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BASIDIOMYCETE WOOD DECAY FUNGI FROM
***PINUS RADIATA*;**
BIOLOGY AND BIOLOGICAL CONTROL

A thesis

submitted for the requirements of the degree of

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**BASIDIOMYCETE WOOD DECAY FUNGI FROM *PINUS RADIATA*;
BIOLOGY AND BIOLOGICAL CONTROL**

by Annette Ah Chee

A collection of 38 basidiomycete cultures, from New Zealand *Pinus radiata* and resident fruiting bodies, were screened for wood decay potential using assays of cellulolytic and ligninolytic enzymes in liquid media, wood weight loss, and growth rates on artificial and wood media. Within each assay, there was significant variation observed between the basidiomycete fungi and between isolates of the same species. Correlation analyses found that the predictors of wood weight loss were wood moisture content and external fungal biomass, rot type (brown or white rot) and the production of ligninolytic, but not cellulolytic, enzymes. Isolates of *Schizophyllum commune* produced high cellulolytic activities but did not cause significant wood weight losses. Although *Gloeophyllum sepiarium* isolates caused amongst the highest weight losses of *P. radiata*, they had relatively slow growth rates on artificial and wood media.

The brown rot fungus *G. sepiarium* isolate 5 and the white rot fungi *Phlebiopsis gigantea* isolate 104 and *S. commune* isolate 3 were further characterised in growth and wood decay assays. Growth on agar was higher in darkness than in the light, with optimal temperatures of 25 to 30 °C. Growth in agar and liquid medium, particularly of *G. sepiarium*, resulted in significant reductions in pH. Low decay hazard conditions were found to be where wood was not in contact with a moisture- or nutrient-holding substrate whereas high hazard conditions involved green wood or that in soil contact. The three fungi caused little or no weight loss of *Eucalyptus regnans* or *Pseudotsuga menziesii*.

Biological control systems against basidiomycete fungi were investigated using a collection of 136 *Trichoderma* fungi from soils of forestry, mill and bush sites in the central North Island of New Zealand, growth media of edible fungi and a HortResearch culture collection. The *Trichoderma* cultures produced high cellulolytic activities and had fast growth rates on

artificial and wood media, but they did not cause significant wood weight losses. Dual culture agar and *Pinus radiata*-agar assays identified 23 *Trichoderma* cultures that exhibited antagonism against *G. sepiarium* and gave significant reductions in *Pinus radiata* weight loss in wood biological control assays. Less antagonism was observed towards *P. gigantea* and *S. commune*. Bioprotection resulted in limited basidiomycete colonisation of *Trichoderma*-precolonised blocks and neighbouring sterile blocks. Biological control resulted in the loss of basidiomycete viability in *G. sepiarium*-precolonised blocks.

Modes of antagonism were investigated for *T. crassum* isolate 26, *T. sp. 'viride'* isolate 38 and *T. viride* isolate 101 cultures which had shown biological control potential against *G. sepiarium*. *Trichoderma crassum* 26 had also shown bioprotection potential against *Phlebiopsis gigantea*. HPLC and GC-MS analyses found that *T. viride* 101 and *T. sp. 'viride'* 38 produced 6-pentyl- α -pyrone in low nutrient liquid medium, although no significant volatile inhibition of *G. sepiarium* was observed in high or low nutrient agar assays. Inhibitory non-volatile compounds were produced on agar, *Pinus radiata*, and in solvent extracts and fractions of supernatant from *Trichoderma* cultures grown in low nutrient liquid medium but GC-MS analyses failed to identify any of the compounds. The presence of *G. sepiarium* was not required to elicit production of these compounds. *Trichoderma crassum* gave the greatest inhibition of *G. sepiarium* in bioactivity assays and there was evidence of antibiosis in mycoparasitism assays. Field and controlled temperature room trials showed that significant decay of *P. radiata* by *G. sepiarium* was possible over the period of eight months, but biological control interactions were not observed as competition by other microorganisms had limited colonisation by the *Trichoderma* fungi.

This thesis study has shown that basidiomycete fungi from *P. radiata* can have varied wood decay potential, and significant antagonism can be exhibited towards basidiomycete species by New Zealand isolates of *Trichoderma* fungi, in artificial and wood media.

Keywords: Basidiomycete fungi, biological control, bioprotection, New Zealand, *Pinus radiata*, *Trichoderma*, wood decay.

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ABBREVIATIONS

General

BCA	Biological control agent
CHH	Carter Holt Harvey
CMC	Carboxymethylcellulose
EA	Ethyl acetate
GC-MS	Gas Chromatography-Mass Spectrophotometry
HPLC	High Pressure Liquid Chromatography
OH [•]	Hydroxyl radicals
LiP	Lignin peroxidase
MnP	Manganese peroxidase
6PAP	6-pentyl- α -pyrone
SEM	Scanning electron microscopy
WHC	Water holding capacity

Media

BA	Benomyl agar
gPDA	Guaiacol potato dextrose agar
gSA	Guaiacol sawdust agar
LNA	Low nutrient agar
LNM	Low nutrient medium
MA	Malt extract agar
SMA	Skim milk agar
TSA	<i>Trichoderma</i> -selective agar
WA	Water agar
YM	Yeast extract-malt extract medium
YMA	Yeast extract-malt extract agar

Fungal species

<i>A. carbonica</i>	<i>Antrodia carbonica</i> (Overh.) Ryvarden & R.L. Gilbertson
<i>A. serialis</i>	<i>Antrodia serialis</i> (Fr.) Donk
<i>A. vaillantii</i>	<i>Antrodia vaillantii</i> (DC.:Fr.) Ryvarden
<i>C. olivacea</i>	<i>Coniophora olivacea</i> (Pers.:Fr.) P. Karst.
<i>C. puteana</i>	<i>Coniophora puteana</i> (Schumach.:Fr.) P. Karst.
<i>G. sacratum</i>	<i>Gloeocystidiellum sacratum</i> (G. Cunn.) Stalpers & P.K. Buchanan
<i>G. sepiarium</i>	<i>Gloeophyllum sepiarium</i> (Wulfen:Fr.) P. Karst.
<i>G. trabeum</i>	<i>Gloeophyllum trabeum</i> (Pers.: Fr.) Murrill
<i>H. rutilans</i>	<i>Hapalopilus rutilans</i> (Pers.: Fr.) Murrill
<i>H. annosum</i>	<i>Heterobasidion annosum</i> (Fr.:Fr.) Bref.
<i>J. vincta</i>	<i>Junghuhnia vincta</i> (Berk.) I. Hood & M. Dick
<i>L. edodes</i>	<i>Lentinus edodes</i> (Berk.) Singer
<i>N. lepideus</i>	<i>Neolentinus lepideus</i> (Fr.:Fr.) Redhead & Ginns
<i>P. chrysosporium</i>	<i>Phanerochaete chrysosporium</i> Burdsall in Burdsall & Eslyn
<i>P. velutina</i>	<i>Phanerochaete velutina</i> (DC.:Fr.) P. Karst.
<i>P. gigantea</i>	<i>Phlebiopsis gigantea</i> (Fr.:Fr.) Juelich
<i>P. pulmonarius</i>	<i>Pleurotus pulmonarius</i> (Fr.) Quel.
<i>P. ostreiformis</i>	<i>Polyporus ostreiformis</i> Berk.
<i>P. placenta</i>	<i>Postia placenta</i> (Fr.) M. Larsen & Lombard
<i>P. coccineus</i>	<i>Pycnoporus coccineus</i> (Fr.) Bondartsev & Singer
<i>R. bicolor</i>	<i>Resinicium bicolor</i> (Albertini & Schwein.:Fr.) Parmasto
<i>S. commune</i>	<i>Schizophyllum commune</i> Fr.:Fr.
<i>S. dimidiatum</i>	<i>Scytalidium dimidiatum</i> (Penz.) Sutton & Dyko
<i>S. hexagonoides</i>	<i>Serpula hexagonoides</i> (Burt) W.B. Cooke
<i>S. brinkmannii</i>	<i>Sistotrema brinkmannii</i> (Bres.) J. Eriksson
<i>S. sapinea</i>	<i>Sphaeropsis sapinea</i> (Fr.:Fr.) Dyko and Sutton in Sutton
<i>S. sanguinolentum</i>	<i>Stereum sanguinolentum</i> (Albertini & Schwein.:Fr.) Fr.
<i>T. versicolor</i>	<i>Trametes versicolor</i> (L.:Fr.) Pilat
<i>T. aureoviride</i>	<i>Trichoderma aureoviride</i> Rifai
<i>T. crassum</i>	<i>Trichoderma crassum</i> ined.
<i>T. hamatum</i>	<i>Trichoderma hamatum</i> (Bonord.) Bainier
<i>T. harzianum</i>	<i>Trichoderma harzianum</i> Rifai

<i>T. koningii</i>	<i>Trichoderma koningii</i> Oudem.
<i>T. polysporum</i>	<i>Trichoderma polysporum</i> (Link) Rifai
<i>T. longibrachiatum</i>	<i>Trichoderma longibrachiatum</i> Rifai
<i>T. virens</i>	<i>Trichoderma virens</i> (Miller, Giddens and Foster) von Arx
<i>T. viride</i>	<i>Trichoderma viride</i> Pers.:Fr.
<i>V. bulbillosum</i>	<i>Verticillium bulbillosum</i> Gams and Malla

Tree species

<i>E. regnans</i>	<i>Eucalyptus regnans</i> F.J. Mueller
<i>P. radiata</i>	<i>Pinus radiata</i> D. Don
<i>P. menziesii</i>	<i>Pseudotsuga menziesii</i> (Mirb.) Franco

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Chapter 1 INTRODUCTION, AIM AND OBJECTIVES, AND

LITERATURE REVIEW

1.1 INTRODUCTION

In New Zealand, there are around 1.5 million hectares of *Pinus radiata* D. Don production forest and exports of forest products are estimated to be \$2.4 billion (NZ 1999). The growth rates of *P. radiata* achieved under New Zealand climatic and soil conditions are amongst the highest in the world where mature trees can be achieved in 28 years but a greater depth of sapwood is produced than in many Northern Hemisphere softwoods (Cown 1992). One of the limitations to the export potential of New Zealand *P. radiata* timber is the susceptibility of sapwood to detrimental fungi including decay, soft rot, mould and sapstain fungi. This thesis research is concerned with the fungi that can potentially result in the greatest damage and loss of value of *P. radiata* timber, the wood decay fungi.

International research in the field of wood decay fungi has sought to understand the decay processes involved and to evaluate various methods of effective control by chemical or biological means. New Zealand research in the last 20 years has focussed on chemical wood preservation (Butcher et al. 1977; Drysdale and Preston 1982; Hedley and Armstrong 1975) and it is only recently that there has been interest in the decay fungi affecting *P. radiata* (Kreber and van der Waals 1999) although some observations of microbial succession were carried out in the 1960's (Butcher 1968a). The wood decay fungi used in most studies are standard cultures isolated from a range of wood species and sourced from American or European culture collections. The majority of wood decay fungi are basidiomycete fungi. In New Zealand, 90% of the national production forest is *P. radiata* but there has been limited research on the basidiomycete fungi affecting this resource. One of the aims of this thesis research was to study the wood decay potential of a range of basidiomycete fungi isolated from *P. radiata*. In addition to the fundamental findings of the research, these fungi could be used in future New Zealand studies on the *P. radiata*-wood decay fungi system, alongside the international standard cultures.

Chemical preservative treatments are currently required for long term durability of *P. radiata* and the Ministry of Forestry estimates that 1 million m³ of New Zealand timber

is treated each year (Heron 1994). Due to the widespread practices of wood preservation in the last 30 to 40 years, there is little literature on the decay of untreated timber. Delays between felling and processing can result in localised loss of wood moisture that may allow fungal colonisation. Mould, sapstain and some decay fungi can become established in *P. radiata* within a few weeks of felling, in the forest or at the mill (Butcher and Drysdale 1991; Keirle 1981). Dietz and Wilcox (1997) proposed that much of the decay occurring in buildings is from preinfection of green wood. Butcher (1967) suggested that there was a risk of infection of *P. radiata* by decay fungi throughout the whole year but Williams (1987) found that colonisation was greatest in the autumn period. There is an air-seasoning period for logs before preservative treatment and any wood degradation during this period can significantly reduce preservative fixation. The incidence of fungal decay in treated timber is very low but may occur where there has been substandard treatment, leaching of preservative, or the decay fungus has a degree of tolerance or the ability to degrade the preservative.

The current wood preservatives have broad spectrum toxicity, although Greaves et al. (1988) found in laboratory assays that no single preservative controlled all of the examined fungal species. Water-soluble preservatives are the main type of treatment in New Zealand. Copper chrome arsenate is used in 60% of the treated timber and different retentions (depending on the wood application) give varying shades of green. Ammoniacal copper quaternary is a less toxic preservative with the elimination of chromium and arsenic emissions but there are greater ammonia emissions (Chen and Randall 1998). Boric compounds are also of lower toxicity than copper chrome arsenate and are used in 37% of the treated timber. Other water-soluble preservatives are the light organic solvent preservatives (used in 3% of the treated timber) such as creosote which are used under pressure to treat timber for above ground purposes. The use of water-soluble preservatives is restricted to wood that is protected from the weather as they can be diluted and leached by water. Protein borates have been developed to reduce the leaching problem and have proven to give protection comparable to copper chrome arsenate (Thevenon et al. 1998).

Alternatives to preservative treatments for control of wood decay were investigated in the 1960's (Ricard et al. 1969) but there was limited progress as the forest industry and the public were content with the ease of use, fast action and reasonable cost of chemical control. Research into biological control of wood decay fungi has occurred in the last 10 to 20 years in parallel to growing international concern and awareness for health and the

environment. Some markets in Europe and the U.S.A. require independent certificates to ensure that imported timber comes from sustainably managed forests and environmentally friendly processing systems. In New Zealand, there is pressure not only on the Government for appropriate legislation but also on the forest industry e.g. the Resource Management Act 1991 and the Occupational Safety and Health Act 1992. The Resource Management Act regulates the use of preservatives and there are detailed protocols for the design and operation of wood treatment plants. There are concerns that residues from spillage and waste disposal may be contaminating soil and waterways, particularly in older designed plants. Ironically, the potential of wood decay fungi for bioremediation of these sites is being investigated (Eschenbach et al. 1995; Fahr et al. 1999; Ullah and Evans 1999). A range of oxidative and reductive processes are involved in wood decay and many of the enzymes are extracellular so wood decay fungi are able to degrade toxic or insoluble chemicals more efficiently than other microorganisms (Cameron et al. 2000). The Occupational Safety and Health Act requires a company to establish practices to protect the health of employees involved in the use of preservatives. There has been research to develop new and less toxic wood treatments e.g. copper 8-quinolinolate (Butcher and Drysdale 1978), tertiary amine acetates (Butcher and Preston 1978) and prophylactic chemicals (Butcher 1973).

To maximise the potential of increasingly discerning export markets, New Zealand needs to develop reliable, environmentally sustainable and ecologically sound production and processing systems for high quality wood products that are resistant to detrimental fungi. Biological control using naturally antagonistic microorganisms, either alone or in an integrated control programme with other non-chemical methods or less toxic chemicals, can result in the reduced use of preservatives and in some cases eliminate the requirement altogether. This would be complemented with wood handling and storage protocols to minimise the incidence or severity of fungal decay. Systems have been studied for the protection of living trees and stumps against basidiomycete fungi but research is less advanced for the protection of processed wood products. The natural origin of a biological control agent (BCA) implies that biological control is not harmful to the environment and has the potential for long-term, self replicating control given the right conditions. Bruce et al. (1990) suggested that there was significant potential for biological control of fungal decay as the wood environment was relatively stable and supported a less diverse microbial community than, for example, soil especially if the wood has previously been

preservative treated. Biological control may be important in limiting the development of resistance that can result from prolonged use of chemical preservatives.

Biocontrol and bioprotection were two concepts defined by Freitag et al. (1991) and both were investigated in this thesis research. Biocontrol was defined as the use of antagonistic microorganisms to restrict the growth or eradicate established decay fungi and to stop decay before significant damage occurred. Biocontrol organisms were stress-tolerant, competitive and active wood colonisers. Bioprotection was defined as the prevention of colonisation of decay fungi by antagonistic microorganisms that colonised and dominated the wood environment without themselves exerting any undesirable characteristic such as decay or wood discolouration. Bioprotection may have more chance of success in biological control systems and the control of wood decay in above ground situations (low to medium decay hazard) may be more feasible than in ground contact situations (high decay hazard).

Trichoderma species are the most extensively researched fungi for the biological control of detrimental wood fungi (Chet 1993; Lumsden 1992; Papavizas 1985). This is due to a number of reasons that include their ubiquitous nature, relatively fast growth rates and their range of possible modes of antagonism. This thesis research investigates the biological control potential of *Trichoderma* species, isolated from New Zealand soils, against basidiomycete fungi, isolated from New Zealand-grown *P. radiata*.

1.2 AIM AND OBJECTIVES

1.2.1 Aim

The aim of this thesis research was to characterise a range of basidiomycete fungi from New Zealand-grown *Pinus radiata* by studying their wood decay potential and some of the factors influencing decay, and to investigate the potential for biological control of fungal decay in *P. radiata* timber using New Zealand *Trichoderma* species.

1.2.2 Objectives

Chapter 2

- To collect a range of New Zealand basidiomycete fungi from *P. radiata*, screen them for wood decay potential in enzyme, wood decay and growth assays, and use the results to select cultures for further characterisation and biological control studies.
- To assess the variation in decay potential between the basidiomycete fungi, and between isolates of the same species, for each of the screening assays.
- To determine if there are any significant correlations between parameters of the screening assays and whether any of the parameters can be used to predict weight loss of *P. radiata* or if the culture is a brown rot or white rot fungus.

Chapter 3

- To determine the influence of light, temperature and pH on growth of the selected basidiomycete fungi from Chapter 2, and the influence of assay temperature and pH on decay enzyme activity, for consideration in the design of biological control assays.
- To characterise growth of the basidiomycete fungi in liquid medium (by accumulated fungal biomass and changes in medium pH, glucose and protein) in comparison to the measurement of radial colony extension growth on agar or *P. radiata*.
- To determine the weight loss by the basidiomycete fungi of dried *P. radiata* heartwood and sapwood, green *P. radiata* sapwood, dried *Pseudotsuga menziesii* (Mirb.) Franco and *Eucalyptus regnans* F.J. Mueller sapwood, and the influence of soil or vermiculite assay substrate.

Chapter 4

- To collect a range of New Zealand *Trichoderma* species, screen them for antagonism against the brown rot fungus *Gloeophyllum sepiarium* (Wulfen:Fr.) P. Karst. in dual

culture agar and wood-agar assays and for the ability to grow on agar and on *Pinus radiata*, and use the results to select *Trichoderma* species for further biological control studies.

Chapter 5

- To screen the selected *Trichoderma* species from Chapter 4 for biological control potential against *G. sepiarium* and the white rot fungi *Phlebiopsis gigantea* (Fr.:Fr.) Juelich and *Schizophyllum commune* Fr.:Fr. in the laboratory using wood biological control assays and decay enzyme assays, and in field and controlled temperature room trials.

Chapter 6

- To study the possible modes of antagonism by the selected *Trichoderma* species against *G. sepiarium*, *P. gigantea* and *S. commune*, in laboratory assays for the production of inhibitory volatile or non-volatile compounds, or evidence of mycoparasitism.

1.3 LITERATURE REVIEW

In the following literature review, there are two main sections that provide a broad background to the research area and some validation of the thesis objectives. The first section covers the wood-fungi system, from describing the structure and composition of *Pinus radiata*, to the range of detrimental wood fungi, the ecological requirements for colonisation, growth and decay, and the biochemistry involved in the degradation process. The second section covers aspects of biological control, from biological control agent (BCA) screening, to the modes of antagonism, enhancement of biological control systems and examples of BCA's that are active against wood decay fungi.

1.3.1 Wood structure and composition

In *P. radiata*, there are three cell types (tracheids, parenchyma and epithelial cells) which are arranged into axial cells (that are of longitudinal orientation and contribute to wood strength) and ray cells (that are of transverse orientation and involved in nutrient storage and transport and control of wood swelling). The growth period of *P. radiata* is generally

restricted to the spring and summer. Tracheids formed during the early part of this period (earlywood tracheids) have a relatively large diameter with thin walls and function in water and nutrient transport. Latewood tracheids have a relatively small diameter and thick walls and make up the denser part of the annual ring and contribute to wood strength. The ray parenchyma have a number of physiological functions in addition to carbohydrate storage. Epithelial cells are thin walled cells associated with resin and gum production. Bordered pits are present in adjacent axial tracheids and between axial and ray tracheids. Half bordered pits are present in cell walls between axial tracheids and ray parenchyma.

The major chemical components of *P. radiata* sapwood are cellulose (40%), hemicelluloses (31%), lignin (27%) and extractives (2%) (Uprichard 1991). Cellulose, hemicelluloses and lignin make up the structure of wood cell walls (Fig. 1.1). The outermost wall layer is the primary wall which in combination with the middle lamella surrounding the cell forms the compound middle lamella. Within this lies the secondary wall which is comprised of three layers, the outer S1, middle S2 and inner S3.

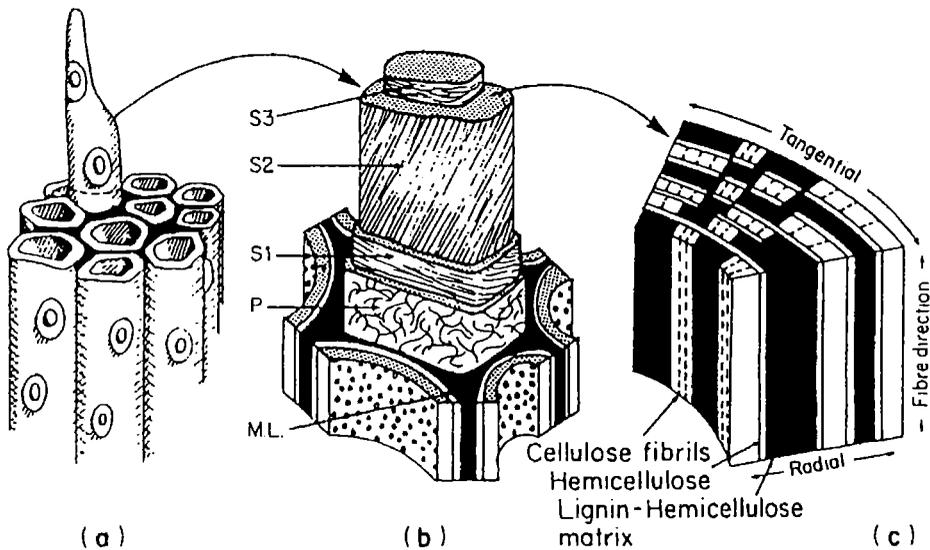


Fig. 1.1 Gymnosperm wood structure showing (a) Tracheid cells with bordered pits, (b) Cell wall structure where S1, S2 and S3 layers form the secondary wall; P is the primary wall; and ML is the middle lamella (Kirk 1983) and (c) A model of the relationship between cellulose, hemicellulose and lignin in the secondary cell wall (Goring 1977).

Cellulose is a long linear polysaccharide molecule of β -1,4-linked anhydro-glucopyranose units (Fig. 1.2). The β -1,4-glucosidic bonds contribute to the stability and insolubility

1.3.2 Wood fungi

Although this thesis research focusses on basidiomycete fungi, there are a range of other detrimental fungi that interact with the decay fungi within the wood substrate and cause damage to wood cell walls (Fig. 1.6).

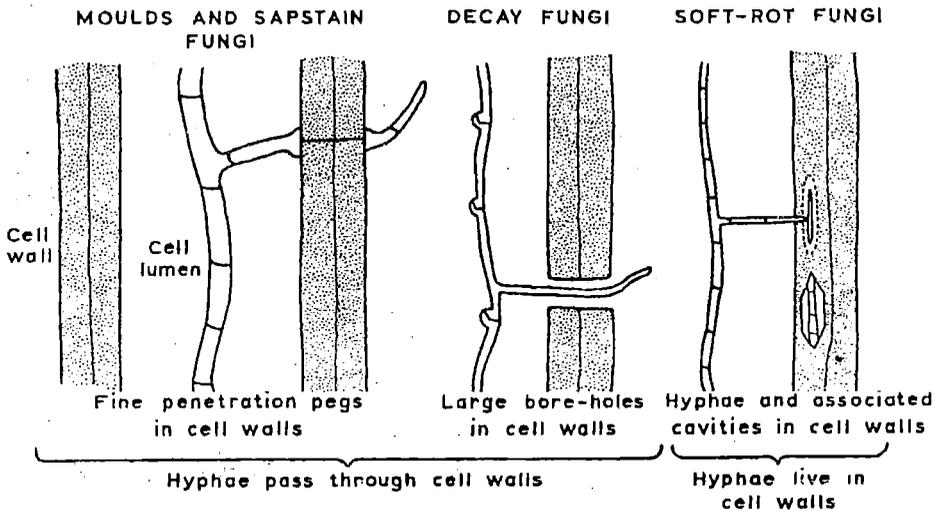


Fig. 1.6 Effect of mould, sapstain, decay and soft rot fungi on wood cell walls (Butcher 1974).

1.3.2.1 Sapstain fungi

In New Zealand, the predominant sapstain species is *Sphaeropsis sapinea* (Fr.:Fr.) Dyko and Sutton in Sutton and members of the Ophiostomataceae are also commonly observed (Kay et al. 1997). They are a diverse group of fungi that can cause a penetrating discolouration to wood from pigmentation of the hyphae, secretion of soluble pigments or the formation of coloured deposits. The pigmentation ranges from red-brown, grey-green to black and is cosmetic. No significant wood weight loss or reduction in strength properties has been attributed to sapstain fungi (Schirp et al. 1999) as simple non-structural wood components are utilised for growth. Fungal colonisation of wood cells occurs primarily through the ray parenchyma and hyphae spread through the pits or by forming penetration holes.

1.3.2.2 Mould fungi

The moulds are a large group of saprophytes that are generally ascomycete or deuteromycete fungi. The production of coloured conidia results in a superficial discolouration to wood that can be brushed or planed off. The mould fungi utilise simple non-structural wood components with no significant wood weight loss or reduction in strength properties (Hulme and Shields 1970). The presence of some mould fungi can cause increases in wood permeability during the air-seasoning period of pine logs. This increase may allow greater uptake of preservatives (Schoeman and Dickinson 1992) but also greater reabsorption of water and thus a more favourable moisture content for colonisation by decay fungi. Fungal colonisation of wood cells occurs primarily through the tracheids and hyphae spread through the pits or by forming penetration holes.

1.3.2.3 Soft rot fungi

Although the majority of soft rot fungi are ascomycete or deuteromycete fungi, soft rot has been exhibited by some basidiomycete fungi (Daniel et al. 1992; Schwarze et al. 1995). Soft rot decay results in wood of a spongy texture that, when dried, has an appearance similar to that degraded by brown rot fungi (see 1.3.2.4). Soft rot fungi are often described as primary colonisers of wood as they can attack wood of a relatively high moisture content. 'Type 1' soft rot is the most common in softwoods and is characterised by chains of cavities in the S2 cell wall layer. Fungal colonisation of the cell lumen is followed by the production of fine penetration hyphae into the S2 layer. The release of degradation enzymes results in cavitation and localised loss of wood strength. Many soft rot fungi can degrade or cause demethylation of lignin.

1.3.2.4 Wood decay fungi

There are two main types of basidiomycete decay fungi, white rot and brown rot, which are categorised according to the wood components that they degrade. It is frequently stated that white rot fungi are predominately found on hardwoods and brown rot fungi on softwoods but Highley (1978) showed that many decay fungi can equally degrade both wood types. White rot fungi can degrade cellulose and hemicellulose and, most importantly, the lignin component of wood. The residual wood is typically fibrous with a whitish yellow to tan colouration due to the removal of lignin (Photo 1.1 a). Brown rot fungi can cause the

extensive degradation of cellulose and hemicellulose and the modification, but not degradation, of lignin. The residual wood is a brown lignin framework that is characterised by cubicular shrinkage, cracking and crumbling (Photo 1.1 b). Brown rot is the most destructive form of wood decay as a significant reduction in strength properties can be incurred at low wood weight losses (Wilcox 1978).

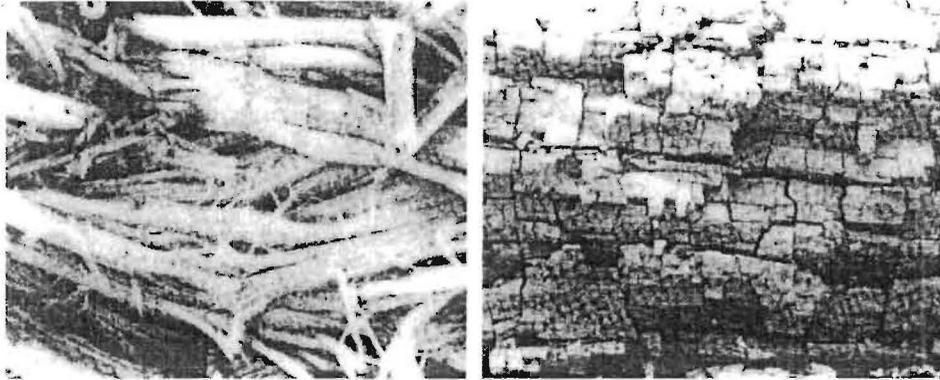


Photo 1.1 Residual wood degraded by (left) a white rot fungus and (right) a brown rot fungus (Alexopoulos et al. 1996).

Fungal colonisation of wood cells occurs primarily through the ray parenchyma. The major longitudinal pathways are through the pits of the earlywood tracheids. In the initial stages, there is passive vegetative growth during which the substrate is rapidly and extensively colonised utilising simple non-structural wood components. Both brown rot and white rot fungi produce pectinases and the hydrolysis of pits may be a key event during colonisation (Green and Clausen 1999). This is followed by enzymatic attack, primarily of the hemicelluloses and amorphous regions of cellulose in the cell wall (Cowling 1961; Ritschkoff et al. 1992). In later stages of decay, the secretion of enzymes facilitates further colonisation by forming bore holes (Eriksson et al. 1980).

Fungal colonisation of wood may be obvious externally as visible mycelium and fruiting bodies, and changes in wood colour and integrity. Internal fungal growth can be determined by destructive means such as culturing techniques, DNA-based assays (Jasalavich et al. 2000; Jellison and Jasalavich 2000), polarised light microscopy (Wilcox 1968), scanning electron microscopy (SEM) (Blanchette 1980a; Blanchette 1980b) and transmission electron microscopy (Nilsson and Daniel 1983). Non-destructive means include the measurement of fungal biomass (Jones and Worrall 1995), immunofluorescence labelling and confocal laser scanning microscopy (Xiao et al. 1999), respirometry (Smith 1975),

microcalorimetry (Bjurman and Wadso 2000), ATP photometry (Nieto et al. 1997), nuclear magnetic resonance (Gilardi et al. 1994), electrical conduction or resistance (Tattar et al. 1972), x-ray microdensitometric assay (Bucur et al. 1997), acoustic emission (Raczkowski et al. 1999), ultrasonic wave propagation (Obuekwe and Ejechi 1998), vibration reverberation time (Ouis 2000) and the electronic nose (Nilsson 1998).

Indicators of fungal wood decay include wood weight loss, reduction in strength and integrity (Kim et al. 1996; Nicholas and Jin 1996), changes in chemical composition (Kirk and Highley 1973), a decrease in pH (Micales and Highley 1989) and an increase in solubility characteristics (Wong and Wilkes 1988b). Immunodiagnosics have been used as a rapid, sensitive, inexpensive and simple method to detect incipient decay (i.e. the presence of wood decay fungi prior to significant structural damage) by enzyme-linked immunosorbant assay (Clausen 1997; Kim et al. 1991; Palfreyman et al. 1998) and particle capture immunoassay (Clausen and Ferge 1995).

Wood decay results from the activity of successive microbial populations rather than the invasion by an individual fungus (Butcher 1968b). A number of studies have observed infection processes in *P. radiata* and succession by decay and non-decay fungi (Butcher 1967; Butcher 1968b; Butcher and Drysdale 1991). It is important to gain an understanding of the natural microbial succession that may occur in wood and the interactions between the different populations (Rayner and Boddy 1988) in order to understand the involvement of basidiomycete fungi in the decay process. The concept of successional stages of microbial infection and decay on felled timber has been generally accepted. Stage one involves the colonisation by 'pioneer' sapstain and mould fungi. Insects and a range of bacteria are also frequently observed. Wood decay fungi follow in stage two with brown rot and soft rot fungi, and in stage three with white rot fungi. These stages are not mutually distinct but are influenced by ecological factors such as wood nutrient status and moisture content (Garrett 1963). Microbial succession in preservative-treated wood is similar to that in untreated wood but occurs at a slower rate depending on the levels of preservative resistance. Some mould fungi have been found to degrade preservatives and allow subsequent colonisation by wood decay fungi (Eveleigh 1986).

1.3.3 Ecological requirements of wood decay fungi

Ecological factors can determine the distribution and prevalence of basidiomycete fungi, and, therefore, aspects of their colonisation, growth and decay ability. Wood decay fungi have fairly basic requirements and are reported to tolerate a wide range of conditions (Xie et al. 1997). The major factors are water, oxygen, temperature, pH and nutrients. These may be interdependent and the relative importance of each factor may change during colonisation and growth of the fungi and as decay progresses.

1.3.3.1 Wood moisture

Wood moisture has a number of roles in the decay process that include: swelling the cellulose molecules so that they are more susceptible to enzymatic attack; providing a diffusion medium for the extracellular decay enzymes and partial degradation products; and as a direct requirement for fungal growth. Water moves through wood cells mainly by capillary flow through bordered pits and diffusion through the cell wall. Moisture is held in wood as free water in the cell lumen and as bound water within the cell wall. Loss of bound water may result in shrinkage or swelling of the cell and changes in strength properties. Water adsorption and desorption by pine sapwood has been studied by Grant (1975) and Hedley (1977).

