
<td>Discostroma tricellulare</td>
<td>AF377285.1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>292</td>
<td>Leaf litter ascomycete strain ITS-295 isolate</td>
<td>AF502810.1</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>294</td>
<td>Penicillium brevicipactum</td>
<td>AF373898.1</td>
<td>94</td>
</tr>
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<td>1</td>
<td>294</td>
<td>Penicillium brevicipactum</td>
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<td>306</td>
<td>Williopsis californica</td>
<td>Z93883</td>
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<td></td>
<td>1</td>
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<td>AY310443</td>
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</tr>
<tr>
<td></td>
<td>1</td>
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<td>Mortierella alpina</td>
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<td>366</td>
<td>Salal associated fungal clone</td>
<td>AY112929</td>
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</table>

Most sequences from the P2 clone library (Table 6.3) were related to fungi in the Ascomycota and Basidiomycota clades. Within the Ascomycota group there were 30 clonal sequences distributed between 22 species with 6 clonal sequences of 346 bp being most closely related (84-96%) to a *Lecythophora* sp. (NCBI Blast accession number: AY219880). There were 16 clones distributed between 15 Basidiomycete species, however, these clonal sequences only shared a relatively low homology with their closest matches (Table 6.3). There were also 3 clonal sequences that were most closely related to: an unknown ectomycorrhizal fungal species; *Sphaerothecum destruens* **n. g., n. sp.** - a Dermocystida (unique group at evolutionary stage of
fungal-animal transition); and *Cyphomyrmex rimosus* Spinola - a subterranean ant species that cultivates fungi.

**Table 6.3** Closest matches in NCBI BLAST database to sequences from P2 (ITS1) clone library.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Number of clones</th>
<th>Sequence length (bp)</th>
<th>Closest match</th>
<th>Accession number</th>
<th>Homology (%)</th>
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<td><strong>Ascomycota</strong></td>
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<td></td>
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<td>Leaf litter ascomycete AF502734</td>
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<td><em>Cyphomyrmex rimosus</em> Note: fungi cultivating ant species from South America</td>
<td>AF079693</td>
<td>78</td>
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</tbody>
</table>

In both clone libraries there were a number of clones represented by the same sequence lengths, but with different sequences, that aligned with fungal species either within the same genus or with distantly related genera (Tables 6.2 and 6.3).
In the remaining results sections, the P1 primer pair was used for soil fungal community DNA LH-PCR analysis. As the P1 primers specifically amplify DNA from the ITS2 region, the term “ITS2 LH-PCR” was adopted in place of soil fungal community DNA LH-PCR.

6.3.2 Verification of ITS2 LH-PCR data

No contamination was detected in any of the negative water controls. The Bray-Curtis similarity value between ITS2 LH-PCR profiles for positive controls was between 78% and 95%. In a separate experiment 6 replicate aliquots from a single PCR sample were run simultaneously on the ABI 3730. Prior to normalising data (by calculating relative ratios) for each replicate profile, the level of variability was moderate with a coefficient of variation of 15% for the mean (n = 6) accumulated peak height. After normalising the data and removing OTUs that contributed <0.5% to the accumulated peak height, the level of Bray-Curtis similarity between replicate profiles was >95%. These results suggest that during a single run on the ABI 3730, replicate ITS2 LH-PCR profiles (each replicate taken from the same PCR sample) may differ by up to 5%. A further 17% (based on Bray-Curtis similarity) dissimilarity between replicate ITS2 LH-PCR profiles (taken from the same soil community DNA sample) may accumulate during separate PCR runs, dilution and handling, and separate runs on the ABI 3730. Therefore, in subsequent analyses differences between samples were determined where ITS2 LH-PCR profiles shared <78% Bray-Curtis similarity (determined from the lowest similarity value for the positive controls).

6.3.3 Fungal ITS2 LH-PCR profiles from Experiment 1 soil samples

MDS analysis based on Bray-Curtis similarity did not discriminate between avocado orchard and rainforest remnant soil samples that yielded DSRs of 3 or less (low DSR) and soil samples that yielded DSRs above 3 (high DSR). ANOSIM confirmed that there were no significant differences (Global R = 0.015) between the 2 groups. In addition, the MDS stress value of 0.24 (Figure 6.2) indicated that the placement of data points in the plot was close to random. There were no other patterns revealed by
the MDS analysis that suggested ITS2 LH-PCR profiles were associated with any of the variables considered (Section 6.2.10).

![Figure 6.2 Multidimensional scaling plot based on Bray-Curtis similarities of ITS2 LH-PCR profiles for soil samples with different DSRs collected for Experiment 1.](image)

Figure 6.2 Multidimensional scaling plot based on Bray-Curtis similarities of ITS2 LH-PCR profiles for soil samples with different DSRs collected for Experiment 1.

○ = DSR <3  
● = DSR >3  
(DSR = disease severity rating)

Cluster analysis using Bray-Curtis similarity values also failed to reveal any meaningful patterns in the data (Figure 6.3). Three main clusters with distantly related constituents were formed, however, separation into clusters did not represent high or low DSR yielding soils or any of the other variables that were considered (Section 6.2.10). Overall, fungal ITS 2 LH-PCR profiles were relatively dissimilar between sampling locations, with the majority of sampling locations sharing <65% similarity (Figure 6.3).
Figure 6.3 Single linkage cluster analysis based on Bray-Curtis similarities of ITS2 LH-PCR profiles with data for soil samples collected for Experiment 1. LH-PCR data for 4 replicate samples from each location were pooled prior to analysis. Als = Alstonvale; Cud = Cudgen; Eur = Eureka; Tu1 = Tuckombil 1; Ta2 = Tamborine Mountain 2; Pr1 = Pretty Gully 1; Trf = Tamborine Mountain rainforest; Prf = Pretty Gully rainforest; Ura = Uralba; Tu2 = Tuckombil 2; Ta1 = Tamborine Mountain 1; Pr2 = Pretty Gully 2.

SIMPER analysis indicated that there was a high level of variability within both low DSR and high DSR groups (mean similarity values were 41% and 43%, respectively) and between soil samples from within the same sampling location (42% to 66% mean similarity). Further analysis with SIMPER showed that there were no OTUs unique to low DSR yielding soil samples or to high DSR samples. The total number of fungal ITS2 OTUs (with relative fluorescence intensity >0.5%) detected in each of the 48 soil samples ranged between 33 and 82 OTUs with a mean of 55 OTUs. Mean OTU richness was 52 OTUs for low DSR soil samples and 58 OTUs for soil samples that yielded high DSRs. Shannon diversity indices suggested high levels of OTU diversity with a mean $H' = 3.5$ for low DSR samples and a mean $H' = 3.7$ for high DSR samples. The Simpson index indicated a high level of evenness for both low DSR and high DSR samples, with average index values of 0.95 and 0.96, respectively. According to ANOVA, the Shannon diversity values for the high DSR soils were significantly higher (p<0.05) than the low DSR soils. However, differences for OTU richness and the Simpson index values were not significant.
6.3.4 Fungal community ITS2 LH-PCR profiles from Experiment 3 soil samples

**Week 0**

There was considerable variability in fungal ITS2 LH-PCR profiles among soil samples from the 4 different orchard locations at week 0 (Figure 6.4). Within group variability (determined by SIMPER analysis) was highest for the irradiated (average similarity 27%), irradiated + suppressive soil (average similarity 29%), and the suppressive avocado soil treatments (average similarity 39%). The average within group similarity value for the conducive pasture soil was much higher at 68%. The conducive pasture soil was also closely associated with and overlapped the data points representing the conducive pasture + suppressive soil treatments in the MDS plot (Figure 6.4). Despite high levels of within group variability for at least 3 of the treatment groups, the MDS analysis returned a stress value of 0.14, which indicates that there was a high probability that the observed patterns were true.

ANOSIM returned a Global R statistic of 0.264 at a significance level of p<0.05, which indicates that there were minor differences between groups that were significant. ANOSIM pairwise comparisons showed that separation between the conducive pasture soil and the conducive pasture + suppressive soil treatments was moderate with a pairwise R statistic = 0.406 (p<0.05). Differences between the suppressive orchard soil and the conducive pasture soil were slightly greater with a pairwise R statistic = 0.648 (p<0.05). There was less difference between the suppressive orchard soil and the conducive pasture + suppressive soil treatments (pairwise R statistic = 0.352; p<0.05). Differences between the suppressive orchard soil, irradiated soil or the irradiated + suppressive soil treatments were not significant.
Figure 6.4 Multidimensional scaling plot based on Bray-Curtis similarities of ITS2 LH-PCR profiles for suppressive avocado orchard, irradiated orchard, irradiated + suppressive, conducive pasture and conducive pasture + suppressive soil treatments collected from pots at week 0 during Experiment 3. Circled data points highlight groupings as discussed in the text.

- ▲ Suppressive orchard
- ● Irradiated orchard
- ▼ Irradiated + suppressive
- ■ Conducive pasture
- ♦ Conducive + suppressive

Week 2
The high level of variability within the irradiated and irradiated + suppressive soil treatment groups observed at week 0 persisted in samples collected from pots at week 2 (similarity values ranged between 32% for the irradiated soils and 56% for the conducive pasture soil). As occurred at week 0, MDS analysis showed that ITS2 LH-PCR profiles obtained from the replicate conducive pasture soil sub-samples clustered and the conducive pasture + suppressive soil treatments also grouped and overlapped with the conducive pasture soil (Figure 6.5). The level of variability observed among the 4 suppressive avocado orchard soils remained high, with 47% average within group similarity (according to SIMPER analysis). Despite this high level of variability, the MDS analysis (Figure 6.5) was able to separate the suppressive orchard soil group from the conducive pasture soil and return a stress
value of 0.14. ANOSIM showed that the overall differences between groups were small but significant (Global R statistic = 0.391; p<0.01). ANOSIM pairwise comparisons confirmed that differences between the suppressive orchard soils and the conducive pasture soil were moderate (pairwise R statistic = 0.594; p<0.05). No significant differences were detected between the conducive pasture soil and the conducive pasture + suppressive soil treatments. This was also the case when the suppressive orchard soil was compared with the conducive pasture + suppressive soil treatments, the irradiated soils and the irradiated + suppressive soil treatments.

**Figure 6.5** Multidimensional scaling plot based on Bray-Curtis similarities of ITS2 LH-PCR profiles for suppressive avocado orchard, irradiated orchard, irradiated + suppressive, conducive pasture and conducive pasture + suppressive soil treatments collected from pots at week 2 during Experiment 3. Circled data points indicate groupings as discussed in the text.