Wood moisture content may increase with the progression of decay and the subsequent lowering of wood density (Butcher 1968b). The moisture content of wood is expressed as a percentage of dry weight. A term commonly used is the fibre saturation point which is defined as the moisture content, usually around 30%, at which the cell walls are fully saturated but there is no free water in the cell lumen. Below the fibre saturation point, water is tightly absorbed and less available to most fungi. As moisture content increases above the fibre saturation point, water replaces the air in the cell lumen and is available for fungal growth. The availability of water to fungi is affected by water potential which is the sum of matric potential and osmotic potential. Matric potential is a result of the forces associated with the interfaces between the air and the wood components and osmotic potential is a result of solute concentration in the water. Water potential was found to affect growth of wood decay fungi which are generally intolerant to drying conditions (Mswaka and Magan 1998; Whiting and Rizzo 1999). Wilson and Griffin (1979) studied a range of

soil basidiomycete fungi and found some could grow at water potentials below -10 MPa and others could not grow below -3 MPa. The equilibrium moisture content of air-seasoned *P. radiata* depends on the prevailing temperature and relative humidity conditions but is generally around 12%. This figure can be affected by wood processing such as kiln-drying and preservative treatment. Moisture gradients can occur between inner and outer zones of wood resulting in variable susceptibility to decay. The decay threshold moisture content for pine sapwood has been reported to be 26.5% (BRE 1975), 31% (Ammer 1964) and 35% (Cartwright and Findlay 1958).

Wood moisture content can influence the species of decay fungi that can colonise as well as the extent of decay. Brown rot fungi are reported to require less moisture for decay in comparison to white rot fungi (Highley and Scheffer 1970) and some brown rot fungi were found to survive for up to 10 years in air-seasoned wood (Scheffer and Chidester 1948). The minimum moisture requirement for growth of brown rot fungi is close to the fibre saturation point (Boddy 1986) and the maximum moisture level is determined by tolerance to low oxygen or high carbon dioxide (Boddy 1983a).

1.3.3.2 Oxygen and carbon dioxide

Most wood fungi are obligate aerobes and so are strongly influenced by wood moisture which can limit oxygen diffusion in wood (Agosin et al. 1989). Relatively low levels of oxygen around 1% are reported to be required for wood decay fungi (Scheffer 1986) which may be related to the high surface area to volume ratio of hyphae for gaseous exchange. Kazemi et al. (1998) found that 5% oxygen was more favourable for growth of basidiomycete fungi on wood and concentrations of less than 1% resulted in decreased growth. Toole (1973) observed that oxygen use peaked in the early stages of decay and then decreased with the progression of decay. Low oxygen levels were found to limit fungal decay in closed assay chambers (Hedley 1977) although variation in tolerance to low oxygen has been observed between species of fungi (Scheffer 1986).

Optimum conditions for growth and wood decay occur when oxygen concentrations are high and carbon dioxide concentrations are low (Highley and Kirk 1979). Small amounts of carbon dioxide (0.05%) are required for fatty acid synthesis in fungi (Griffin 1981) but higher concentrations may cause toxicity, acidity effects and inhibit fruiting in basidiomycete fungi (Taber 1966). However, basidiomycete fungi are often tolerant of high

atmospheric carbon dioxide and at concentrations of around 10%, growth may be stimulated (Hintikka and Korhonen 1970; Schanel 1976).

1.3.3.3 Temperature

Temperature directly affects the many integrated enzymatic processes of decay fungi up to limits that cause denaturation of enzymes or where the reactions become rate limiting. Wood decay fungi generally have optimum temperatures for growth of 20 to 30°C (Boddy 1983b) but they can tolerate a range from 15 to 45°C (Highley and Kirk 1979). Cartwright and Findlay (1958) observed that the optimal temperature for fungal growth on agar may be 2 to 3°C more than that for growth on wood. Humphrey and Siggers (1933) determined minimal, optimal and maximal temperatures for growth of wood decay fungi and found significant variation in growth rates between and within species for a given temperature and on a given assay medium. The growth-temperature relationships of some basidiomycete fungi have been correlated to their geographic distribution (Mswaka and Magan 1998).

Time-temperature studies have been carried out to determine the feasibility of wood sterilisation (Chidester 1939) although heat treatment has been shown to result in loss of strength in *P. radiata* sapwood (Kim et al. 1998). Temperatures of 55 to 60°C eliminate most basidiomycete fungi and microfungi such as *Trichoderma* species but some brown rot fungi are heat resistant and can survive 12 h at 60°C (Cooper et al. 1998). Temperature, relative humidity and exposure time were found to be critical interdependent factors to the development of decay (Viitanen 1997).

Most of the research on temperature, and the effects on fungal growth and decay, have been carried out under constant conditions. Reduced wood decay is likely in the field situation where fluctuating temperatures are the norm (Micka and Reinprecht 1999).

1.3.3.4 Light

Light has generally been assumed to be detrimental to the growth of wood decay fungi due to the inhibitory effects of ultraviolet at high light intensities but there has been little research in this area. Duncan (1967) found that periodic exposure to light increased the rate of decay but this may have been caused by a light-associated increase in temperature.

1.3.3.5 Wood pH

Although the optimum pH for fungal growth is around 5 (Yamaguchi et al. 1997), a lower pH appears to promote wood decay (Agosin et al. 1989). Wood decay fungi tend to decrease the pH of wood during degradation. This is in part due to the production and secretion of organic acids such as oxalic acid and the presence of certain gases such as carbon dioxide. The pH of wood is generally 4 to 6 (Rayner and Boddy 1988) but wood degraded by brown rot fungi may have a pH of less than 2.5 (Micales and Highley 1989). Brown rot fungi have a greater tolerance to low pH, with optima for growth of around pH 3. Growth of brown rot fungi is more sensitive to high pH than white rot fungi (Kaarik 1974) which may be related to the effects of pH on decay enzymes (Highley 1976).

1.3.3.6 Wood nutrients

The nutrients required for growth of wood decay fungi may include nitrogen compounds, vitamins and essential elements.

The requirements for nitrogen by wood decay fungi may be relatively high. The nitrogen content of spores is around 3% and some species produce large chitinous basidiocarps. Only small amounts of nitrogen (0.03 to 0.1%) are present in wood which has a carbon:nitrogen ratio of 350 to 1250:1 (Cowling and Merrill 1966). Levi (1969) observed that an increase in the ratio resulted in a decrease in the rate of fungal decay and an increase in the production of cellulolytic enzymes. Colonising fungi may follow nitrogen pathways or gradients (Cowling 1961). Protein is the primary nitrogen source in wood so assimilation of nitrogen by decay fungi may be dependant on the production of extracellular proteinases (Sauter et al. 1989) or on lignin degradation as 50% of the total nitrogen in wood is bound to lignin (Dill et al. 1984). Boyle (1998) studied the nutritional requirements of white rot fungi in wood and found that nitrogen availability limited growth but the availability of carbohydrates, micronutrients or vitamins had little effect on growth.

Vitamins and minerals are required for fungal enzyme systems. Some fungi can synthesise vitamins but others need external sources. Thiamine is the only essential vitamin for growth of most decay fungi and is present in wood (Jennison 1952). Wood is naturally low in mineral element concentration (Young and Guinn 1966). Levels of iron, manganese and other transition metals are often below 2 μ M in non-degraded wood (Jellison et al. 1993)

and are bound to cell wall components or in an insoluble form. Wong and Wilkes (1988a) found that levels of nitrogen, phosphorus and potassium significantly affected the rate of wood decay. Jellison et al. (1997) reviewed the influence of cations such as iron, manganese, zinc, copper, calcium, magnesium, potassium and metals on colonisation and decay by brown rot fungi. This followed a study by Jellison et al. (1992) on the changes in overall cation ratios or concentrations in brown rotted wood. Wood decay fungi are capable of selectively uptaking mineral nutrients from soil into wood and translocating interfering quantities of others out of wood (Connolly and Jellison 1997). Calcium is primarily associated with pectin which is found in pit membranes, the middle lamella and ray parenchyma and the removal of calcium is necessary for hydrolysis of pectin. Green et al. (1997) showed that treatment of wood blocks with a calcium-precipitating agent inhibited fungal decay by preventing the biochemical reactions involved in colonisation.

Iron plays an important role in wood decay as an essential component of the extracellular haem enzymes in white rot decay (Paszczynski et al. 1988) and possibly in brown rot decay during non-enzymatic cellulose degradation (Murmanis et al. 1988a). Ferric iron is the form normally present in wood. Siderophores are extracellular, low molecular weight, high affinity iron-binding compounds that sequester iron as chelate-metal complexes which bind to specific receptor sites on the fungal hyphae membrane. Reduction processes occur to release the iron, or the complex, intracellularly for use in metabolic functions. The production of siderophores occurs through secondary metabolic pathways and multiple forms may be produced by one fungus. Decay fungi produce primarily phenolate-type siderophores (in addition to hydroxamate-type siderophores) which show affinity to a wide variety of metals including manganese (Jellison et al. 1990).

1.3.4 Biochemistry of wood decay

Most ecological factors affecting fungal colonisation, growth and decay can be related to their effects on fungal enzymes and processes and on the susceptibility of wood cells and their components. Brown and white rot fungi are differentiated on the basis of their degradation systems although the actual mechanisms involved in cellulose and lignin degradation may be similar (Highley et al. 1998). The degradation mechanisms for lignin and cellulose are still not clear and are the subject of considerable research and debate.

1.3.4.1 Lignin modification and degradation

Brown rot fungi do not degrade lignin (Kirk and Highley 1973) but numerous studies have indicated that the residual lignin is modified by demethylation (Cowling 1961) and by oxidation (Kirk 1975). Aromatic hydroxylation and formation of carboxyl groups has also been reported (Kirk 1971) and limited lignin depolymerisation (Kirk et al. 1970).

White rot fungi utilise a complex extracellular multi-enzymatic system which allows for simultaneous degradation of lignin and polysaccharide components or selective degradation of lignin (Leonowicz et al. 1999). Many white rot fungi show simultaneous and sequential lignin degradation on the same substrate (Blanchette 1984a; Blanchette 1984b) depending on factors such as temperature and nitrogen. The ligninolytic enzymes involved are the phenol oxidases of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. The peroxidases are haem-containing enzymes with typical catalytic cycles. The range of reducing substrates is different for the two peroxidases as they have different substrate binding sites. LiP can oxidise a number of aromatic compounds but MnP oxidises almost exclusively Mn^{2+} to Mn^{3+} , which then degrades phenolic compounds such as lignin. MnP has been found to be an efficient degrader of fungal melanins and can act in a biological control capacity by reducing the resistance of melanised plant pathogen propagules (Butler and Day 1998). Laccases are copper-containing oxidases which reduce oxygen to water and oxidise phenolic compounds. Laccase has diverse physiological functions such as oxidation, demethylation, polymerisation or depolymerisation with a broad substrate specificity (Thurston 1994). The ligninolytic enzymes may have feedback mechanisms and function separately or cooperatively. Isoenzymes have been reported and may act synergistically (Farrell et al. 1989). White rot fungi can be divided into groups according to which combinations of lignin enzymes they produce (Morais et al. 1996; Tuor et al. 1995). Vares and Hatakka (1997) found that some white rot fungi had a LiP dominating ligninolytic system and others a MnP dominating system.

Interactions between brown rot and white rot fungi, from different successional stages of wood decay, were studied by Iakovlev and Stenlid (2000) by measuring laccase activity in qualitative and quantitative assays. They found that the high combative ability of late succession stage fungi may partly be related to laccase activity involved in the detoxification of defence phenolic compounds produced by combatants. Arora and Sandhu (1985) observed a correlation between laccase production and lignin degradation.

Aerial mycelium produced at the interface between interacting fungi is often associated with peroxidase activity (White and Boddy 1992) whereas laccase activity is associated with suppression of aerial mycelium (Rayner et al. 1994).

Schoemaker et al. (1991) proposed a lignin degradation system where lignin depolymerisation was an oxidative process and the metabolism of lignin fragments was a combination of oxidative and reductive processes catalysed by LiP and MnP. An oxidative process was supported by Chen and Chang (1985) who found that the oxygen content from decayed wood was higher than sound wood. Call and Mucke (1997) reviewed the role of laccase in the degradation of lignin. Using immuno-electron microscopy, Goodell et al. (1998) showed that laccase did not penetrate into the wood cell wall, despite extensive lignin degradation, which suggested that compounds of a lower molecular weight were involved. The involvement of reduced, highly reactive oxygen species such as hydroxyl radicals (OH^\cdot) was questioned (Faison and Kirk 1983; Kirk et al. 1983) although Backa et al. (1993) found that growth of white rot fungi was associated with the production of OH^\cdot . Tanaka et al. (1999) and Hilden et al. (2000) suggested that OH^\cdot production, by an extracellular low molecular weight substance, via hydrogen peroxide (H_2O_2) resulted in new phenolic substructures on the lignin polymer making it more susceptible to degradation by MnP or laccase. Guillen et al. (2000) demonstrated the production of OH^\cdot during lignin oxidation by laccase, with synergism observed with another ligninolytic enzyme, aryl alcohol oxidase. H_2O_2 production has been detected by a number of white rot fungi (Highley and Murmanis 1985; Koenigs 1972).

Zapanta and Tien (1997) reviewed the proposed roles of veratryl alcohol and oxalic acid in LiP and MnP activity, respectively. The roles of sugar oxidoreductases and veratryl alcohol oxidase were reviewed by Ander and Eriksson (1977). In the white rot system, oxalic acid may play multiple roles in the regulation of LiP and as a metal chelator (Shimada et al. 1997). Manganese is thought to play a major regulating role in lignin depolymerisation in the presence of oxalic acid (Daniel et al. 1997; Hames et al. 1998; Kurek and Gaudard 2000) and can be observed as black deposits on the surface of wood degraded by white rot fungi (Blanchette 1984b). Watanabe et al. (1998) introduced a new hypothesis of non-enzymatic lignin degradation by the use of copper complexes and lipid hydrogen peroxide in the presence of pyridine, a metabolite of ligninolytic fungi. Kapich and Shishkina (1995) found that peroxidation of lipids of basidiomycete fungi may have an important role in lignin degradation and hypothesised a coupling between the processes.

1.3.4.2 Hemicellulose degradation

The hemicellulases are comprised of endo- and exo-enzyme types, the former being the most commonly found. Degradation of wood xylans requires endo-1,4- β -D-xylanases, β -xylosidases, α -L-arabinosidase, α -D-glucuronidase and acetyl xylan esterase. Degradation of wood mannans requires endo-1,4- β -mannanase, β -mannosidase, α -glucosidase, β -galactosidase and acetyl galactoglucomannan esterase. Assays for these enzymes were reviewed in Eriksson et al. (1990). A study of the relative rate of removal of cellulose, xylan, mannan and lignin during fungal decay of pine showed that xylan and mannan were degraded at a faster rate than cellulose and lignin (Breccia et al. 1997; Kirk and Highley 1973). The initial degradation of hemicelluloses may be a critical step required to increase the accessibility of cellulose and lignin and presents a potential target for the control of fungal decay (Highley 1987a; Micales et al. 1987).

1.3.4.3 Cellulose degradation

The cellulase degradation system of most brown rot fungi is comprised of an endoglucanase which randomly hydrolyses β -1,4-glucosidic linkages in the cellulose microfibril, and a β -D-glucosidase which hydrolyses cellobiose or other oligosaccharides to glucose. Multiple isozyme forms of cellulases may exist (Clausen 1995). The cellulases of wood decay fungi are relatively stable to denaturing conditions as their extensive glycosylation may give protection to the protein component (Sharon and Lis 1982).

The cellulase system of white rot fungi has an additional enzyme, an exoglucanase which hydrolyses cellobiose units from the non-reducing ends of cellulose chains that are exposed from the action of endoglucanases and acts to disrupt hydrogen bonding. Wood (1975) showed that some cross-synergism may occur between endo- and exo-glucanases from different fungi. An exoglucanase has not been found in most brown rot fungi but it appears that the synergistic endoglucanase-exoglucanase action is not essential as brown rot fungi clearly have the ability to degrade cellulose in wood. Cellulase of the exo-type in most brown rot fungi was found to be associated with growth on lignified cellulose substrates such as wood or filter-paper and was dependent on the presence of wood components such as hemicelluloses or lignin (Highley 1988; Nilsson 1974).

Studies of the regulation of cellulases and xylanases are often complicated by their simultaneous production when grown on cellulose and also the fact that these two different enzymes can hydrolyse their respective primary substrates. Gong et al. (1981) found that when grown on xylan alone, some fungi produce xylanases with little or no cellulases. Several endoglucanases have been purified and characterised (Kleman-Leyer and Kirk 1994; Schmidhalter and Canevascini 1992) although they were difficult to purify and found not to be substrate specific. So-called purified endoglucanases often show xylanase activity due to difficulties in separation (Herr et al. 1978) but this may be solved using “hydrophobic interaction chromatography” (Clausen 1995). Reports in the literature vary as to whether cellulolytic and xylanolytic systems are under independent or common regulatory control. The highest cellulase activities are generally found with cellulose as a carbon source and in the presence of simple sugars such as cellobiose or glucose (Cotoras and Agosin 1992; Highley 1973). Glucose at 0.1% resulted in repression of cellulase and xylanase synthesis until concentrations dropped which indicated that their synthesis was under common regulatory control. Carbohydrate-degrading enzymes are typically produced in low quantities by brown rot fungi and attempts to increase production have had limited success as the enzymes appear to be largely constitutive (Highley 1976). This was supported by Elisashvili (1993) who examined a range of basidiomycete fungi and proposed that the cellulases and xylanases of the white rot fungi were inducible enzymes and all β -glucosidases and β -xylosidases and the cellulases and xylanases of the brown rot fungi were constitutive enzymes.

Wood cell wall degradation by brown rot fungi is initiated by the fungal hyphae within the cell lumen (Wilcox 1970). The S1 and S2 layers are extensively degraded in a non-uniform manner prior to attack on the S3 layer and compound middle lamella. The form of the cell remains relatively intact due to the structural characteristics of lignin until late in decay when collapse rather than thinning of the wall occurs. Immuno-electron microscope studies showed that decay enzymes and metabolites from brown rot fungi were not capable of penetrating the cell wall in early stages of decay (Blanchette 1995; Cowling and Brown 1969; Kim et al. 1993). It would appear that enzymatic attack is not localised near the hyphae but the enzymes would have to diffuse to the site of decay. In elution experiments, Green (1980) observed that brown rot fungi did not bind their cellulases to the mycelium as did the white rot fungi. The mechanism by which brown rot fungi can achieve rapid and extensive depolymerisation of cellulose and hemicelluloses, in the presence of lignin, and

in parts of the cell wall not in close proximity to the hyphae, has not been established but may involve both enzymatic and non-enzymatic agents and processes.

Halliwell (1965) was the first to propose a non-enzymatic cellulolytic mechanism involving hydrogen peroxide and ferrous iron ($\text{H}_2\text{O}_2/\text{Fe}^{2+}$; Fenton's reagent) that resulted in the production of OH^\cdot which have the capability of initiating the oxidative depolymerisation of lignocellulose within the wood cell wall. This was supported by observations that the initial degradation of wood was closely related to OH^\cdot formation (Backa et al. 1992; Hirano et al. 1997; Wetzstein et al. 1997). Murmanis et al. (1988a) concluded that $\text{H}_2\text{O}_2/\text{Fe}^{2+}$ may by itself degrade wood, and it was not possible to determine whether there was a synergistic relationship between $\text{H}_2\text{O}_2/\text{Fe}^{2+}$ and the cellulases. Flournoy (1994) found that the effects of Fenton's reagent on wood components were not duplicated by many brown rot fungi. The OH^\cdot are non-specific and non-diffusible so reactions were proposed to be facilitated by the fungi hyphal sheath (Green et al. 1990) which may penetrate the cell wall layers and facilitate the transport of enzymes to the substrate and the uptake of nutrients to the fungal protoplast (Chou and Levi 1971; Hirano et al. 2000). Using SEM, Larsen and Green (1992) observed extracellular mycofibril structures embedded within the glucan sheath. Further research into the factors influencing formation of the hyphal sheath may provide information for use in the development of strategies for controlling wood decay.

The $\text{H}_2\text{O}_2/\text{Fe}^{2+}$ system was studied by a number of researchers (Green and Highley 1997; Koenigs 1974; Ratto et al. 1997). There were conflicting reports on whether brown rot fungi produce extracellular H_2O_2 . However, if H_2O_2 was the substrate for OH^\cdot production it may be produced in small amounts at particular stages of hyphal growth and rapidly utilised and so not detected in some assays. Highley (1987b) found low nitrogen and carbohydrates stimulated H_2O_2 production by brown rot fungi but had little effect on white rot fungi. Espejo and Agosin (1991) suggested that brown rot fungi may produce H_2O_2 from an oxalic acid oxidase, although extracellular oxidase production by brown rot fungi has rarely been reported. In a model proposed by Hyde and Wood (1995) and Hyde and Wood (1997), a pH-dependent auto-oxidation of Fe^{2+} produced H_2O_2 at a distance without damage to the hyphae, and this initiated Fenton's reaction and the formation of OH^\cdot . However, there was no proposed mechanism that would direct a site-specific reaction of Fe^{2+} and H_2O_2 within the wood cell wall.

Flournoy et al. (1991) proposed that a small diffusible agent initiated cellulose depolymerisation by causing erosion and enlargement of wood pores. Several extracellular organic acids are produced by brown rot fungi that could cause localised swelling of the secondary wall and increased accessibility to enzymatic attack. Oxalic acid may have roles in the hydrolytic or oxidative depolymerisation of cellulose (Jordan et al. 1996a) and hemicellulose (Green et al. 1991; Shimada et al. 1994), and function in iron chelation, calcium precipitation, and the formation of OH^- and a pH gradient (Goodell et al. 1997). Shimada et al. (1991) and Son et al. (1996) questioned the involvement of Fenton's reagent as they found that oxalic acid was capable of directly attacking wood cellulose.

Schmidt et al. (1981) proposed a model involving oxalic acid, H_2O_2 , Fe^{2+} and OH^- (Fig. 1.7). They found that brown rot fungi produced and accumulated oxalic acid which may function to reduce Fe^{3+} to Fe^{2+} that can react with H_2O_2 to produce OH^- which have the capability of initiating the oxidative depolymerisation of lignocellulose in the wood cell wall. However, Hyde and Wood (1995) found that oxalic acid does not reduce Fe^{3+} except as a reaction in the presence of light. Internal wood decay normally occurs under relatively low light levels so this reaction may not play a significant role. Production and accumulation of oxalic acid can depend on carbon and nitrogen sources (Micales 1995) and may be stimulated by certain acids and sugars from hemicellulose degradation (Green et al. 1992). In general, brown rot fungi accumulate oxalic acid during growth and white rot fungi do not unless supplemented with calcium carbonate (Dutton et al. 1993). White rot fungi do not accumulate oxalic acid due to degradation by oxalate decarboxylase. The regulation and metabolism of oxalic acid in brown rot fungi is not well understood. Brown rot fungi can produce oxalate decarboxylase particularly under low pH conditions to facilitate metal reduction, OH^- production and the decay process by preventing over-accumulation of oxalic acid that inhibits the Fenton's reaction (Micales 1997).

Siderophores have been proposed to play a role in cellulose degradation (Chandhoke et al. 1991) and have been found in a number of brown rot fungi (Fekete et al. 1989; Jellison et al. 1997). A non-enzymatic oxidation of cellulose was proposed to involve the action of low molecular weight diffusible metal chelators. The mechanism(s) for production of these chelators in wood decay fungi has not been determined. Paszczynski et al. (1999) isolated two new compounds, a catechol and a hydroquinone which were proposed to serve as Fe^{3+} chelators, oxygen reducing agents and redox-cycling molecules including electron transport carriers to Fenton's reactions. This was supported by work by Kerem et al. (1999) who

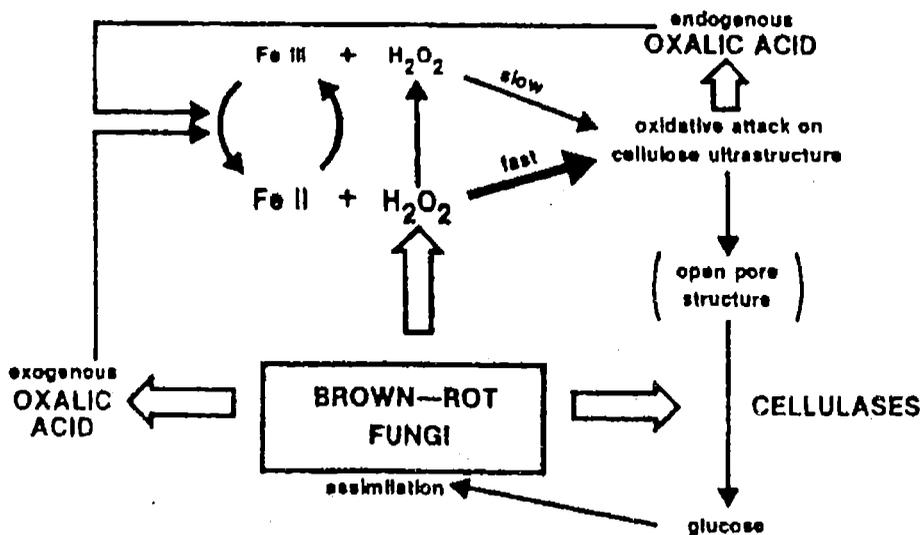


Fig. 1.7 Model of cellulose degradation, including the role of oxalic acid in the initial non-enzymatic oxidation (Schmidt et al. 1981).

proposed a hydroquinone-driven Fenton's reaction. Other hypotheses include mechanisms involving glycopeptide compounds that both reduce and oxidise iron (Enoki et al. 1989). Phenolate-type siderophores have been shown in enzyme-linked immunosorbant and immuno-electron microscope studies to be of sufficiently low molecular mass to penetrate wood cell walls (Jellison et al. 1991; Jordan et al. 1996b). Oxalic acid can also act as a weak chelator by sequestering iron from relatively insoluble iron hydroxide complexes and then allowing the chelators to access the iron. The chelators have a high affinity for Fe^{3+} and will mediate its reduction in redox cycling processes at pH values below neutrality. It has been proposed that the chelators are either converted to depolymerising agents or via Fenton reactions they result in OH^- production (Goodell et al. 1998; Highley and Flourney 1994; Illman and Highley 1989).

1.3.5 Biological control of wood decay

In their review of biological control agents (BCA's) and application to forest products, Schoeman et al. (1999) concluded that successful biological control of wood decay relies on appreciating the limitations of biological treatments and understanding the mechanisms involved. One of the greatest obstacles is the variability of performance that may be caused by a number of factors including incomplete BCA colonisation, adverse environmental conditions, competitive resident microorganisms, non-persistent inhibitory compounds, BCA mutations and natural selective pressure that result in ineffective cultures, and BCA

death. The requirements for effective biological control of wood decay were reviewed by Bruce and Palfreyman (1998) and the major influencing factors included physical and chemical conditions at application and biological factors between the BCA, wood decay fungi, other microorganisms and the wood substrate.

1.3.5.1 Biological control agent (BCA) screening

Laboratory assays are necessary to screen large numbers of potential BCA's against the target decay fungi but the antagonistic interactions demonstrated using pure cultures and under a controlled environment and nutrient rich conditions often do not translate to the field situation. The complexity of microbial communities in wood, environmental effects and the different physiological conditions are such that these interactions are less obvious if present at all. Wood-based BCA screening assays may simulate the field situation more closely than agar assays but the results can be difficult to interpret. A compromise may be the use of a wood extract or low nutrient medium (Leben 1978) or one with an amino acid profile representing the major constituents present in the test wood substrate (Tucker and Bruce 1995). Mercer and Kirk (1984a) carried out an initial BCA screen on nutrient agar and selected the most promising cultures for wood block assays on agar and then on glass beads. Subsequent field trials showed that the better BCA's had also done well in the laboratory but the reverse did not necessarily follow (Mercer and Kirk 1984b).

Dual culture agar assays are the most commonly used screen for potential BCA's (Bruce and Highley 1991; Croan and Highley 1990; Highley and Ricard 1988) and can show interactions between the BCA and the decay fungus that include competition, antibiosis and parasitism. Agar block and soil block decay assays have been adapted for the evaluation of potential BCA's against wood decay fungi (Freitag and Morrell 1989; Hedley and Foster 1972). The wood blocks are usually sterilised so that the influences of naturally occurring microbial populations are excluded and the effects of the potential BCA are artificially enhanced. Leben (1978) developed a relatively simple and effective screen for BCA's using surface-sterilised wood blocks. The screening medium should provide equal growth opportunities for both BCA and wood decay fungi (Benko and Highley 1990a; Benko and Highley 1990b). For use in integrated control strategies, the BCA's would need to be resistant to the preservatives and this could be either screened on agar containing preservatives (Abd-El Moity et al. 1982), or developed by gamma irradiation (Troutman and Matejka 1978) or ultraviolet radiation (Papavizas et al. 1982).

There are diverse opinions on the best approach to the collection of potential BCA's. Preston et al. (1982) suggested that the reason that a biological control system for wood decay fungi had not yet been developed was that research depended on the investigation of a limited number of microorganisms, ones that have been shown to have potential in previous studies, and that these were isolated from a narrow range of sources. They proposed that isolation from aged but sound wood could give cultures that are responsible for the absence of decay. Other researchers believe it is important to obtain BCA's from the site of origin at which the problems are occurring (Schwarz 1992). Yet others believe isolations away from the site of origin may provide useful cultures e.g. potential BCA's against wood decay fungi have been isolated from contaminated growth medium of edible fungi (Doi and Mori 1993). Regardless of their source, potential BCA's would need the ability to utilise and occupy the same ecological niche as decay fungi (Shukla and Rana 1995). If BCA's cannot colonise sites in which the decay fungi colonise and grow, their ability to control the fungi will be limited.

1.3.5.2 BCA modes of antagonism

The success of a BCA will depend on its antagonistic abilities and also its ecological compatibility in the wood environment. Where the mode of antagonism is unknown, it cannot be assumed safe on the basis that the system occurs in nature. There is a poor understanding of the modes of action employed by most potential BCA's in wood so the selection of suitable media to screen for these mechanisms is currently not feasible. The conditions under which "mode-of-action" studies are carried out may determine which mechanisms are activated or excluded. The nutrient composition of media may selectively favour particular modes of antagonism. Many "mode-of-action" theories are based on autecological rather than synecological studies and therefore may not accurately reflect the mechanisms that occur within microbial communities in wood.

Antagonistic mechanisms can be categorised in terms of competition for nutrients and space (Hulme and Shields 1970), production of volatile or non-volatile inhibitory compounds (fungistatic and fungitoxic), mycoparasitism (Schoeman et al. 1999), hyphal interference (Boddy 2000) and the production of siderophores (Srinivasan et al. 1994). These categories are not mutually exclusive and their relative importance *in vivo* is not clear. The means by which biological control is expressed is not a simple process which is produced by a single metabolite but is likely to be a number of mechanisms mediated by

specific compounds which may be genetically or enzymatically regulated. Unlike most chemicals, environmental conditions may determine the relative importance of each mechanism. The ability to exhibit the different modes of antagonism may vary between and within BCA species.

BCA's that are effective competitors may help to control sapstain and mould fungi but the basidiomycete fungi which can utilise structural components of wood may be less successfully excluded on this basis. Competition for iron in wood may be a possibility for BCA's with siderophores with an equal or greater affinity constant than the wood decay fungus (Bruce et al. 1995). Successful BCA's would require chelators with an equal or greater affinity for iron than the decay fungi.

Inhibitory compounds produced by BCA's against wood decay fungi on agar and in liquid culture are not necessarily effective on wood (Highley et al. 1997; Lundborg and Unestam 1980). The compounds need to show stability and persistence e.g. resist leaching (Morris et al. 1986). The production of antibiotics requires substantial resources and may be limited by available carbon and minerals (Duffy and Defago 1999). One of the major problems in registration and subsequent commercialisation of fungal BCA's has been risk assessment, particularly with respect to extracellular metabolites (Strasser et al. 1999). There has been progress in the identification of secondary metabolites produced by BCA's and the effects on wood decay fungi (Ayer and Trifonov 1999).

Mycoparasites are fungi that parasitise other fungi by a number of mechanisms including invasion of host cells by the production of lytic enzymes (such as chitinases and laminarinases) or by mechanical means, coiling around the host hyphae and the action of agglutinins. Basidiomycete fungi have a microfibrillar structural cell wall component composed of chitin (Bartnicki-Garcia 1968) that is typically 7 to 24% (of the dry weight of the water-insoluble portion of the hyphal cell wall) (Johnson and Chen 1983). To select for potential mycoparasites against a specific wood decay fungus, organisms can be screened for high constitutive levels of lytic enzymes in the presence of the decay fungus cell wall material (Bruce et al. 1995).

Synergism is also possible where the BCA may provide trace essential nutrients to the wood decay fungus e.g. bacteria may provide thiamine (Henningsson 1967). The BCA may utilise nutrients provided by the wood decay fungus that would otherwise be operating in a

feedback mechanism and controlling wood decay (Hulme and Stranks 1970), or mixtures of enzymes from the BCA and the wood decay fungus are more effective than each alone (Wood 1969). Competition between BCA's can result in less control than each BCA alone (Thrane et al. 2000).

1.3.5.3 Enhancement of biological control systems

There have been a number of approaches used to enhance the effectiveness of BCA's in inoculum formulation, timing of application, delivery systems and understanding the influence of the BCA on the natural microbial succession in wood systems. The limitation to biological control of wood decay may be the ability of the BCA to fully colonise the wood substrate (Bruce et al. 1989; Morrell and Sexton 1990; Thrane et al. 2000) and thus confer maximal protection. Some wood decay fungi form pseudosclerotial plates, a morphological and chemical barrier to protect and maintain nutrients and resources within the area of wood that is colonised. Improved colonisation may be achieved by altering the wood environment to stimulate growth of the BCA or to limit colonisation and growth of the decay fungi and other competing organisms. The incorporation of specific food bases (Canessa and Morrell 1997; Morris 1983) or a favourable pH (Ejechi and Obuekwe 1994) into the inoculum may improve the BCA colonisation and survival. Results from these studies were not promising as there was either no effect or a simultaneous stimulation of the decay fungi. However, biological control activity of *Trichoderma viride* Pers.:Fr. has been enhanced by the addition of extraneous nutrient (Ejechi and Obuekwe 1994; Seaby 1977). As part of an integrated control strategy (Papavizas 1981), the BCA could be applied to wood following a compatible chemical treatment such as fumigants (Morrell 1990), fluoride and boron based waterborne chemicals (Dawson Andoh and Morrell 1992a; Giron and Morrell 1989), or heat and steam pasteurisation (McAfee and Gignac 1997).

The ideal BCA for wood decay would be one that mimics typically broad-spectrum chemical treatments, by controlling a range of decay fungi species and with the ability to colonise rapidly, survive and retain effectiveness over a period that may range from a few months to 40 years. For the effective control of more than one species of decay fungi it is likely that an inoculum of several BCA's, which act via different mechanisms, would be necessary. This may also limit the development of resistance by the wood decay fungi to a single BCA. Repeated application of BCA's to wood in-service may be required during conditions favourable to wood decay fungi (Ejechi 1997).

Genetic engineering could be used to enhance the antagonistic attributes or growth and survival capabilities of BCA's in wood. This may be carried out through non-specific genetic manipulations and gene transfer using fungal transformation. Traditional approaches have used ultraviolet irradiation, chemical mutagenesis, hybridisation and protoplast fusion (Haran et al. 1993; Harman and Hayes 1993). Little is known about the genes, gene products and regulatory systems involved in the expression of biological control activity in wood.

1.3.5.4 Examples of BCA's that are active against wood decay fungi

Phlebiopsis gigantea

One of the first examples of biological control of wood decay was the control of the root rot fungus *Heterobasidion annosum* (Fr.:Fr.) Bref. by *P. gigantea* (Rishbeth 1963). Registration of this BCA only occurred after 30 years of field use and there are three different formulations commercially available in Europe (Pratt et al. 1999). *Phlebiopsis gigantea* can only partially replace established *H. annosum* so tree stumps are inoculated straight after felling. Colonisation by *H. annosum* is prevented by competition for resources and hyphal interference. The effectiveness of control is influenced by wood moisture content and a number of environmental and silvicultural conditions (Sierota 1997). The use of *P. gigantea* was shown to cause no obvious damage to ground vegetation and soil properties around the tree stumps, in comparison to urea or borate treatments (Westlund and Nohrstedt 2000).

To avoid the risk of decay associated with *P. gigantea*, *Trichoderma* species such as *Trichoderma harzianum* Rifai (Anselmi et al. 1997; Capretti and Mugnai 1989; Seaby 1977) and *Resinicium bicolor* (Albertini & Schwein.:Fr.) Parmasto (Holmer and Stenlid 1997) were evaluated against *H. annosum* with varying levels of inhibition. More recently, Nicolotti et al. (1999) had success using *Phanerochaete velutina* (DC.:Fr.) P. Karst. and *Verticillium bulbillosum* Gams and Malla against *H. annosum*.

***Trichoderma* species**

The antagonistic effects of *Trichoderma* species on detrimental fungi and investigations of their potential as BCA's were reported more than 60 years ago (Chidester 1940; Weindling

1934) and since then have been studied by many researchers (Hjeljord and Tronsmo 1998; Li and Hood 1992; Lindgren and Harvey 1952). The most commonly reported species in the literature are *T. harzianum* and *T. viride*. An important step in assessing *Trichoderma* for biological control potential is to identify them to the species level using taxonomic schemes (Bisset 1991a; Bisset 1991b) and molecular techniques (Hermosa et al. 2000).

In recent years, commercial biological control preparations have become available which include Binab spore pellets and wettable powder, containing *T. harzianum* and *Trichoderma polysporum* (Link) Rifai, and Trichodex 20P (Makhteshim-Agan) containing *T. harzianum*, with other products on the path to commercialisation (Messner et al. 1996). Trichodex was the first BCA commercialised, and is used for management of greenhouse diseases such as grey mould, white mould, leaf mould and powdery mildews in combination with fungicides. *Trichoderma* species have been studied in integrated control strategies as they can be tolerant of wood preservatives (Wallace et al. 1992).

Schoeman and Dickinson (1992) proposed that biological control by *Trichoderma* species was, at best, transient and useful only in the short-term. However, depending on the target situation, initial BCA colonisation may be adequate to exclude decay fungi during a high risk period. Wood has been found to be still resistant to decay seven years after inoculation with *Trichoderma* fungi which retained viability (Bruce et al. 1989). Studies by Highley (1997) found that bioprotection of pine wood blocks was only effective by live cultures of *Trichoderma virens* (Miller, Giddens and Foster) von Arx. In many cases, decay was reduced by *Trichoderma* species but not completely inhibited (Canessa and Morrell 1997).

Biological control by *Trichoderma* species has been found to be target specific to the strain level of brown rot fungi (Bruce and Highley 1991; Highley and Ricard 1988) and is less effective against white rot fungi (Highley 1997; Tucker and Bruce 1995). Morrell (1990) observed that Binab was most effective for brown rot fungi in above ground situations and there was little effect on white rot fungi. In the wood system, the control of brown rot fungi may leave the white rot fungi with less competition for resources and greater potential for decay. Highley (1997) found that production of the antibiotic gliotoxin by *T. virens* was necessary to inhibit decay of pine blocks by the white rot fungi examined but not the brown rot fungi. In studies on *T. virens* culture filtrates, Highley (1997) found that inhibition of white rot fungi was greater when *T. virens* was grown on low nitrogen medium and

inhibition of brown rot fungi was greater when *T. virens* was grown on high nitrogen medium.

Trichoderma species have a range of possible modes of antagonism including the production of volatile inhibitory compounds (Dennis and Webster 1971b), non-volatile inhibitory compounds (Dennis and Webster 1971a), competition for substrate and available nutrients (Hulme and Shields 1970) and mycoparasitism (Chet et al. 1990; Elad et al. 1982; Lumsden 1992). Antagonistic mechanisms may interact e.g. the *Trichoderma* fungus may weaken the wood decay fungus by the production of antibiotics and thus increase their susceptibility to mycoparasitism. *Trichoderma* fungi may deplete the easily accessible nutrients in wood (Hulme and Shields 1972) and then use a combination of antibiosis and parasitism to prevent colonisation by decay fungi and germination of basidiospores. Smith et al. (1981) proposed that the replacement of 'pioneer' fungi by *T. harzianum* led to changes in the organic environment of the wood cell that were less conducive to colonisation by decay fungi. A review by Ghisalberti and Sivasithamparam (1991) showed evidence for the involvement of antibiotics in the biological control activity of a number of *Trichoderma* species e.g. *T. viride* was reported to produce 6-PAP, dermadin, trichoviridin, heptelidic acid, trichodermin and octadecapeptides. There are three categories of antibiotics produced by *Trichoderma* fungi: volatile, non-volatile (water-soluble) and peptabiols (metabolites that are able to interact with membranes).

Anke et al. (1991) reported the production of hydroxamate type siderophores by *Trichoderma* species that have lower iron binding ability than the phenolate-type siderophores which are primarily produced by decay fungi (Hider 1984). The importance of siderophore production by *Trichoderma* species in the biological control of wood decay fungi was investigated by Srinivasan et al. (1994). Ratto et al. (1996) found that siderophores produced by *T. harzianum* showed growth inhibition of brown rot fungi. A *T. harzianum* culture was shown to solubilise insoluble minerals by chelation and reduction which may be part of a multiple component action to achieve effective biological control under a range of environmental conditions (Altomare et al. 1999). The production of chitinolytic enzymes by *Trichoderma* species has been studied (Liu and Morrell 1997; Lorito et al. 1993; Tronsmo and Harman 1993). Bruce et al. (1995) found significant differences in production of chitinases and laminarinases depending on the decay fungus cell wall material used.

There have been a number of ecological studies on *Trichoderma* fungi. Danielson and Davey (1973a) proposed that *Trichoderma* species from forest soils had temperature optima and maxima that reflected their geographical origins. They found that *T. viride* and *T. polysporum* were restricted to low temperature areas, *T. harzianum* in warm areas and *Trichoderma hamatum* (Bonord.) Bainier and *Trichoderma koningii* Oudem. were found in diverse climatic conditions. Assays involving *Trichoderma* species are generally run at 20°C (Schoeman and Dickinson 1993), 22°C (Tucker and Bruce 1995) or 23°C (Bruce and Highley 1991). Sierota (1976) found that *T. viride* produced the highest biomass in liquid medium with a pH of 2.5 to 3.1 and the optimum inhibition of basidiomycete fungi occurred at a pH of 2 to 3.5. However, Ejechi et al. (1996) proposed that *T. viride* grew better in liquid medium as the pH approached neutral, and the highest biomass was produced at pH 5. Danielson and Davey (1973b) observed that the optimal growth pH of *Trichoderma* species was 3.7 to 4.7 although some cultures could grow well at around pH 6. Hintikka and Korhonen (1970) showed that *T. viride* had an exceptionally high tolerance to carbon dioxide in wood, such that when carbon dioxide levels were elevated it became the dominant species.

Trichoderma fungi are sensitive to high wood moisture content and tend to colonise superficially which may be related to the reduced gaseous exchange at depth (Dowding 1985). Although *Trichoderma* fungi may be relatively poor primary colonisers of fresh pine sapwood (Bruce and King 1986a), the rapid superficial colonisation may be all that is required to exclude colonisation by decay fungi. Although not physically present in depth, some *Trichoderma* species produce inhibitory metabolites or lytic enzymes that diffuse into the wood and act on decay fungi (Ejechi and Obuekwe 1996). Although they are not effective colonisers of living wood, *Trichoderma* species have been used to exclude basidiomycete fungi from tree wounds (Pottle et al. 1977; Woodgate-Jones and Hunter 1983). Genetic improvement of *Trichoderma* species using protoplast fusion can combine desirable traits (Stasz et al. 1988) although there may be problems with somatic compatibility.

There are some negative aspects to the use of *Trichoderma* species in control of wood decay. Some species e.g. *Trichoderma longibrachiatum* Rifai, *T. viride* and *T. koningii* are used for commercial cellulase production (Bollok and Reczey 2000). Chidester (1941) showed that *T. viride* reduced strength properties of pine sapwood, although they were not significant compared to those caused by basidiomycete fungi. Many *Trichoderma* species

produce masses of coloured spores so the production of albino mutants may be necessary to avoid undesirable discolouration of treated timber. This would not be necessary for some applications such as utility poles since preservative treatments already result in discolouration. Freitag and Morrell (1992) and Canessa and Morrell (1997) observed that *T. harzianum* significantly induced laccase production by the white rot fungus *Trametes versicolor* (L.:Fr.) Pilat and thus potentially stimulating wood decay.

***Scytalidium* species**

Scytalidium fungi were first investigated as BCA's against *Poria* species in *Pseudotsuga menziesii* (Ricard 1966) and have since been shown to exhibit antagonism towards a range of wood decay fungi (Klingstrom and Johansson 1973) although some researchers have had less success (Morris and Dickinson 1981). *Scytalidium dimidiatum* (Penz.) Sutton & Dyko was less effective in wood against established decay fungi unless the infection was at an early stage (Unligil 1978). Cease et al. (1989) observed the formation of pseudosclerotial plates by white rot fungi in response to *Scytalidium* species. There have been some difficulties in establishing *Scytalidium* fungi in the field (Graham 1973). Some *Scytalidium* species have been reported to reduce wood brightness and result in wood weight loss (Nilsson 1973) although this may be isolate dependent (Highley 1990).

The first commercial biological control formulation was developed for protection against the brown rot fungus *Neolentinus lepideus* (Fr.:Fr.) Redhead & Ginns in Scots pine poles. Binab FYT pellets (containing *Trichoderma harzianum*, *T. polysporum* and a *Scytalidium* species) are applied into multiple inoculation holes drilled into the poles. Bruce and King (1983) detected a residual toxic effect of Binab on *N. lepideus* from wood blocks after sterilising and leaching. There are a number of modes of antagonism observed by *Scytalidium* species including the production of antibiotics such as scytalidin (Klingstrom and Johansson 1973), lytic enzymes (Highley 1990) and hyphal coiling (Von Aufsess 1976).

Other fungi

Biological control of wood decay fungi using decay fungi seems incongruous and in most cases is unsuitable. However, even the use of non-decay fungi may initiate community development involving decay fungi. Some decay fungi such as *Gloeophyllum sepiarium*,

commonly found on *Pinus radiata*, have been investigated for control of the root rotting *Armillaria* fungi (Sokolov 1964).

Besides *Trichoderma*, investigations of mould fungi as potential BCA's for decay fungi have included *Penicillium* and *Aspergillus* (Bruce et al. 1984; Doi and Mori 1995). Some mould species were shown to produce cellulases and several have been identified as human allergy risk genera e.g. *Penicillium*, *Cladosporium*, *Aspergillus*, *Mucor*, *Trichoderma* and *Phoma* (Simeray et al. 1997) and so may not be acceptable for some wood applications.

A discomycete *Coryne* species, commonly associated with decayed wood in standing trees, has been shown to produce antibiotics *in vitro* (Etheridge and Craig 1973) and inhibit growth of *Stereum* species (Whittaker 1962). *Cryptosporiopsis* species produce a broad spectrum antibiotic that has been shown to inhibit the growth of a range of basidiomycete fungi (Stillwell and Hodgson 1968).

Bacteria

Benko and Highley (1989) reported that a solution of *Streptomyces*, *Pseudomonas*, *Streptoverticillium* and *Xenorhabdus* prevented degradation of pine by *Postia placenta* (Fr.) M. Larsen & Lombard in agar block assays. Actively growing bacteria may be necessary for effective control (Benko and Highley 1990b). *Streptomyces* species have been shown to produce antifungal metabolites (Croan and Highley 1994). De Groot (1971) found that *Streptomyces* species inhibited growth of decay fungi on nutrient agar but in wood decay assays, greater weight losses resulted in the presence of *Streptomyces*.

Bacillus species have shown antagonism towards wood decay fungi (Mercer and Kirk 1984a) by the production of volatile and non-volatile inhibitory compounds (Dass and Teyegaga 1996) including the antibiotics bacillomycin, mycocerein, fengmycin and bacilysin (Loeffler et al. 1986) and lytic enzymes (Preston et al. 1982). Volatile compounds were the primary means of antagonism of some yeast and bacteria BCA's against sapstain and mould fungi in *Pinus* blocks (Payne et al. 2000).

Natural products

The effects of classical enzyme inhibitors such as metals, phenolics and wood extracts on decay enzymes vary between species of wood decay fungi and between isolates of a species (Highley 1975a; Highley and Micales 1990; Kennedy et al. 1995). A number of natural product compounds have been found to show inhibitory activity against wood decay fungi and these include water-soluble lichen extracts (Land and Lundstrom 1998), chitin synthesis inhibitors (Johnson 1986), and green tea and coffee extracts (Arora and Ohlan 1997). Green (2000) screened 11 chemical compounds for inhibition of weight loss in cotton cellulose and found that antioxidants were generally only effective against brown rot fungi but some compounds such as ruthenium red inhibited weight loss by all of the decay fungi tested.

In *Pinus* species, sapwood has been found to have up to 0.5% monoterpene content and heartwood up to 1.1% (Englund and Nussbaum 2000), but the composition of this component is variable. Hill et al. (1997) screened terpene fractions of pine oil for inhibitive activity against sapstain fungi in agar and wood assays. They found that carvacrol had higher inhibition than alpha-terpineol, but the oxygenated terpenoids had high levels of inhibitive activity compared to bicyclic structures such as borneol. Their results showed that it was the oxygenated terpene fractions, rather than the terpene hydrocarbons, that had the highest activity against fungi. The structural features that gave this observed activity were not clear. On agar, alpha-terpineol and terpinen-4-ol resulted in inhibition that increased with treatment concentration. This work was supported by thesis screening of a number of oxygenated monoterpene compounds for inhibition of *Gloeophyllum sepiarium* growth on agar (Appendix 3).

Chapter 2 WOOD DECAY POTENTIAL OF BASIDIOMYCETE FUNGI FROM *PINUS RADIATA*

2.1 INTRODUCTION

The objective of Chapter 2 was to collect a range of basidiomycete fungi from New Zealand-grown *Pinus radiata* and to screen them for wood decay potential in the following assays:

- 1) Cellulase, xylanase and mannanase enzymes in liquid medium
- 2) Laccase, peroxidase and proteinase enzymes on agar
- 3) Wood weight loss of *P. radiata* in a low decay hazard assay
- 4) Fungal growth on agar and on *P. radiata*

The variation in wood decay potential between the basidiomycete fungi, and between isolates of the same species, was assessed for each of the assays. Regression analyses were carried out to determine if there were any significant correlations between the parameters of the assays and whether they were correlated to rot classification (brown rot or white rot fungi). The results of these assays were used to select basidiomycete cultures for further characterisation and biological control studies.

2.2 MATERIALS AND METHODS

2.2.1 Collection of basidiomycete fungi

Basidiomycete fungi were isolated from *P. radiata* and resident fruiting bodies obtained from forestry sites in the central North Island and South Island, and from HortResearch log and timber trials in Hamilton. Eleven fungi that had been isolated from *P. radiata* were sourced from a culture collection at Forest Research (Rotorua) and two fungi from a culture collection at Landcare Research (Auckland).

The majority of basidiomycete fungi are resistant to the fungicide benomyl so this was used as the first screening criterion. Fragments of wood, mycelium or fruiting body were placed onto benomyl agar (BA; Appendix 1.1) and the Petri dishes were incubated at 25°C. Emerging colonies were subcultured onto malt extract agar (MA; Appendix 1.7) until pure cultures were obtained.

Macroscopic observations such as gross colony morphology on agar were noted and the cultures were examined under a light microscope (Zeiss) for the presence of other characteristics of basidiomycete fungi such as clamp connections. Hyphal morphology and clamp distribution were examined microscopically by mounting mycelium in 10% ammonia and staining with 1% Congo Red. Basidiomycete culture record sheets (Appendix 4) provided by Geoff Ridley (Forest Research, Rotorua) were used to number and collect details of each culture (not shown). Fruiting bodies were identified using field guides to New Zealand wood decay fungi (Buchanan 1989; Hood 1992). The field cultures were not formally identified prior to screening for wood decay potential but some were tentatively assigned to species with the assistance of Geoff Ridley. The naming authorities for the identified cultures are given in the Abbreviations section. The remaining cultures were not identified due to the absence of fruiting bodies and distinctive cultural characteristics necessary to follow the detailed procedures and keys of Nobles (1965), Grant and Savory (1969), Stalpers (1978) and Rayner and Boddy (1988). Observations of fungal growth on *P. radiata* and wood decay (2.2.2.3) were used to tentatively classify the cultures into brown rot or white rot fungi.

All basidiomycete cultures were maintained on glycerol at -80°C (Appendix 2.2), 1.5% MA slopes in 20 mL glass vials at room temperature and at 4°C, and MA and BA Petri dishes at 4°C. Freeze-drying was attempted but some cultures did not survive the technique, as observed by Croan (2000), so the method was discontinued.

2.2.2 Wood decay potential

2.2.2.1 Cellulase, xylanase and mannanase enzymes

The 38 basidiomycete cultures collected in 2.2.1 were screened for activity of the decay enzymes cellulase, xylanase and mannanase. The enzyme assays (Appendix 2.1) were

adapted from those used at the University of Waikato that were based on Lever (1973). The carbohydrate sources for production of cellulase, xylanase and mannanase were carboxymethylcellulose (CMC; Aldrich Chemical Co., U.S.A.), oat spelts xylan (Sigma Chemical Co., U.S.A.) and locust bean galactoglucomannan (Sigma Chemical Co., U.S.A.), respectively, at a concentration of 0.2% in tryptone-yeast extract nutrient medium (Appendix 1.11).

The accumulated fungal biomass at 8 d was extracted from the liquid medium by vacuum filtration onto Whatman #2 filter-papers (dried at 80°C to a constant weight), dried at 80°C to a constant weight and averaged over the replicate flasks. Colony morphology and pigmentation of the test fungi in each medium was noted but not characterised (Appendix 5). To check for contamination, 200 µL of culture supernatant from each flask was inoculated onto MA, in duplicate Petri dishes, and incubated at 25°C for up to 7 d. The treatments were repeated where any contamination was apparent.

The results were analysed statistically using analysis-of-variance. Transformation of the data was required because of the heterogeneity of variation (Nancy and Amburgey 1976) e.g. an increase in variation with increasing data values required a logarithmic transformation. Analysis of enzyme activity used square-root transformed data for the CMCase and mannanase assays, and logarithmic transformed data for the xylanase assay. Analysis of fungal biomass used logarithmic transformed data for the CMCase assay and square-root transformed data for the xylanase and mannanase assays. Comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level.

2.2.2.2 Laccase, peroxidase and proteinase enzymes

Laccase assay

The presence of laccase during growth of the basidiomycete fungi on MA was detected using α -naphthol as a colour indicator (Gramss et al. 1998). Two separate 20 µL drops of α -naphthol solution (1.44% in 96% ethanol) were placed on the growing edge of 8 d old cultures. There were triplicate Petri dishes for each fungus. The Petri dishes were incubated at 25°C for a minimum of 24 h until there was no further colour change in the cultures showing a positive reaction.

The stain intensity of each drop was rated on a scale of 0 to 5 (Photo 2.1).

0 = no stain

1 = very faint purple tinge around drops

2 = faint but diffuse purple tinge around drops

3 = still diffuse but darker purple haloes around drops

4 = more defined and darker purple haloes

5 = defined purple-black drops

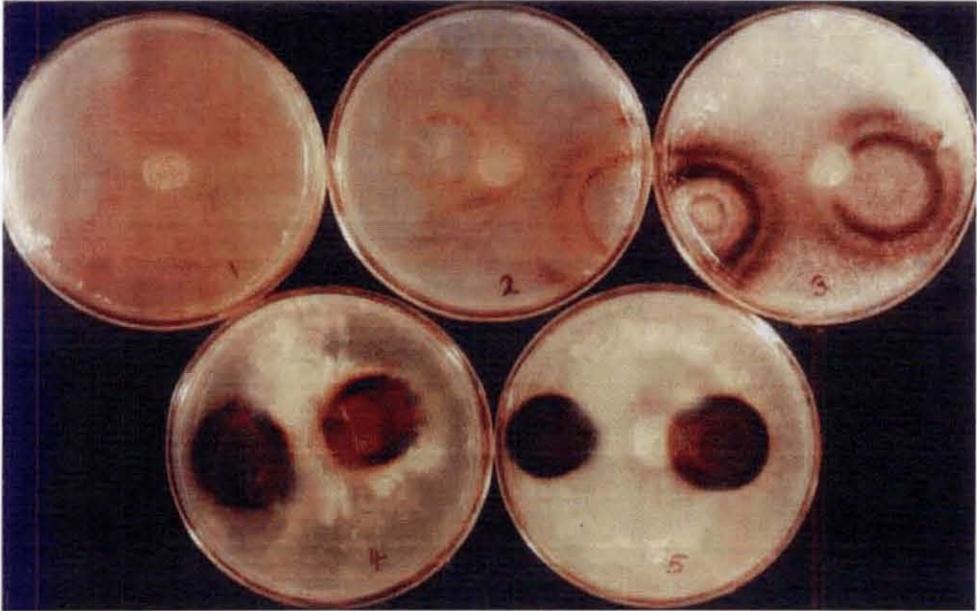


Photo 2.1 Examples of α -naphthol stain intensity ratings for fungal laccase on malt extract agar.

Peroxidase assay

The presence of peroxidase during growth of the basidiomycete fungi was detected using guaiacol (0.01%) as a colour indicator incorporated into two agar media (Jung et al. 1995), guaiacol potato dextrose agar (gPDA; Appendix 1.4) and guaiacol sawdust agar (gSA; Appendix 1.5), herein described as peroxidase (gPDA) and peroxidase (gSA). There were triplicate Petri dishes of each medium for each fungus. A 6 mm agar core of the test fungus (8 d old cultures) was inoculated in a central position on each Petri dish and incubated for up to 14 d at 25°C. The maximum stain intensity over the incubation period was recorded.

The stain intensity was rated on a scale from 0 to 5, with a range in colour response within each rating (Photo 2.2).

0 = no stain

1 = faint pink halo or tinge around core only

2 = diffuse pink over colony or dark red-brown halo around core only

3 = pink-brown over colony and dark red halo around core

4 = red-brown all over colony

5 = dark red-brown all over colony

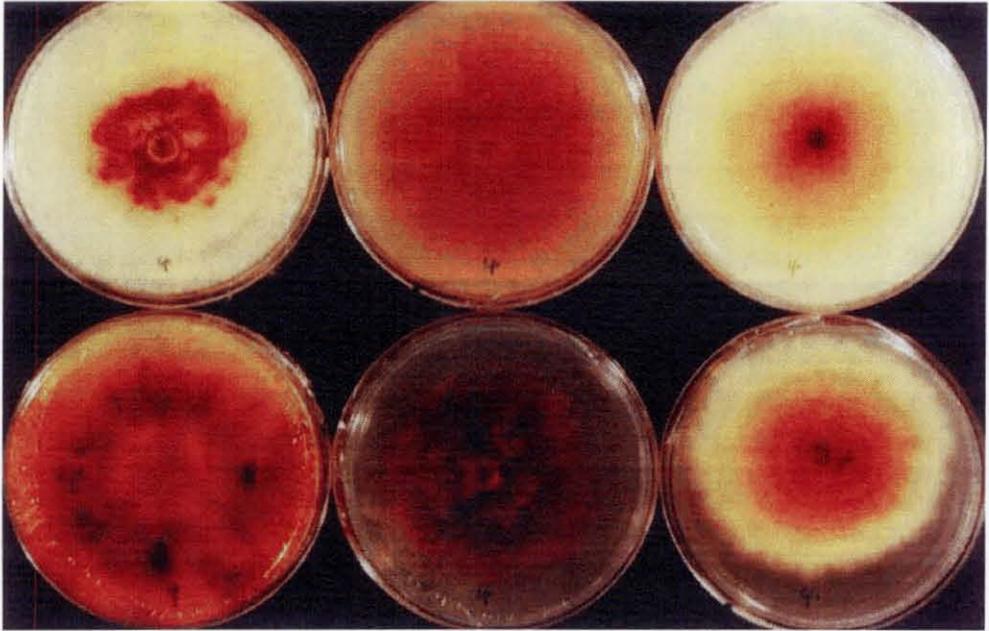


Photo 2.2 The range in colour response of basidiomycete fungi to guaiacol for a given stain intensity rating, in this case 4, on potato dextrose agar.

Proteinase assay

The ability of the basidiomycete fungi to produce proteinases was detected by the production of clearing zones during growth on skim milk agar (SMA; Appendix 1.8). There were triplicate Petri dishes for each fungus. A 6 mm agar core of the test fungus (8 d old cultures) was inoculated in a central position on each Petri dish and incubated for up to 10 d at 25°C. The maximum width of clearing zones over the incubation period was recorded.

2.2.2.3 Wood decay

The 38 basidiomycete cultures were subjected to a low decay hazard assay to assess their ability to cause weight loss of *P. radiata* over a period of 18 weeks at 25°C. The assay was carried out in 500 mL glass jars with triplicate jars for each fungus and six uninoculated control jars. A Whatman #2 filter-paper was placed on the bottom of each jar with 10 mL of distilled water. A galvanised steel stand was placed over the filter-paper. The jar lids had 3.5 mm holes filled with non-absorbent cottonwool for aeration. The jars were autoclaved for 20 min at 121°C.

Wood blocks (20 x 20 x 20 mm) were produced from freshly harvested *P. radiata* sapwood obtained from Carter Holt Harvey (CHH) Timber Putaruru Mill. The blocks were labelled and equilibrated to a constant weight at 25°C to reduce the variation in moisture content between blocks. The blocks were then sterilised by gamma irradiation (27.7 kGy; Schering Plough Animal Health Ltd., Upper Hutt). Isolations onto MA from internal and external sections of a sample of 20 blocks (duplicate Petri dishes per block) showed effective sterilisation as there was no visible growth after 14 d at 25°C.

Observations of the wood blocks showed that there were marked differences in fresh weight. A preliminary experiment was carried out to determine whether blocks of a certain range of initial weights should be used to reduce variability in final results. The wood blocks were separated into 'high density' (HD) and 'low density' (LD) blocks. The HD blocks had four or more growth rings in cross-section and the LD blocks had less than four growth rings. The mean initial fresh weight of 50 HD blocks was 7.74 ± 1.242 g compared to 4.46 ± 0.380 g for 50 LD blocks. After 2 d equilibration at 25°C, the mean weight of 50 HD blocks was 4.44 ± 0.282 g compared to 3.67 ± 0.256 g for 50 LD blocks, although the mean moisture content of both sets of blocks was $14 \pm 0.4\%$. The water application required to bring the blocks up to the 26.5% decay threshold moisture content for pine sapwood (BRE 1975) was determined for HD and LD blocks. The treatments were 1, 2, 3 and 4 mL of water applied per block, with three blocks of each wood density per treatment and six blocks of each wood density for the control treatment. After application, the blocks were incubated at room temperature for 30 min, drained for 2 min, weighed and dried at 80°C to a constant weight. For the 1, 2, 3 and 4 mL treatments, the HD blocks increased in moisture content to 41, 63, 82 and 77% and the LD blocks to 35, 35, 35 and 39%, respectively. This experiment was carried out in Petri dishes which allowed the blocks to

reabsorb any runoff water. In the wood decay assay, the moisture contents achieved would be less as any excess water would be lost to the bottom of the jar. It was decided that HD blocks would be used for all future decay assays to ensure an adequate wood moisture content for colonisation, growth and decay.

Three wood blocks were placed onto the stand in each jar and 3 mL of sterile distilled water were applied to each block. The same treatment applied to a sample of 50 blocks, showed that the mean moisture content increased from $14 \pm 0.4\%$ to $54 \pm 7.6\%$. A 14 mm agar core of the test fungus (14 day old cultures) was inoculated onto the cross-cut surface of each block. Sterile agar cores were inoculated onto blocks of the uninoculated control treatment. The jars were incubated at 25°C.

After 18 weeks, all wood blocks were removed from the jars, weighed and dried at 80°C to a constant weight. The external fungal biomass was then carefully brushed off and each block redried at 80°C to a constant weight. The dry weight fungal biomass was averaged over the nine blocks for each test fungus. The mean dry weight of the blocks from each treatment was converted to dry weight loss (%). Colony morphology of the fungi on the wood blocks was noted on the basidiomycete culture record sheets (not shown). Examination of the blocks was used to tentatively assign the unidentified basidiomycete cultures as brown rot or white rot fungi using the characteristics given in Nobles (1965).

Additional jars were used to monitor wood weight loss and moisture content and jar oxygen concentration over the course of the experiment for three test fungi. One was a brown rot field isolate (*Gloeophyllum sepiarium* 5) and the other two were white rot fungi from Forest Research and Landcare Research culture collections (*Phlebiopsis gigantea* 119 and *Pycnoporus coccineus* 124, respectively). A single jar was examined for each fungus at 4, 8, 12 and 16 weeks incubation. The blocks were harvested as described above after each incubation period. Oxygen concentration was measured using an oxygen meter (Illinois instruments 3500) with a probe inserted through the lid aeration hole. At 8 weeks, one wood block colonised by each fungus was sent for SEM by Professor Robert A. Blanchette (University of Minnesota, U.S.A.).

The results were analysed statistically using analysis-of-variance. Logarithmic transformation of the wood moisture content data and angular transformation of the fungal biomass data was required. All data was adjusted using the initial equilibrium weight as a

covariate to take account of the initial block to block variation in weight due to size and density. Comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level.

2.2.2.4 Growth on agar and on *Pinus radiata*

The 38 basidiomycete cultures were assessed for the ability to grow on MA, low nutrient agar (LNA; Appendix 1.6), BA and *P. radiata*. There were triplicate Petri dishes of each medium for each fungus.

For the agar medium, a 6 mm agar core of the test fungus (7 d old cultures) was inoculated in a central position on each Petri dish and incubated at 25°C. Colony diameters were measured in two predetermined directions every second day for up to 14 d after inoculation. The colony morphology of each fungus was noted at 14 d.

Due to the time-consuming measurements of growth on wood, two consecutive assays were carried out with 18 cultures in one assay and 20 cultures in another. Wood blocks (40 x 40 mm cross-cut x 5 mm thick) were produced from freshly harvested *P. radiata* sapwood obtained from CHH Timber Putaruru Mill. The blocks were sterilised by gamma irradiation (51.4 kGy) and placed in independent sterile Petri dishes. A 6 mm agar core of the test fungus (9 d old cultures) was inoculated onto the edge of each block so that growth would occur from outer to inner growth rings (as might occur in the natural colonisation of logs). The Petri dishes were incubated at 25°C. External hyphal extension growth across the wood block was observed under a stereo-microscope (Zeiss) and the leading edge of the colony was measured every 2 d after inoculation until growth had reached the end of the block (or a maximum of 28 d). The colony morphology of each fungus was noted at 28 d.

For each measurement, the growth rate was expressed as mm d^{-1} and averaged over the replicates and the assay period. The results for each medium were analysed statistically using analysis-of-variance. The wood assay used square-root transformed data. Comparisons between individual means for each medium were carried out using Tukey's least-significant-difference at the 5% significance level.

2.2.2.5 Regression analyses

Standard regression and correlation analyses (Pearson's test) were carried out to determine if there were any significant correlations between the parameters measured in the enzyme, wood decay and growth assays and whether the parameters were correlated to rot classification. There were separate analyses for the brown rot and the white rot fungi.

2.3 RESULTS

2.3.1 Collection of basidiomycete fungi

There were 38 basidiomycete cultures collected from sites that ranged from Wenita Otago Coast Forest in the central South Island to the Waipoua State Forest in the upper North Island (Table 2.1). The 11 cultures from Forest Research were isolated during the period 1994 to 1996 and the two cultures from Landcare Research were isolated in 1985. The 25 field cultures were collected during 1996, and of these, 11 cultures were tentatively identified and 14 were not identified. The identified cultures were from the Order Aphyllophorales (Class Hymenomycetes) and there were 15 species represented. There were equal numbers of brown rot and white rot fungi and equal numbers of cultures that had been collected from wood and from fruiting bodies. There were five species (one brown rot fungus and four white rot fungi) with more than one isolate. The brown rot fungus was *Gloeophyllum sepiarium* (four isolates) and the white rot fungi were *Phlebiopsis gigantea* (four isolates), *Pycnoporus coccineus* (two isolates), *Resinicium bicolor* (two isolates) and *Schizophyllum commune* (two isolates).

The laccase assay was the first enzyme assay to be completed and the results were used to assign basidiomycete record (BR) numbers to each test fungus. Each record number corresponded to a basidiomycete culture record sheet as outlined in 2.2.1. Fungi with two digit numbers tested negative for laccase and fungi with three digit numbers tested positive for laccase.

Table 2.1 Identification and isolation details of the basidiomycete fungi studied.