▲ Suppressive orchard
● Irradiated orchard
▼ Irradiated + suppressive
■ Conducive pasture
♦ Conducive + suppressive
Variability observed among samples taken at week 0 and week 2, from pots containing the suppressive orchard, irradiated and irradiated + suppressive soil treatments, was still evident at week 14 (similarity values ranged between 30% for the irradiated soils to 43% for irradiated + suppressive soil treatments). Despite high within group variability, the differences between treatment groups were sufficient for the MDS analysis to define loosely associated clusters representing each group (MDS stress value of 0.18; Figure 6.6). The irradiated soils separated from each of the other treatment groups and there was clear discrimination between the conducive pasture soil and the suppressive orchard soils. The conducive pasture + suppressive soil treatments overlapped with both the conducive pasture soil and the suppressive orchard soils. The irradiated + suppressive soil cluster also overlapped with the suppressive orchard soils. ANOSIM confirmed that there were moderate dissimilarities among the treatment groups after returning a Global R statistic of 0.417 (p<0.01). ANOSIM pair-wise comparisons (Table 6.4) suggested that the largest differences were between the suppressive orchard soils and the conducive pasture soil (R statistic = 0.635; p<0.05) and the conducive soil and the irradiated + suppressive soil treatments (R statistic = 0.833; p<0.05). Differences between the suppressive orchard soils and the irradiated + suppressive soil treatments were not significant. No significant differences were observed between the conducive pasture soil and the conducive pasture + suppressive soil treatments. Differences between all other treatment groups were moderate and statistically significant (R statistics ranged between 0.333 and 0.479; p<0.05; Table 6.4).
Figure 6.6 Multidimensional scaling plot based on Bray-Curtis similarities of ITS2 LH-PCR profiles for suppressive avocado orchard, irradiated orchard, irradiated + suppressive, conducive pasture and conducive pasture + suppressive soil treatments collected from pots at week 14 during Experiment 3. Circled data points indicate groupings as discussed in the text.

▲ Suppressive orchard
● Irradiated orchard
▼ Irradiated + suppressive
■ Conducive pasture
♦ Conducive + suppressive
Table 6.4 ANOSIM results for fungal community ITS2 LH-PCR profiles obtained from pots sampled during Experiment 3 at week 14. Analysis of similarity was calculated based on 4 replicate treatments within each treatment category. * indicates R statistic values for pairwise tests with a significance level p<0.05. † indicates R statistic values that are not statistically significant.

<table>
<thead>
<tr>
<th>Pairwise treatment comparisons</th>
<th>R statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressive orchard v irradiated orchard</td>
<td>0.417*</td>
</tr>
<tr>
<td>Suppressive orchard v irradiated + suppressive</td>
<td>0.135†</td>
</tr>
<tr>
<td>Suppressive orchard v conducive pasture</td>
<td>0.635*</td>
</tr>
<tr>
<td>Suppressive orchard v conducive pasture + suppressive</td>
<td>0.365*</td>
</tr>
<tr>
<td>Irradiated orchard v irradiated + suppressive</td>
<td>0.250*</td>
</tr>
<tr>
<td>Irradiated orchard v conducive pasture</td>
<td>0.396*</td>
</tr>
<tr>
<td>Irradiated orchard v conducive pasture + suppressive</td>
<td>0.333*</td>
</tr>
<tr>
<td>Conducive pasture v irradiated + suppressive</td>
<td>0.833*</td>
</tr>
<tr>
<td>Conducive pasture v conducive pasture + suppressive</td>
<td>0.210†</td>
</tr>
<tr>
<td>Conducive pasture + suppressive v irradiated + suppressive</td>
<td>0.479*</td>
</tr>
</tbody>
</table>

Results from SIMPER analysis at week 14

Similarity percentage analysis (SIMPER) was carried out on fungal community ITS2 LH-PCR data from week 14. The level of variability observed between replicates for each of the 5 treatments groups was high (Section 6.3.4, week 14). In accordance with low within group similarity, SIMPER analysis showed that pairwise similarities between treatments (Table 6.5) were also low (28% - 49%). Mixing the conducive and irradiated soils with suppressive orchard soils slightly increased the level of similarity between these and the suppressive soils (Table 6.5).
Table 6.5 Results from SIMPER pairwise similarity comparisons between treatments. Values represent % similarity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Suppressive</th>
<th>Irradiated</th>
<th>Irradiated + suppressive</th>
<th>Conducive pasture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiated</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irradiated + suppressive</td>
<td>39</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conducive pasture</td>
<td>36</td>
<td>32</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Conducive pasture + suppressive</td>
<td>42</td>
<td>31</td>
<td>40</td>
<td>49</td>
</tr>
</tbody>
</table>

Further analysis with SIMPER showed that 9 OTUs accounted for 50% of the dissimilarity observed between the suppressive orchard soils and the conducive pasture soil, which was primarily due to differences in OTU relative abundance (data not shown). Of these, 8 OTUs were present in the suppressive, conducive pasture and conducive pasture + suppressive soil treatments and 1 OTU (297 bp) was absent from the conducive pasture soil in week 14. This OTU (297 bp) accounted for 5% of the dissimilarity between the suppressive and conducive soils. A total of 16 OTUs accounted for 50% of the dissimilarity between the suppressive orchard soils and the irradiated soil treatments. Several of these OTUs were present in suppressive soil and absent from the irradiated soils but none appeared in the irradiated + suppressive soil treatments. At week 14, the relative abundance of the 297 bp OTU was very low in the irradiated soils (mean relative abundance = 0.8%), was approximately 7 fold higher in the suppressive orchard soils (mean relative abundance = 6.12%) and increased 3 fold in the irradiated + suppressive soil treatments (mean relative abundance 3.73%).

Results from diversity analyses at week 14
Average OTU richness ($S$ = total number of OTUs) and values for Shannon diversity were higher for the suppressive orchard soils ($S = 46$ OTUs; $H' = 3.4$) than for the conducive pasture soil ($S = 36$ OTUs; $H' = 3.0$) (Figure 6.7). However, there were no significant differences between $S$, $H'$ or $1-\lambda$ for any soil treatment. Simpson index values were the same for each treatment (Simpson index value = 0.9).
Figure 6.7 Mean OTU richness, Shannon diversity and Simpson indices for Experiment 3, week 14 data. Data points represent pooled means for each diversity measure. Bars on either side of the means represent 95% confidence intervals. Error bars represent 95% confidence intervals for each mean.

6.4 Discussion

All 3 primer pairs assessed for use in soil fungal LH-PCR analysis in this study successfully amplified soil community DNA samples. Primer pairs P1 (ITS86-F and ITS4-R) and P2 (ITS1-F and ITS86-R) synthesised PCR products of less than 500 bp. This indicated that amplicon separation could be carried out using the ABI 3730 automated analyser without reduction of amplicon length by restriction enzymes. Therefore, both P1 and P2 primer pairs were potentially suitable for fungal community DNA LH-PCR analysis.

PCR products produced by P1 and P2 primer pairs were cloned and sequenced and the cloned sequences were aligned with published sequences in the NCBI BLAST nucleotide database. P1 primers amplified DNA from 3 fungal clades (Ascomycota, Basidiomycota, Zygomyecota) and P2 primers amplified DNA from 2 fungal clades
Ascomycota, Basidiomycota). However, the P2 clone library also produced at least 2 clonal sequences that aligned with non-fungal DNA. One cloned sequence of 606 bp was 96% homologous to S. destruens. S. destruens lives in aquatic habitats and is a fish pathogen (Arkush et al. 2003). It is a member of the Dermocystida order, a unique phylogenetic group at an evolutionary point of fungal-animal transition (Mendoza et al. 2002). The closest match (79%) for the cloned sequence with 440 bp was an arthropod C. rimosus from South America (Murakami and Higashi 1997). This mycophagous ant species cultivates fungi (Murakami and Higashi 1997) and therefore it is possible that the sequence published for C. rimosus is actually from fungal DNA that may have contaminated the C. rimosus DNA sample. Considering the low sequence similarity of 76% between the cloned sequence and the sequence assumed to be from C. rimosus, the 440 bp cloned ITS2 fragment is most probably from an unknown fungal species. However, the decision was made not to use the P2 primer pair in this study due to the uncertainty surrounding the identity of the 2 clonal sequences. The P1 primer pair was favoured because: 1) cloned sequences from the P1 library aligned with fungal sequences only; 2) the P1 primer pair amplified fungal DNA from 3 fungal clades (which suggests that this primer pair may be more “universal” than the P2 primer pair); 3) all cloned DNA fragments analysed from the P1 library were less than 500 bp (the P2 primer pair produced several amplicons that were greater than 500 bp).

Prior to this study only one other study had used a rDNA profiling method to examine the structure of fungal communities associated with P. cinnamomi suppressive and conducive soils (Borneman and Hartin 2000). Borneman and Hartin (2000) designed 2 primer pairs homologous to fungal specific oligonucleotide sequences located within the eukaryotic small-subunit rRNA genes (in the IGS region between the 18S and 28S rRNA genes). They used these primers to amplify DNA extracted from soil sampled from 2 avocado orchards, one orchard with P. cinnamomi suppressive soil and the other with P. cinnamomi conducive soil. The PCR products produced by these primers were used to construct clone libraries. The clonal DNA was sequenced and then the cloned sequences were aligned with published sequences. Borneman and Hartin (2000) found that both primer pairs were fungal specific and amplified DNA from 4 major fungal clades (Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota). Ten species were identified among
62 cloned DNA fragments from both soils, with 4 species exclusive to the suppressive soil (*Exophiala jeanselmei* [Langeron] McGinnis and Padhye, *Nectria cinnabarina* [Tode] Fr., *Verticillium dahliae* Klebahn, *Coccodinium bartschii* Massal). Borneman and Hartin (2000) only compared samples from 2 orchards and therefore it is not known whether these species were common to other avocado soils suppressive and conducive to *P. cinnamomi* within the same geographical location (i.e. same avocado growing region within California, USA). In addition, while the primers used by Borneman and Hartin (2000) can theoretically be used to produce a fungal community DNA profile with DGGE, TRFLP or ARISA, no fungal community DNA profiles were generated in their study.

In the present study, ITS2 LH-PCR was used to profile fungal community DNA in 48 soil samples from 10 avocado orchards and 2 rainforest remnants that demonstrated varying abilities to reduce *P. cinnamomi* root rot in inoculated lupin seedlings (Chapter 3). The total number of fungal ITS2 OTUs (with relative fluorescence intensity >0.5%) detected in each of the soil samples averaged 55 (range 33 - 82 OTUs), which was more than 5 times the number of taxonomic units detected by Borneman and Hartin (2000). Of the 70 OTUs identified within the ITS2 LH-PCR profile for the suppressive soil sample used for DNA template positive controls (suppressive soil sample #38 from Tuckombil 2), 32 OTUs were represented by the 53 clones that were successfully sequenced (60 clones were sequenced but data for 7 clones were unusable). While beyond the scope of this study, all 70 OTUs from positive controls may have been accounted for if a greater number of clones had been sequenced. The primary purpose for the sequencing work was to confirm the fungal specificity of the primers used in this study, not to provide a comprehensive assessment of phylogenetic structure and diversity. However, information from the small number of clones that were sequenced can also be used for making general inferences with regard to possible OTU identities.

The 32 OTUs that were sequenced were most closely aligned with 28 different fungal species. However, 13 clones shared low sequence homology (<90%) suggesting that they were from fungal groups that have not previously been sequenced. This was not surprising considering that only 5 - 10% of an estimated 1.5 million fungal species have been described (Hawksworth and Rossman 1997;
Hawksworth 2001). Of the 28 fungal species identified, only 2 genera were in common with those found in Borneman and Hartin’s (2000) suppressive soil (\textit{V. dahliae} and \textit{N. cinnabarina}). These included several \textit{Verticillum} species, which were prominently represented among the P1 clones, and one 294 bp sequence, which aligned with a \textit{Nectria} species.

ITS2 LH-PCR profiles from the field soils collected for Experiment 1 did not reveal any patterns that were unique to soils that demonstrated the highest potential for \textit{P. cinnamomi} suppression during the glasshouse assay. Of the 3 biodiversity measures applied to the data, the only differences observed between low DSR soils and high DSR soils was for the Shannon diversity index, which was higher in the high DSR soils. However, while statistically significant, the differences between the 2 soils were small, with both groups demonstrating high Shannon diversity indices (mean Shannon diversity indices were 3.5 for the low DSR soils and 3.7 for the high DSR soils). Therefore, it appears that neither the gross community structure (based on composition and relative abundance of fungal community OTUs) nor OTU diversity (based on OTU richness and Shannon and Simpson diversity indices) of fungal communities in the soil samples were factors associated with reduced severity of root rot in the lupin seedlings during Experiment 1.

However, results from the ITS2 LH-PCR analyses carried out for this study should be interpreted conservatively due to the extent of variability encountered within the data. The variability encountered in the fungal community ITS2 LH-PCR data for both Experiment 1 and Experiment 3 samples was in contrast to the high level of similarity observed between bacterial community 16S rDNA LH-PCR profiles reported in Chapter 5. Comparison of 6 positive control PCR samples amplified from the same community DNA template (Section 6.3.2) suggested that errors accumulated during PCR, PEG precipitation and dilution of PCR products, and during separate runs on the ABI 3730, accounted for dissimilarities between profiles of up to 24% (based on analysis using the Bray-Curtis similarity algorithm). During Experiment 3 (Chapter 3), within group variability was greatest during the first 2 weeks of the experiment but was lower by 14 weeks, which suggested that fungal communities in potted soils had partially recovered and stabilised following the initial disturbance that occurred when the pot experiment was established. However,
at 14 weeks there was only 30% mean similarity among the irradiated soil samples and 41% mean similarity within the suppressive avocado orchard soils group. Variability was lower among replicate extractions from the conducive pasture soil sample, which showed 62% similarity.

The occurrence of micro- and macro-spatial heterogeneity in soil biotic properties and soil microbial community structure is widely recognised (Ettema and Wardell 2002; Franklin and Mills 2003; Ranjard et al. 2003; Stark et al. 2004). The results presented here also indicate that there may have been a high level of spatial heterogeneity among the soil fungal communities within and between sampling locations and also within a single soil sample (i.e. the conducive pasture soil sample). However, it is also possible that a substantial proportion of the variability can be attributed to the mass of soil from which the community DNA sample was extracted. In a publication released after most soil DNA extractions for this study had been completed, Ranjard et al. (2003) demonstrated that DNA extracted from soil samples <1 g were suitable for profiling bacterial community DNA, but samples ≥ 1 g were required to obtain reproducible soil fungal community DNA profiles using ARISA. In the present study, DNA samples used to profile both soil bacterial (Chapter 5) and fungal communities were extracted from 0.2 g soil.

Despite high levels of variability for Experiment 3 samples, ITS2 LH-PCR analysis was able to discriminate between the suppressive avocado orchard soils, the irradiated orchard soils and the conducive pasture soil, particularly in samples collected from pots at week 14. Similarity between the irradiated + suppressive soil treatments and the suppressive orchard soils also increased by week 14. This result indicates that the structure of fungal communities in the irradiated soil was partially restored by adding non-irradiated suppressive orchard soil. Mixing the conducive soil with 10% v/v suppressive soil also resulted in a minor shift in fungal community structure away from the conducive pasture soil and toward the suppressive orchard soils. The minor shifts in community structure observed in the mixed soil treatments were not accompanied by significant increases in species richness, diversity or evenness.
Further analysis using SIMPER showed that, of the OTUs that contributed to dissimilarities between the 5 treatment groups, only a single OTU with length 297 bp was absent from the conducive pasture soil but present in the suppressive and irradiated + suppressive soil treatments at week 14. The relative abundance of this OTU was also very low in the irradiated soil but its abundance increased 7 fold in response to mixing the irradiated soil with 10% suppressive soil. However, at weeks 0 and 2 the 297 bp OTU (which may represent more than one species) was present in the conducive soil and therefore it was not unique to the suppressive avocado orchard soils. A 297 bp sequence was also present in the P1 clone library constructed from community DNA extracted from suppressive soil sample #38. The closest nucleotide database match for this sequence was *Verticicola caudatus* Hyde, Wong and Ranghoo. *V. caudatus* is a recently described species isolated from decaying wood submerged in fresh water in Hong Kong and the Phillipines (Ranghoo et al. 2000). However, considering the low homology (79%) between the cloned sequence and *V. caudatus* it is more likely that the 297 bp OTU represented one or more uncultured or unknown species from the ascomycete group. Further study would be required to determine the identity of this OTU and also whether the presence and/or abundance of this OTU is associated with *Phytophthora* suppression.

Of the many fungal species that have been associated with *P. cinnamomi* suppression or identified as having potential for biological control (Marx 1969; Malajczuk 1988; Gees and Coffey 1989; Mass and Kotze 1989; Casale 1990; Duvenhage et al. 1990; Duvenhage and Kotze 1993; McLeod et al. 1995; Costa et al. 1996; Erwin and Ribeiro 1996), only 2 relevant genera were detected in the P1 clone library from suppressive soil sample #38. The clonal sequence with 294 bp was 97% homologous with *Pencillium brevicompactum* Dierckx and 91% of the clonal sequence with 303 bp was homologous to *Myrothecium verrucaria* (Albertini and Schweinitz:Fr.) Ditmar. However, neither of these 2 OTUs made significant contributions to the dissimilarities observed between the 5 main treatment groups at week 14 and therefore it is unlikely that they were involved in suppressing the development of disease in avocado seedlings during Experiment 3.

The most meticulous approach to characterising the structure of soil microbial communities with current technologies would involve amplifying whole community
DNA using universal primers, constructing large clone libraries and then sequencing the cloned DNA until there was no further increase in the number of new sequences isolated from the library. However, the costs, labour requirements and data handling involved in such an undertaking impose obvious practical limitations, especially where there are a large number of samples that require analysis (thousands of clones would need to sequenced for each sample to obtain a true assessment of structure and diversity; discussed in Section 1.9.1). A well known limitation of TRFLP, ARISA and LH-PCR is that unrelated species which share the same sequence length will be represented by a single OTU in the profile (Section 1.9.1). This artefact emerged from the LH-PCR clone libraries presented in this study (Tables 6.2 and 6.3). In addition, the clonal libraries indicated that variations in sequence length can occur within the same species which may result in a single species being represented by more than one OTU. Despite these shortcomings, community DNA profiling methods such as LH-PCR provide a more practical and efficient means (than constructing large clone libraries) of determining whether there are structural characteristics of interest within the soil community under study. Where required, further cloning and sequencing work can be undertaken. Previously LH-PCR has only been applied to analysis of bacterial communities. This study identified ITS2 primers that enable the LH-PCR method to be extended to analysis of fungal communities in environmental samples. A framework for application of the methodology (including multivariate analysis) to identify patterns in fungal communities was demonstrated within the context of *P. cinnamomi* suppressive soils. Overall, the results presented in this chapter do not support the hypothesis that gross fungal community structure is a critically important factor determining a soil’s ability to suppress *P. cinnamomi*. Also, OTU richness and evenness did not appear to be important factors associated with *P. cinnamomi* suppression. However, minor shifts in community structure were observed when suppressive soil was added to a conducive soil and irradiated soils, and this coincided with a reduction in disease severity in avocado seedlings during Experiment 3 (Chapter 3). This minor shift in community structure was partially explained by an increase in the relative abundance of OTUs that were absent or low in relative abundance in the conducive dairy pasture soil and the irradiated soils. This result supports the hypothesis that an abundance of specific, presumably antagonistic, fungi within soil microbial communities is an
important factor determining the ability of the soil to suppress *P. cinnamomi*. Many of these fungi may (at this stage) be unculturable and therefore future research should continue to focus on identifying OTUs and DNA sequence information that represent these fungi. Once these are known discovery of the conditions that promote their presence, abundance and disease suppressive functions should be pursued. The results presented in this study were largely affected by a high level of variability that was due to a number of factors, one of which may have been related to the mass of soil from which the community DNA was extracted. Therefore, future studies involving profiling of soil fungal community DNA may benefit from a more robust sampling strategy that includes DNA extractions from larger soil sub-samples.
Chapter 7

General Discussion

_Phyllophthora cinnamomi_ suppressive soils were first reported in the early 1970s in an avocado orchard located on the east coast of Australia (Broadbent and Baker 1974a). Since then, there has been ongoing interest in understanding the processes that result in _P. cinnamomi_ suppression. In Australia, research to determine these processes has mostly been undertaken in native plant communities (Chapter 1). Elsewhere, the focus has been on avocado production systems with a significant proportion of work undertaken in California (USA) and South Africa (Chapter 1). Prior to this study, naturally-occurring _P. cinnamomi_ suppressive avocado soils within the Australian context had only been investigated by Broadbent and Baker (1974a; 1974b) and then almost 2 decades later by Stirling et al. (1992). You and Sivasithamparam (1994; 1995) and You et al. (1996) also investigated _P. cinnamomi_ suppressive mulches applied to avocado orchards in Western Australia. From these and other studies, _P. cinnamomi_ suppression was known to be biologically mediated in nearly all cases where _P. cinnamomi_ suppression was observed (Section 1.7).