Culture identification	Rot	Source	Year	Origin	Site in New Zealand
<i>Antrodia serialis</i> 1	brf	LR	1985	wood	Waipoua State Forest
<i>Coniophora olivacea</i> 19	brf	FR	1995	wood	CHH Kaingaroa Forest
<i>Ganoderma</i> sp. 123	wrf	FR	1995	fruiting body	Te Wera Forest
<i>Gloeocystidiellum sacratum</i> 118	wrf	FR	1996	wood	Velva Std
<i>Gloeophyllum sepiarium</i> 4	brf	AAC	1996	fruiting body	Hamilton-HR log trial
<i>Gloeophyllum sepiarium</i> 5	brf	AAC	1996	fruiting body	Hamilton-HR log trial
<i>Gloeophyllum sepiarium</i> 14	brf	AAC	1996	fruiting body	Hamilton-HR log trial
<i>Gloeophyllum sepiarium</i> 15	brf	AAC	1996	fruiting body	Hamilton-HR timber trial
<i>Hapalopilus rutilans</i> 122	wrf	FR	1996	wood	Parengarenga
<i>Junghuhnia vincta</i> 121	wrf	FR	1996	wood	Matahina
<i>Oligoporus</i> sp. 117	brf	FR	1995	wood	Omataroa
<i>Phlebiopsis gigantea</i> 103	wrf	AAC	1996	fruiting body	CHH Kinleith Mill
<i>Phlebiopsis gigantea</i> 104	wrf	AAC	1996	fruiting body	FCF Waipa Mill
<i>Phlebiopsis gigantea</i> 119	wrf	FR	1995	wood	Berwick Forest
<i>Phlebiopsis gigantea</i> 130	wrf	AAC	1996	wood	CHH Kinleith Forest
<i>Poria</i> sp. 17	brf	FR	1994	wood	Wenita Otago Coast Forest
<i>Pycnoporus coccineus</i> 101	wrf	AAC	1996	fruiting body	East Coast
<i>Pycnoporus coccineus</i> 124	wrf	LR	1985	wood	Waipoua State Forest
<i>Resinicium bicolor</i> 108	wrf	AAC	1996	fruiting body	CHH Kinleith Forest
<i>Resinicium bicolor</i> 120	wrf	FR	1996	wood	Kaingaroa Forest
<i>Schizophyllum commune</i> 2	wrf	AAC	1996	fruiting body	FCF Waipa Mill
<i>Schizophyllum commune</i> 3	wrf	AAC	1996	fruiting body	FCF Waipa Mill
<i>Sistotrema brinkmannii</i> 18	wrf	FR	1995	wood	CHH Kinleith Forest
<i>Stereum sanguinolentum</i> 116	wrf	FR	1995	wood	Port Charles- Sandy Bay
BR 6	wrf	AAC	1996	wood	Hamilton-HR timber trial
BR 7	wrf	AAC	1996	fruiting body	CHH Kinleith Forest
BR 8	brf	AAC	1996	fruiting body	East Coast
BR 9	brf	AAC	1996	fruiting body	Hamilton-HR log trial
BR 10	brf	AAC	1996	fruiting body	Mamaku Forest
BR 11	brf	AAC	1996	fruiting body	Hamilton-HR log trial
BR 12	brf	AAC	1996	wood	Hamilton-HR timber trial
BR 106	brf	AAC	1996	fruiting body	CHH Kinleith Forest
BR 107	brf	AAC	1996	wood	CHH Kinleith Forest
BR 109	brf	AAC	1996	fruiting body	CHH Kinleith Forest
BR 110	brf	AAC	1996	wood	CHH Kinleith Forest
BR 111	brf	AAC	1996	wood	CHH Kinleith Forest
BR 112	brf	AAC	1996	fruiting body	Hamilton-HR timber trial
BR 113	wrf	AAC	1996	wood	CHH Kinleith Forest

BR=Basidiomycete Record number

brf= brown rot fungus; wrf= white rot fungus

LR= Landcare Research; FR= Forest Research; HR= HortResearch; AAC= Annette Ah Chee

CHH= Carter Holt Harvey; FCF= Fletcher Challenge Forests

2.3.2 Wood decay potential

2.3.2.1 Cellulase, xylanase and mannanase enzymes

Cellulase assay

All 38 basidiomycete cultures produced CMCase activity under the given assay conditions (Table 2.2). There were significant differences between the fungi in CMCase activity ($P < 0.001$) which ranged from 0.001 to 0.328 micromoles glucose $\text{min}^{-1} \text{mL}^{-1}$ (IU mL^{-1}). Five white rot cultures (*Schizophyllum commune* 2 and 3, *Phlebiopsis gigantea* 119, *Pycnoporus coccineus* 124 and BR 113) had the highest activities of more than 0.173 IU mL^{-1} ($P < 0.05$) but five white rot cultures (*Hapalopilus rutilans* 122, *Ganoderma* sp. 123, *Gloeocystidiellum sacratum* 118, and *Resinicium bicolor* 108 and 120) also had the lowest activities of less than 0.018 IU mL^{-1} ($P < 0.05$). Significant differences in CMCase activity were found between the *Gloeophyllum sepiarium* isolates (0.030 to 0.155 IU mL^{-1}) and the *Phlebiopsis gigantea* isolates (0.074 to 0.218 IU mL^{-1}) but not between the isolates of *Pycnoporus coccineus*, *R. bicolor* or *S. commune* ($P < 0.05$).

There were significant differences in accumulated fungal biomass after 8 d growth on CMC medium ($P < 0.001$). Seventeen cultures had the highest biomasses of 0.63 to 1.31 mg mL^{-1} ($P < 0.05$) (including *S. commune* 2 and 3, *G. sepiarium* 14 and 15, *Coniophora olivacea* 19, *Junghuhnia vincta* 121 and *Ganoderma* sp. 123). The fungi BR 106, *H. rutilans* 122, *Poria* sp. 17 and *Oligoporus* sp. 117 had the lowest biomasses of less than 0.16 mg mL^{-1} ($P < 0.05$). Significant differences in fungal biomass were found between the *Gloeophyllum sepiarium* isolates (0.43 to 0.97 mg mL^{-1}) but not between the isolates of the white rot species ($P < 0.05$).

Xylanase assay

There were significant differences between the basidiomycete fungi in xylanase activity ($P < 0.001$). There were 16 cultures that produced the highest xylanase activities of 4.190 to 11.024 IU mL^{-1} ($P < 0.05$) (Table 2.2). Five cultures (*J. vincta* 121, *R. bicolor* 108 and 120 and BR 11 and 107) did not produce any significant xylanase activity under the given assay conditions and the remaining 17 cultures had activities of less than 1.529 IU mL^{-1} .

Significant differences in xylanase activity were found between the *G. sepiarium* isolates (0.044 to 6.573 IU mL⁻¹) and the *Phlebiopsis gigantea* isolates (0.035 to 6.649 IU mL⁻¹) but not between the isolates of *Pycnoporus coccineus*, *R. bicolor* or *S. commune*.

There were significant differences in accumulated fungal biomass after 8 d growth on xylan medium. Six cultures (*S. commune* 3, *J. vincta* 121, *Phlebiopsis gigantea* 104 and BR 7, 109 and 113) had the highest biomasses of 1.68 to 2.65 mg mL⁻¹ (P<0.05) and five of these were white rot cultures. Of the 27 cultures that had the lowest biomasses of less than 1.00 mg mL⁻¹ (P<0.05) 16 were brown rot cultures. Significant differences in fungal biomass were found between the *P. gigantea* isolates (0.32 to 1.68 mg mL⁻¹) and the *S. commune* isolates (0.23 and 1.80 mg mL⁻¹) but not between the isolates of *G. sepiarium*, *Pycnoporus coccineus* or *R. bicolor*.

Mannanase assay

There were significant differences between the basidiomycete fungi in mannanase activity (P<0.001). Eighteen cultures had the highest mannanase activities of 0.140 to 0.722 IU mL⁻¹ (P<0.05) and 11 of these were brown rot cultures (Table 2.2). Of the remaining 20 cultures that had activities of less than 0.132 IU mL⁻¹, 11 were white rot cultures. Significant differences in mannanase activity were found between the *S. commune* isolates (0.001 and 0.299 IU mL⁻¹) but not between the isolates of *G. sepiarium*, *Phlebiopsis gigantea*, *Pycnoporus coccineus* or *R. bicolor*.

There were significant differences in accumulated fungal biomass after 8 d growth on mannan medium. Six cultures had the highest biomasses of 1.39 to 2.01 mg mL⁻¹ (P<0.05) and five of these were white rot cultures. Nine cultures had the lowest biomasses of less than 0.53 mg mL⁻¹ (P<0.05) and seven of these were brown rot cultures. Significant differences in fungal biomass were found between the *G. sepiarium* isolates (0.34 to 0.89 mg mL⁻¹), the *Phlebiopsis gigantea* isolates (0.72 to 1.48 mg mL⁻¹), the *Pycnoporus coccineus* isolates (0.42 and 0.88 mg mL⁻¹) and the *S. commune* isolates (0.61 and 2.00 mg mL⁻¹) but not between the *R. bicolor* isolates.

Table 2.2 CMCase, xylanase and mannanase activity and accumulation of fungal biomass by a range of basidiomycete fungi in liquid medium.

Culture identification	Rot	CMC medium		Xylan medium		Mannan medium	
		CMCase ¹	BM	Xylanase ¹	BM	Mannanase ¹	BM
<i>Antrodia serialis</i> 1	brf	0.037	0.44	0.029	0.54	0.693	0.59
<i>Coniophora olivacea</i> 19	brf	0.022	1.31	0.018	0.28	0.086	0.31
<i>Ganoderma</i> sp. 123	wrf	0.015	1.05	0.018	0.38	0.323	0.62
<i>Gloeocystidiellum sacratum</i> 118	wrf	0.021	0.61	7.158	0.52	0.000	0.57
<i>Gloeophyllum sepiarium</i> 4	brf	0.155	0.49	6.573	0.29	0.489	0.89
<i>Gloeophyllum sepiarium</i> 5	brf	0.090	0.43	0.114	0.46	0.528	0.40
<i>Gloeophyllum sepiarium</i> 14	brf	0.039	0.97	0.044	0.38	0.404	0.34
<i>Gloeophyllum sepiarium</i> 15	brf	0.030	0.79	0.481	0.69	0.413	0.81
<i>Hapalopilus rutilans</i> 122	wrf	0.001	0.16	1.529	1.50	0.132	1.24
<i>Junghuhnina vincta</i> 121	wrf	0.147	0.82	0.000	2.54	0.012	1.25
<i>Oligoporus</i> sp. 117	brf	0.073	0.10	7.145	0.54	0.008	0.24
<i>Phlebiopsis gigantea</i> 103	wrf	0.133	0.50	6.649	0.32	0.232	0.72
<i>Phlebiopsis gigantea</i> 104	wrf	0.077	0.72	0.279	1.68	0.071	1.48
<i>Phlebiopsis gigantea</i> 119	wrf	0.218	0.54	0.059	0.63	0.041	0.81
<i>Phlebiopsis gigantea</i> 130	wrf	0.074	0.50	0.035	1.00	0.023	1.15
<i>Poria</i> sp. 17	brf	0.057	0.16	4.766	0.35	0.001	0.27
<i>Pycnoporus coccineus</i> 101	wrf	0.097	0.55	0.013	1.59	0.180	0.88
<i>Pycnoporus coccineus</i> 124	wrf	0.221	0.48	0.048	0.58	0.094	0.42
<i>Resinicium bicolor</i> 108	wrf	0.018	0.32	0.000	0.75	0.141	1.01
<i>Resinicium bicolor</i> 120	wrf	0.022	0.50	0.000	0.48	0.052	1.05
<i>Schizophyllum commune</i> 2	wrf	0.279	0.92	6.137	0.23	0.001	0.61
<i>Schizophyllum commune</i> 3	wrf	0.328	1.23	4.672	1.80	0.299	2.00
<i>Sistotrema brinkmannii</i> 18	wrf	0.036	0.62	7.293	0.56	0.640	0.70
<i>Stereum sanguinolentum</i> 116	wrf	0.062	0.52	6.778	0.59	0.006	0.31
BR 6	wrf	0.147	0.84	1.011	1.21	0.415	1.44
BR 7	wrf	0.045	0.26	11.024	2.65	0.000	1.39
BR 8	brf	0.091	0.41	0.030	0.30	0.545	0.57
BR 9	brf	0.037	0.81	6.808	0.54	0.482	1.63
BR 10	brf	0.145	0.53	6.620	0.52	0.217	1.02
BR 11	brf	0.026	0.77	0.000	0.34	0.003	0.68
BR 12	brf	0.117	0.25	7.088	0.63	0.623	0.63
BR 106	brf	0.044	0.08	6.843	1.56	0.014	1.23
BR 107	brf	0.105	0.74	0.000	1.27	0.001	0.96
BR 109	brf	0.133	0.61	0.439	2.12	0.246	1.29
BR 110	brf	0.035	0.58	7.548	0.51	0.005	0.53
BR 111	brf	0.077	0.63	0.057	0.45	0.025	0.63
BR 112	brf	0.075	0.45	*4.190	0.92	0.722	0.45
BR 113	wrf	0.173	1.24	0.377	2.14	0.056	2.01
sed (P<0.05)		0.0278	0.151	0.5371	0.282	0.1124	0.115

BR=Basidiomycete Record number

brf= brown rot fungus; wrf= white rot fungus

¹ Micromoles glucose min⁻¹ mL⁻¹ (IU mL⁻¹)

BM= Milligrams dry weight fungal biomass mL⁻¹ after 8 d incubation at 25°C

* Mean of inconsistent replicate flasks, sed does not apply

2.3.2.2 Laccase, peroxidase and proteinase enzymes

Laccase assay

There were 21 basidiomycete cultures that tested positive for laccase (Table 2.3). As mentioned in 2.3.1, the basidiomycete record numbers with three digits indicate a laccase-positive culture and two digits indicate a laccase-negative culture. White rot fungi generally had a positive reaction and brown rot fungi a negative reaction. The exceptions were seven laccase-positive brown rot cultures (*Oligoporus* sp. 117, and BR 106, 107, 109, 110, 111 and 112) and five laccase-negative white rot cultures (*Schizophyllum commune* 2 and 3, *Sistotrema brinkmannii* 18, and BR 6 and 7). The laccase-positive brown rot fungi generally had lower stain intensity ratings than the laccase-positive white rot fungi. The highest ratings were produced by *Ganoderma* sp. 123, *Gloeocystidiellum sacratum* 118, *Junghuhnia vincta* 121 and BR 11. Stain intensity ratings were consistent between the isolates of *Phlebiopsis gigantea* (rating of 3), *Pycnoporus coccineus* (4) and *Resinicium bicolor* (4) and the isolates of *Gloeophyllum sepiarium* and *Schizophyllum commune* were laccase-negative.

Peroxidase assay

The fungi that had tested positive for laccase, along with *S. commune* 2 and 3, tested positive for peroxidase on one or both of the guaiacol-amended agar media (Table 2.3). Growth of the fungi was faster on gPDA and of a more fluffy or thick farinaceous morphology compared to the sparse growth on gSA. Stain intensity was generally higher on gSA than on gPDA for any given fungus. On gSA, 15 of the 23 cultures had reached maximum staining intensity by 5 d compared to only ten cultures on gPDA. The lower nutrient conditions in gSA may have induced fungi that have the capability, to produce peroxidases for degradation of the sawdust and thus the higher staining intensities on gSA. The exceptions were the white rot cultures *H. rutilans* 122, and *S. commune* 2 and 3 (Photo 2.3) who gave no staining on gSA but a stain intensity of 4 on gPDA. There were six white rot cultures (*R. bicolor* 108 and 120, *J. vincta* 101, *P. coccineus* 124, *Ganoderma* sp. 123 and *Gloeocystidiellum sacratum* 118) that showed maximum staining intensity on both guaiacol media.



Photo 2.3 The guaiacol peroxidase assay for *Schizophyllum commune* 3 on potato dextrose agar (left) and sawdust agar (right).

Proteinase assay

There were 18 basidiomycete cultures (nine brown rot and nine white rot cultures) that tested positive for proteolytic activity where growth resulted in a zone of clearing in the SMA (Table 2.3). The brown rot fungi had zones of 1 to 7 mm and included *Antrodia serialis* 1, *C. olivacea* 19, *Poria* sp. 17, and BR 8, 10, 106, 107, 110 and 111 (Photo 2.4). The white rot fungi had zones of 2 to 13 mm and included *Ganoderma* sp. 123, *R. bicolor* 108 and 120,, *Sistotrema brinkmannii* 18, *Stereum sanguinolentum* 116 and BR 6. The *Phlebiopsis gigantea* and *Schizophyllum commune* isolates resulted in indistinct zones.



Photo 2.4 Proteolytic activity shown by, clockwise from top left, BR 10, *Sistotrema brinkmannii* 18, *Ganoderma* sp. 123 and BR 111 on skim milk agar.

Table 2.3 Laccase, peroxidase and proteinase activity during growth of a range of basidiomycete fungi in agar-based assays.

Culture identification	Rot	Stain intensity rating			Clearing zone (mm) Proteinase
		Laccase	Peroxidase		
			gPDA	gSA	
<i>Antrodia serialis</i> 1	brf	0	0	0	1
<i>Coniophora olivacea</i> 19	brf	0	0	0	2
<i>Ganoderma</i> sp. 123	wrf	5	5	5	5
<i>Gloeocystidiellum sacratum</i> 118	wrf	5	5	5	0
<i>Gloeophyllum sepiarium</i> 4	brf	0	0	0	0
<i>Gloeophyllum sepiarium</i> 5	brf	0	0	0	0
<i>Gloeophyllum sepiarium</i> 14	brf	0	0	0	0
<i>Gloeophyllum sepiarium</i> 15	brf	0	0	0	0
<i>Hapalopilus rutilans</i> 122	wrf	4	4	0	0
<i>Junghuhnia vincta</i> 121	wrf	5	5	5	0
<i>Oligoporus</i> sp. 117	brf	1	0	5	0
<i>Phlebiopsis gigantea</i> 103	wrf	3	0	5	0
<i>Phlebiopsis gigantea</i> 104	wrf	3	4	5	5
<i>Phlebiopsis gigantea</i> 119	wrf	3	1	4	0
<i>Phlebiopsis gigantea</i> 130	wrf	3	1	3	2
<i>Poria</i> sp. 17	brf	0	0	0	1
<i>Pycnoporus coccineus</i> 101	wrf	4	4	5	0
<i>Pycnoporus coccineus</i> 124	wrf	4	5	5	0
<i>Resinicium bicolor</i> 108	wrf	4	5	5	10
<i>Resinicium bicolor</i> 120	wrf	4	5	5	6
<i>Schizophyllum commune</i> 2	wrf	0	4	0	0
<i>Schizophyllum commune</i> 3	wrf	0	4	0	2
<i>Sistotrema brinkmannii</i> 18	wrf	0	0	0	9
<i>Stereum sanguinolentum</i> 116	wrf	4	2	5	6
BR 6	wrf	0	0	0	13
BR 7	wrf	0	0	0	0
BR 8	brf	0	0	0	3
BR 9	brf	0	0	0	0
BR 10	brf	0	0	0	6
BR 11	brf	0	0	0	0
BR 12	brf	0	0	0	0
BR 106	brf	4	4	5	6
BR 107	brf	2	3	5	2
BR 109	brf	2	4	5	0
BR 110	brf	2	2	5	2
BR 111	brf	5	4	5	7
BR 112	brf	1	1	5	0
BR 113	wrf	4	4	5	0

BR=Basidiomycete Record number

brf= brown rot fungus; wrf= white rot fungus

gPDA= Guaiacol potato dextrose agar; gSA= Guaiacol sawdust agar

2.3.2.3 Wood decay

There were significant differences between the basidiomycete fungi in mean wood dry weight loss, moisture content and external fungal biomass ($P<0.001$) (Table 2.4). No weight loss or fungal growth occurred in the uninoculated control treatment.

The additional jars monitored at 4, 8, 12 and 16 weeks incubation showed an increase in wood weight loss and moisture content with time (data not shown). Oxygen concentrations in the jars were the same as atmospheric levels.

Table 2.4 Wood weight loss, moisture content and external fungal biomass of *Pinus radiata* blocks for a range of basidiomycete fungi in a low decay hazard assay after 18 weeks at 25°C.

Culture identification	Rot	Wt loss	MC	BM
<i>Antrodia serialis</i> 1	brf	15.9	49.8	49.2
<i>Coniophora olivacea</i> 19	brf	7.7	35.9	21.4
<i>Ganoderma</i> sp. 123	wrf	6.6	34.2	14.6
<i>Gloeocystidiellum sacratum</i> 118	wrf	4.3	33.6	18.7
<i>Gloeophyllum sepiarium</i> 4	brf	19.3	49.8	88.8
<i>Gloeophyllum sepiarium</i> 5	brf	20.9	52.5	99.6
<i>Gloeophyllum sepiarium</i> 14	brf	20.1	49.4	112.8
<i>Gloeophyllum sepiarium</i> 15	brf	22.7	51.8	90.1
<i>Hapalopilus rutilans</i> 122	wrf	3.8	29.1	16.2
<i>Junghuhnia vincta</i> 121	wrf	6.2	34.7	33.0
<i>Oligoporus</i> sp. 117	brf	14.3	58.3	46.7
<i>Phlebiopsis gigantea</i> 103	wrf	4.6	33.3	8.4
<i>Phlebiopsis gigantea</i> 104	wrf	7.0	35.1	13.5
<i>Phlebiopsis gigantea</i> 119	wrf	6.9	32.8	18.2
<i>Phlebiopsis gigantea</i> 130	wrf	6.2	32.2	9.3
<i>Poria</i> sp. 17	brf	12.7	41.7	22.8
<i>Pycnoporus coccineus</i> 101	wrf	5.1	33.0	22.0
<i>Pycnoporus coccineus</i> 124	wrf	4.2	28.1	24.1
<i>Resinicium bicolor</i> 108	wrf	3.6	32.3	12.2
<i>Resinicium bicolor</i> 120	wrf	3.4	32.8	21.5
<i>Schizophyllum commune</i> 2	wrf	0.7	31.0	11.2
<i>Schizophyllum commune</i> 3	wrf	0.5	33.6	8.8
<i>Sistotrema brinkmannii</i> 18	wrf	1.7	31.4	12.3
<i>Stereum sanguinolentum</i> 116	wrf	6.4	35.0	42.6
BR 6	wrf	0.0	31.2	5.1
BR 7	wrf	0.7	29.2	7.8
BR 8	brf	9.5	40.8	10.4
BR 9	brf	19.5	47.3	84.6
BR 10	brf	19.1	40.1	37.2
BR 11	brf	4.1	27.6	3.2
BR 12	brf	27.1	78.2	19.5
BR 106	brf	2.7	31.2	14.4
BR 107	brf	3.7	30.9	11.2
BR 109	brf	4.2	36.6	12.2
BR 110	brf	6.9	34.1	14.3
BR 111	brf	2.2	35.2	13.9
BR 112	brf	13.6	37.5	18.0
BR 113	wrf	1.2	31.5	20.4
sed (P<0.05)		1.50	4.15	9.26

BR=Basidiomycete Record number

brf= brown rot fungus; wrf= white rot fungus

Wt loss= Wood dry weight loss (%)

MC= Wood moisture content (%)

BM= Milligrams dry weight external fungal biomass per wood block

Wood weight losses ranged from 0 to 27.1%. Of the 20 cultures that resulted in significant weight losses of 5.6% or greater, 14 were brown rot cultures with weight losses of 6.9 to 27.1% (*Gloeophyllum sepiarium* 4, 5, 14 and 15, *Oligoporus* sp. 117, *Poria* sp. 17, *C. olivacea* 19, *A. serialis* 1, and BR 8, 9, 10, 12, 110 and 112) and six were white rot cultures with weight losses of 6.2 to 7.0% (*Phlebiopsis gigantea* 104, 119 and 130, *Stereum sanguinolentum* 116, *J. vincta* 121 and *Ganoderma* sp. 123). Of the 18 cultures that resulted in insignificant weight losses, 13 were white rot and five were brown rot cultures. Wood moisture content ranged from 27.6 to 78.2%. The highest moisture contents were associated with the blocks that had experienced significant weight losses. Mean external fungal biomass ranged from 3.2 to 112.8 mg per wood block. The four *Gloeophyllum sepiarium* isolates and BR 9 had the highest biomasses of 84.6 to 112.8 mg.

There were no significant differences in wood weight loss, moisture content or external fungal biomass between isolates of *G. sepiarium*, *P. gigantea*, *R. bicolor*, *Pycnoporus coccineus* or *Schizophyllum commune*. The four white rot species resulted in lower wood moisture contents (28.1 to 35.1%) than *G. sepiarium* (49.4 to 52.5%).

SEM of wood blocks from the additional jars after 8 weeks incubation showed early stages of wood decay in *G. sepiarium* 5 and *Phlebiopsis gigantea* 119 blocks (Photo 2.5) but not *Pycnoporus coccineus* 124 blocks (not shown). Decay is shown by the loss of cell wall integrity and cell separations from sectioning of weakened cells.

2.3.2.4 Growth on agar and on *Pinus radiata*

There were significant differences between the basidiomycete fungi in growth rates on each medium ($P < 0.05$) (Table 2.5). The fungi showed a range of colony morphologies which were similar for each fungus on agar and wood medium. Descriptions were noted on the basidiomycete culture record sheets for each fungus and photos were taken showing the range in growth forms (not shown).

Growth rates on the agar medium ranged from 2.20 to 24.21 mm d⁻¹ (MA), 2.01 to 25.42 mm d⁻¹ (LNA) and 0.69 to 17.26 mm d⁻¹ (BA). Growth rates were generally similar on MA and LNA, and lower on BA. The fungi with the highest growth rates included the isolates of *Phlebiopsis gigantea*, *Pycnoporus coccineus* and *S. commune*, *J. vincta* and BR 107, 109, 110, 111, 112 and 113.

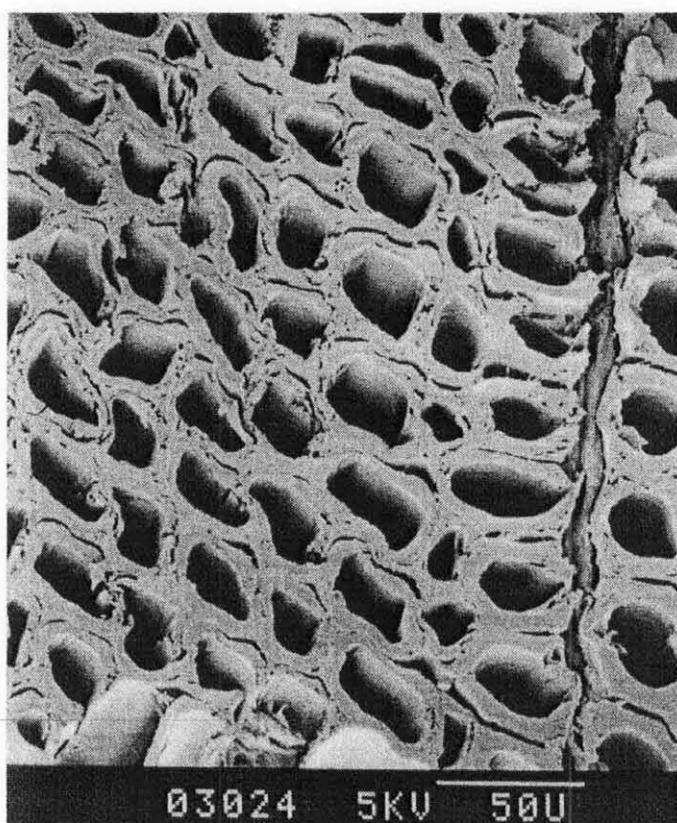
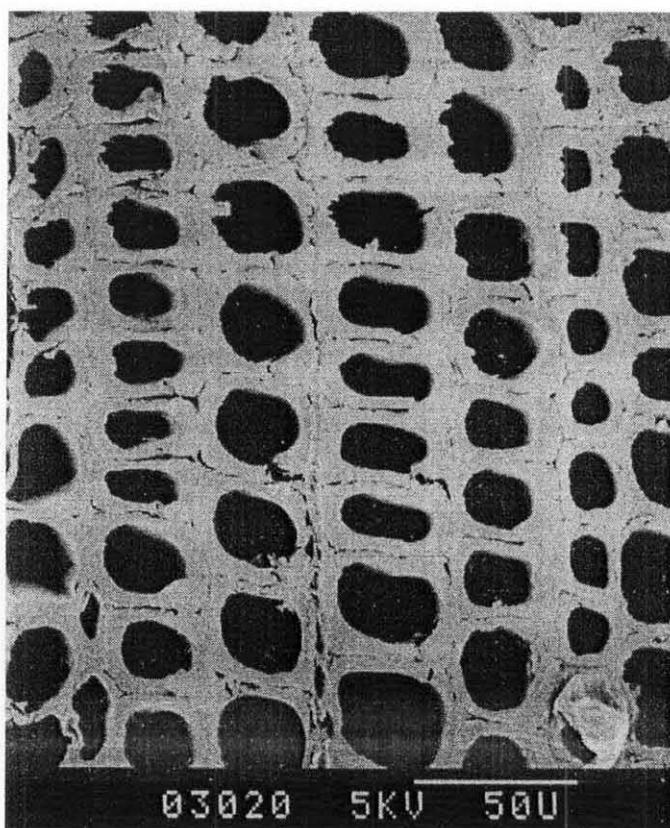


Photo 2.5 Early stages of decay in *Pinus radiata* by *Gloeophyllum sepiarium* 5 (top) and *Phlebiopsis gigantea* 119 (bottom) after 8 weeks incubation in a low decay hazard assay. Transverse surface; SEM.

Table 2.5 Growth rates (mm d⁻¹) of a range of basidiomycete fungi on three agar media and on *Pinus radiata* at 25°C.

Culture identification	Rot	MA	LNA	BA	<i>P. radiata</i>
<i>Antrodia serialis</i> 1	brf	4.54	3.18	3.93	0.22 a
<i>Coniophora olivacea</i> 19	brf	3.92	7.71	1.40	0.00 a
<i>Ganoderma</i> sp. 123	wrf	3.32	2.01	1.15	1.14 a
<i>Gloeocystidiellum sacratum</i> 118	wrf	5.95	6.01	5.10	0.52 a
<i>Gloeophyllum sepiarium</i> 4	brf	5.68	4.67	4.96	1.20 a
<i>Gloeophyllum sepiarium</i> 5	brf	4.91	4.06	5.14	2.04 b
<i>Gloeophyllum sepiarium</i> 14	brf	5.14	4.21	5.73	1.34 a
<i>Gloeophyllum sepiarium</i> 15	brf	5.72	4.38	5.55	0.09 b
<i>Hapalopilus rutilans</i> 122	wrf	2.20	2.77	2.28	0.58 a
<i>Junghuhnia vincta</i> 121	wrf	13.94	15.38	7.03	4.59 a
<i>Oligoporus</i> sp. 117	brf	2.93	2.05	0.69	0.00 a
<i>Phlebiopsis gigantea</i> 103	wrf	13.41	14.04	9.97	4.68 a
<i>Phlebiopsis gigantea</i> 104	wrf	10.87	11.25	8.45	2.83 b
<i>Phlebiopsis gigantea</i> 119	wrf	12.88	12.73	4.07	5.21 a
<i>Phlebiopsis gigantea</i> 130	wrf	12.52	10.01	9.63	5.66 a
<i>Poria</i> sp. 17	brf	7.34	4.39	0.94	0.49 a
<i>Pycnoporus coccineus</i> 101	wrf	8.75	8.32	2.22	0.00 b
<i>Pycnoporus coccineus</i> 124	wrf	8.30	8.83	1.73	1.30 a
<i>Resinicium bicolor</i> 108	wrf	5.97	5.82	3.77	0.85 b
<i>Resinicium bicolor</i> 120	wrf	6.00	6.40	4.18	0.94 a
<i>Schizophyllum commune</i> 2	wrf	9.25	7.08	7.60	4.66 a
<i>Schizophyllum commune</i> 3	wrf	8.58	9.47	8.21	2.35 b
<i>Sistotrema brinkmannii</i> 18	wrf	5.93	8.86	6.00	1.26 a
<i>Stereum sanguinolentum</i> 116	wrf	4.09	2.04	3.49	1.02 a
BR 6	wrf	4.89	6.16	5.89	0.00 b
BR 7	wrf	5.66	5.25	3.34	0.00 b
BR 8	brf	4.06	4.45	6.03	0.84 b
BR 9	brf	7.34	4.47	7.34	2.22 b
BR 10	brf	5.77	4.03	6.70	1.36 b
BR 11	brf	5.69	6.47	6.85	1.05 b
BR 12	brf	5.76	5.54	5.63	1.24 b
BR 106	brf	7.14	7.16	6.05	0.36 b
BR 107	brf	14.91	11.18	5.58	3.03 b
BR 109	brf	7.42	7.80	8.83	2.54 b
BR 110	brf	15.57	11.93	7.42	2.85 b
BR 111	brf	13.67	11.03	8.44	0.00 b
BR 112	brf	15.84	12.33	13.33	2.69 b
BR 113	wrf	24.21	25.42	17.26	0.00 b
sed (P<0.05)		0.239	0.238	0.288	^a 0.395 ^b 0.233

BR=Basidiomycete Record number

brf= brown rot fungus; wrf= white rot fungus

MA= Malt extract agar; LNA= Low nutrient agar; BA= Benomyl agar

a 18 culture assay; b 20 culture assay

The fastest growing fungi on the wood blocks were generally those that had tested positive for laccase and peroxidase activity. In the assay with 18 cultures, five white rot cultures (*Phlebiopsis gigantea* 103, 119 and 130, *S. commune* 2 and *J. vincta* 121) had the highest growth rates of 4.59 to 5.66 mm d⁻¹ (P<0.05). In the assay with 20 cultures, *P. gigantea*

104, *G. sepiarium* 5, *S. commune* 3, and BR 9, 107, 109, 110 and 112 had the highest growth rates of 2.04 to 3.03 mm d⁻¹ (P<0.05). Across the two assays, *Pycnoporus coccineus* 101, *C. olivacea* 19, *Oligoporus* sp. 117, and BR 6, 7, 111 and 113 did not show any growth on the wood blocks over the 28 d assay period. Maximum growth rates for the fastest growing fungi occurred at 5 to 7 d (18 culture assay) and 6 to 13 d (20 culture assay). Maximum growth rates for the slower growing fungi occurred at 10 to 27 d (18 culture assay) and 10 to 21 d (20 culture assay).

Significant differences in growth rates on each agar medium were found between the *Phlebiopsis gigantea* isolates, 10.87 to 13.41 mm d⁻¹ (MA), 10.01 to 14.04 mm d⁻¹ (LNA) and 4.07 to 9.97 mm d⁻¹ (BA), and between the *S. commune* isolates on LNA (7.08 and 9.47 mm d⁻¹) but not between the isolates of *G. sepiarium*, *Pycnoporus coccineus* or *R. bicolor*. There were significant differences in growth rates on *Pinus radiata* between the *G. sepiarium* isolates (0.09 to 2.04 mm d⁻¹), the *Phlebiopsis gigantea* isolates (2.83 to 5.66 mm d⁻¹) and the *S. commune* isolates (2.35 and 4.66 mm d⁻¹). The *Pycnoporus coccineus* and *R. bicolor* isolates grew poorly, if at all, on the wood blocks.