Downer et al. (2001a; 2001b) implied that cellulase and laminarinase were important in general suppression of _P. cinnamomi_ induced by applying organic mulches under avocado trees, but their results only provided associative evidence and the direct relationship between these enzymes and _P. cinnamomi_ suppression was never confirmed. Microorganisms isolated from various soils were found to antagonise _P. cinnamomi_ in vitro but their role as agents of specific suppression was never confirmed in field studies (Section 1.7). Several studies have provided an inventory of bacteria, actinomycetes and fungi isolated from suppressive soils but these were limited to culturable soil bacteria, actinomycetes and fungi (Sections 1.7.2 and 1.7.3). To overcome limitations imposed by culture-based studies, Yang et al. (2001) used DGGE (Section 1.9) to compare bacterial communities associated with healthy and diseased avocado roots. Borneman and Hartin (2000) designed fungal specific primers and used these to compare fungal communities in 2 avocado soils with differing abilities to suppress Phytophthora root rot. The combined evidence from
these studies indicated that specific metabolic functions (such as the production of cellulolytic enzymes) and soil microbial community structure and diversity (defined in Section 1.9) may be important in *P. cinnamomi* suppression. However, the role of cellulase and laminarinase, and microbial community structure and diversity, in *P. cinnamomi* suppressive soils required further investigation.

In the present study, field surveys and glasshouse assays with soils sampled from 10 avocado orchards and 2 rainforest remnants were undertaken to determine their suppressive capacity and to: i) confirm biological suppression in those soils where suppression was observed, ii) confirm whether *P. cinnamomi* suppression resulted from soil cellulase and laminarinase activities and iii) determine whether microbial community structure and diversity (defined in Section 1.9) were important attributes of the *P. cinnamomi* suppressive soils examined during this study. The principal findings of the study were: i) identification of biological suppression in some eastern Australian avocado orchard soils, ii) that cellulase and laminarinase activities did not appear to be the primary mechanisms involved in suppression, iii) neither the gross community structure nor diversity of soil bacteria or fungal communities appeared to be critically important in *P. cinnamomi* suppression and iv) that several bacterial OTUs and at least one fungal OTU were associated with transferred suppression during Experiment 3. Key findings relating to enzyme activities and bacterial and fungal community structure and diversity have been summarised in Table 7.1.

Early in this project, 58 avocado growers within the south-eastern Qld and north-eastern NSW regions of Australia were informally interviewed by telephone to aid in identifying locations with soils potentially suppressive or conducive to *P. cinnamomi*. Using leads provided by growers, 10 avocado orchards and 2 rainforest remnants were selected for a suppressive soil screening survey. Soils sampled from each location varied in their ability to reduce the severity of root rot in lupins during a glasshouse bioassay. Disease severity was consistently low (DSR <3) in lupin seedlings grown in soil samples from 5 orchards (Section 3.3.2). Four of these orchards were sampled for a second glasshouse bioassay to confirm that the soils were suppressive and that suppression resulted from biological factors. On this occasion, a sub-sample from each soil sample was γ-irradiation sterilised to eliminate biological activity. Once again, non-irradiated soil samples from each of the orchards
Table 7.1 Summary of key findings from this study

<table>
<thead>
<tr>
<th>Key findings</th>
</tr>
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<tbody>
<tr>
<td><strong>Biological suppression of <em>P. cinnamomi</em></strong></td>
</tr>
<tr>
<td>• Several avocado orchards with <em>P. cinnamomi</em> suppressive soils were identified</td>
</tr>
<tr>
<td>• γ-irradiation of soils destroyed each soil’s ability to suppress <em>P. cinnamomi</em>, thus confirming biological suppression in 4 avocado orchard soils</td>
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<td>• Suppression was observed in the presence of both lupins and avocado seedlings</td>
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<td>• Suppression was partially transferred by mixing irradiated and conducive soils with 10% suppressive avocado soils</td>
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<td><strong>Involvement of cellulase and laminarinase in <em>P. cinnamomi</em> suppression</strong></td>
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<tr>
<td>• Cellulase and laminarinase activity was not associated with disease severity in lupins</td>
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<tr>
<td>• Reductions in disease severity achieved by mixing irradiated and conducive soils with suppressive soils was not accompanied by increased enzyme activities</td>
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<td>• Cellulase and laminarinase added to sterile sand did not reduce root rot in lupins and was phytotoxic at higher concentrations</td>
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<tr>
<td><strong>Structure and diversity of bacterial communities in <em>P. cinnamomi</em> suppressive soils</strong></td>
</tr>
<tr>
<td>• Neither suppressive nor conducive soils had characteristic 16S rDNA LH-PCR profiles</td>
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<td>• 16S rDNA LH-PCR discriminated between avocado soils and a dairy pasture soil</td>
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<td>• Minor shifts were observed in the structure of bacterial communities in response to mixing conducive and irradiated soils with suppressive soils</td>
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<td>• Several OTUs were unique to the suppressive soils and were associated with decreased disease severity in avocado seedlings grown in irradiated and conducive soils mixed with suppressive soil treatments</td>
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<td>• Bacterial diversity was moderate to high in both suppressive and conducive soils</td>
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<td>• Decreases in disease severity in irradiated and conducive soils mixed with suppressive soil treatments were not accompanied by changes in bacterial diversity</td>
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<tr>
<td><strong>Structure and diversity of fungal communities in <em>P. cinnamomi</em> suppressive soils</strong></td>
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<tr>
<td>• Neither suppressive nor conducive soils had characteristic ITS2 LH-PCR profiles</td>
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<tr>
<td>• ITS2 LH-PCR discriminated between avocado soils and a dairy pasture soil</td>
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<td>• Structure of fungal communities shifted slightly in response to mixing conducive and irradiated soils with suppressive soils</td>
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<tr>
<td>• At least one OTU was abundant in the suppressive soils, less abundant in the conducive soil and was associated with decreased disease severity in avocado seedlings grown in irradiated and conducive soils mixed with suppressive soil treatments</td>
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<tr>
<td>• Fungal diversity was moderate to high in both suppressive and conducive soils</td>
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<tr>
<td>• Decreases in disease severity in irradiated and conducive soils mixed with suppressive soil treatments were not accompanied by changes in fungal diversity</td>
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<tr>
<td>• ITS2 LH-PCR data were highly variable possibly due to the small sample size used for DNA extractions</td>
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performed well in terms of reducing disease severity in lupins, and disease severity in lupins increased significantly in response to γ-irradiation of these samples (Section 3.3.3). These findings were consistent with published studies that have reported loss of *P. cinnamomi* suppression in response to pasteurising or sterilising suppressive soils using steam / heat, fumigation or γ-irradiation (Section 1.7).

In the third glasshouse experiment, the same 4 orchard soils were sampled and portions of each sample were γ–irradiated. A dairy pasture soil, found to be conducive in preliminary work, was also included in the experiment. The irradiated soils and the conducive pasture soil were mixed with the suppressive avocado orchard soils to give 10% suppressive soil. Each soil was inoculated with *P. cinnamomi* and each pot was planted with avocado seedlings. Each of the 4 suppressive avocado orchard soils yielded low DSRs. Biological suppression was confirmed with disease severity increasing significantly after these soils were γ–irradiated. High DSRs recorded for the pasture soil confirmed the conduciveness of this soil. A reduction in disease severity in response to mixing the conducive and irradiated soils with suppressive soils indicated that suppression was partially restored in the irradiated soil and was partially transferred to the conducive soil. This finding was consistent with Malajczuk et al. (1977), who demonstrated that *P. cinnamomi* suppression was restored to soils treated at 100°C by adding a small amount of suppressive soil. The findings were also consistent with studies of other soilborne plant pathogens (e.g. *G. graminis* and *F. oxysporum*) which have demonstrated that suppression can be restored in sterile soil or transferred to a conducive soil by adding suppressive soil (Alabouvette et al. 1979; Hornby 1979).

Cellulase and laminarinase activities were measured in soils from the 4 orchards sampled for the second glasshouse assay. However, there was no indication that the activities of either enzyme were related to disease severity. During the third glasshouse assay, decreases in disease severity in the irradiated mixed with suppressive soil treatments and the conducive mixed with suppressive soil treatments were not accompanied by significant increases in enzyme activity. Together, these results indicate that there was no relationship between cellulase and laminarinase activities and *P. cinnamomi* suppression. These findings contrast with those of Downer et al. (2001a) which indicated an association between activities of both
enzymes and significant decreases in *P. cinnamomi* inoculum and avocado root infection under organic mulches. Application of organic mulches are known to increase microbial and enzyme activities within the mulch itself and also within the top few centimetres of the soil (Nesbitt et al. 1979; Hankin 1982; Henis 1986; You and Sivasithamparam 1994; 1995; Yang et al. 2003; Yao et al. 2005). There is also a body of evidence suggesting that the application of various organic materials may contribute toward inducing *P. cinnamomi* suppression (Broadbent and Baker 1974a; Nesbitt et al. 1979; You and Sivasithamparam 1994; 1995; Costa et al. 1996; Aryantha et al. 2000; Vawdrey et al. 2002). Therefore, it is not surprising that Downer et al. (2001a) found an association between higher cellulase and laminarinase activities and lower incidence and severity of avocado root rot within the mulched plots when compared with unmulched soil. However, on their own these findings do not confirm that the occurrence of *P. cinnamomi* suppression is dependent on the level of cellulase and laminarinase activities in the soil.

In a separate series of experiments, Downer et al. (2001b) demonstrated that at certain concentrations (Section 1.8.2) cellulase and laminarinase had a negative impact on the production and viability of various *P. cinnamomi* propagules. However, Downer et al. (2001b) did not calibrate the enzyme concentrations used in their study with the enzyme activities associated with their suppressive mulch (Downer et al. 2001a). Therefore, the relationship between soil enzyme activities and concentrations used in their laboratory experiments was not clear. This was also the case in the present study in which cellulase and laminarinase were added to sterile sand at concentrations similar to that used by Downer et al. (2001b). At lower concentrations (Section 4.3.4) both enzymes failed to protect lupins from infection by *P. cinnamomi*. Higher concentrations had a phytotoxic effect on the lupin roots. Despite effects on plant growth, *P. cinnamomi* was isolated from lupin root tips grown in pots receiving higher enzyme concentrations. These results indicate that the level of cellulase and laminarinase activity required to degrade *P. cinnamomi* propagules will also affect plant growth. Based on these data, it appears unlikely that *P. cinnamomi* suppression results from an accumulation of these enzymes in soil. However, the industrial grade enzyme preparations used in this study (Section 4.2.4) contained impurities which may have been responsible for the effects on plant growth. Future studies undertaken with the aim of determining the role of cellulase
and laminarinase in suppressive soils should include similar pot trials to those reported here but enzyme preparations of a higher purity should be used at concentrations calibrated to that measured in suppressive and conducive soils.