2.3.2.5 Regression analyses

Regression analyses between the parameters of the enzyme, wood decay and growth assays showed a number of significant correlations for brown rot and white rot fungi (Appendix 6). The relevance of some of the correlations is obscure and further research would be required to understand their significance or relevance.

All fungi showed significant correlations between the presence of the two ligninolytic enzymes and the presence of laccase and peroxidase (gSA) was correlated to wood weight loss but similar correlations were not found for the cellulolytic enzymes (CMCase, xylanase or mannanase). Wood weight loss was correlated to moisture content and external fungal biomass. Brown rot fungi had significant correlations between growth on *Pinus radiata* and the three agar media, and between growth on LNA and wood weight loss.

There were significant correlations between rot classification and the parameters of the enzyme, wood decay and growth assays.

The following parameters were indicative of brown rot fungi (in order of decreasing significance):

- relatively high wood weight loss, moisture content and external fungal biomass.
- the absence of laccase and peroxidase (gPDA).
- high mannanase activity.
- low fungal biomass on xylan and mannan medium.

Conversely, the parameters indicative of white rot fungi were as follows:

- relatively low wood weight loss, moisture content and external fungal biomass.
- the presence of laccase and peroxidase (gPDA).
- low mannanase activity.
- high fungal biomass on xylan and mannan medium.

2.4 DISCUSSION

The basidiomycete fungi that were collected are only a representation of the range of species that may occur on New Zealand *Pinus radiata*. A more accurate representation could only have been achieved by isolations from intensive sampling of *P. radiata* logs, timber and fruiting bodies over all four seasons, from a large number and range of sites, and by the use of a variety of isolation protocols. This was beyond the scope of this thesis research. The presence or absence of particular fungal species may reflect the time and nature of wood exposure and be influenced by seasonal effects relating to temperature and wood moisture content. The culture media and isolation protocol would have directly influenced the basidiomycete species collected (Greaves and Savory 1965). The presence of benomyl-resistant *Mucor* species and other fast growing fungi had been a problem for isolation of the relatively slow growing basidiomycete fungi.

The identification of basidiomycete fungi is recognised as being difficult because most do not form fruiting bodies in the laboratory (and if they do, they may not be typical of those formed in the field situation) and cultures can lack a number of characteristics that are necessary to follow identification keys. Commonly isolated brown rot fungi from

P. radiata are *Gloeophyllum trabeum* (Pers.:Fr.) Murrill, *Antrodia serialis*, *Antrodia vaillantii* (DC.:Fr.) Ryvarden, *Poria* species and *Coniophora* species and common white rot fungi are *Phlebiopsis gigantea*, *Schizophyllum commune*, *Trametes versicolor* and *Pycnoporus coccineus* (Butcher and Drysdale 1991). Some of these species were represented in this study, and a number of the identified cultures and ones from the culture collections, were genera or species that are commonly studied internationally e.g. *Gloeophyllum sepiarium* and *S. commune*. Ideally, New Zealand studies of *Pinus radiata* decay would challenge wood with a wide range of New Zealand decay fungi under the conditions they are likely to encounter in the field situation. Fungi to be used in decay assays should have consistent decay and preservative tolerance, and high decay ability (Levi 1978; Unligil 1972).

Decay enzymes

There were significant differences between the basidiomycete fungi in CMCase, xylanase and mannanase activity and in the ability to grow on the liquid media. As an initial screen, it was not possible to run the enzyme assays under differing conditions to optimise enzyme production for each individual test fungus. The assay conditions (50°C and pH 6) were chosen as an incubation temperature of 50°C is commonly used in cellulase and hemicellulase assays (Mandels et al. 1974) and the majority of cellulase pH optima were around 6.0 (Wood et al. 1988). The low enzyme activities measured for some fungi may be due to instability at 50°C and/or insufficient incubation time of enzyme with substrate (Bailey et al. 1991). The culture supernatant may have contained a mixture of cellulases, mannanase, xylanase, β -glucosidase and other hydrolases. The assays determined the accumulated enzyme at the time of sampling and did not take into account any loss of enzyme activity or fluctuation in activity due to, for example, feedback inhibition or the production of proteases (Hummell et al. 1998). Culture conditions can affect quantitatively and qualitatively the enzymes that are produced by wood decay fungi. These conditions include sources and concentrations of carbon and nitrogen (Highley 1976) and aeration (King and Smith 1973). As the substrates and cultural conditions differ markedly, the enzymes produced in liquid medium may be different to those produced in wood. Highley et al. (1981) compared the enzymes produced by *Postia placenta* in liquid medium and in decayed wood, and found that there were quantitative differences but the enzyme complexes were structurally similar and considered to be the same enzymes. There are other methods of measuring enzyme activity including the use of fungal fruiting body

explants (Agerer et al. 2000). There are few reports in the literature on the production of decay enzymes by the identified fungal species. Research has focussed on fungi that are important in biotechnological applications, for example, *G. trabeum* and *S. commune*. This thesis study and other reports in the literature have shown that there is a wide variation in CMCase, xylanase and mannanase activities between species of basidiomycete fungi and between isolates of a species. In comparison to the enzyme activities observed for the four *G. sepiarium* isolates, Ritschkoff et al. (1992) measured very low activities, particularly of CMCase (up to 0.018 IU mL⁻¹) and mannanase (up to 0.004 IU mL⁻¹), and found that activities were similar between *G. sepiarium* and *G. trabeum*. In contrast, Mansfield et al. (1998) measured high enzyme activities for the two *Gloeophyllum* species, particularly of CMCase and mannanase (up to 25 and 11 IU mL⁻¹, respectively), and found that CMCase and xylanase activities were similar but mannanase activity was much higher for *G. sepiarium* (14 IU mL⁻¹) compared to *G. trabeum* (1 IU mL⁻¹). Schmidt and Liese (1980) measured CMCase and xylanase activities for *S. commune* that were comparable to *S. commune* 2 and 3, but Haltrich and Steiner (1994) measured higher activities particularly of xylanase (285 IU mL⁻¹). Haltrich et al. (1993) had also measured very high activities, particularly of xylanase (5740 IU mL⁻¹), by a wild *S. commune* strain that was acknowledged to have exceptional enzyme production. The different mannanase activities that were measured for *S. commune* 2 and 3 (0.001 and 0.299 IU mL⁻¹, respectively) may have been because galactomannan is a poor inducer of mannanase (Haltrich and Steiner 1994).

Comparison of enzyme activities is difficult, not only because of the variation between fungal isolates but also the different assay methods and substrates that are used. The most common method is the dinitrosalicylic acid (DNSA) assay but there are different substrates and concentrations and differing assay pH, temperature and incubation times. Bailey et al. (1992) carried out a survey of 20 laboratories who analysed the same enzyme sample for xylanase activity using their usual method as well as a standard assay. The variation between the laboratories was reduced as the assay method, and also assay substrate, was standardised. A degree of variation is always present using xylan and mannan substrates as they are only partially soluble and form heterogeneous, viscous solutions. Nilsson (1974) found that the loss of crystalline cellulose in liquid medium was not correlated to cellulase production. Alternative cellulose substrates are water-soluble substituted derivatives or regenerated cellulose such as CMC (Highley 1973), but the CMC assay is not specific for

endoglucanases as exoglucanases and non-cellulolytic microorganisms can also degrade CMC (Reese et al. 1950).

The agar-based assays for laccase and peroxidase were simple and rapid screens for the presence of these ligninolytic enzymes but they may be less reliable than spectrophotometric assays (Abdel-Raheem 1997). Laccase was tested on MA although Abdel-Raheem (1997) found that many fungi produced laccase in low nitrogen liquid medium but not in high nutrient medium. Jung et al. (1995) found a positive correlation between the stain zone width on gSA and Klason lignin loss of *Pinus* sawdust. It was decided to assess the stain intensity as, in many cases, the stain zone was large but the colour was diffuse. The measurement of both parameters would have allowed comparisons to be made with Jung et al. (1995) and Nishida et al. (1988). Stain intensity has been found to vary between fungal species and several that are high lignin degraders have a negative or low reaction for ligninolytic enzymes (Ander and Eriksson 1977).

The presence of laccase and peroxidase were correlated and both were generally present in white rot fungi and absent in brown rot fungi, as found by Fahr et al. (1999). The two *S. commune* isolates tested laccase-negative but peroxidase-positive, which Morais et al. (1996) also observed for some species of white rot fungi. Out of 49 *S. commune* cultures studied by Schmidt and Liese (1980), there were two cultures that were laccase-negative and peroxidase-positive, 14 cultures that were laccase-positive and peroxidase-negative, 29 cultures that were laccase- and peroxidase-positive, and four cultures that were laccase- and peroxidase-negative.

Gramss et al. (1998) carried out α -naphthol reactions for laccase on agar and defined extracellular enzyme to be produced when the stain diffused beyond the fungal colony and intracellular enzyme to be produced when the stain was restricted to the colony. Stain intensity ratings for laccase could be interpreted in a similar manner where ratings of 1, 2 or 3 suggest extracellular enzyme production and ratings of 4 and 5 suggest intracellular enzyme production. The peroxidase stain intensity ratings were not so easily differentiated. The laccase-positive brown rot fungi generally had lower stain intensity ratings which suggested an extracellular laccase compared to the laccase-positive white rot fungi that suggested an intracellular laccase.

There were no reports of brown rot fungi producing laccase until Score et al. (1997) observed that *Coniophora puteana* (Fries) P. Karsten produced laccase in pure culture, probably induced by components in the medium, and *Serpula hexagonoides* (Burt.) W.B. Cooke was found to produce laccase in response to the presence of *Trichoderma* fungi (Eriksson et al. 1990). Ejechi et al. (1996) found an isolate of *G. sepiarium*, from mahogany wood, that tested positive for laccase and they proposed that this was associated with the degradation of extractives. Similarly, there were no reports of LiP or MnP production by brown rot fungi until Dey et al. (1994) observed peroxidase production by *Polyporus ostreiformis* Berkeley.

The proteinase assay was a qualitative one and no investigations were carried out to determine whether the width of clearing zone on SMA was correlated to proteinase activity. North (1982) screened a wide range of fungi for proteinase production and found that the enzyme was produced by fungi of all major taxonomic groups, although some did not exhibit proteinase activity in the presence of easily assimilable nitrogen sources (Abraham et al. 1993).

Wood decay

In the wood decay assay, no soil, agar or vermiculite base substrate was used, agar inoculum was applied directly to the wood blocks, and the blocks were sterilised by gamma irradiation. These assay conditions were closer to those likely to be encountered by *Pinus radiata* timber in above ground, relatively low decay hazard situations where biological control systems have a greater chance of success in comparison to ground contact situations. In the standard American Society for Testing and Materials assay (ASTM 1981), soil is used to maintain moisture and act as a nutrient source, inoculum blocks are preinoculated with the decay fungus and test blocks are steam sterilised. In some decay assays, the soil is exchanged for agar or vermiculite. A nutrient solution is often added to the vermiculite to assist fungal colonisation and growth to a critical biomass from which it can start to degrade the test blocks. For a low decay hazard assay, Magasi (1975) had eliminated extraneous nutrients with a sand substrate but still used an inoculum block which provided a greater potential for rapid colonisation of test blocks that were placed on them, in comparison to the individual block inoculation used in this thesis study. In many experiments, wood blocks are steam sterilised or autoclaved and under these conditions, the cell wall structure may be disrupted and prone to fungal attack. Autoclaving can

mobilise further nutrients in wood for use in fungal colonisation and growth (Bender et al. 1970). The dose of gamma irradiation for sterilisation (27.7 kGy) was higher than the 15 kGy recommended by Freitag and Morrell (1998) and Pointing et al. (1999). The latter researcher found that doses of up to 100 kGy had no adverse effects on physical wood properties. Mean wood moisture contents were above the reported decay threshold of 26.5% (BRE 1975), the incubation temperature of 25°C was controlled, and the oxygen concentrations in the assay jars were not limiting decay potential.

The wood decay assays throughout the thesis research did not allow for fungal biomass in the final dry weights of test blocks. This can contribute to an under-estimation of weight loss as fungal biomass can contribute up to 50% of final wood dry weight (Swift 1973). Anagnost and Smith (1997) found that decay by the brown rot fungus *Postia placenta*, but not the white rot fungus *Trametes versicolor*, reduced wood hygroscopicity so the use of blocks at equilibrium moisture content or dried can contribute to an over-estimation of wood weight loss.

The wood weight losses were less than those commonly reported from agar block or soil block assays that simulated more severe decay hazard situations. Merrill and Cowling (1966) measured decay of individually inoculated wood blocks incubated in a humid environment with no extraneous nutrient addition and similarly observed reduced weight losses. Giron and Morrell (1989) equated conditions in the agar block assay to wood that was not in ground contact and under less hazard of decay than the soil block assay which paralleled wood in ground contact. Even the type of soil used in soil block assays can significantly influence fungal wood decay (Amburgey 1978; Terziev and Nilsson 1999).

Kreber and van der Waals (1999) carried out soil block assays to determine the *Pinus radiata* decay potential of nine *Phlebiopsis gigantea* isolates and a number of reference wood decay fungi, including *Gloeophyllum trabeum* and *Schizophyllum commune*. The *P. gigantea* 103, 104, 119 and 130 cultures were included in the nine isolates and they gave the highest wood weight losses of 12.5% (*P. gigantea* 103) to 26.5% (*P. gigantea* 104). The *G. trabeum* reference culture, a British Standard (BS) commonly used in wood durability and preservative assays, resulted in a 71.2% weight loss. Some cultures of *G. trabeum* have been shown in other studies to have lesser abilities to degrade *Pinus radiata* e.g. 34% (Juacida and Villanueva 1997). The *S. commune* reference culture resulted in a comparable weight loss (0.8%) to *S. commune* 2 and 3. Schmidt and Liese

(1980) also measured only 0.5% weight loss of *Pinus* species by *S. commune*. Butcher (1967) studied two *Phlebiopsis gigantea* isolates that resulted in weight losses of 44 and 46% for *Pinus radiata* blocks that were buried in the soil, and 2 and 12% for blocks that were above the soil. Hedley and Foster (1972) found that weight losses of *P. radiata* by *Pycnoporus coccineus* and *Coniophora olivacea* in soil block assays were around twice those observed for *P. coccineus* 101 and 124 and *C. olivacea* 19.

As for the enzyme assays, the wood decay assay was carried out under one set of conditions so the decay potential of each test fungus may not have been achieved. To determine the decay potential of both brown rot and white rot fungi, Hedley and Foster (1972) modified the ASTM soil block assay to include a relatively low decay hazard situation (blocks on the soil surface) and a high decay hazard situation (blocks buried in soil) as they found that buried blocks had higher weight loss than those above soil, particularly for white rot fungi. This was supported by Highley (1978) who observed higher decay occurring in inoculum blocks, particularly with white rot fungi, and suggested that decay by white rot fungi required a higher wood moisture in comparison to brown rot fungi. Hedley and Foster (1972) showed that brown rot fungi can tolerate lower wood moisture content for decay than white rot fungi. Highley and Scheffer (1970) used a filter-paper in place of the inoculum block which allowed for higher moisture contents of test blocks and found that decay by white rot fungi was also favoured by nutrient supplementation. *Pinus radiata* has been reported to be subject to higher decay by brown rot than white rot fungi (Butcher and Drysdale 1991). Assay conditions appeared to favour decay by the brown rot over the white rot fungi. Lower wood moisture contents may restrict the extent of growth and decay for some fungi and this may have been a limiting factor for the white rot fungi. Wood moisture content was not limiting decay as the lowest mean moisture content of 27.6% was above the reported threshold moisture content of 26.5% for decay of pine sapwood (BRE 1975).

The basidiomycete fungi that were studied had not previously been tested for wood decay potential. The majority of decay studies used standard reference fungal cultures selected for their virulence. The lower decay ability of field isolates was also observed by Butcher (1968a) who collected a range of basidiomycete fungi from *P. radiata* sapwood and, in soil block assays, found less than 12% weight loss after 10 weeks. Ohta (1997) noted that mycelial growth rates of basidiomycete fungi may be less than a half of those measured for standard reference cultures. Troya and Navarrete (1992) showed that loss of virulence in

standard cultures was related to the enzymatic inhibition produced in successive subculturing and, in some cases, could be restored by growing in different media. Hesse (1991) found that the virulence of basidiomycete fungi may be subject to rhythms of the natural growing season despite controlled laboratory conditions.

As for the enzyme results, comparison of wood weight losses with those reported in the literature is difficult. Variability in wood decay ability and fungal growth rate occurs between basidiomycete species and between isolates of a species (Esllyn 1986). Decay assay methods of different laboratories, although based on standard methods, are often modified to suit available resources or incorporate minor executional differences. The decay fungi may come from varied sources and origins. In many cases, the incubation chambers that are available may determine the dimensions of the wood blocks and this can result in significant differences in decay (Smith 1970). Ofosu-Asiedu and Smith (1973) found that weight loss was related to the volume of wood blocks rather than the surface area.

Regression analyses showed correlations for the fungi between wood weight loss, moisture content and fungal biomass. Wood moisture content is influenced by metabolic water from fungal growth and decay. Hedley and Foster (1972) estimated that 50% weight loss of a 19 mm wood cube would result in the production of 1 mL of water. Brown rot fungi are reported to be more effective water conductors than white rot fungi (De Groot 1975). The brown rot fungi, in particular the *Gloeophyllum sepiarium* isolates, had fruiting body-like mycelial aggregations on the wood blocks at harvest. There were significant correlations between wood weight loss and external fungal biomass, particularly for brown rot fungi. However, Onosode and Sokolov (1973) found in decay assays of Russian wood species by *Coniophora puteana* (Schumach.:Fr.) P. Karst., that weight loss was not related to the development of external fungal biomass on wood.

Fungal growth

The MA medium had a C:N ratio of 50:1, and to address concerns associated with measuring fungal growth under relatively high nitrogen conditions, a LNA medium was also used that had a C:N ratio of 410:1 that was within the range found in wood (350 to 1250:1) (Cowling and Merrill 1966). Fungal growth was also measured on BA, the original basidiomycete isolation medium, to assess the degree of fungal inhibition from the

fungicide benomyl. Of the 38 basidiomycete cultures, the growth of 13 brown rot and 16 white rot cultures were inhibited to some degree.

There were correlations between growth rates on the three agar media for each fungus. The comparable growth rates on MA and LNA indicated that the basidiomycete fungi were well adapted to growth on a minimal medium. However, the ability of a fungus to grow well on LNA did not necessarily indicate an ability to grow well on *P. radiata*. Growth rates on agar were correlated to growth on *P. radiata* only for brown rot fungi. BR 107, 109, 110 and 112 and *Phlebiopsis gigantea* 110 grew well on both agar and wood but BR 113 which grew the fastest on the agar media was one of five cultures that failed to grow on wood. Yang (1999) found that, for many sapstain fungi, there was no correlation between growth rates on MA and on wood.

Stalpers (1978) developed an identification key for about 550 species of Aphyllophorales. One of the diagnostic characters was the colony radius after two weeks growth on 2% MA at room temperature (17 to 20°C) in diffuse daylight. Converted growth rates of the basidiomycete cultures on MA, from mm d^{-1} to colony radius after two weeks, showed that nine of the 15 identified fungi had comparable growth rates within the ranges given in the species key. *Antrodia serialis*, *Gloeocystidiellum sacratum* and *Junghuhnia vincta* were not described in the keys, and the *Ganoderma*, *Oligoporus* and *Poria* cultures were not identified to species level. Eslin (1986) observed growth rates of 3.4 to 5.0 mm d^{-1} for eight *G. sepiarium* isolates on MA at 25°C, which was comparable to 4.9 to 5.7 mm d^{-1} for *G. sepiarium* 4, 5, 14 and 15.

The measurement of radial colony extension growth on agar is not necessarily correlated with an increase in fungal biomass (White and Boddy 1992) but it gives relative growth responses under different treatments and can be combined with observations of colony morphology. This is the most common and practical assay for large-scale experiments.

The measurement of growth on *Pinus radiata* was based on visible external fungal growth, which may not reflect growth within the wood block. Future assays could assess internal fungal growth using light microscope and SEM, or measure growth on filter-paper discs over a two week period whilst still retaining a cellulose substrate (Dickinson 1974) or growth on 1 to 2 mm thick wood veneer (Levi 1969).

Regression analyses

The parameters in the enzyme, wood decay and growth assays were evaluated as predictors for wood weight loss i.e. a more simple, faster or precise assay or combination of assays such as growth on LNA or production of a particular enzyme may be a possible alternative to the 16 week wood decay assay. The following parameters were correlated to wood weight loss: wood moisture content and external fungal biomass, laccase, peroxidase (SA) and, additionally for brown rot fungi, growth on LNA, peroxidase (PDA) and proteinase.

The lack of correlations between CMCCase, xylanase and mannanase activities and wood decay has been observed by other researchers. The enzyme activities measured in liquid media may or may not be comparable to wood decay but the presence of activity showed that the fungus had the capability to produce the enzyme under some conditions. Tanaka et al. (1999) measured phenol oxidase activity and wood decay by *Phanerochaete chrysosporium* Burdsall in Burdsall & Eslyn and found no clear correlations between the two parameters. However, Mswaka and Magan (1998) found a wide range in cellulase activity for ten basidiomycete species where high activity was correlated to high wood weight loss. The *Schizophyllum commune* isolates had relatively high CMCCase and xylanase activities but caused no significant weight loss of *Pinus radiata*. Highley (1988) found no correlation between production of cellulase and wood weight loss but Jakucs and Vetter (1992) observed correlations between the presence of endoglucanase and weight loss of sawdust for 55 fungal species. Growth on MA has not been found to be correlated to the decay potential of wood decay fungi (Cartwright and Findlay 1934; Eslyn 1986; Thornton and Wazny 1986) and Kirk and Highley (1973) found no consistent correlations between fungal growth on wood and wood weight loss.

Variation between isolates of basidiomycete species

Variation between isolates of *G. sepiarium*, *Phlebiopsis gigantea*, *Pycnoporus coccineus*, *Resinicium bicolor* and *S. commune* was observed over all the assays. For the CMCCase, xylanase and mannanase assays, the most variation was in the ability to grow on mannan medium. Isolates of *Phlebiopsis gigantea* and *S. commune* also showed variation in growth on xylan medium. Isolates of *G. sepiarium* and *P. gigantea* showed variation in CMCCase and xylanase activity. In the agar-based enzyme assays, there were different reactions between *P. gigantea* isolates for peroxidase (particularly on gPDA). Only two *P. gigantea*

isolates and one *S. commune* isolate showed evidence of proteinase. There was no variation between isolates for the wood decay assay parameters. For *G. sepiarium*, there are reports of variation between isolates in the ability to degrade *Pinus* species e.g. 45 to 65% (Highley et al. 1998), 21 to 45% (Eslyn and Highley 1976), 20 to 38% (De Groot 1975) and 25 to 60% (Eslyn 1986). There are also reports of variation in wood decay between *Phlebiopsis gigantea* isolates e.g. 2 to 13% (De Groot 1975) and 12 to 25% ((Eslyn 1986). There was variation in growth rates between *G. sepiarium* isolates on wood, *P. gigantea* isolates on the three agar media and wood, and *S. commune* isolates on LNA and wood.

Basidiomycete cultures for further studies

It was primarily on the basis of the wood decay assay that the brown rot fungus *G. sepiarium* 5 and the white rot fungi *P. gigantea* 104 and *S. commune* 3 were selected for further studies (Photo 2.6). Descriptive culture information on these fungi is given in Appendix 7. The three species are commonly reported on *Pinus radiata* (Butcher 1968a) and they were all isolated from fruiting bodies, *G. sepiarium* 5 from a HortResearch log trial and *P. gigantea* 104 and *S. commune* 3 from timber at Waipa Mill.

The four *G. sepiarium* isolates caused relatively high wood weight losses of 19.28 to 22.66%. BR 12 had the highest weight loss (27.1%) but an identified fungus was selected so that results could be compared to the literature. *G. sepiarium* 5 was selected for its ability to grow on wood and form fruiting body-like mycelial aggregations. This fungus caused a wood weight loss of 20.9%, had average CMCase activity, insignificant xylanase activity, and high mannanase activity, tested negative for laccase, peroxidase and proteinase, and had average growth rates on agar and *Pinus radiata*.

The *P. gigantea* 104 isolate was selected as it had caused the highest wood weight loss of the identified white rot fungi (7.0%), had average CMCase activity, and insignificant xylanase and mannanase activity, tested positive for laccase, peroxidase and proteinase, and had high growth rates on agar and on *Pinus radiata*.

Of the two *S. commune* isolates, *S. commune* 3 had the higher CMCase and mannanase activities, but an insignificant wood weight loss of 0.5%, tested negative for laccase and peroxidase (gSA), positive for peroxidase (gPDA) and proteinase, and had high growth rates on agar and *Pinus radiata*.

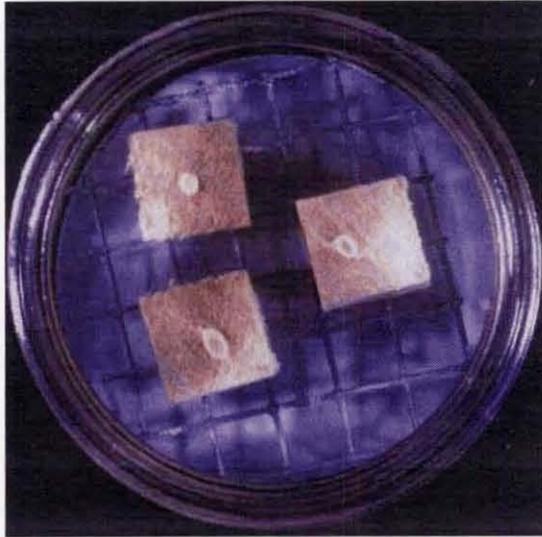
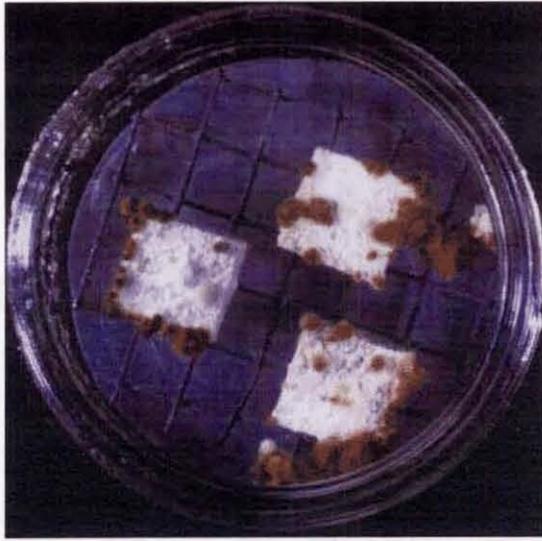


Photo 2.6 Aerial view of the wood decay assay jar showing (top) the dense white mycelium and brown mycelial aggregations of *Gloeophyllum sepiarium* 5, (middle) the farinaceous mycelium of *Phlebiopsis gigantea* 104 and (bottom) the fine fluffy mycelium of *Schizophyllum commune* 3.

Chapter 3 **GROWTH AND WOOD DECAY OF BASIDIOMYCETE FUNGI**

3.1 INTRODUCTION

The three basidiomycete fungi *Gloeophyllum sepiarium* 5, *Phlebiopsis gigantea* 104 and *Schizophyllum commune* 3 selected from the results of Chapter 2 (herein described as *G. sepiarium*, *P. gigantea* and *S. commune*) were further characterised in the following growth and wood decay assays:

- 1) Light, temperature and pH influences on fungal growth on agar.
- 2) Assay pH and temperature influences on cellulase, xylanase and mannanase activity.
- 3) Wood decay assay including treatments of green and dried *Pinus radiata* sapwood, *P. radiata* heartwood, *Pseudotsuga menziesii* and *Eucalyptus regnans* sapwood, and soil or vermiculite assay substrate.

An alternative to measuring fungal extension growth on agar or wood was investigated by assessing growth in liquid medium by accumulated fungal biomass and changes in medium pH, glucose and protein concentration. The objective of Chapter 3 was to gain a greater understanding of factors influencing growth and wood decay ability of the basidiomycete fungi, to assist in the design of appropriate assays and screens for the biological control studies.

3.2 MATERIALS AND METHODS

3.2.1 Agar assays

Growth responses of *G. sepiarium*, *P. gigantea* and *S. commune* under light, temperature and pH treatments were quantified by measuring radial colony extension growth on malt extract agar (MA; Appendix 1.7). There were five replicate Petri dishes for each treatment.

A 6 mm agar core of each test fungus (8 d old cultures) was inoculated in a central position on each Petri dish. Colony diameters were measured in two predetermined directions every 2 d after inoculation until the Petri dish was covered (or up to 14 d). Treatment effects on colony morphology and pigmentation of the fungi were noted but not characterised. The results were analysed statistically using analysis-of-variance and comparisons between individual means were carried out using Fisher's protected least-significance-difference at the 5% significance level.

3.2.1.1 Light

The influence of light or dark on fungal growth was assessed on standard MA (pH 5.5) at 25°C by leaving the Petri dishes exposed in the incubator to continuous light or by covering the Petri dishes with tinfoil. The incubator was lit by a single top-mounted 4000 k fluorescent lamp with a radiant flux of 14 000 mW.

3.2.1.2 Temperature

The influence of six incubation temperature treatments (5, 10, 20, 25, 30 and 37°C) on fungal growth was assessed on standard MA (pH 5.5) in the dark. The 20, 25, 30 and 37°C treatments were repeated to check reproducibility of the experiment.

3.2.1.3 pH

The influence of five pH treatments (3.5, 4.5, 5.5, 6.5 and 7.0) on fungal growth was assessed on MA, that was adjusted using 5 M NaOH and HCl (conc.) and incubated at 30°C in the dark. At the conclusion of the experiment, the agar pH was measured using pH indicator papers (Merck, Germany).

3.2.2 Liquid medium assay

Growth parameters of accumulated fungal biomass and medium pH, glucose and protein concentrations were determined for *G. sepiarium*, *P. gigantea* and *S. commune* in yeast extract-malt extract medium (YM; Appendix 1.14). Each flask was inoculated with 5 mL of the test fungus (3 d old cultures in YM) and incubated at 25°C and 140 rpm. There were

triplicate flasks for each incubation time interval; 0, 6, 12, 24, 36, 48, 72, 96 and 144 h from inoculation. At each incubation time, three flasks were removed and the pH of the medium was measured using a pH electrode (Mettler, Delta 350). Samples of supernatant were stored at -20°C for subsequent glucose and protein analyses. Accumulated fungal biomass was extracted from the liquid medium by vacuum filtration onto Whatman #2 filter-paper (dried at 80°C to a constant weight) and dried at 80°C to a constant weight. Colony morphology and pigmentation of the fungi was noted but not characterised.

Glucose concentration was determined using a glucose assay (Boehringer Mannheim) based on a glucose oxidase method using azinobis(3 ethylbenzothiazoline-6-sulfonic acid) as a hydrogen peroxide indicator. Glucose standards were 0, 0.101, 0.202, 0.303, 0.404 and 0.505 mmol L^{-1} . The samples were assayed in triplicate using $40\text{ }\mu\text{L}$ of supernatant. Some samples required a 1:10 dilution with sterile distilled water.

Total extracellular protein was determined using a commercial protein assay (Bio-Rad Laboratories; Sigma) which was based on the Bradford method (Bradford 1976) and used Coomassie Brilliant Blue G-250 dye as a protein indicator. Bovine serum albumin was used as the standard at concentrations of 0, 1.45, 2.9, 4.35, 5.8, 7.25 and $8.7\text{ }\mu\text{g mL}^{-1}$. The standards were run with each assay to give a calibration curve for protein calculations. The microassay procedure described in the assay kit was carried out using $800\text{ }\mu\text{L}$ of supernatant. Some samples required a 1:4 dilution with sterile distilled water.

The results were analysed statistically using analysis-of-variance. The fungal biomass, glucose and protein data required logarithmic transformation because of the heterogeneity of variation. Comparisons between individual means were carried out using Fisher's protected least-significance-difference at the 5% significance level.

3.2.3 Decay enzyme assays

The influence of assay pH and temperature on cellulase, xylanase and mannanase activities was investigated for *G. sepiarium*, *P. gigantea* and *S. commune*. Aliquots with the highest activity for each enzyme, for each fungus, were retrieved from storage at -20°C . The samples were reassayed for enzyme activity (Appendix 2.1) under four pH treatments of 4, 5, 6 and 7 in 100 mM citrate buffer at an incubation temperature of 50°C .

The pH that resulted in the highest enzyme activity for each fungus was used in a subsequent assay with three incubation temperature treatments of 25, 37 and 50°C.

3.2.4 Wood decay assay

The wood decay assay was modified in a number of ways to that used in Chapter 2. Polycarbonate jars (67 x 67 x 110 mm) with a polyethylene lid replaced the glass jars which had broken after repeated autoclaving. Vermiculite or soil was used as a base substrate to maintain the moisture content of wood blocks. The soil was also a source of nutrients for fungal growth. Preinoculated *Pinus radiata* sapwood blocks provided an inoculum source for the test blocks that were placed on them. All test blocks were dried at 80°C except those for a green *P. radiata* sapwood treatment. Dried blocks were used as the temperature and relative humidity facilities available were not adequate for the use of “equilibrium moisture content” blocks as in the American Society for Testing and Materials assay (ASTM 1981). The moisture content of dried blocks was not a limiting factor for decay as a sample of untreated blocks placed under the new jar system showed that the reported decay threshold moisture content of 26.5% (BRE 1975) was reached within a week of incubation at 30°C.

There were six treatments for each basidiomycete fungus, and an uninoculated control treatment, with triplicate jars for each treatment. Five treatments used vermiculite as the base substrate and test blocks of: green *P. radiata* sapwood; dried *P. radiata* sapwood and heartwood; and dried *Pseudotsuga menziesii* and *Eucalyptus regnans* sapwood. The sixth treatment used soil as the base substrate and dried *Pinus radiata* sapwood test blocks. There were three treatments for the inoculum wood blocks: inoculum blocks in vermiculite supporting dried test blocks; inoculum blocks in vermiculite supporting green *P. radiata* sapwood; and inoculum blocks in soil supporting dried test blocks.