While the results presented in this study provide little support for the hypothesis that cellulase and laminarinase are principal mechanisms involved in *P. cinnamomi* suppressive soil (Downer et al. 2001a; 2001b), the hypothesis should not be rejected. There is certainly sufficient evidence from *in vitro* studies that cellulolytic metabolites produced by a number of bacteria and fungi affect *P. cinnamomi* reproduction and survival (Section 1.8). For example, cellulolytic *Trichoderma* spp. are known to parasitise and lyse *Phytophthora* hyphae (Malajczuk 1983) and stimulate *Phytophthora* to form homothallic oospores (Pratt et al. 1972; O’Brien 1991), and cellulolytic bacteria such as *Bacillus*, *Pseudomonas*, *Rhizobium*, *Flavobacterium* and several actinomycetes have been isolated from and observed swarming on hyphae and abortive sporangia (Broadbent and Baker 1974a; Malajczuk et al. 1983; Mass and Kotze 1989; Stirling et al. 1992). Therefore, it is possible that β-1,3- and β-1,4 glucanases, and possibly other microbial metabolites (e.g. Brown et al. 1987; Budi et al. 2000), are involved in antagonism when the cellulolytic antagonist is in direct or close contact with *P. cinnamomi* propagules, but may be too dilute in the universal soil matrix to be specifically responsible for the phenomenon of *P. cinnamomi* suppressive soil. This implies that soil cellulases and laminarinases may just be 2 contributing factors among many that ultimately result in general suppression of *P. cinnamomi*.

Many of the studies that have provided insight into the structural organisation of microbial communities in *P. cinnamomi* suppressive soils have been culture-based (Sections 1.7 and 1.9). However, the results from these studies were limited to analysis of <1% to 10% of the soil microbial community (Atlas and Bartha 1997; Head et al. 1998) and were most likely biased toward readily culturable groups. Current understanding concerning microbial community DNA analytical techniques indicates that, while there may be some bias associated with preferential amplification during polymerase chain reaction (Suzuki et al. 1998; Anderson et al. 2003), both culturable and non-culturable organisms can be accessed by direct extraction and PCR amplification of microbial community DNA and, therefore, a
more comprehensive assessment of microbial community structure and diversity can be achieved (van Elsas et al. 1997; Head et al. 1998). In this study, the community DNA profiling method known as LH-PCR was used to analyse both bacterial and fungal communities in *P. cinnamomi* suppressive and conducive soils. This is the first time that this method has been used to profile microbial communities in disease suppressive soil.

The structural characteristics of bacterial communities (determined by 16S rDNA LH-PCR) in soils that yielded low DSRs during the first glasshouse assay (Experiment 1) did not differ significantly from the more conducive soils (Section 5.3.2). Similarly, there was insufficient evidence to suggest that disease severity in lupin seedlings during the same experiment was related to gross fungal community structure (determined by ITS2 LH-PCR) (Chapter 6). These findings do not support those of previous studies (Sections 1.7.2, 1.7.3 and 1.9.2) which indicated that an abundance of several bacterial and fungal strains within microbial communities may be important in the development of *P. cinnamomi* suppressive soils. However, suppression of other soil-borne plant pathogens such as *Pythium* spp. may be influenced by different communities (Kowalchuk et al. 2003) and therefore it is also possible that microbial communities with different taxonomic composition and/or species abundance distribution may influence suppression of *P. cinnamomi*.

The LH-PCR results presented for Experiment 1 (Section 3.3.2) were based on an analysis of microbial communities in bulk soil taken from the feeder root zone beneath avocado trees. Analyses of avocado rhizosphere communities may also have been possible (Yang et al. 2001). However, resource constraints limited the number of analyses that could be undertaken and therefore it was necessary to develop a rationale for deciding whether to target rhizosphere communities or microbial communities within the universal soil matrix. Microbial communities within the universal soil matrix surrounding the root mat were targeted for LH-PCR analysis for the following reasons: i) microbial antagonists identified during culture-based studies were mainly isolated from bulk soil, which indicated that the universal soil matrix potentially harboured agents responsible for *P. cinnamomi* suppression, ii) microbial communities in the universal soil matrix of *P. cinnamomi* suppressive soils had not previously been investigated using community DNA profiling methods, and iii) the
feeder roots from the avocado trees used in this study did not form a rhizosheath thus making collection of rhizosphere soil impractical.

As soil was collected from within close proximity to the root mat it would be expected that the sample would contain rhizosphere microorganisms. However, results from the LH-PCR analysis of bacterial communities in field soils during the present study, contrasted with results from a study by Yang et al. (2001) in which bacterial community DGGE profiles (Section 1.9.1) acquired from healthy avocado roots were similar to each other but distinctly different to those from diseased roots. While the results from this study and the study by Yang et al. (2001) were carried out in different systems using different methods and therefore are not directly comparable, the disparity between the results from the 2 studies indicates that \( P. cinnamomi \) suppression may involve rhizosphere bacteria rather than free-living bacteria within the universal soil matrix. If this is the case, then future studies may benefit from including analyses of both rhizosphere and free-living microbial communities.

Using fungal specific primers to amplify fungal community DNA and cloning and sequencing techniques, Borneman and Hartin (2000) found differences in the composition of fungal communities in bulk soil samples from beneath healthy avocado trees and from beneath diseased trees. However, Borneman and Hartin (2000) compared a limited number of sequences from only 2 avocado orchards, which compromised the robustness of their findings. In the present study, fungal community DNA was analysed in soil from 10 avocado orchards and 2 rainforest remnants. The only discriminating variables for these were associated with some of the sampling locations. However, it should be noted that high levels of variability encountered in the ITS2 LH-PCR data reduced the discriminatory power of the method. During the third glasshouse experiment, the variability encountered with field samples collected for the first glasshouse assay persisted. Despite this, ITS2 LH-PCR was able to discriminate between the dairy pasture soil and the avocado orchard soils, especially when measured at week 14. The fact that the method was able to discriminate between soils of the same type (red ferrosol) but supporting different plant production systems (i.e. pasture vs. avocado trees) indicates that the
method had the capacity to detect gross differences between soil samples in Experiment 1 based on low or high DSRs if such differences had existed.

Data from the bacterial 16S rDNA LH-PCR method were more reliable and clearly demonstrated differences in bacterial community structure in the dairy pasture soil and the avocado soils at each time point during Experiment 3 (Section 3.3.4). Mixing the suppressive avocado soils with the conducive dairy pasture soil and the irradiated soils resulted in a minor shift in the overall structure of the bacterial communities in these treatments toward the 16S rDNA LH-PCR profiles from the suppressive soils. Small shifts in community structure in the same direction were also observed for ITS2 LH-PCR profiles for the same treatments. This indicates that bacteria and fungi from the suppressive soil established or increased in abundance in the irradiated and conducive soils. This coincided with decreased disease severity in the mixed soil treatments. Therefore, it is reasonable to suggest that these colonising bacteria and fungi may have been involved in the development of suppressive conditions within the mixed soil treatments.

SIMPER analysis was useful in identifying OTUs that contributed most to differences between treatment groups and also those OTUs that contributed to shifts in community structure. Several bacterial OTUs that were absent in the conducive soil and the irradiated soils were transferred to the conducive soil or were restored in the irradiated soil through addition of suppressive soil. This indicates that there were specific bacteria represented by these OTUs that were associated with transferred suppression. Only one fungal OTU that was 297 bp in length was present in the suppressive soil, absent in the conducive soil (at week 14 only) and established and increased in abundance in the mixed soil treatments. Cloning and sequencing of fungal amplicons was carried out for one suppressive avocado soil during assessments of primers for use in fungal community LH-PCR. This confirmed that the selected primers were fungal specific and also allowed inferences with regard to the possible phylogenetic identity of fungal OTUs of interest. A clonal sequence of 297 bp was detected in the clone library and was most closely matched to *V. caudatus*. However, this clonal sequence only shared 79% similarity with *V. caudatus* and therefore was more likely to belong to an unknown fungal taxon. Only 2 other clonal sequences were of interest due to their similarity with fungal
sequences from genera known to antagonise *P. cinnamomi* (Section 1.7.3). These were the clonal sequences of 294 bp, which was 97% homologous with *P. brevicompactum*, and 303 bp, which shared 91% sequence similarity with *M. verrucaria*. Whether the fungi and bacteria represented by the OTUs associated with decreased disease severity in Experiment 3 were directly responsible for suppression could not be determined during this study.

Neither bacterial nor fungal diversity (assessed by OTU richness and by Shannon and Simpson’s diversity indices) appeared to be critically important in determining disease severity in lupins or avocado test plants during the first and third glasshouse assays. Species richness (number of OTUs in the LH-PCR profile), Shannon diversity index and Simpson’s diversity (evenness) index did not differ significantly between any of the main treatment groups. On average, Shannon diversity was moderate to high and Simpson’s index was mostly high for soils that yielded both low and high DSRs. According to the results from this study, it appears that moderate to high microbial diversity is a characteristic of *P. cinnamomi* suppressive soils but can also be characteristic of more conducive soils. This contrasts with studies that have shown that higher microbial diversity is an attribute of soils that suppress other soil-borne phytopathogens such as *R. solani* (van Elsas et al. 2002; Garbeva et al. 2006). However, while maintaining high diversity in soils would be expected to provide a higher potential for general suppression than a soil that has extremely low microbial diversity, evidence from several culture-based studies (Baker 1978; Malajczuk and McComb 1979; Mass and Kotze 1989) suggests that population densities of specific bacteria (including actinomycetes) and fungi that are antagonistic toward *P. cinnamomi* may be more important than species richness.

While the results from the LH-PCR method presented here do not provide a comprehensive description of soil microbial communities in *P. cinnamomi* suppressive and conducive soils, they do demonstrate the potential for molecular methods such as LH-PCR to detect constituents of microbial communities that cannot be detected with existing culture-based approaches. With adequate resources, LH-PCR and other community DNA profiling techniques could be applied to provide a detailed description of microbial communities within *P. cinnamomi* suppressive and conducive soils. However, as revealed during this study in relation to variability
in the LH-PCR data (especially the ITS2 LH-PCR), molecular community DNA profiling methods have a number of technical limitations that require attention to improve their reliability. The variability encountered in the ITS2 LH-PCR data during this study was attributed to 2 major contributing factors. The first of these is related to the variable uptake of samples by the ABI 3730 capillary tubes (Section 6.4). This is an inherent issue with the ABI 3730 that can only be resolved by the manufacturer. In this study, the problem was ameliorated by normalising the data by calculating relative ratios of peak height to total fluorescence and then using a minimum relative ratio cut-off (1% for 16S rDNA LH-PCR data and 0.5% for IT2 LH-PCR data). The second contributor to variability was possibly attributed to extraction sample size (Section 6.4). Future applications of soil fungal community ITS2 LH-PCR in similar studies may benefit from extracting community DNA from larger sample volumes than were used in this study (Ranjard et al. 2003 suggested >1g soil).

In conclusion, during this study new locations suitable for studying *P. cinnamomi* suppressive soils were identified on the east coast of Australia. This study also provided further evidence for the involvement of biological processes in *P. cinnamomi* suppressive soils. There was little evidence supporting the hypothesis that *P. cinnamomi* suppression is principally caused by an accumulation of cellulase and laminarinase activities in soil. However, other studies have provided evidence for the role of these enzymes in antagonism of *P. cinnamomi* (Section 1.8.2) and therefore their involvement in suppression should be investigated further using approaches suggested in this discussion (see above). The results from bacterial and fungal community LH-PCR analysis indicated that neither gross microbial community structure nor microbial diversity were critically important in *P. cinnamomi* suppressive soils. However, LH-PCR analysis of soil samples collected during Experiment 3 indicated that there were several bacteria and at least one fungal taxon (represented by OTUs) associated with transferred suppression. This implies that *P. cinnamomi* suppression may be influenced by the presence and abundance of specific bacteria and/or fungi.
Future work

Both the community DNA extracted from soil samples and PCR samples amplified from the community DNA during this study have been cryogenically stored (at NSW Department of Primary Industries, Wollongbar) and further analysis can be undertaken using these samples. To determine the identities of bacterial OTUs associated with transferred suppression in Experiment 3, amplicons within the original PCR sample need to be cloned, sequenced and analysed in silico for homology with published sequences. The length (bp) of the sequences can then be compared with the length of OTUs in the LH-PCR profiles to determine which OTUs represented which phylogenies. Inferences made with regard to the phylogenetic identity of fungal OTUs in this study were based on sequences obtained from only one suppressive field sample collected for Experiment 1. Therefore, further cloning and sequencing will also need to be carried out on PCR samples from Experiment 3 to determine the identity of fungal OTUs associated with transferred suppression, the 297 bp OTU in particular. Where bacterial and fungal sequences match culturable organisms, selective media can be used to harvest these from the soil. Interactions between these organisms and *P. cinnamomi* can then be studied in vitro and in vivo and introduced to sterile or conducive soil to determine whether they are agents of *P. cinnamomi* suppression. Colonisation of their populations in the sterile and conducive soils could also be monitored using both culture-based enumeration and LH-PCR analysis. Evidence indicating their increasing dominance in the soil would provide a strong indication for their involvement in *P. cinnamomi* suppression.

Where sequences match unculturable or unknown taxa, demonstrating their role in suppression is made more difficult. Attempts could be made to isolate the organism by baiting with multiple substrates and then comparing sequences from the isolates that grow on the substrates with sequences from OTUs in the LH-PCR profile (a similar approach was used by Yin et al. 2004). Alternatively, further surveys for suppressive soil in other locations combined with LH-PCR (using the same primer sets used in this study) to determine the consistency in the presence and abundance of these OTUs in *P. cinnamomi* suppressive soils may provide some indication of their involvement. Undertaking experiments that involve manipulations to induce biological suppression (e.g. application of various organic amendments: Broadbent and Baker 1974a; Nesbitt et al. 1979; You and Sivasithamparam 1994, 1995; Costa
et al. 1996; You et al. 1996; Downer et al. 1999; Aryantha et al. 2000; Downer et al. 2001a; Vawdrey et al. 2002) and using LH-PCR (with the same primers used in this study) to monitor shifts in microbial populations may provide further evidence for the involvement of the bacteria and fungi represented by the OTUs identified in this study.
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Appendix 1

Draft manuscript

Extraction of Soil Enzymes by Mechanical Disruption
and Microplate Determination of
Soil Cellulase and Laminarinase Activity

The manuscript contained within this appendix will be submitted to the journal Soil Biology and Biochemistry.
Extraction of Soil Enzymes by Mechanical Disruption and Microplate Determination of Soil Cellulase and Laminarinase Activity

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Abstract
Published assays for determining the activities of glucanases in soil almost certainly underestimate the true cellulytic potential of the soil. The colorimetric methods used for quantifying reducing sugars released during enzyme-substrate reactions are also subject to interferences by other soil compounds. In this paper, a new method for extracting soil enzymes is described, followed by a microplate method for assaying soil β-1,4-glucanases (cellulases) and β-1,3-glucanases (laminarinases). Soil samples were mechanically disrupted to produce a crude enzyme extract of which the total protein concentration was determined using a commercially available microplate assay. The enzyme extracts were diluted to standardise the concentration of total protein and then incubated in a microplate containing either CM-cellulose substrate to determine cellulase activity or laminarin substrate to determine laminarinase activity. Enzyme activity was terminated by heating and the resulting glucose was measured using the fluorometric Amplex® Red glucose assay. Soils with high and low enzyme activity were used to evaluate the effectiveness of the method. We found that the method was reproducible, could be completed in one day and measured twice as much enzyme activity than the standard passive soil enzyme extraction procedure. The principles of the new method described in this paper may be useful in measuring other soil enzymes.

Keywords: Soil Enzymes, Enzyme Extraction, β-1,4-glucanase, β-1,3-glucanase, Cellulase, Laminarinase, Amplex Red.
A-1 Introduction

The soil environment is a dynamic ecosystem in which all biochemical processes are controlled by enzymatic reactions (Tabatabai 1994). One of the key functions of soil enzymes is the decomposition of organic matter (Kiss et al. 1978). Soil organic matter is principally comprised of plant, microbe and animal residues, which mainly consist of polymers and polysaccharides. These compounds are broken down by cellulases, laminarinases, xylanases, chitinases and amylases (Wirth and Wolf 1992).

The most abundant plant derived compounds are cellulose and hemicelluloses (Aro et al. 2001; Criquet 2002), with cellulose comprising half of the biomass on earth (Erikson et al. 1990). The degradation of cellulose plays an important role in the carbon cycle (Begün and Aubert 1994), supporting the growth, reproduction and activities of soil microorganisms (Deng and Tabatabai 1994a; Gander et al. 1994). The capacity of a soil to degrade cellulose provides an indication of soil biological activity and biomass turnover potential, which are important measures of soil health in sustainable agriculture. In addition, enzymes that degrade β-1,3-glucan (laminarin) and β-1,4-glucan (cellulose) may be involved in the suppression of soilborne phytopathogens such as Phytophthora cinnamomi Rands (Downer et al. 2001) and Fusarium culmorum Smith (Rasmussen et al. 2002).

There are several methods for estimating cellulase activity in soil. Standard methods typically involve passive extraction of enzymes by incubating the soil suspended in a buffered cellulosic substrate solution such as carboxymethylcellulose (CM-cellulose) (e.g. Schinner and von Mersi 1990; Deng and Tabatabai 1994a; Gander et al. 1994; Criquet 2002) or Avicel (Benefield 1971; Hope and Burns 1987). These extraction methods access the extracellular enzymes that are present in soil as free enzymes released from living cells, disintegrating cells and cell fragments. However, enzymes are also bound to cell constituents and organic compounds within the soil matrix and are present within both proliferating and non-proliferating cells (Tabatabai 1994). Therefore, it is highly likely that passive approaches to extracting enzymes from soil result in an underestimate of the soils true enzymatic potential. This limitation is of particular importance in soils where enzyme activity is below the chosen assay limits of sensitivity or in soils where, at the time of sampling, microbial synthesis of
extracellular enzymes is affected by limiting factors such as soil moisture and temperature.

Following the combined extraction-substrate hydrolysis step of the standard approach, cellulase activity is typically estimated by using one of several colorimetric methods to determine reducing sugars (which mostly consist of glucose with smaller fractions of cellobiose and other higher molecular weight oligosaccharides - Deng and Tabatabai 1994a) released during the reaction (e.g. Deng and Tabatabai 1994b). Colorimetric methods for determining reducing sugars commonly involve phenol-sulphuric acid, anthrone-sulphuric acid, dinitrosalicylic acid (DNS), alkaline copper, ferric-ferrocyanide (Dygert et al. 1965; Schinner and von Mersi 1990; Deng and Tabatabai 1994b; Gander et al. 1994) or a glucose oxidase:peroxidase system (Benefield 1971). As discussed by Gander et al. (1994) and Deng and Tabatabai (1995) these methods vary with respect to sensitivity and reliability and all are affected by interferences from within the soil-enzyme complex including pigments, trace elements, and organic matter. In their multi-enzyme microplate colorimetric assay, which includes assays for both cellulases and laminarinases, Wirth and Wolf (1992) took an alternative approach by agitating soil in a buffer solution and then transferring aliquots of the supernatant to microplate wells containing soluble remazol brilliant blue (RBB) dye-labelled substrates. While a useful innovation, Wirth and Wolf’s (1992) approach still relies on passive extraction of free enzymes and a colorimetric based assay.

A possible solution to issues associated with colorimetric assays is the use of fluorometric methods. One such method is the Amplex© Red glucose assay developed and supplied in kit form by Molecular Probes (USA). During the Amplex Red glucose assay glucose oxidase reacts with D-glucose to form hydrogen peroxide (H₂O₂) which, in the presence of horseradish peroxidase (HRP), oxidises the Amplex Red reagent to generate highly fluorescent red-resorufin (Zhou et al. 1997; Molecular Probes 2002). Fluorescent resorufin produced during the reaction is determined using excitation wave lengths near 530 nm and emission maxima of approximately 590 nm. Amplex Red is photo- and chemically stable, which allows the reaction to occur with minimal background. Due to the spectral properties of the oxidised resorufin product from the Amplex Red reagent, the assay avoids interferences from
autofluorescence when testing biological and environmental samples (Zhou et al. 1997; Molecular Probes 2002). Therefore, the assay should circumvent issues affecting colorimetric methods for estimating reducing sugars released during substrate hydrolysis.

Our aim was to develop a cellulase and laminarinase assay that: (1) maximises access to both free and immobile enzymes by using a disruptive strategy to extract enzymes from the soil matrix, (2) utilises the Amplex Red glucose assay to quantify glucose liberated following substrate hydrolysis, and (3) can be completed in a day and allows high throughput analysis. To address these aims we developed a method based on mechanical disruption for isolating enzymes from the soil matrix. We also adapted the enzyme-substrate reaction step, used in standard cellulase assays (Deng and Tabatabai 1994a), so that it could be carried out in smaller volumes in microtitre plates. Glucose produced following hydrolysis of CM-cellulose and laminarin substrates was measured using the Amplex Red glucose microplate assay kit. The enzyme assays developed in this study were evaluated using a dairy pasture soil and a garden soil found to have low and high (respectively) cellulase and laminarinase activities during preliminary work using the standard passive extraction approach (Deng and Tabatabai 1994a). These 2 soil samples were selected to facilitate an assessment of assay performance under conditions of both high and low enzyme activity.