Vermiculite or soil (100 mL) was placed into each jar together with an aliquot of sterile distilled water. The volume of water applied to the vermiculite (medium grade, Nuplex Industries Ltd., Auckland) was 25 mL, which saturated the vermiculite without any excess water. The soil was a clay loam from HortResearch (Hamilton) which was passed through a 2 mm sieve prior to determination of pH (5.2; Appendix 2.4), moisture content (31.6%)

and water-holding-capacity (WHC; 45.9%, Appendix 2.5). The volume of water to produce a WHC of 130%, as specified in ASTM (1981), was 15 mL.

Inoculum blocks (50 x 50 mm cross-cut x 5 mm thick) were produced from freshly harvested *P. radiata* sapwood obtained from CHH Timber Putaruru Mill. The blocks were dried at 80°C to a constant weight and placed on the vermiculite or soil within the jars. The jars were autoclaved for 20 min at 121°C. The soil treatment jars were reautoclaved after a 16 h interval to ensure sterilisation. Each block was inoculated with four 6 mm agar cores of the test fungus (9 d old cultures) and then incubated for 28 d at 30°C in the dark.

Log sections of freshly harvested *P. radiata*, *Pseudotsuga menziesii* and *E. regnans* were obtained from CHH Timber Putaruru Mill. Test blocks (20 x 20 x 20 mm) were produced from *Pinus radiata* sapwood and heartwood, and *Pseudotsuga menziesii* and *E. regnans* sapwood. All test blocks, excluding those for the green *Pinus radiata* sapwood treatment, were labelled and dried at 80°C to a constant weight. All blocks were sterilised by gamma irradiation (53.1 kGy; Schering Plough Animal Health Ltd., Upper Hutt). Four test blocks were placed on each precolonised inoculum block and then incubated at 30°C in the dark.

After 16 weeks, all blocks were removed from the jars, the external fungal biomass was carefully brushed off and each block was weighed and dried at 80°C to a constant weight. The mean wood dry weight loss was calculated as a percentage of the initial block dry weight for the dried block treatments. For the green *P. radiata* sapwood treatment, weight loss was calculated using the mean initial dry weight of the dried *P. radiata* sapwood blocks.

Additional jars were used to monitor wood weight loss and moisture content for the three basidiomycete fungi over the course of the assay. In each jar, one test block each of *P. radiata* sapwood and heartwood, and *Pseudotsuga menziesii* and *E. regnans* sapwood was placed on the precolonised *Pinus radiata* sapwood inoculum block. Single jars for each fungus and an uninoculated control were harvested at 4, 8 and 12 weeks incubation using the same procedure as at 16 weeks. The results of this monitoring were for observational purposes only and the lack of replication meant that statistical analyses were not possible.

One wood block of each treatment was examined by SEM at the Waikato Microscope Unit using techniques based on Exley et al. (1974) and Exley et al. (1977). The blocks were incubated in wood fixative (Appendix 1.13) for 7 d and then stored in 30% ethanol. Longitudinal sections were cut from each block and mounted using double-sided tape onto a specimen stub. The specimens were dehydrated through a graded ethanol series and critical-point dried with ethanol as the transitional fluid in a Polaron instrument. They were platinum/palladium coated in a Hitachi E1030 Ion Sputter Coater and examined using a Hitachi S4000FE-SEM.

The results were analysed statistically using analysis-of-variance of angular transformed data. The non-transformed data and the maximum standard-error of the-difference (sed) for the different analyses is presented in the results table. Comparisons between individual means were carried out using Tukey's least-significance-difference at the 5% significance level. Standard regression analyses were carried out to determine if there were significant correlations between wood weight loss and final wood moisture content for test and inoculum blocks.

3.3 RESULTS

3.3.1 Agar assays

3.3.1.1 Light

Growth of *P. gigantea* and *S. commune* was significantly greater in the dark than in the light over the entire assay period but differences in growth of *G. sepiarium* were only significant over the first 6 d ($P < 0.05$) (Appendix 8). Growth of *G. sepiarium* in the light had a yellow-brown pigmentation to the agar but the colony morphology of *P. gigantea* and *S. commune* was similar between the light and dark treatments.

3.3.1.2 Temperature

The greatest growth of *G. sepiarium* was at 30°C, followed by 25°C (Fig. 3.1; Photo 3.1). At 37°C, growth was initially as high as at 30°C but decreased after 8 d to below that observed at 25°C. There was no significant growth at 5°C and relatively slow growth at

10°C. A repeat of the 20, 25, 30 and 37°C treatments showed the same trends (data not shown). *Gloeophyllum sepiarium* had appressed cream mycelial clumping around the agar core and farinaceous colony growth with a yellow-brown pigmentation (similar to that observed in the light experiment) at 30°C which was accentuated at 37°C.

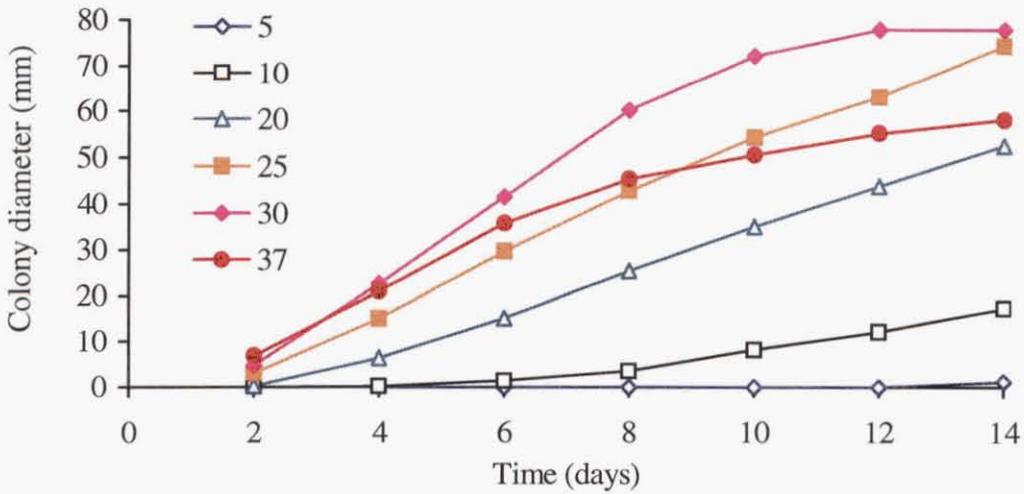


Fig. 3.1 Growth responses of *Gloeophyllum sepiarium* to temperature on malt extract agar. sed ($P < 0.05$) 2.13.

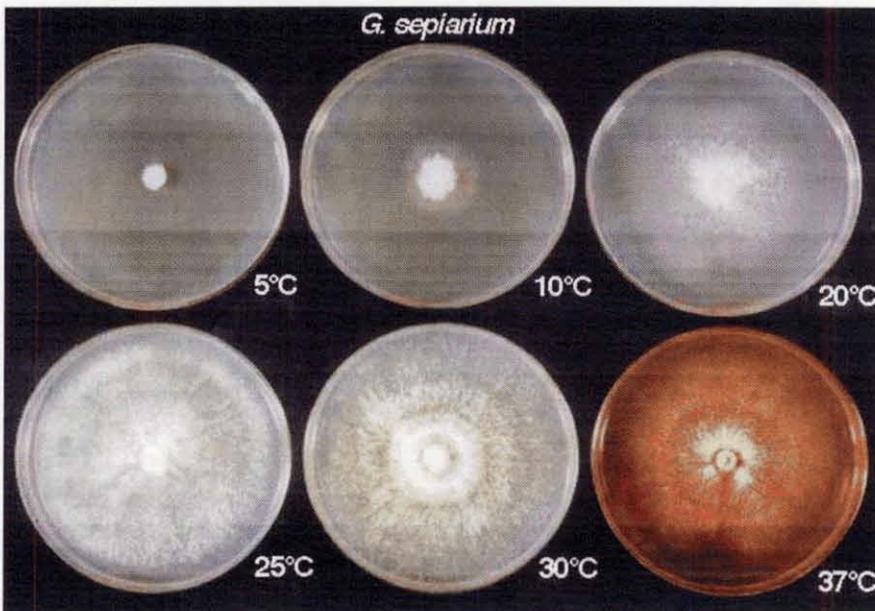


Photo 3.1 The influence of temperature on growth and morphology of *Gloeophyllum sepiarium* on malt extract agar, at 14 d after inoculation.

The greatest growth of *P. gigantea* was at 25°C, followed by 20°C (Fig. 3.2; Photo 3.2). In comparison, growth at 30°C was relatively slow for the first 6 d and then below that observed at 20°C. The initial setback in growth at this temperature may have been due to a

change in incubators that was necessary at that time. There was relatively slow growth at 5°C and no growth at 37°C. A repeat of the 20, 25 and 30°C treatments showed greater growth with increased temperature and there was no significant difference in growth at 25 or 30°C (data not shown). *Phlebiopsis gigantea* had a thick white floccose morphology which grew less dense at 30°C.

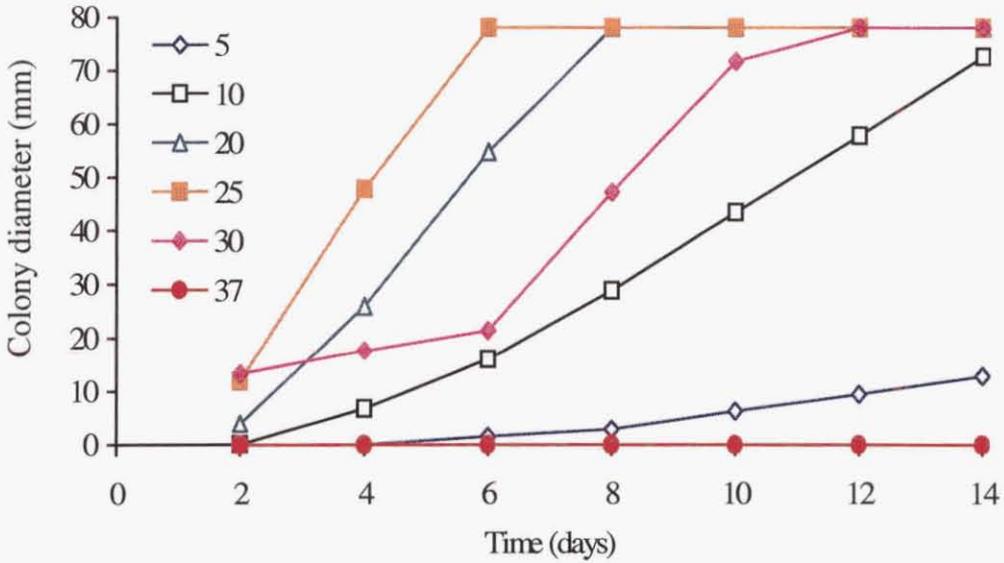


Fig. 3.2 Growth responses of *Phlebiopsis gigantea* to temperature on malt extract agar. sed ($P < 0.05$) 3.27.

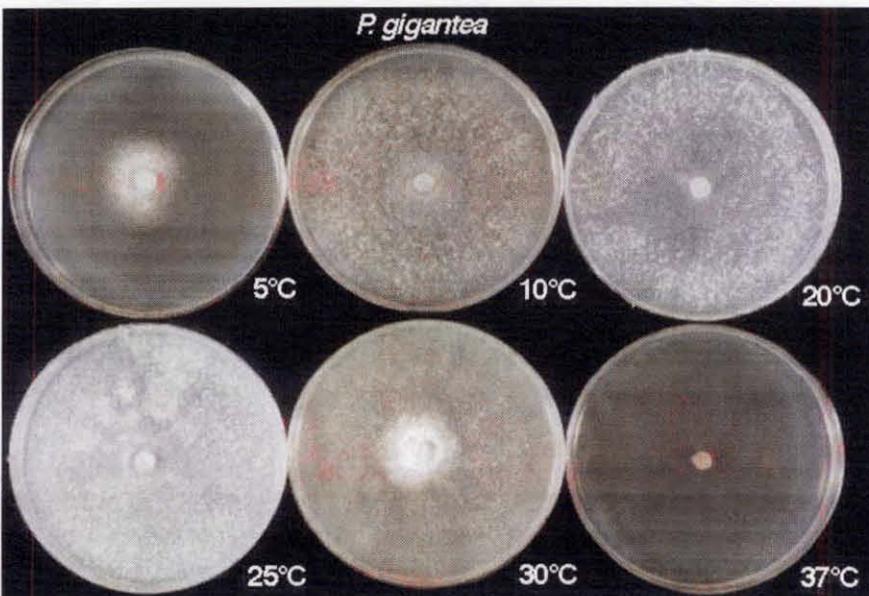


Photo 3.2 The influence of temperature on growth and morphology of *Phlebiopsis gigantea* on malt extract agar, at 14 d after inoculation.

The greatest growth of *S. commune* was at 25 and 30°C (which were not significantly different), followed by 20°C (Fig. 3.3; Photo 3.3). Colony growth at 37°C was below that observed at 20°C. There was relatively slow growth at 10°C and no significant growth at 5°C. A repeat of the 20, 25 and 30°C treatments showed the same trends but growth was significantly higher at 30°C (data not shown). *Schizophyllum commune* had a wispy white mycelial growth at temperatures up to 30°C but at 37°C, there was fine, dense fluffy growth around the agar core.

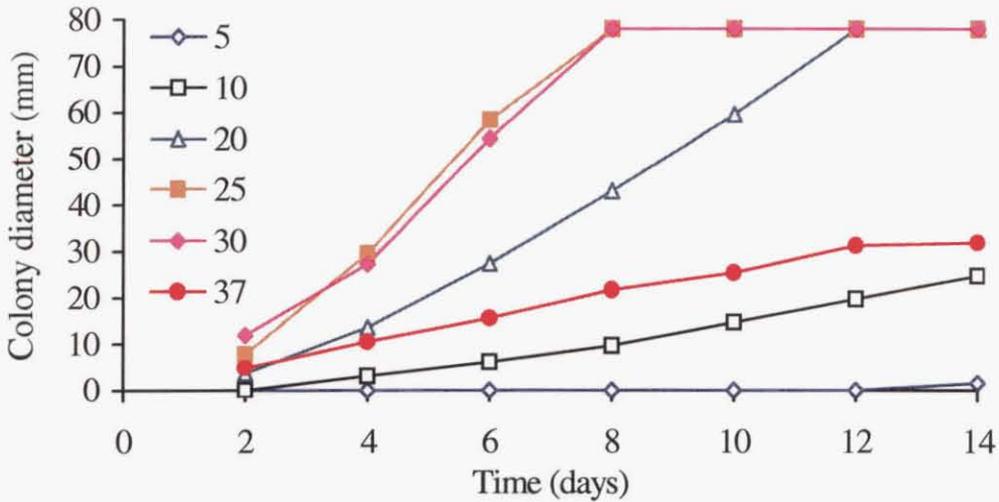


Fig. 3.3 Growth responses of *Schizophyllum commune* to temperature on malt extract agar. sed ($P < 0.05$) 1.74.

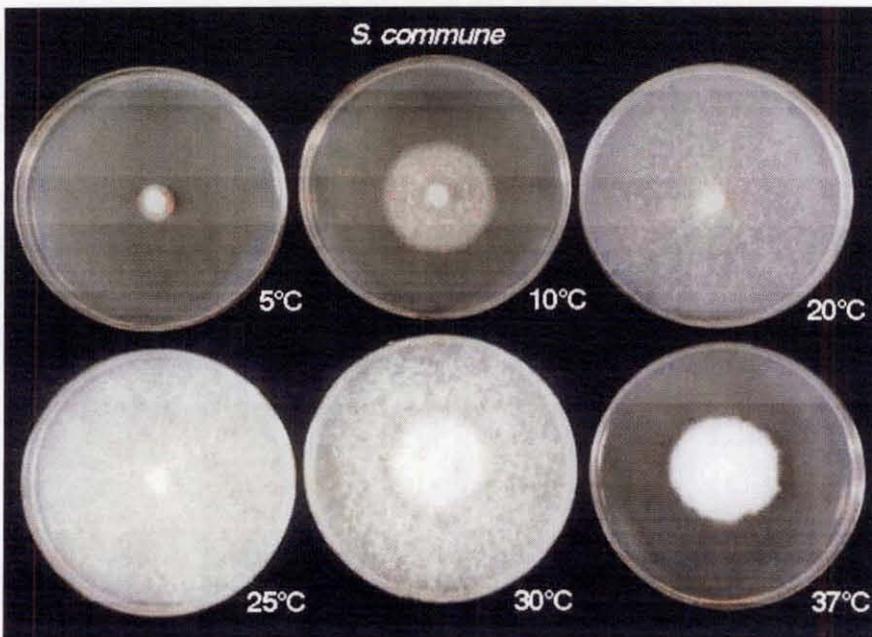


Photo 3.3 The influence of temperature on growth and morphology of *Schizophyllum commune* on malt extract agar, at 14 d after inoculation.

3.3.1.3 pH

The basidiomycete fungi showed significantly lower growth at pH 3.5 but there were no consistent significant differences between the other pH treatments (Appendix 9). Within each pH treatment, fungal growth resulted in a decrease in agar pH. In the pH 3.5 treatment, there was little change in pH, which was probably due to the inhibition of fungal growth in this treatment. Growth in the pH 4.5 treatment resulted in a final pH of less than 3.5 (*G. sepiarium*) and 4 (*P. gigantea* and *S. commune*). Growth in the pH 5.5, 6.5 and 7.0 treatments resulted in a final pH of 3.8 (*G. sepiarium*), 4.7 to 4.9 (*P. gigantea*) and 5.5 (*S. commune*).

3.3.2 Liquid medium assay

Growth of *S. commune* in YM was significantly higher than *G. sepiarium* and *P. gigantea* from around 12 h after inoculation ($P < 0.001$) (Fig. 3.4). There were no significant differences in growth of *G. sepiarium* and *P. gigantea* in YM. At 144 h after inoculation, the accumulated fungal biomass of *G. sepiarium*, *P. gigantea* and *S. commune* was 210, 190 and 630 mg mL⁻¹, respectively.

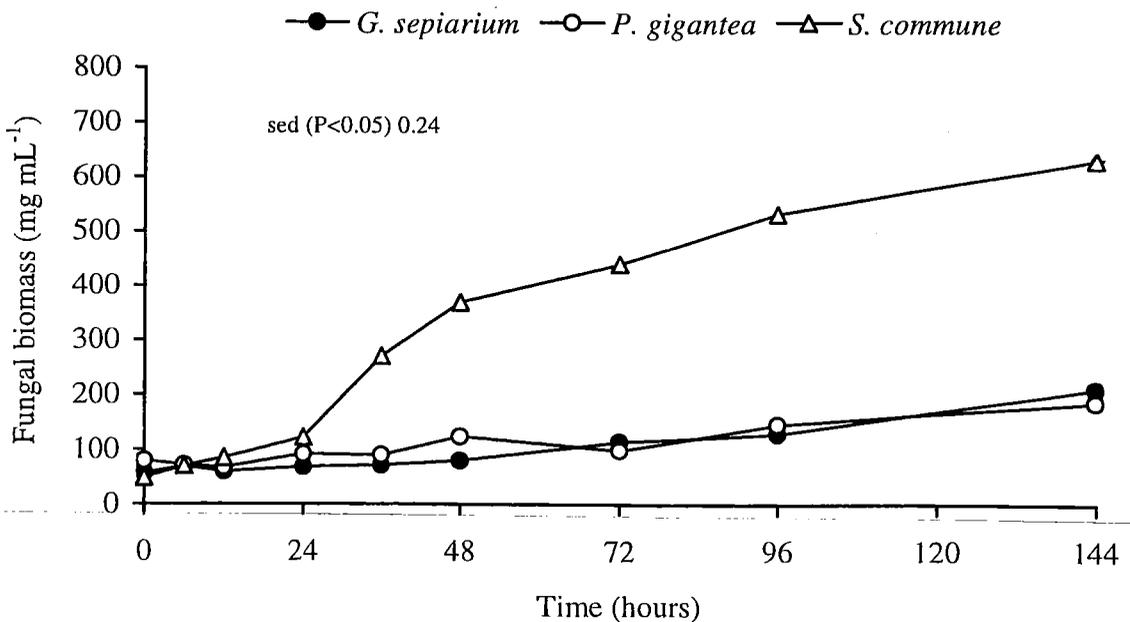


Fig. 3.4 Changes in accumulated fungal biomass of *Gloeophyllum sepiarium*, *Phlebiopsis gigantea* and *Schizophyllum commune* in yeast extract-malt extract medium.

Growth of the fungi in YM resulted in significant changes in the medium pH (Fig. 3.5). The initial pH was 5.2 to 5.5 until around 36 h after inoculation. The pH of the *G. sepiarium* and *P. gigantea* media both decreased to pH 4.2 to 4.3 and were significantly lower than the *S. commune* medium. For most of the assay period, there were no significant differences in pH of the *G. sepiarium* and *P. gigantea* media. The *S. commune* medium then increased in pH to 5.8 to 6 for the remainder of the assay period.

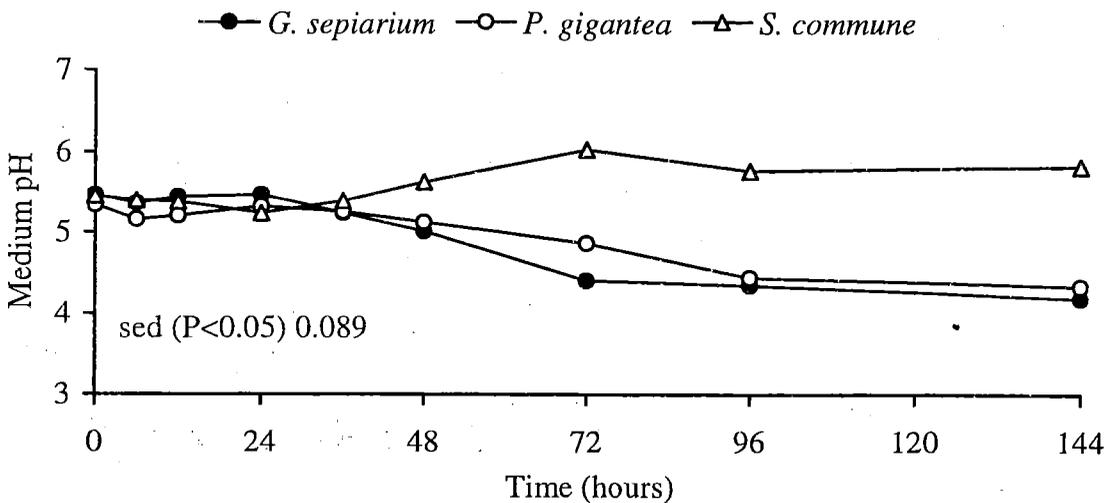


Fig. 3.5 Changes in pH of yeast extract-malt extract medium with growth of *Gloeophyllum sepiarium*, *Phlebiopsis gigantea* and *Schizophyllum commune*.

There were significant differences between the fungi in glucose, but not protein, concentration during growth in YM (Fig. 3.6). The initial glucose concentration was 170 to 190 mM. In the *G. sepiarium* medium, there was little change in glucose concentration over the assay period. In the *P. gigantea* medium, the glucose concentration increased with time up to 410 mM. The *S. commune* medium had the lowest glucose concentration of around 20 mM by 48 h after inoculation and less than 12 mM at the end of the assay period. The initial YM protein concentration was around 12 $\mu\text{g mL}^{-1}$. In the *G. sepiarium* and *P. gigantea* media, the protein concentrations were not significantly different and decreased with time to 6 to 7 $\mu\text{g mL}^{-1}$. The protein concentration of the *S. commune* medium was variable over the assay period.

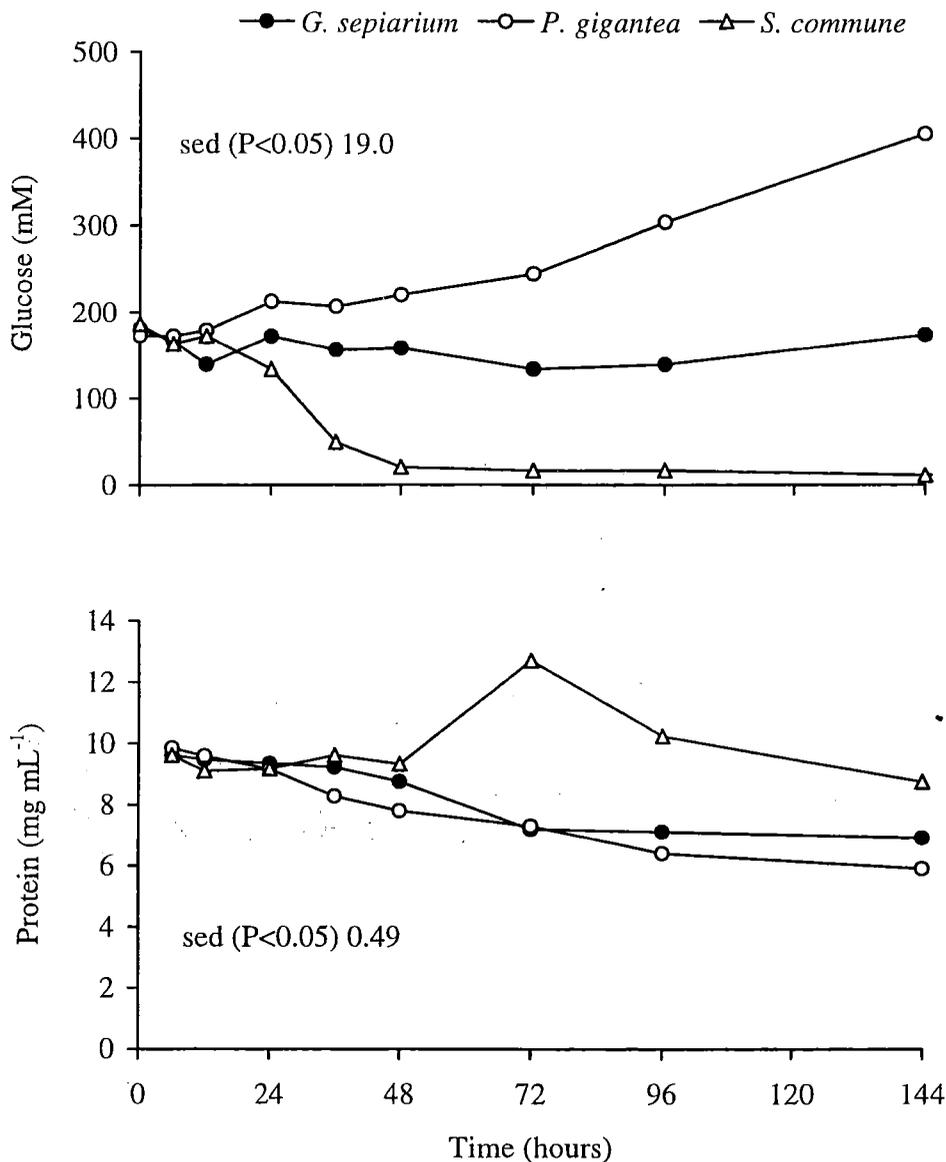


Fig. 3.6 Changes in yeast extract-malt extract medium of (top) glucose concentration and (bottom) protein concentration during growth of *Gloeophyllum sepiarium*, *Phlebiopsis gigantea* and *Schizophyllum commune*.

3.3.3 Decay enzyme assays

The assay pH of 6 used in the original assays was not optimal for enzyme activities of the three basidiomycete fungi (Appendix 10 a). The highest CMCase activity occurred at pH 4 for *G. sepiarium* and *P. gigantea* and at pH 6 for *S. commune*. The highest xylanase activity occurred at pH 5 for *S. commune* and was not detectable for *G. sepiarium* and *P. gigantea*. The highest mannanase activity occurred at pH 4 for *G. sepiarium* and *S. commune* and was not detectable for *P. gigantea*. Of the detectable activities, the assay incubation temperature of 50°C in the original assay was optimal for CMCase, xylanase

and mannanase activities of the three fungi (Appendix 10 b). There were differences in enzyme activities compared to those in the original assays, and these were attributed to variability in the assay (e.g. reagents) and the use of samples that had been reconstituted from freezer storage.

3.3.4 Wood decay assay

For all treatments, *G. sepiarium* caused significantly higher mean wood weight losses and moisture contents than *P. gigantea* or *S. commune* ($P < 0.05$) (Table 3.1). If weight losses of less than 2% were considered insignificant (Hedley and Foster 1972), *S. commune* did not cause significant weight losses in test or inoculum blocks of any treatment. There were no weight losses observed in the uninoculated control treatment.

Gloeophyllum sepiarium caused the highest test block weight losses in the green *Pinus radiata* sapwood treatment (33.9%) and the soil treatment (34.7%), followed by *P. radiata* heartwood, *P. radiata* sapwood and *Pseudotsuga menziesii*, with no significant weight loss of *E. regnans*. *Phlebiopsis gigantea* resulted in the highest test block weight losses in the green *Pinus radiata* sapwood treatment (14.6%) and the soil treatment (6.5%), followed by *P. radiata* heartwood, with no significant weight loss of *P. radiata* sapwood, *Pseudotsuga menziesii* or *E. regnans*. The highest mean weight losses of the *Pinus radiata* inoculum blocks were in the soil treatment (61.3% for *G. sepiarium* and 50.3% for *P. gigantea*), followed by blocks on vermiculite supporting green test blocks and then blocks supporting dried test blocks.

The additional jars harvested at 4, 8 and 12 weeks incubation showed an increase with time, in weight loss of test and inoculum blocks inoculated with *G. sepiarium* or *P. gigantea* (data not shown). *Schizophyllum commune* did not cause any significant weight losses.

Table 3.1 Wood weight loss and moisture content of test and inoculum block treatments inoculated with *Gloeophyllum sepiarium*, *Phlebiopsis gigantea* or *Schizophyllum commune* after 16 weeks at 30°C.

Treatment	<i>G. sepiarium</i>		<i>P. gigantea</i>		<i>S. commune</i>	
	Wt loss	MC	Wt loss	MC	Wt loss	MC
Test block						
<i>Pinus radiata</i> green sapwood on verm. ¹	33.9	78	14.6	42	0.0	40
<i>P. radiata</i> dried sapwood on verm. ²	9.5	8	1.5	7	1.1	8
<i>P. radiata</i> dried heartwood on verm. ³	12.8	8	2.2	9	0.9	8
<i>Pseudotsuga menziesii</i> dried sapwood on verm. ⁴	2.2	8	0.0	7	0.0	8
<i>Eucalyptus regnans</i> dried sapwood on verm. ⁵	0.4	10	0.1	8	0.1	8
<i>P. radiata</i> dried sapwood on soil ⁶	34.7	23	6.5	17	1.6	16
sed (P<0.05)	3.3	7.2	3.3	7.2	3.3	7.2
Inoculum block						
In verm. - green test blocks ¹	45.5	103	14.7	40	0.2	32
In verm. - dried test blocks ^{2,3,4,5}	27.0	8	11.1	8	0.9	8
In soil - dried test blocks ⁶	61.3	28	50.3	18	1.0	16
sed (P<0.05)	2.88	3.8	2.88	3.8	2.88	3.8

Wt loss= Wood dry weight loss (%)

MC= Wood moisture content (%)

Verm.= vermiculite

^{1,2,3,4,5,6} Corresponding treatments for test and inoculum block treatments.

The following photos show the assay jars of the green *Pinus radiata* sapwood-vermiculite treatment (Photo 3.4) and the dried *P. radiata* sapwood-soil treatment (Photo 3.5) for the three basidiomycete fungi together with the test and inoculum blocks after 16 weeks incubation. High external fungal biomass was produced by *G. sepiarium* on green *P. radiata* test blocks in vermiculite compared to the dried blocks in soil but the weight losses were similar.

The mean moisture contents of test and inoculum blocks were similar for each treatment. Final block moisture contents were very low for the dried block-vermiculite treatments (less than 10%) and also the soil treatment (less than 28%). Only the green *P. radiata* sapwood treatment had block moisture contents of 32 to 103%. Regression analysis showed that there was a significant correlation between wood weight loss and final wood moisture content for both test and inoculum blocks (P<0.001).

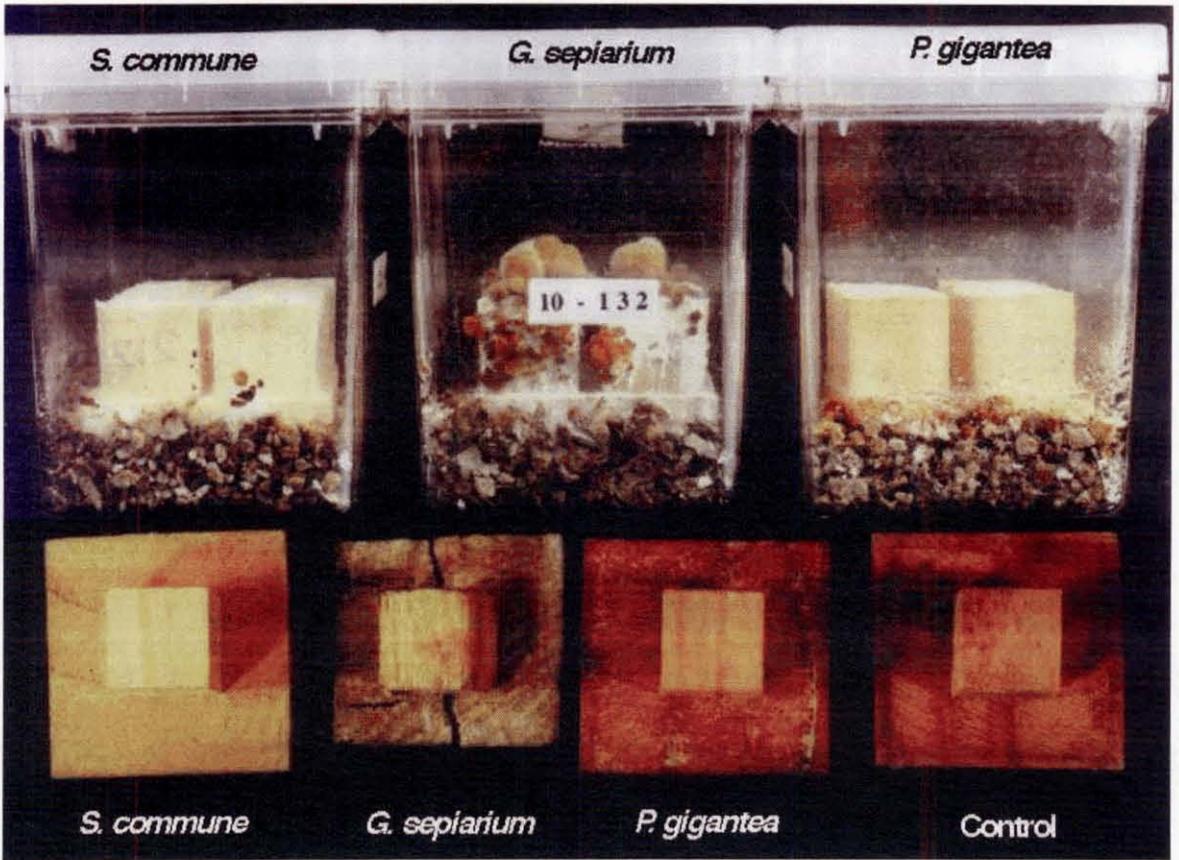


Photo 3.4 The green *Pinus radiata* sapwood-vermiculite treatment showing (top row) the assay jars of *Schizophyllum commune*, *Gloeophyllum sepiarium* and *Phlebiopsis gigantea*, and (bottom row) the test and inoculum blocks for *S. commune*, *G. sepiarium*, *P. gigantea* and the uninoculated control after 16 weeks incubation.

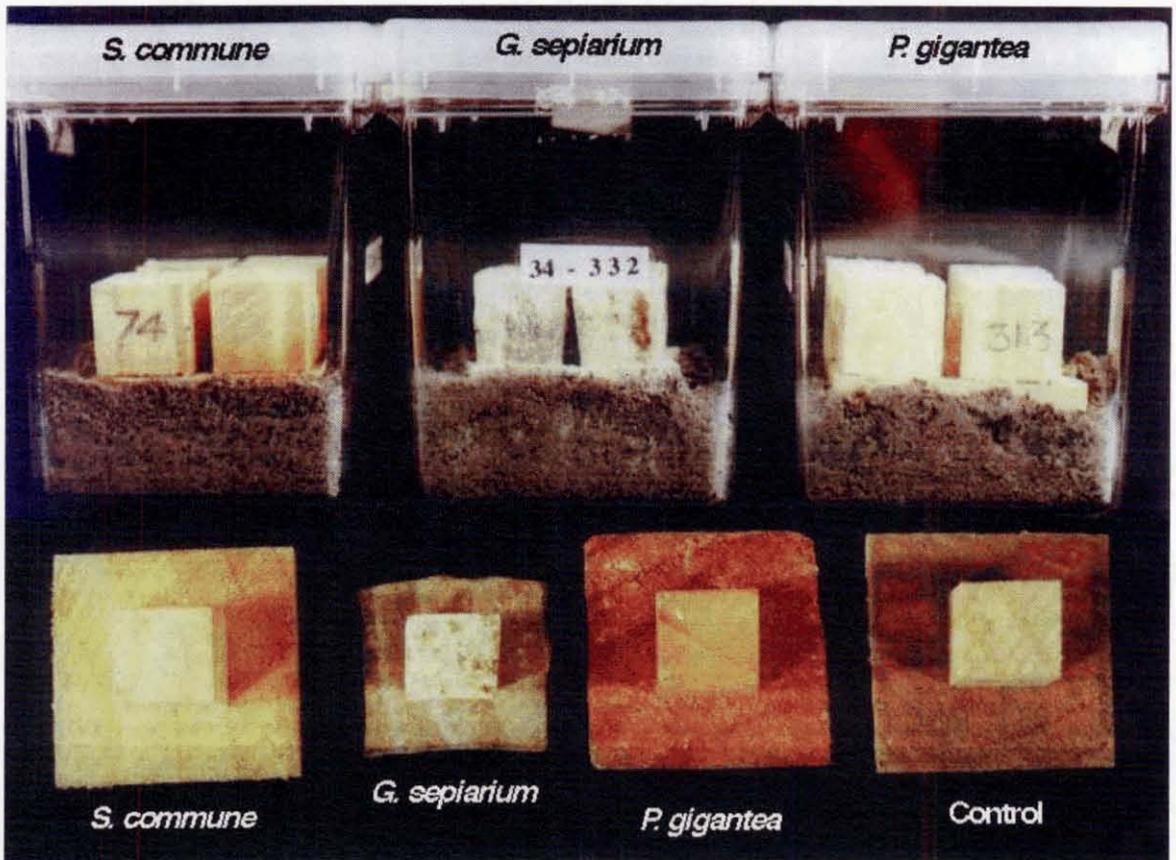


Photo 3.5 The dried *Pinus radiata* sapwood-soil treatment showing (top row) the assay jars of *Schizophyllum commune*, *Gloeophyllum sepiarium* and *Phlebiopsis gigantea*, and (bottom row) the test and inoculum blocks for *S. commune*, *G. sepiarium*, *P. gigantea* and the uninoculated control after 16 weeks incubation.

The SEM work showed extensive *G. sepiarium* colonisation of blocks from the *P. radiata* treatments but little colonisation of *Pseudotsuga menziesii* or *E. regnans* blocks (Photo 3.6). The lumen cell walls of the three wood species were smooth and showed no signs of degradation, even in the *Pinus radiata* blocks with a weight loss of around 35%. Colonisation of blocks by *P. gigantea* and *S. commune* was less extensive, and the uninoculated control blocks showed that there was no contamination (photos not shown).

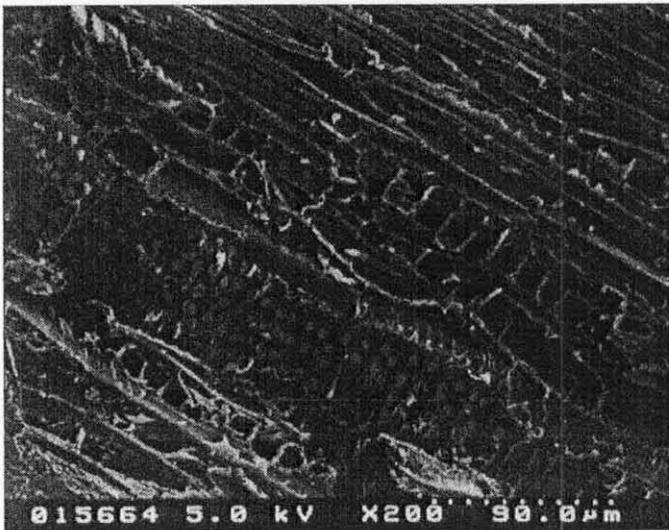
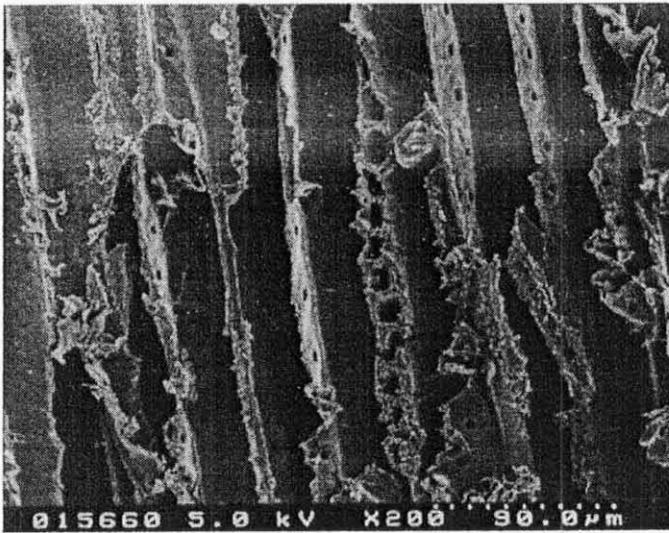
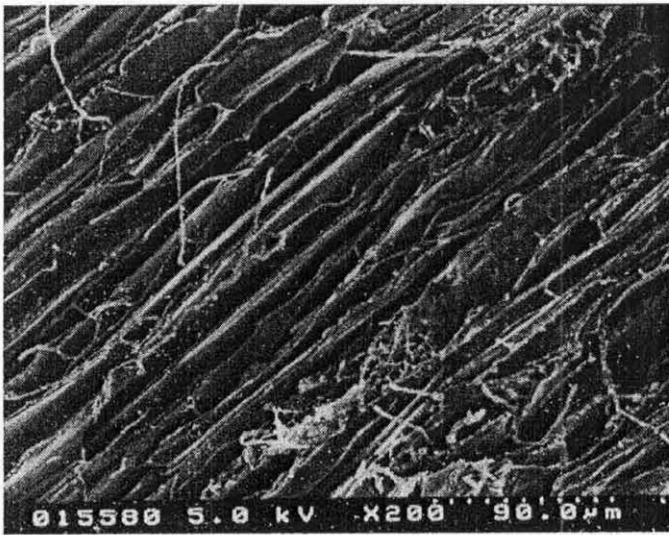


Photo 3.6 *Gloeophyllum sepiarium* colonisation of sapwood blocks of (top) *Pinus radiata*, (middle) *Pseudotsuga menziesii* and (bottom) *Eucalyptus regnans* after 16 weeks incubation on vermiculite. Longitudinal surface; SEM.

3.4 DISCUSSION

Growth of the three basidiomycete fungi was characterised by assays on agar and wood and in enzyme liquid medium (Chapter 2) and the agar and liquid medium assays in the current chapter. *Phlebiopsis gigantea* grew the fastest of the three fungi on agar and wood, and *S. commune* was the fastest grower in liquid medium. *Gloeophyllum sepiarium* was the slowest grower on all three media. *Gloeophyllum sepiarium* and *S. commune*, but not *P. gigantea*, had higher accumulated biomass on YM than on the enzyme liquid media. Measurements of radial colony extension growth on agar may be misleading as it does not consider mycelial density that may be greater, for example, at some temperatures. The fungal growth parameters measured in YM showed some correlations to wood weight loss. For *G. sepiarium* and *P. gigantea*, who caused significant wood weight losses, there was relatively low accumulation of fungal biomass in YM, a decrease in medium pH and protein concentration, and no change or an increase in glucose concentration. The little change in glucose concentration in the *G. sepiarium* medium suggested that glucose was not being utilised for growth. The increase in glucose concentration in the *P. gigantea* medium may have resulted from the autolysis of fungal cells through the production of extracellular proteinases. In comparison, *S. commune*, who caused no significant weight losses, had relatively high accumulation of fungal biomass, no decrease in medium pH, a decrease in glucose concentration and variable protein concentration.

Light

Growth of the three basidiomycete fungi was greater in the dark than the light treatment but the differences were not sufficient to justify the exclusion of light from Petri dishes, flasks and wood decay jars. Light has generally been assumed to be detrimental to the growth of basidiomycete fungi and this has been related to the inhibitory effects of ultraviolet radiation at high light intensities. There are few publications on the effect of light on fungal wood decay. Duncan (1967) found that periodic exposure to light increased the rate of decay although this may have been caused by associated effects on temperature. The effect of light on temperature was not measured in this thesis study. De Groot (1975) found in soil block assays, that the rate of wood decay by *G. sepiarium* was unaffected by light, and decay by *P. gigantea* was reduced in the presence of light if wood moisture

content was low. Hegarty and Curran (1985) found that wood decay by a range of fungi was unaffected by light.

Temperature

Wood decay fungi have been reported to degrade wood more efficiently at temperatures close to the optimum for growth (Eslyn 1986). There were differences in optimal growth temperatures for the three basidiomycete fungi. The optimum temperature for *G. sepiarium* growth was 30°C, which is comparable with reports in the literature: 30°C (Cartwright and Findlay 1934; Loman 1962); 30 to 35°C (Eslyn 1986); 32 to 35°C (Lindgren 1933); 35°C (Hulme and Stranks 1976); and 36°C (Humphrey and Siggers 1933). Lethal temperatures were not determined but Xie et al. (1997) found the lethal temperature for *G. sepiarium* was 60 to 65°C. Following exposure to high temperatures, recovery time was necessary for the resynthesis of heat sensitive enzymes. The optimal temperature for *P. gigantea* growth was 25°C, which is comparable with reports in the literature: 25°C (Hulme and Stranks 1976); 28°C (Wazny and Grzywacz 1981); and 30°C (Humphrey and Siggers 1933). The optimal temperature for *S. commune* growth was 25 or 30°C, which was lower than the 30 to 35°C observed by Eaton and Hale (1993). *S. commune* has been shown to be tolerant to high temperatures (Cartwright and Findlay 1934; Nicolotti et al. 1998) and respond to heat stress by producing heat-shock proteins (Higgins and Lilly 1993). The recommended growth temperatures for *G. sepiarium*, *P. gigantea* and *S. commune* cultures from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig) are 30, 20 and 30°C, respectively. This supports the lower optimal growth temperature that was observed for *P. gigantea*.

An incubation temperature of 25°C was used in further assays as the basidiomycete fungi grew relatively well at this temperature and the incubator and controlled temperature room facilities were readily available. The use of 30°C was considered but there were changes in colony morphology of *P. gigantea* and the release of a brown pigment by *G. sepiarium*.

pH

The basidiomycete fungi had significantly lower growth on agar with a pH of 3.5. Basidiomycete fungi have been reported to grow only at pH less than 6.5 (Kaarik 1974) but growth was observed at an initial pH of 6.5 and 7. A reduction in pH was measured

following growth on agar and in liquid medium. This was, in part, attributed to the production and secretion of organic acids and indicated that the medium had insufficient buffering capacity. In this study, changes in wood pH were not measured. Regardless of the initial pH of MA, growth of *G. sepiarium* resulted in a final pH of less than 3.8, *P. gigantea* in less than 4.9 and *S. commune* in less than 5.5. Over 6 d, the pH of YM decreased during growth of *G. sepiarium* and *P. gigantea* from an initial pH of 5.5 to pH 4.2 and 4.4, respectively. Jellison et al. (1997) found that growth of *Gloeophyllum trabeum* in liquid medium over 3 weeks decreased the pH to 2.4 to 3.0. During growth of *S. commune* on YM, the medium pH increased which indicated that breakdown products such as alkali compounds were produced and secreted into the liquid medium and again there was insufficient buffering capacity. Hummell et al. (1998) found that growth of *S. commune* lowered the pH of minimal medium.

Gloeophyllum sepiarium CMCase and mannanase activity, *P. gigantea* CMCase activity and *S. commune* mannanase activity was found to be optimal at pH 4 but growth of the fungi on MA was decreased at pH 3.5. In agreement with the result for *G. sepiarium*, Highley (1975b) found that the pH optima for endoglucanases of brown rot fungi were around 2 to 4. Further studies could investigate whether the pH changes in agar, liquid medium and wood as a result of basidiomycete growth, could be a feasible method of rapidly determining fungal decay potential.

Wood decay

The highest weight losses of test blocks by *G. sepiarium* and *P. gigantea* were in the green *Pinus radiata* and the soil treatments. For *G. sepiarium*, the mean weight losses were not significantly different between the two treatments and were comparable to those measured under similar assay conditions by Eslyn (1986) and Kreber and van der Waals (1999). The weight losses of dried *P. radiata* sapwood by *G. sepiarium* (9.5%) were lower than those observed in Chapter 2 (21%). Both were low decay hazard assays, but the method of fungal inoculation was different and the conditions in the current assay had resulted in very low wood moisture contents which may have restricted decay. The moisture contents of the test and inoculum blocks had shown a decrease with time, which was attributed to the new assay jars which were not sufficiently airtight for the assay period. The higher moisture content in wood blocks degraded by *G. sepiarium* may be due to water produced during fungal metabolism, and also the fact that brown rot fungi are generally more

effective water conductors than white rot fungi (De Groot 1975; Highley and Scheffer 1970).

For *P. gigantea*, the mean weight loss of green *Pinus radiata* on vermiculite was significantly higher than of dried *P. radiata* on soil. Colonisation and decay by *P. gigantea* may be favored more by high initial wood moisture content and unspirated pits than the presence of soil as a nutrient and water source. When green sapwood is dried, bordered pits aspirate and this closure is usually irreversible which results in lower permeability that can restrict rewetting and fungal colonisation (Phillips 1933). Significant microscopic damage in wood cell wall layers can be induced by the drying process (Kifetew et al. 1998). The use of green wood has been observed not to increase decay by brown rot or white rot fungi (Hall and Leben 1985; Highley 1978; Schmidt and Liese 1980). *Phlebiopsis gigantea* produced significant decay of *Pinus radiata* in the soil treatment although the weight losses were lower than for *G. sepiarium* but were comparable to those measured under similar assay conditions by Butcher (1967).

The highest weight losses by *G. sepiarium* and *P. gigantea* were in the *Pinus radiata* inoculum blocks, particularly in the soil treatment. The relatively high weight losses of inoculum blocks may be related to the preinoculation time of 28 d and the higher wood moisture content but also to the use of autoclaving for sterilisation rather than gamma irradiation and closer contact with the base substrate. The use of vermiculite or soil as assay base substrates with widely different water-holding-capacities, resulted in wood blocks of the same equilibrium moisture content if the substrates were of the same moisture content (Carey and Grant 1975).

Despite the higher moisture content in green blocks, *S. commune* produced no significant weight losses and was not influenced by wood moisture content and soil nutrients as was *G. sepiarium* or *P. gigantea*. The lack of wood decay ability by *S. commune* is supported by reports in the literature (Butcher 1967; Kreber and van der Waals 1999).

Brown rot fungi have been reported to produce significant decay in both hardwoods and softwoods (Esllyn and Highley 1976; Peterson and Cowling 1964). White rot fungi have been reported as being ineffective in decaying conifer wood (Peterson and Cowling 1973; Scheffer and Cowling 1966) and have a greater capacity to degrade hardwoods than softwoods (Esllyn and Highley 1976). There was no significant decay of the exotic

hardwood *E. regnans* by either basidiomycete fungus. This observation was supported by other reports in the literature (de Lima et al. 1997). The decay resistance of *Eucalyptus* species has been attributed to a deficiency of essential growth factors (Esllyn and Highley 1976) or the presence in sapwood of inhibitory compounds (Hillis and Yazaki 1973). However, there have been reports of *Eucalyptus* weight losses of up to 9% by *G. sepiarium* (Highley 1978). *Gloeophyllum sepiarium* and *Phlebiopsis gigantea* caused less than 2% weight loss of the exotic softwood *Pseudotsuga menziesii*. This was supported by Esllyn (1986) who measured 0 to 2% weight loss by *P. gigantea*, but relatively high weight losses of *Pseudotsuga menziesii* by *G. sepiarium* have been reported e.g. 9 to 36% (Esllyn 1986), 23% (Esllyn and Highley 1976) and 57% (Highley 1978). It is not known whether the fact that the basidiomycete fungi were isolated from *Pinus radiata* was related to the inability to cause weight losses of *E. regnans* or *Pseudotsuga menziesii*.

Further assays with the basidiomycete fungi on agar, liquid medium and wood, were carried out using unbuffered medium, in the light, at 25°C. The measurement of pH changes during fungal growth in agar, liquid medium, or on wood could be a useful indicator of wood decay potential but this would require further investigation. The wood decay assay showed that *G. sepiarium*, *P. gigantea* and *S. commune* were capable of causing weight losses of *Pinus radiata* that were comparable to those reported in the literature but they were unable to cause significant weight losses in *E. regnans* or *Pseudotsuga menziesii*.

Chapter 4 **ANTAGONISM OF *TRICHODERMA* CULTURES AGAINST BASIDIOMYCETE FUNGI**

4.1 *INTRODUCTION*

The studies of basidiomycete fungi in Chapters 2 and 3 formed the basis for an investigation into the potential for biological control or biological protection against these fungi. Within the scope of this thesis, it was decided to focus on *Trichoderma* fungi which have been shown to exhibit antagonism against basidiomycete fungi but systems have not yet been developed that give reliable control in a field situation. The objective of the remaining research was to develop a greater understanding of the biological control potential of the *Trichoderma*-basidiomycete fungi system.

The objective of Chapter 4 was to collect a range of New Zealand *Trichoderma* species and to screen them for antagonism against *Gloeophyllum sepiarium*. This fungus was selected on the basis that brown rot fungi had resulted in the highest wood weight losses in previous decay assays, and that *Trichoderma* fungi have been reported to be more effective antagonists against brown rot fungi. The two white rot fungi, *Phlebiopsis gigantea* and *Schizophyllum commune*, were included at a later stage in the research. Antagonism was screened for in dual culture *Trichoderma*-*G. sepiarium* assays on agar and in a *Pinus radiata* wood-agar assay. *Trichoderma* cultures which showed antagonism against *G. sepiarium* in one or more of these assays were then assessed for the ability to grow on agar and on *P. radiata*.

4.2 *MATERIALS AND METHODS*

4.2.1 *Collection of Trichoderma cultures*

New Zealand *Trichoderma* species were collected from the following sources:

- i) Soil samples from Tokoroa, at the CHH Kinleith Mill and log yard, and areas in the surrounding *P. radiata* forest, including a skid site and young and mature plantings.

- ii) Soil samples from Rotorua, at the CHH Kaingaroa and View Road Mills, Fletcher Challenge Forests Waipa Mill, Mamuku Forest, scenic reserves, Forest Research tree reserve and Redwood State Forest park.
- iii) Soil samples from a HortResearch (Hamilton) trial site and bush reserve.
- iv) Contaminated growth medium of the edible fungi *Lentinus edodes* (Berk.) Singer (Shiitake mushroom) and *Pleurotus pulmonarius* (Fr.) Quel. (Phoenix mushroom) from Landcare Research (Auckland).
- v) A HortResearch (Hamilton) culture collection of fungi with general disease suppression properties.

Wood blocks of *Pinus radiata* were used to selectively bait for *Trichoderma* fungi that were capable of colonisation and growth. The blocks (20 x 20 x 20 mm) were produced from freshly harvested *P. radiata* sapwood obtained from CHH Timber Putaruru Mill and sterilised by gamma irradiation (53.1 kGy; Schering Plough Animal Health Ltd., Upper Hutt). Four blocks were placed in each soil sample (3 litres) and then the bags were sealed and incubated at room temperature. Over the following 14 d, single blocks from each bag were removed, flame-sterilised, split in half and internal fragments placed onto *Trichoderma*-selective agar (TSA; Appendix 1.9). The Petri dishes were incubated at 25°C. Emerging *Trichoderma* colonies were transferred onto MA and stored at 4°C. The *Trichoderma* cultures were recognised by macroscopic characteristics that included rapid growth on agar, sparse aerial mycelium and the production of distinctive white or green conidiogenous pustules. Identification of the *Trichoderma* species was attempted but the process is recognised as being difficult and time-consuming even with the detailed taxonomic schemes available (Bisset 1991a). It was decided to number the *Trichoderma* cultures, carry out the biological control screening assays and then send the most promising cultures to Gary Samuels (USDA-ARS, U.S.A.) for expert identification (see Chapter 5).

4.2.2 Dual culture *Trichoderma-Gloeophyllum sepiarium* assays

There were 136 *Trichoderma* cultures collected for screening in dual culture agar and wood-agar assays against *G. sepiarium*. The cultures identified as potential antagonists were freeze-dried in skim milk (Appendix 2.3) and stored at room temperature.

4.2.2.1 Agar assays

The agar dual culture assays were carried out on MA and LNA with triplicate Petri dishes of each medium for each *Trichoderma-G. sepiarium* treatment and the single and dual-inoculated *G. sepiarium* control treatments. The Petri dishes were inoculated on one side with a 6 mm agar core of *G. sepiarium* (7 d old culture) and incubated for 4 d (MA) or 6 d (LNA) at 25°C. The other side of the Petri dish was then inoculated with a 6 mm agar core of the test *Trichoderma* (7 d old cultures). The Petri dishes were incubated at 25°C. Observations of pigment production, *Trichoderma* sporulation and the interaction between the two fungi were carried out at 7, 14 and 21 d after inoculation of *G. sepiarium*. The challenge period of up to 17 d (MA) and 15 d (LNA) was sufficient to observe both the interaction between each *Trichoderma* culture and *G. sepiarium*, and the growth of *G. sepiarium* in comparison to the control treatments which had grown to fill the Petri dishes within this period.

There were four categories of interaction.

A: the *Trichoderma* colony completely overgrew the *G. sepiarium* colony.

B: the *Trichoderma* colony partially overgrew the *G. sepiarium* colony.

C: the two colonies met with no overgrowth or inhibition zone.

D: there was an inhibition zone and the two colonies did not meet.

At 21 d, the viability of *G. sepiarium* was assessed by reisolation onto BA. A 6 mm agar core was taken from an area on the *G. sepiarium* colony distal to the *Trichoderma* colony. The Petri dishes were incubated at 25°C. Growth of *G. sepiarium* was assessed at 7, 14 and 21 d and *Trichoderma* cultures were identified as potential antagonists if there was no *G. sepiarium* growth after 21 d. Loss of *G. sepiarium* viability was the only criterion used for selection of antagonistic *Trichoderma* cultures, and observations of the interactions between the fungi were used in a supportive role e.g. to indicate the mode of antagonism.

4.2.2.2 *Pinus radiata*-agar assay

The wood-agar dual culture assay (Etheridge and Craig, 1973; Schoeman, 1994) screened the *Trichoderma* cultures for the ability to produce soluble and diffusible inhibitory compounds during growth in *P. radiata*, by assessing the subsequent growth response of *G. sepiarium* on an agar overlay of the wood blocks.

Wood blocks (50 x 50 mm cross-cut x 5 mm thick) were produced from freshly harvested *P. radiata* sapwood obtained from CHH Timber Putaruru Mill, sterilised by gamma irradiation (56.4 kGy) and then placed in independent sterile Petri dishes (90 mm diameter x 20 mm high). There were triplicate Petri dishes for each *Trichoderma* culture and nine replicates for the uninoculated control treatment. The blocks were inoculated with four 6 mm agar cores of the test *Trichoderma* (10 d old cultures) and incubated at 25°C. After 21 d, an overlay of molten BA (27 mL) was poured into each Petri dish to cover the block and inhibit further *Trichoderma* growth (Davet et al., 1981). After the agar had set, the Petri dishes were reincubated at 25°C to allow soluble and diffusible inhibitory compounds produced by the *Trichoderma* fungi during growth in the wood block to be released into the agar. After 5 d, each Petri dish was inoculated with two 6 mm agar cores of *G. sepiarium* (7 d old culture) and incubated at 25°C. One core was placed on the agar in a central position above the block and the other was placed on the agar to one side of the block. Observations of pigment production and growth of *G. sepiarium* was assessed at 7, 14 and 21 d after inoculation and *Trichoderma* cultures were identified as potential antagonists if there was no *G. sepiarium* growth after 21 d.

4.2.3 Growth of *Trichoderma* cultures on agar and on *Pinus radiata*

The dual culture assays identified 95 *Trichoderma* cultures as potential antagonists of *G. sepiarium* and these were assessed for the ability to grow on MA, LNA and *P. radiata*.

There were triplicate Petri dishes of MA and LNA for each *Trichoderma* culture. A 6 mm agar core of the *Trichoderma* (7 d old cultures) was inoculated in a central position on each Petri dish and incubated at 25°C. Colony diameters were measured in two predetermined directions on a daily basis up to 8 d after inoculation, when colony morphology was noted.

Due to the time-consuming measurement of growth on wood blocks, two consecutive assays were carried out with 40 *Trichoderma* cultures in one assay and 55 cultures in another. Wood blocks (50 x 50 mm cross-cut x 5 mm thick) were produced from freshly harvested *P. radiata* sapwood obtained from CHH Timber Putaruru Mill, sterilised by gamma irradiation (56.4 kGy) and then placed in independent sterile Petri dishes. There were five Petri dish replicates for each *Trichoderma* culture. A 6 mm agar core of the test *Trichoderma* (7 d old cultures) was inoculated onto the edge of each block so that

growth would occur from outer to inner growth rings (as might occur in the natural colonisation of logs). The Petri dishes were incubated at 25°C. External hyphal extension growth across the wood block was observed under a stereo-microscope (Zeiss) and the leading edge of the colony was measured on a daily basis up to 8 d after inoculation, when colony morphology was noted.

For each measurement, the growth rate was expressed as mm d^{-1} and averaged over the replicates and the assay period. The results for each medium were analysed statistically using analysis-of-variance. Comparisons between individual means for each medium were carried out using Tukey's least-significance-difference at the 5% significance level. The data for the two wood assays were analysed separately, and the 40 culture assay required square-root transformation because of the heterogeneity of variation. Standard regression analyses was carried out to determine if there were any significant correlations between growth rates on the agar and wood media.

4.3 RESULTS

4.3.1 Collection of *Trichoderma* cultures

There were 136 *Trichoderma* cultures collected for screening, and these were sourced from Tokoroa (51), Rotorua (36), Hamilton (15), *Lentinus edodes* medium (19), *Pleurotus pulmonarius* medium (8) and the HortResearch culture collection (7). There was a wide range of colony and spore morphology observed amongst the *Trichoderma* cultures on MA.

4.3.2 Dual culture *Trichoderma-Gloeophyllum sepiarium* assays

From the dual culture agar and wood-agar assays, a total of 95 *Trichoderma* cultures were identified as potential antagonists of *G. sepiarium* and selected for further study (Table 4.1). The origins of the remaining 41 *Trichoderma* cultures is given in Appendix 11.

Table 4.1 Origin and growth rates of 95 *Trichoderma* cultures identified as potential antagonists of *Gloeophyllum sepiarium* in agar and *Pinus radiata*-agar assays.

T sp.	Origin	Growth rates (mm d ⁻¹)			Antag. of <i>G. sepiarium</i> ¹		
		MA	LNA	<i>P. radiata</i>	MA	LNA	<i>P. radiata</i> -agar
1	CHH K Mill	16.1	15.3	5.3^{A1}	*	*	
2	CHH K Mill	25.3	17.7	4.5 ^{A2}	*		
3	CHH K Mill	24.8	18.5	4.5 ^{A2}	*		
4	CHH K Mill	25.6	25.6	2.9 ^{A1}	*	*	
5	CHH K Mill	16.4	8.5	8.3 ^{A2}	*		
6	CHH K Mill	15.9	12.3	7.2 ^{A2}	*		
7	CHH K Logyard	15.9	11.6	9.4 ^{A2}	*		
8	CHH K Logyard	18.7	13.3	9.2 ^{A2}	*		
9	CHH K Logyard	19.3	10.0	9.2 ^{A2}	*		
10	CHH K Logyard	19.8	11.4	7.4 ^{A2}	*		
11	CHH K Logyard	13.2	10.5	8.4 ^{A2}	*		
12	CHH K Logyard	14.2	12.4	9.3 ^{A2}	*		
13	CHH K Logyard	18.3	13.6	7.2 ^{A2}	*		
14	CHH K Logyard	16.8	15.2	7.7 ^{A2}	*		
15	CHH K F-Seagull Rd.	24.6	22.9	4.0^{A1}	*	*	
16	CHH K F-Seagull Rd.	25.1	19.5	3.3 ^{A1}	*	*	
17	CHH K F-Seagull Rd.	20.4	14.5	7.5 ^{A2}	*		
18	CHH K F-Seagull Rd.	18.0	14.2	9.3 ^{A2}	*		
19	CHH K F-Seagull Rd.	28.0	26.0	3.4 ^{A1}	*	*	
20	CHH K F-Seagull Rd.	26.0	23.6	4.0 ^{A1}	*	*	
21	CHH K F-Dave Rd. skid site	26.2	23.8	3.0 ^{A1}	*	*	
22	CHH K F-Dave Rd. skid site	27.1	26.2	3.2 ^{A1}	*	*	
23	CHH K F-Loop Rd. young <i>P. radiata</i>	19.8	18.4	4.0^{A1}	*	*	
24	CHH K F-Loop Rd. mature <i>P. radiata</i>	25.2	19.4	3.1^{A1}	*	*	
25	CHH K F-Maiden/Tram Rd. graveyard	25.2	22.2	5.1 ^{A2}	*		
26	CHH K F-Maiden/Tram Rd. graveyard	26.8	27.3	3.1^{A1}	*	*	
29	Hamilton-HR trial site	13.9	12.7	7.6 ^{A2}	*		
30	Hamilton-HR trial site	18.0	14.0	5.5 ^{A2}	*		
31	Hamilton-HR trial site	21.7	13.4	7.5 ^{A2}	*		
33	Hamilton-HR trial site	24.3	21.5	3.5^{A1}	*	*	
34	Hamilton-HR trial site	15.7	11.4	8.1 ^{A2}	*		
35	Hamilton-HR trial site	16.8	11.6	9.5 ^{A2}	*		
36	Hamilton-HR trial site	20.5	11.0	9.3 ^{A2}	*		
37	CHH K Mill	16.0	9.9	8.6 ^{A2}	*		
38	CHH K Mill	19.1	15.6	5.0^{A1}		*	
39	CHH K Mill	16.8	11.4	6.3 ^{A2}	*		
41	CHH K logyard	13.1	12.1	9.1 ^{A2}	*		
42	CHH K F-Osprey Rd. young <i>P. radiata</i>	27.5	28.5	3.6 ^{A1}	*	*	
45	CHH K F-Seagull Rd.	24.4	21.6	3.1 ^{A1}	*	*	
46	CHH K F-Seagull Rd.	24.8	23.0	3.9^{A1}		*	
47	CHH K F-Dave Rd. skid site	25.2	22.4	3.8 ^{A1}	*	*	
48	CHH K F-Loop Rd. young <i>P. radiata</i>	15.0	13.4	3.3 ^{A1}	*	*	
49	FCF Waipa Mill-Top Hill	17.0	9.3	8.8 ^{A2}	*		
50	CHH Kaingaroa Mill	17.8	12.7	5.8 ^{A2}	*		
52	Hamilton-HR trial site	18.8	12.5	4.3^{A1}	*		*
55	CHH K F-Maiden/Tram Rd. graveyard	20.3	11.6	4.8 ^{A2}	*		
58	Hamilton-HR trial site	19.1	13.8	5.0 ^{A2}	*		
59	Hamilton-HR trial site	16.8	10.4	4.8 ^{A2}	*		
61	Rotorua-Mamuku cutover	18.3	9.4	6.8 ^{A2}	*		
63	Rotorua-Mamuku-mature <i>P. radiata</i>	26.0	23.8	3.5 ^{A1}	*	*	
64	CHH Mill-View Rd.	17.8	15.9	2.5 ^{A2}	*		
65	CHH Mill-View Rd.	16.3	12.8	3.3 ^{A2}	*		
66	CHH Mill-View Rd.	16.0	14.2	9.0 ^{A2}	*		

67	CHH Mill-View Rd.	20.7	10.1	5.1 ^{A2}	*		
68	CHH Mill-View Rd.	15.7	10.7	8.7 ^{A2}	*		
69	Rotorua scenic reserve	20.6	12.0	5.1 ^{A2}	*		
70	Rotorua scenic reserve	21.4	12.4	5.0 ^{A2}	*		
72	Rotorua scenic reserve-<i>Quercus</i> sp.	24.7	22.2	3.7^{A1}	*	*	*
75	Rotorua Redwood Grove-SFP	26.2	23.9	3.5 ^{A1}		*	
78	FR- <i>Eucalyptus</i> sp.	20.9	15.4	3.8 ^{A1}	*	*	
79	FR- <i>Dacrydium</i> sp.	28.6	25.7	3.1 ^{A1}	*	*	
80	FR-<i>Dacrydium</i> sp.	24.4	19.2	3.8^{A1}	*	*	
82	FR- <i>Pseudotsuga</i> sp.	27.0	25.3	3.1 ^{A1}	*	*	
83	FR- <i>Pseudotsuga</i> sp.	24.1	23.6	3.5 ^{A1}	*	*	
88	FR-mature <i>P. radiata</i>	27.1	23.7	3.6 ^{A1}	*	*	
89	Hamilton bush reserve	16.6	10.5	4.8 ^{A2}	*		
90	Hamilton bush reserve	16.1	11.1	6.4 ^{A2}	*		
91	CHH K Logyard	17.5	12.4	7.1 ^{A2}	*		
92	CHH K Logyard	22.2	10.8	5.8 ^{A2}	*		
94	CHH K Logyard	13.7	8.8	5.9 ^{A2}	*		
95	CHH K Logyard	16.5	10.3	4.9 ^{A2}	*		
96	CHH K Logyard	16.5	10.3	7.5 ^{A2}	*		
98	CHH K Mill	17.7	12.4	5.7 ^{A2}	*		
99	CHH K Mill	16.0	12.0	4.9^{A1}	*		*
101	CHH Mill-View Rd.	19.4	16.4	4.2^{A1}	*	*	
102	Rotorua-Redwood Grove-SFP	20.1	21.8	5.0 ^{A2}	*		
111	LR- <i>Lentinus edodes</i> medium	18.5	15.4	0.7 ^{A2}	*		
113	LR- <i>L. edodes</i> medium	17.8	11.7	0.0 ^{A2}	*		
115	LR-<i>L. edodes</i> medium	21.0	18.7	4.3^{A1}	*	*	
116	LR-<i>L. edodes</i> medium	24.2	16.9	4.4^{A1}			*
117	LR-<i>L. edodes</i> medium	21.4	14.7	3.5^{A1}			*
118	LR-<i>L. edodes</i> medium	11.0	9.8	2.1^{A1}			*
119	LR-<i>L. edodes</i> medium	8.5	8.5	1.8^{A1}			*
120	LR-<i>L. edodes</i> medium	14.5	12.3	1.9^{A1}			*
121	LR-<i>L. edodes</i> medium	9.1	8.0	4.6^{A1}	*		*
122	LR-<i>Pleurotus pulmonarius</i> medium	12.6	13.1	3.7^{A1}			*
123	LR- <i>P. pulmonarius</i> medium	18.7	14.3	0.0 ^{A2}	*		
124	LR- <i>P. pulmonarius</i> medium	19.3	16.5	2.7 ^{A2}	*		
126	LR-<i>P. pulmonarius</i> medium	16.5	15.4	3.8^{A1}	*		*
127	LR-<i>P. pulmonarius</i> medium	14.6	14.4	4.3^{A1}	*		*
128	LR- <i>P. pulmonarius</i> medium	15.3	11.9	1.1 ^{A2}	*		
129	LR- <i>P. pulmonarius</i> medium	16.5	14.2	3.6 ^{A2}	*		
133	HR culture collection TBHPP7	16.4	13.2	5.5 ^{A2}	*		
134	HR culture collection THF2/3	19.2	12.2	4.4 ^{A2}	*		
135	HR culture collection HPP1	17.0	11.2	2.1 ^{A2}	*		
sed (P<0.05)		0.66	0.59	^{A1} 0.39			
				^{A2} 0.73			

¹Defined as no *G. sepiarium* growth 21 d after reisolation from the dual culture assays.