A-2 Materials and Methods

A-2.1 Soil samples

Two soil samples were collected from the NSW Department of Primary Industries Wollongbar Agricultural Institute (WAI), which is located on the Alstonville Plateau, North Coast region, NSW, Australia. Both sampling points occurred on red Ferrosol (previously red Krasnozem; Isbell 1996) soil of the Wollongbar series. The red Ferrosols of the Wollongbar series are typically deep, well drained clay loam to light-medium clay soils derived from weakly weathered basalt (Morand 1994). One sample (high enzyme activity sample) was collected from a long-established garden with a thick layer of leaf litter (~ 15 cm). This sample was taken by removing the
surface litter and scraping at the soil-mulch interface to a depth of ~ 2 cm. The second soil sample (low enzyme activity) was taken from the centre of a large bare-soil patch within a dairy pasture to a depth of 10 cm. Each soil sample was sieved (using a 2 mm mesh) immediately after sampling. The soil was stored in plastic bags at 4°C overnight and then returned to room temperature before extractions were carried out the following day.

A-2.2 Soil enzyme extractions

Enzymes were released from the soil matrix and from microbial cells within the soil matrix by mechanical disruption. For each soil sample, 300 mg soil was transferred to autoclave sterilised 2 mL plastic screw-cap tubes (Scientific Specialities Incorporated, USA) containing 0.8 g of 1 mm acid washed ceramic beads (Saint Gobain, USA) and 0.3 g acid washed 0.1 mm glass beads (Sigma, USA). To each tube, 600 µL of sterile sodium phosphate buffer (0.1 mol L⁻¹; pH 7.0) was added and tubes were briefly vortexed. The soil sample was then disrupted by bead-beating using a FP120 FastPrep© Instrument (Bio101-Savant Instruments, USA).

Six processing times were assessed by varying the number of processing cycles (1-6 cycles). A processing cycle is defined as 30 s of bead-beating at 5.5 m s⁻¹ followed by 1 min incubation in an ice bath. Tubes were placed on ice to avoid over-heating and inactivation of the enzymes in the extract. Three replicate extractions of each soil sample were prepared for each number of processing cycles. A zero cycle time served as a blank control for protein estimations (Section A-2.3). Following bead-beating, the tubes were centrifuged (Model 5810R, Eppendorf, Germany) at 4°C and 13,000 revs min⁻¹ for 5 min. Approximately 300 µL of supernatant (crude enzyme extract) was then transferred to sterile 1.7 mL micro-centrifuge tubes (Astral Scientific, Australia) and stored on ice while preparing for protein estimations and enzyme assays reactions.
A-2.3 Protein assay

The protein content of enzyme extracts was determined by using a commercially available bicinchoninic acid (BCA) protein assay reagent kit (Pierce Products, USA) in accordance with the microplate procedure described in the manufacturer’s instructions. The BCA indicator reagent was prepared by mixing 50 parts BCA reagent A (contains sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 mol L\(^{-1}\) sodium hydroxide) with 1 part BCA reagent B (contains 4% cupric sulphate). To each well of a 96 well clear microplate (350 µL wells; Greiner Bio-one, Germany), 25 µL of crude enzyme extract was added to 200 µL of the BCA indicator reagent. Bovine serum albumin (BSA) standards, BSA positive controls (500 µg protein mL\(^{-1}\)), negative controls (no BSA or soil enzyme extract) and zero cycle controls (Section A-2.2) were also added to wells containing 200 µL of the BCA indicator reagent. Enzyme extracts and controls were measured in duplicate on the same plate.

The microplate was sealed and incubated at 37°C for 30 min on an orbital shaker at 180 revs min\(^{-1}\) (Bio-Line, Edwards Instruments, Australia). After standing at room temperature for 10 min, protein concentration was measured against a standard curve using an automated microplate reader (Fluorstar Galaxy, BMG Labtechnologies, Germany) fitted with a 570 nm absorbance filter. The BSA standard curve was constructed using concentrations from 0 µg mL\(^{-1}\) to 2000 µg mL\(^{-1}\) in sodium phosphate buffer (0.1 mol L\(^{-1}\); pH 7.0). Net protein yield was calculated by averaging values for sample duplicates and subtracting average values for the respective zero cycle controls.

A-2.4 Enzyme-substrate reactions

Enzyme-substrate reactions were standardised by diluting the crude enzyme extracts so that each reaction received an equivalent quantity of total protein. Enzyme extracts containing 30 µg protein were diluted with sodium phosphate buffer (0.1 M; pH 7.0) to a final volume of 125 µL and then transferred to a microplate well. This was repeated for each replicate extract (i.e. 1 reaction for each replicate enzyme extract).
For the cellulase assays, 125 µL 2% (w/v) CM-cellulose (low viscosity; Sigma, USA) in 50 mmol L$^{-1}$ acetate buffer (pH 5.5; Deng and Tabatabai 1994a) was added to each well (final concentration 1% CM-cellulose). For the laminarinase assays, 125 µL of 0.2% w/v laminarin (Sigma, USA) in 50 mmol L$^{-1}$ acetate buffer (Downer et al. 2001) was added to each well (final concentration 0.1% laminarin). Sodium nitrite (1 mg mL$^{-1}$) was added to each substrate solution to inhibit microbial growth during the reactions (Wirth and Wolf 1992). Microplates were sealed and incubated at 50°C on an orbital shaker at 180 revs min$^{-1}$. Eight incubation times were assessed including: 30 min; 1 h and then hourly up to 6 h; and then at 24 h. Enzyme-substrate reactions were terminated simultaneously by placing the microplates in a dry oven at 90°C for 30 min. The microplates were then centrifuged (Eppendorf 5810R centrifuge, Eppendorf, Germany) for 10 min (4000 revs min$^{-1}$) at 4°C, and 200 µL of supernatant was transferred to sterile 1.7 mL micro-centrifuge tubes (Astral Scientific, Australia). Completed reactions were placed on ice until glucose yield was determined. Heat inactivated (90°C for 30 min) enzyme extracts were used as blank controls to determine the background levels of glucose in the soil. Background glucose levels were subtracted prior to calculating sample glucose yield.

**A-2.5 Amplex Red glucose assay**

Enzyme activity was determined by measuring the amount of glucose liberated during substrate hydrolysis. Glucose yields were measured using an Amplex Red glucose assay kit according to the manufacturer’s instructions with only minor modifications. Aliquots of 50 µL from terminated enzyme reactions, enzyme inactivated controls, glucose standards and a negative control (no glucose) were dispensed into microplate wells (black microplate used; Bioline, Edwards Instruments, Australia) and mixed with 50 µL working solution (100 µmol L$^{-1}$ Amplex Red reagent, 0.2 U mL$^{-1}$ HRP and 2 U mL$^{-1}$ glucose oxidase in 1 x reaction buffer - supplied with kit). The plates were covered and incubated in the dark at room temperature for 30 min. Samples with glucose concentrations higher than the assay detection limits were diluted 1 in 2 with 1 x reaction buffer and re-assayed. A positive glucose control of known concentration was always included to monitor reproducibility between runs.
Fluorescent resorufin was measured against a glucose standard curve using an automated fluorescence microplate reader (Fluorstar Galaxy, BMG Labtechnologies, Germany) fitted with a 544 nm excitation filter and a 590 nm fluorescence emission detection filter. Glucose standards (0 to 27 µg mL\(^{-1}\)) were prepared in 1 x reaction buffer (supplied with kit) and subjected to the same assay conditions as the samples. A 4 parameter fit (quadratic regression) was used to calculate sample glucose concentrations against the standard curve. Glucose yield for each sample was calculated after subtracting the respective deactivated enzyme extract control. For the purpose of method evaluations, enzyme activity data were expressed as µg glucose mL\(^{-1}\) supernatant from the completed reaction. During routine assays soil enzyme activity was presented as normalised units of µg glucose g\(^{-1}\) dry soil h\(^{-1}\).

A-2.6 Comparison with passive extraction method

Passive extractions were carried out for the purposes of comparison with the mechanical disruption extraction method. Soil was sampled from the same garden as described in Section A-2.1, however, on this occasion the mulch layer was removed and the soil was sampled to a depth of 5 cm. The mechanical disruption extraction method was carried out as described in Section A-2.2 and the enzyme-substrate reaction step for these extracts was carried out as described in Section A-2.4. Four replicate extractions were performed for both the mechanical and passive extraction methods and glucose produced during the enzyme-substrate reactions were determined using the Amplex Red glucose assay as described in Section A-2.5.

The passive extraction method (Deng and Tabatabai 1994a) was carried out as follows: 5 g of each soil sample was added to 20 mL buffered 2 % CM-cellulose substrate solution (prepared as described above) in 50 mL plastic centrifuge tubes (Sarstedt, Australia). After 4 h incubation at 50ºC on an orbital shaker at 180 revs min\(^{-1}\) (Bioline, Edwards Instruments, Australia), the tubes were centrifuged (Model GT-70, Spintron, Australia) at 4000 revs min\(^{-1}\) for 5 min. A 250 µL aliquot of the supernatant from each extract was transferred to a microplate and the reactions were terminated simultaneously by heating the microplate in a dry oven at 90ºC for 30 min. Negative controls were similarly set up, except that no CM-cellulose substrate was present (i.e. 5 g soil in 20 mL acetate buffer).
A-3 Results

A-3.1 Mechanical enzyme extraction efficiency

For the garden soil (Figure A-1), increasing the number of processing cycles above 1 increased the amount of protein extracted during mechanical disruption of soil samples. However, the amount of protein extracted from 2-4 cycles was similar, with only minor gains from additional cycles. For the dairy pasture soil (Figure A-2), protein yield increased during 1-3 cycles, and 4-6 cycles yielded more but the gains were only minor. After only 1 processing cycle the garden soil yielded 771 µg protein mL$^{-1}$ extract, almost 9 times more protein than the dairy pasture soil (88 µg mL$^{-1}$). Six processing cycles yielded 1430 and 440 µg protein mL$^{-1}$ enzyme extract for the garden (Figure A-1) and dairy pasture (Figure A-2) soils, respectively.

![Figure A-1](image_url)  
*Figure A-1* Effect of processing time (number of processing cycles) on protein yield during mechanical extraction of enzymes from the garden soil. Data points represent the mean of 3 replicate extractions. Horizontal bars represent lowest and highest values among the 3 replicate extractions.
Figure A-2 Effect of processing time (number of processing cycles) on protein yield during mechanical extraction of enzymes from the pasture soil. Data points represent the mean of 3 replicate extractions. Horizontal bars represent lowest and highest values among the 3 replicates extractions.

A-3.3 Glucose standard curve

The relationship between the concentrations of glucose used in the standard curve and fluorescence emission at 590 nm was always strong (e.g. Figure A-3; $r^2 = 0.998$). This indicated that sample glucose concentrations within the standards range (0 - 27 µg glucose mL$^{-1}$) could be determined by quadratic regression.
Figure A-3  Typical standard curve for the Amplex Red glucose assay measured using a 544 nm excitation filter and a 590 nm fluorescence emission detection filter.

A-3.2 Cellulase reactions

Cellulase activity was considerably greater in the garden soil than in the dairy pasture soil (Figure A-4). Glucose yield from the garden soil reactions increased linearly for the first 5 h of the assay. Glucose yield from the dairy pasture soil increased linearly for the first 6 h of the assay. For both soils, glucose yield peaked after 6 h incubation (Figure A-4) and dropped to below detection limits by 24 h (data not shown).
Figure A-4 Cellulase activity (glucose yield) measured for the garden soil and the dairy pasture soil extracts over 6 h. Data points represent mean glucose yield of 3 replicate enzyme reactions per sampling time and bars represent standard error of the mean.

A-3.3 Laminarinase reactions

Laminarinase activity in both soils followed a similar trend to the cellulase reactions for the first 5 h of the assay, with linear increases in glucose yield that were greater for the garden soil extract than for the dairy pasture soil extract (Figure A-5). In contrast to the cellulase assay, glucose yield in the garden soil began to decline after 5 h and glucose yield in the dairy pasture soil continued to increase up to 24 h (data not shown). By 24 h, the completed reaction supernatant for the garden soil contained only 5 µg glucose mL\(^{-1}\) whereas the pasture soil supernatant contained 17 µg glucose mL\(^{-1}\).
### A-3.4 Comparison with standard approach

The combined passive extraction/enzyme-substrate reaction method of Deng and Tabatabai (1990) yielded 15.3 µg glucose g\(^{-1}\) dry soil h\(^{-1}\) (range 13.6 – 17.4 µg glucose g\(^{-1}\) dry soil h\(^{-1}\)) from the garden soil. Extraction by mechanical disruption of the garden soil followed by a separate enzyme-substrate reaction assay yielded 31.0 µg glucose g\(^{-1}\) dry soil h\(^{-1}\) (range 30.5 – 31.8 µg glucose g\(^{-1}\) dry soil h\(^{-1}\)).

### A-4 Discussion

Our approach to extracting soil enzymes using mechanical disruption, standardising enzyme-substrate reactions by using equivalent total protein concentrations in each reaction, and assaying glucose produced during the reaction using fluorometric detection, was effective in measuring enzymes in soils with contrasting levels of cellulase and laminarinase activities. We found the method to be reproducible with a 10% relative percentage difference between cellulase assay replicates (9 replicate
soil enzyme extractions with 3 replicate assays per extract) with the lowest and highest values and mean cellulase activity having a coefficient of variation of 7% (data not shown). When compared with the passive extraction approach our method doubled the estimate for soil enzyme activity based on glucose yield g⁻¹ dry soil h⁻¹ for one soil sample. This increased sensitivity was most likely a result of the mechanical extraction procedure, which we hypothesise maximises access to both free and immobile enzymes.

The mechanical soil enzyme extraction method described in this study is similar in principle to mechanical lysis methods used to extract nucleic acids from soil. When extracting nucleic acids from soil using mechanical lysis methods, chemical buffers are used and processing times are kept short to minimise shearing damage (Tein et al. 1999; Bürgmann et al. 2001). The success of the enzyme-substrate reactions during this study indicates that the hydrolytic activities of cellulase and laminarinase enzymes were not affected by mechanical disruption. However, heat is generated as a result of bead-beating and therefore during extended, discontinuous processing times excessive temperature could potentially inactivate the enzymes (Bohinski 1979). To overcome this, we split total processing times into consecutive cycles with each cycle consisting of 30 s bead-beating followed by 1 min incubation in an ice bath.

Total protein yield resulting from the mechanical soil enzyme extraction method increased substantially with more than 1 processing cycle, with the highest mean yield achieved after 6 processing cycles for both the garden soil and the dairy pasture soil. However, only small increases in mean protein yield were achieved after 2 cycles for the garden soil and 3 - 4 cycles for the pasture soil. While this appears to be the ideal number of processing cycles for the soils used in this study, the optimum number of cycles may vary for different soil types and this should be determined before using the extraction procedure in comparative investigations.

Standard cellulase assays based on Schinner and von Mersi (1990) typically involve enzyme-substrate reactions lasting 24 h using a 0.7% CM-cellulose solution (0.35% final reaction concentration). Using a 2% CM-cellulose solution, Deng and Tabatabai (1994a) reported linear increases in glucose yield over 7 d. This indicates that, for 24 h reactions, CM-cellulose concentrations ranging between 0.35% and 2% do not
limit the velocity of the enzyme reaction or result in a build up of reaction products (i.e. cellobiose and glucose) sufficient to inhibit the reaction. We were therefore surprised that the amount of glucose produced during our cellulase reactions showed a linear increase up to 5 h for the garden soil and 6 h for the dairy pasture soil, but by 24 h glucose concentrations were below detection limits for both soils.

Disappearance of glucose from the enzyme reaction between 5 - 6 and 24 h may be attributable to several causes (e.g. failure of the sodium nitrite to inhibit microbial activity in the enzyme reaction and/or glucose catalysis through the action of other enzymes in the crude preparations). Without conducting further experimentation it would be difficult to determine the source of this phenomenon. However, as glucose production was linear from 0 to 5 - 6 h when CM-cellulose and laminarin were used as substrates, it would be possible to avoid this problem by adopting a shorter reaction time. The risk of error increases with increasing incubation time for soil cellulase reactions, and for this reason, shorter reaction times should result in improved reliability (Deng and Tabatabai 1994a). Downer et al. (2001) used 4 h reactions successfully to determine soil cellulase and laminarinase activity using a modification of the Schinner and von Mersi (1990) method. Kanazawa and Miyashita (1986) found that mixing 10 g of soil with 20 mL of 1% CM-cellulose resulted in glucose yields steadily increasing up to 20 h, with the velocity of glucose production slowing but still increasing slightly up to 40 h. They concluded that the optimum incubation time for their assay was in the linear phase between 4 h and 5 h incubation with 4 h incubation recommended for routine applications. This recommendation is supported by our results. However, glucose production continued to increase up to 24 hour during pasture soil enzyme extract-laminarin reactions which indicates that longer reaction times may be useful if soil enzyme extracts with extremely low enzyme activity are encountered. Results from different reaction times can be compared if the data are normalised on a dry soil mass and reaction time basis (i.e. glucose yield g\(^{-1}\) oven dry soil h\(^{-1}\)).

Using the Amplex Red fluorometric glucose assay as a measure of enzyme activity in place of colorimetric determinations of reducing sugars (glucose equivalents) released during the enzyme-substrate reaction provides several advantages. Firstly, the Amplex Red reaction is similar in principle to Benefield’s (1971) colorimetric
glucose oxidase: horseradish peroxidase method, in which glucose oxidase specifically reacts with glucose. This strategy avoids the inaccuracies (usually overestimates) associated with determinations of reducing sugars (glucose equivalents), which incorporate non-glucose products produced during the reaction (e.g. cellobiose and other cellodextrins) (Gander et al. 1994). The reliability and sensitivity of colorimetric methods, which are based on spectrophotometric absorbance readings, are also frequently compromised by interfering materials in the soil extract such as trace elements, ions and pigments from humus and other organic materials (Dygert et al. 1965; Kanazawa and Miyashita 1986; Schinner and von Mersi 1990; Deng and Tabatabai 1994b; Gander et al. 1994). While resorufin (formed during the Amplex Red assay reaction) can be measured using absorbance, the main advantage of resorufin is that it can also be measured fluorometrically, thus eliminating inaccuracies caused by non-specific light absorbance in the presence of interfering materials.

Resorufin has absorption and fluorescence emission maxima of 562 nm and 587 nm, respectively. While fluorometric measurements of environmental samples can be affected by autofluorescence, the spectral properties (long wavelength) of resorufin minimise interference from autofluorescence in biological and environmental samples (Zhou et al. 1997). The Amplex Red glucose assay is highly sensitive and has the capacity to detect glucose concentrations as low as 3 µmol L⁻¹ or 500 ng mL⁻¹ (Molecular Probes 2002). In comparison, the 2 most sensitive colorimetric assays for determining reducing sugars, the Prussian Blue method (as modified by Deng and Tabatabai 1994b) and the Somogyi-Nelson method (as modified by Deng and Tabatabai 1994b), have a working range of 1 to 20 µg glucose equivalents mL⁻¹ and 5 to 100 µg glucose equivalents mL⁻¹, respectively (Deng and Tabatabai 1994b).

We conclude that the mechanical disruption soil enzyme extraction method and the 2 step microplate enzyme assay (enzyme-substrate reactions followed by fluorometric determination of glucose released during the reaction) presented here represent an improvement on approaches to measuring soil cellulases and laminarinases that involve passive extraction of soil enzymes followed by colorimetric determination of reducing sugar. In our laboratory, one person was able to assay both cellulase and laminarinase for up to 32 soil samples (with duplicate extractions for each sample) in
one day. However, our bead-beating machine could only hold 12 extraction tubes and therefore a machine with a larger capacity would increase the volume of samples that could be assayed in a single day.

The principles of the enzyme extraction and enzyme-substrate reaction steps of the method presented here may also be useful in assaying other soil enzymes. The enzyme extraction procedure could be used in multi-enzyme assays (using the same extract) that utilise substrates labelled with coloured dyes (Wirth and Wolf 1992) or fluorescent compounds (e.g. Marx et al. 2001; Vepsäläinen et al., 2001; Ramussen et al. 2002) to detect a range of enzymes in soils and other environmental and biological samples. Microplate methods that utilise substrates labelled with fluorescent compounds such as 4-methylumbelliferone (also known as MUB or MUF) represent a significant improvement on approaches that rely on the measurement of reaction products such as reducing sugars or glucose (Marx et al., 2001; Vepsäläinen et al., 2001). However, at the time of writing, fluorescently labelled substrates suitable for detecting less commonly studied enzymes such as laminarinase were not readily available and therefore our method will be useful where fluorescently labelled substrates for detecting target enzymes are not available and where information or comparisons based on glucose yield are required.

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