^{A1}= 40 culture assay, ^{A2}= 55 culture assay.

MA= Malt extract agar, LNA= Low nutrient agar, CHH = Carter Holt Harvey, FCF= Fletcher Challenge Forests, K= Kinleith, F= Forest, SFP= State Forest Park.

FR= Forest Research, LR= Landcare Research, HR= HortResearch.

Highlighted in bold are the 23 *Trichoderma* cultures selected for further study.

4.3.2.1 Agar assays

The MA assay had 105 *Trichoderma-G. sepiarium* treatments where *G. sepiarium* failed to grow by 7 d after reisolation. This decreased to 86 treatments by 14 and 21 d after reisolation. These 86 *Trichoderma* cultures were selected for further study (Table 4.1). Sources of the cultures were Tokoroa (41), Rotorua (22), Hamilton (10), *L. edodes* medium (4), *P. pulmonarius* medium (6) and HortResearch culture collection (3). The highest number were from Kinleith log yard (14) and Kinleith Mill (10), followed by Kinleith Forest-Seagull Road (7), CHH View Road (6) and Forest Research (6).

The LNA assay had 52 *Trichoderma-G. sepiarium* treatments where *G. sepiarium* failed to grow by 7 d after reisolation. This decreased to 36 treatments by 14 d and 29 treatments by 21 d after reisolation. Of these 29 *Trichoderma* cultures selected for further study (Table 4.1), three were exclusive to the LNA assay and 26 were in common with the MA assay. Sources of the cultures were Tokoroa (17), Rotorua (10), Hamilton (1) and *L. edodes* medium (1) and the highest number were from Kinleith Forest-Seagull Road (6) and Forest Research (6), followed by Kinleith Mill (3) and Kinleith Forest-Dave Road skid site (3).

The categories of interaction observed at 7, 14 and 21 d after *G. sepiarium* inoculation on MA and LNA are shown in Table 4.2. The interactions were consistent across replicate Petri dishes. In some treatments, the interaction changed from a B, C or D to an A over the assay period. After 21 d, the numbers in each category were similar for MA and LNA and they represented the same *Trichoderma* cultures. The majority of the cultures completely overgrew the *G. sepiarium* colony (interaction A) (Photo 4.1 a). Only one or two cultures resulted in partial merging of the fungal colonies (interaction B). Five cultures resulted in no overgrowth or inhibition zones (interaction C). The remaining five or six cultures gave small zones of inhibition (interaction D) (Photo 4.1 b).

On MA, *Trichoderma* sp. 115, 116, 117, 118, 119 and 120 resulted in an interaction D which suggested that these cultures produced a compound(s) inhibitory to *G. sepiarium*. On LNA, the same *Trichoderma* cultures were represented except for *Trichoderma* sp. 115 which changed to an interaction B between 14 and 21 d. However, only *Trichoderma* sp. 115 resulted in the loss of *G. sepiarium* viability on MA and LNA.

Table 4.2 Categories of interaction for 136 dual culture *Trichoderma-Gloeophyllum sepiarium* treatments, with time after inoculation, on malt extract and low nutrient agars.

Days after inoculation of <i>G. sepiarium</i>	Categories of interaction ¹							
	Malt extract agar				Low nutrient agar			
	A	B	C	D	A	B	C	D
7	106	23	6	1	115	10	11	0
14	121	1	5	9	117	5	8	6
21	124	1	5	6	124	2	5	5

¹ A: the *Trichoderma* colony completely overgrew the *G. sepiarium* colony.

B: the *Trichoderma* colony partially overgrew the *G. sepiarium* colony.

C: the two colonies met with no overgrowth or inhibition zone.

D: there was an inhibition zone and the two colonies did not meet.

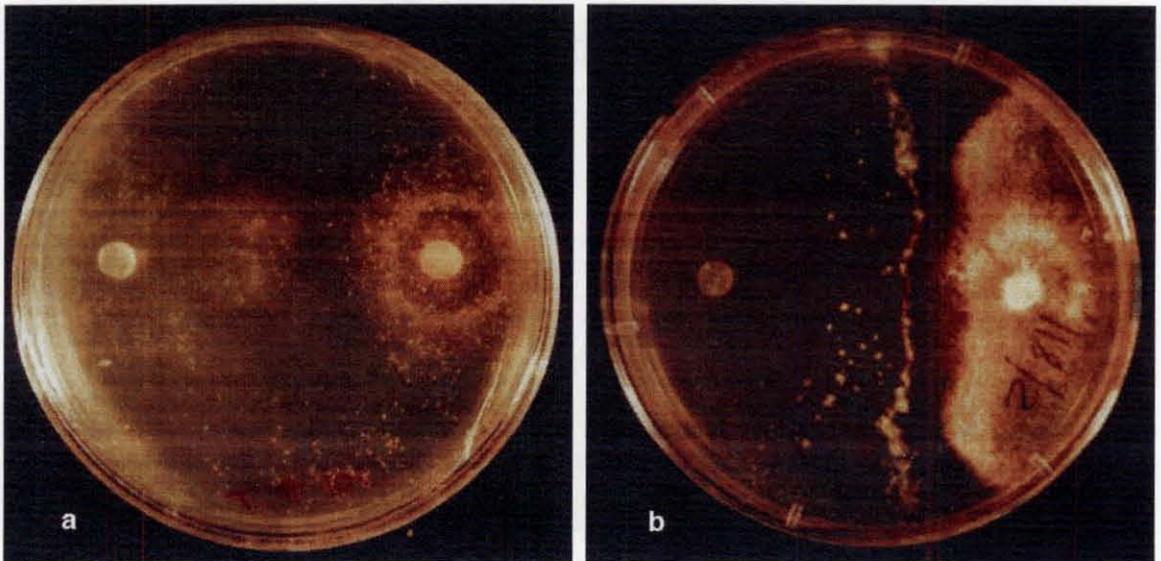


Photo 4.1 Dual culture Petri dishes of a test *Trichoderma* (left core) and *Gloeophyllum sepiarium* (right core), showing (a) interaction A with the *Trichoderma* overgrowing *G. sepiarium* and (b) interaction D with a zone of inhibition between the two colonies.

Some of the treatments resulted in pigmentation of the agar, which was noted but not characterised. In most cases, the pigmentation was red-brown (Photo 4.2) but there was also a yellow pigmentation observed. The presence of pigmentation was not restricted to the treatments where antagonism was observed. The *Trichoderma* cultures produced good sporulation which was heavier sporulation on MA in comparison with LNA.



Photo 4.2 Dual culture Petri dish of a test *Trichoderma* (left core) and *Gloeophyllum sepiarium* (right core) with the release of a red-brown pigment by *G. sepiarium*.

4.3.2.2 *Pinus radiata*-agar assay

The results are based on observations of *G. sepiarium* growth from inoculum cores that were placed on the agar to one side of the wood blocks (Photo 4.3). There were problems with the BA overlay where many of the blocks had floated in the molten agar. This allowed the *Trichoderma* fungi to grow in areas of the block that were exposed and, in some cases, the cores of *G. sepiarium* that were placed on the agar above the blocks had been contaminated by the *Trichoderma* culture. The pigmentation of the agar in these treatments was noted but not characterised, and ranged from colourless, yellow, yellow-green, yellow-brown, brown to red-brown. The presence of pigmentation was again not restricted to the treatments where antagonism was observed.

There were 55 *Trichoderma*-*G. sepiarium* treatments where *G. sepiarium* failed to grow by 7 d after inoculation. This decreased to 12 treatments by 14 and 21 d after inoculation. Of these 12 *Trichoderma* cultures selected for further study (Table 4.1), *Trichoderma* sp. 116, 117, 118, 119, 120 and 122 were exclusive to the *P. radiata*-agar assay. The other six cultures were also represented in the MA dual culture assay but only *Trichoderma* sp. 72 was also represented in the LNA assay. Their sources were Tokoroa (1), Rotorua (1), Hamilton (1), *L. edodes* medium (6) and *Pleurotus pulmonarius* medium (3).

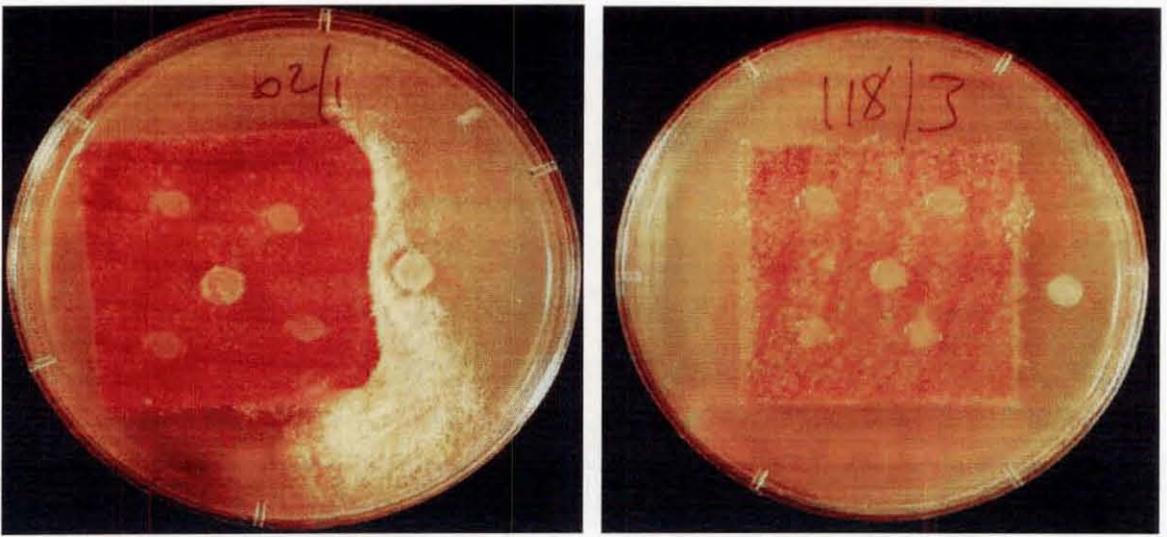


Photo 4.3 Inoculum cores of *Gloeophyllum sepiarium* to one side of wood blocks that were preinoculated with a test *Trichoderma* for 21 d, showing (left) growth of *G. sepiarium* and (right) inhibition of growth.

4.3.3 Growth of *Trichoderma* cultures on agar and on *Pinus radiata*

There were significant differences between the 95 *Trichoderma* cultures in growth rates on MA (8.5 to 28.6 mm d⁻¹), LNA (8.0 to 28.5 mm d⁻¹) and on *P. radiata* (0 to 9.5 mm d⁻¹) (P<0.001) (Table 4.1). The two wood assays gave different seed values but across the assays, there were 23 cultures that had the highest growth rates of 4.3 to 9.5 mm d⁻¹. *Trichoderma* sp. 64, 111, 113, 123, 128 and 135 had no significant growth on the blocks over the assay period. Regression analyses showed a positive correlation between growth rates on the two agar media and there was a greater correlation between growth on wood and LNA (P<0.001) than wood and MA (P<0.01).

The morphology of each *Trichoderma* culture was similar on agar and on *P. radiata*, and consisted of fluffy white mycelium and a range of white, olive, grey-green and dark green sporulation. The reverse of Petri dishes was colourless or yellow in colour.

4.4 DISCUSSION

The *Trichoderma* cultures (87) collected from the Tokoroa and Rotorua mill and forestry areas are only a representation of the range of species present in the soils. The occurrence of individual *Trichoderma* species is influenced by soil conditions and competition with other soil microorganisms, and the ability to compete can be species-dependent (Wardle et al. 1993). There is a seasonality to the occurrence of *Trichoderma* species in forest soils (Widden and Arbitol 1980) and *T. viride* has been reported to be sensitive to high pH soils (Fritze and Baath 1993). Although the scope of the sampling was restricted by time and resources, a range of sites had been covered and *Trichoderma* cultures were successfully isolated. The 49 *Trichoderma* cultures collected from Hamilton, edible fungi media and the HortResearch culture collection widened the source of cultures. The variation in colony morphology on agar and wood indicated that there were a range of species.*

The dual culture agar assays showed evidence of antibiosis occurring in some treatments with the production of a red-brown or yellow pigment and/or small inhibition zones. Visual observations noted that the *Gloeophyllum sepiarium* colony did not grow as extensively as in the control treatments. The red-brown pigment may be stress-related metabolites produced and released into the agar by *G. sepiarium* as a breakdown product from damaged cells, possibly a response to localised antibiotic production by the *Trichoderma* fungi. Bruce et al. (1984) also observed the release of a red pigment in dual cultures of *Lentinus lepideus* and *Trichoderma* fungi, and microscopic studies showed that total disruption and cell wall lysis of *L. lepideus* mycelium had occurred. Hermosa et al. (2000) observed the production of a yellow pigment by *Trichoderma* species in dual culture assays against wood decay fungi. Score and Palfreyman (1994) found yellow and brown pigmentation in *Serpula hexagonoides* in the presence of *Trichoderma* species, which may indicate laccase and peroxidase activity by *S. hexagonoides* to produce melanin for the protection of hyphae.

Inhibition zones were produced in the dual cultures of *Trichoderma* sp. 115, 116, 117, 118, 119 and 120 which had all been isolated from *Lentinus edodes* medium. Although only *Trichoderma* sp. 115 resulted in loss of *G. sepiarium* viability, the other five cultures showed similar antagonism in the wood-agar assay where *Trichoderma* sp. 115 did not. Of the 12 *Trichoderma* antagonists identified in the wood-agar assay, six were isolated

from *L. edodes* medium and three from *Pleurotus pulmonarius* medium. The identification of these cultures from edible fungi medium is unknown but results indicated that they were capable of growing on wood and producing compounds that were inhibitory to *G. sepiarium*. *Trichoderma harzianum* is the most commonly reported fungal contaminant of commercial mushrooms (Chen et al. 1999; Komatsu 1976) and the biotypes of *T. harzianum* were originally based on pathogenicity to mushrooms (Seaby 1987).

In the treatments with no change in pigmentation or no inhibition zones, there was a competitive growth interaction where *G. sepiarium* was overgrown by the test *Trichoderma*. The *Trichoderma* identified as potential antagonists had fungicidal activity against *G. sepiarium*, where no viability was observed 21 d after reisolation from dual culture Petri dishes. Verrall (1966) observed no antagonism between *T. viride* cultures and *G. sepiarium* on MA, with either compatible growth or overgrowth of the *Trichoderma* by *G. sepiarium*, but Highley (1988) found some *Trichoderma* species overgrew a range of brown and white rot fungi on MA and, in many cases, had fungicidal activity.

Schoeman et al. (1994) showed that a better indication of the field performance of *Trichoderma* species was obtained when they were grown on wood than on MA and that there was no correlation between the performance of *Trichoderma* species on the two media. Differences in inhibition of *G. sepiarium* by the *Trichoderma* cultures in the agar and *Pinus radiata*-agar dual culture assays may be due to different metabolites being induced by the wood components or different concentrations of the same metabolites (Doi and Mori 1994). Srinivasan et al. (1992) found that fungicidal effects were more prevalent on MA, particularly against brown rot fungi, but inhibition of the white rot fungi was greater on a minimal medium. The *Trichoderma* cultures which showed inhibition on a minimal medium such as LNA may be more likely to operate on wood. Tucker and Bruce (1995) carried out BCA screening on MA, a minimal medium, and a minimal medium with the major soluble amino acids (phenylalanine, glutamine and arginine) present in Scots pine sapwood. They found that interactions between the *Trichoderma* and wood decay fungi were influenced by the media and proposed that this may be partly due to changes in the modes of antagonism, as suggested previously by Srinivasan et al. (1992). The LNA assay identified fewer potentially antagonistic *Trichoderma* cultures than the MA assay. On LNA, the *Trichoderma* fungi may have been less competitive in terms of growth or have produced fewer inhibitory compounds. The fewer identified antagonists in the

P. radiata-agar assay may be a reflection of low growth rates of the *Trichoderma* on wood, or low production or diffusibility of inhibitory compounds.

An alternative to the *P. radiata*-agar assay was investigated by Dawson Andoh and Morrell (1992b) and Li and Morrell (1999) who used sodium acetate buffer, amended with Tween 80, to extract proteins and enzyme activity from wood blocks dual-inoculated with *Trichoderma* and decay fungi. Murmanis et al. (1988b) incorporated water extracts of dual-inoculated blocks, and also *Trichoderma* culture filtrates, into agar but growth inhibition of decay fungi was not shown although antagonism had been observed by SEM. Further studies could use wood extraction to study effects of the interaction between *G. sepiarium* and *Trichoderma* fungi on the physiological activities of *G. sepiarium*.

An attribute of a successful BCA is the ability to compete for available nutrients and thus colonise and grow faster than the basidiomycete fungus (Lundborg and Unestam 1980). There was a wide range in growth rates of the *Trichoderma* cultures on the two agar media. The range of growth rates were in agreement with Mukherjee and Raghu (1997) who found mean growth rates of 21 mm d⁻¹ at the optimal growth temperature of 30°C and 18 mm d⁻¹ at 25°C. As expected, growth rates were lower on LNA than MA but the differences were not always significant and growth on the two media were correlated.

The range in fungal growth rates on *P. radiata* was smaller and values were around 25 to 35% of those on agar. There was a stronger correlation between growth on wood and LNA, than between wood and MA. Six *Trichoderma* cultures did not show significant external fungal growth on wood over the 8 d assay period, despite being originally isolated from the interior of wood blocks. This may be due to differences in wood moisture content and to the greater surface area of the blocks used in the growth assay (50 x 50 x 5 mm) compared to the soil baiting blocks (20 x 20 x 20 mm). In a soil system, the *Trichoderma* cultures may preferentially colonise the interior of blocks. Future assays may be improved by the assessment of internal fungal growth in wood blocks using light microscope or SEM.

Schoeman et al. (1996) used a similar bilayer assay on agar and wood blocks to screen a number of potential BCA's obtained from freshly felled pine. They found the most antagonistic microorganisms were *T. viride* and *T. harzianum*. *Trichoderma* sp. 101 was identified in Chapter 5 as *T. viride*, and *T.* sp. 38 and 117 as *T.* sp. 'viride'. They had

growth rates on MA at 25°C of 19.1, 19.4 and 21.4 mm d⁻¹, respectively, which were lower than the 27 mm d⁻¹ observed for *T. viride* by Hulme and Shields (1970) at 27°C.

Schoeman and Dickinson (1993) used numerical scoring for a number of BCA screening assays and produced selection indices that indicated the biological control potential of each BCA based on inhibitory activity, spore germination, growth rate on agar and sporulation ability. This approach was not attempted as the computer programmes and expertise necessary to analyse large amounts of data were not available. Further information could have been gathered on the interactions between the *Trichoderma* and *G. sepiarium* fungi e.g. loss of vigour of *G. sepiarium* or sporulation parameters, but there were a large number of dual culture treatments and the results of the assays had given sufficient information on which to make a considered selection of *Trichoderma* cultures for further studies.

Due to limited resources, 23 *Trichoderma* cultures were selected from the 95 identified antagonists for further biological control studies. These cultures are shown highlighted in Table 4.1. The selection process was based on the ability of the *Trichoderma* to exhibit antagonism in the *P. radiata*-agar assay and high growth rates in the LNA assay. It was the intention that eventually all of the 95 identified antagonists would be submitted to the assays in Chapter 5 (although not necessarily within the time-frame of this thesis).

Chapter 5 BIOLOGICAL CONTROL POTENTIAL OF *TRICHODERMA* CULTURES AGAINST BASIDIOMYCETE FUNGI

5.1 INTRODUCTION

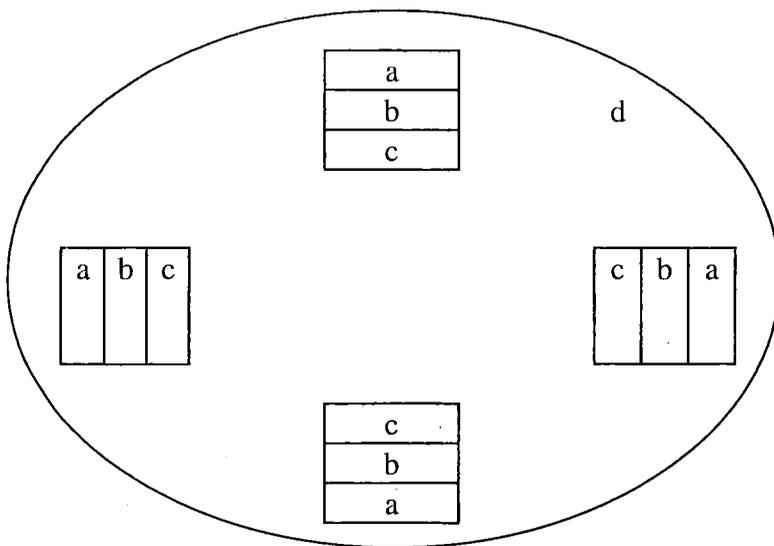
The objective of Chapter 5 was to determine the biological control potential of *Trichoderma* species against *Gloeophyllum sepiarium*, *Phlebiopsis gigantea* and *Schizophyllum commune* in *Pinus radiata* using wood assays that were developed to investigate three aspects of *Trichoderma* antagonism: biological control; bioprotection; and the degree of bioprotection conferred by *Trichoderma* to neighbouring sterile blocks.

The most promising *Trichoderma* cultures were sent for expert identification and their wood decay potential was assessed in enzyme assays and by the determination of wood weight loss. Three *Trichoderma* cultures were further investigated for biological control potential against *G. sepiarium* in field and controlled temperature room trials.

5.2 MATERIALS AND METHODS

5.2.1 Wood biological control assays

The wood biological control assays were based on a soil-wood block method by Freitag and Morrell (1990). There were three types of *P. radiata* test blocks: a 'basidiomycete precolonised block'; a 'sterile block'; and a '*Trichoderma* precolonised block'. The blocks were set up in stacks of the three types, in the order given, with four stacks per Petri dish (Fig. 5.1). The 'basidiomycete precolonised block' assessed the ability of the *Trichoderma* fungus to colonise the block, prevent further wood weight loss and result in loss of basidiomycete viability. The 'sterile block' assessed the ability of the *Trichoderma* fungus to colonise the block and prevent basidiomycete colonisation and decay. The '*Trichoderma* precolonised block' assessed the ability of the *Trichoderma* fungus to prevent basidiomycete colonisation and decay.



- a 'Basidiomycete precolonised block'
- b 'Sterile block'
- c '*Trichoderma* precolonised block'
- d Vermiculite

Fig. 5.1 Experimental Petri dish design of the wood biological control assay.

A series of four assays were carried out. The first assay screened 23 *Trichoderma* cultures (selected from Chapter 4) for biological control potential against *G. sepiarium* with a 21 d precolonisation time, *G. sepiarium* control and uninoculated control treatments, and storage of 'sterile blocks' at 4°C over the precolonisation period. The second assay screened seven selected *Trichoderma* cultures against *G. sepiarium* with a 28 d precolonisation time, control treatments for each fungus and an uninoculated control treatment, inoculation of 'sterile blocks' with sterile agar cores, and wood chemical analyses. The third assay screened three selected *Trichoderma* cultures against *G. sepiarium*, *P. gigantea* and *S. commune* with a 21 d precolonisation time, and control treatments for each fungus and an uninoculated control treatment. The fourth assay screened the three *Trichoderma* and three basidiomycete fungi for wood weight loss over the 21 and 28 d precolonisation times.

Wood blocks (15 x 15 mm cross-cut x 5 mm thick) were produced from freshly harvested *Pinus radiata* sapwood obtained from CHH Timber Putaruru Mill. To check for consistency of wood parameters, the fresh weight, dry weight and moisture content were determined for 50 untreated blocks from each assay. The assay was carried out in vermiculite with no nutrient supplementation and the wood blocks were sterilised by

gamma irradiation to more closely match the conditions that are likely to be encountered in a low decay hazard field situation than the ASTM assay (ASTM 1981).

Assay 1 Screen of *Trichoderma* sp. 1, 15, 23, 24, 26, 33, 38, 46, 52, 72, 80, 99, 101, 115, 116, 117, 118, 119, 120, 121, 122, 126 and 127 for biological control potential against *G. sepium*

Precolonisation Petri dishes were set up for the *Trichoderma* fungi and for *G. sepium*. The wood blocks were labelled and dried at 80°C to a constant weight prior to sterilisation by gamma irradiation (56.2 kGy; Schering Plough Animal Health Ltd., Upper Hutt). Polyethylene Petri dishes (90 mm diameter x 25 mm high) containing 50 mL vermiculite (fine grade, Nuplex Industries Ltd, Auckland) and 20 mL distilled water were autoclaved for 20 min at 121°C. After cooling, 12 blocks were placed on the vermiculite in each Petri dish and each block was inoculated with a 6 mm agar core of the test fungus (7 d old cultures) (Photo 5.1). The Petri dishes were incubated for 21 d at 25°C. The blocks for 'sterile blocks' were stored in sealed plastic bags at 4°C during this period. To check that the moisture content of the dried blocks was not a limiting factor for precolonisation, six additional Petri dishes were set up with 'sterile blocks' and incubated at 25°C. After 7 d, the moisture contents were determined by weighing and then drying blocks at 80°C to a constant weight.

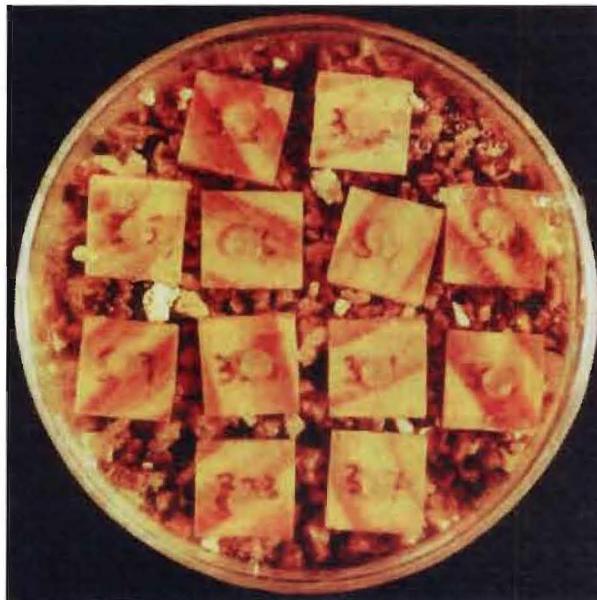


Photo 5.1 Fungal precolonisation of wood blocks for the biological control assays.

Assay Petri dishes were set up with vermiculite and water and autoclaved as described for the precolonisation Petri dishes. There were triplicate Petri dishes for each of the 23 *Trichoderma-G. sepiarium* treatments. The wood blocks were set up in stacks of the three block types. A stack consisted of a '*G. sepiarium* precolonised block', a 'sterile block' and a '*Trichoderma* precolonised block', in the order given. The control treatments each had six Petri dish replicates. The *G. sepiarium* control treatment replaced the '*Trichoderma* precolonised block' with a 'sterile block' and the uninoculated control treatment had all 'sterile blocks'. Due to the number of *Trichoderma* cultures being screened, there were no *Trichoderma* control treatments. Each stack of blocks was placed on its side into the vermiculite so that all three blocks were half immersed. There were four stacks placed equidistant around each Petri dish with the '*G. sepiarium* precolonised block' on the outer edge. The Petri dishes were incubated at 25°C. After 12 weeks, three stacks were removed from each Petri dish, the external fungal biomass was carefully brushed off, and each block was weighed and then dried at 80°C to a constant weight. The wood weight loss for each treatment was calculated as a percentage of the initial block dry weight. The mean wood weight loss and moisture content was calculated separately for the three blocks, across the three stacks and triplicate Petri dishes. Viability assessments of *G. sepiarium* and the *Trichoderma* were carried out for the three blocks in the remaining stack from each Petri dish. The blocks were flame sterilised, placed on water agar and incubated for 21 d at 30°C. Growth of *G. sepiarium* and/or the *Trichoderma* were clearly distinguishable based on fungal growth rate and morphology. Following viability assessment, the wood blocks were dried at 80°C to a constant weight for determination of weight losses.

The results for each block type were analysed statistically using analysis-of-variance. The wood weight loss data required angular transformation because of the heterogeneity of variation. Comparisons between individual means and the uninoculated control treatment were carried out using Dunnett's test at the 5% significance level. Comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level. Standard regression and correlation analyses (Pearson's test) were carried out for wood weight loss, moisture content and fungal viability. Regression analyses were also carried out for the three block types to determine if the wood weight loss in the stack used for viability assessment was correlated to the weight loss in the other three stacks.

Assay 2 Screen of *Trichoderma* sp. 15, 24, 26, 38, 80, 101 and 117 for biological control potential against *G. sepiarium*

The wood blocks were labelled and dried at 80°C to a constant weight prior to sterilisation by gamma irradiation (50.6 kGy). The precolonisation and assay Petri dishes were set up using the same method as Assay 1. There were precolonisation Petri dishes for the 'sterile block' type which were inoculated with cores of sterile MA. The precolonisation Petri dishes were incubated for 28 d at 25°C. In the assay Petri dishes, the *Trichoderma* control treatments replaced the '*G. sepiarium* precolonised block' with a 'sterile block'. The assay Petri dishes were incubated at 25°C and after 12 weeks, the blocks were harvested and the data was statistically analysed as for Assay 1.

Samples of the wood blocks were analysed for wood chemical composition (extractives, Klason and acid-soluble lignin, carbohydrates and nitrogen) by the Analytical Laboratory at Forest Research (Rotorua). The '*G. sepiarium* precolonised blocks' were analysed from the seven *Trichoderma*-*G. sepiarium* treatments and the *G. sepiarium* control treatment, the '*Trichoderma* precolonised block' type from the *Trichoderma* control treatments, and 'sterile blocks' from the uninoculated control treatment. In order to reduce costs, only *Trichoderma* sp. 80 and 101 control treatments were selected for analyses as they had resulted in the highest and lowest weight losses, respectively.

Assay 3 Screen of *Trichoderma* sp. 26, 38 and 101 for biological control potential against *G. sepiarium*, *P. gigantea* and *S. commune*

There were nine *Trichoderma*-basidiomycete fungi treatments, three *Trichoderma* control treatments, three basidiomycete fungi control treatments and an uninoculated control treatment. Precolonisation and assay Petri dishes were set up and blocks were harvested using the same method as previous assays. The precolonisation Petri dishes were incubated for 21 d at 25°C. The results were statistically analysed as for Assay 1, with the analysis-of-variance of the weight loss data using the initial block dry weight as a covariate.

Assay 4 Screen of wood weight losses by *Trichoderma* sp. 26, 38 and 101 and *G. sepiarium*, *P. gigantea* and *S. commune* over the 21 and 28 d precolonisation times

In the previous three assays, the final wood weight losses did not indicate the weight loss caused by the *Trichoderma* or basidiomycete fungi during the precolonisation period. This assay assessed the wood weight losses after the precolonisation times of 21 d (Assay 1 and 3) or 28 d (Assay 2). Precolonisation Petri dishes of *Trichoderma* sp. 26, 38 and 101, *G. sepiarium*, *P. gigantea* and *S. commune*, and an uninoculated control treatment were set up as for Assay 1 and incubated for 21 d at 25°C. Further precolonisation Petri dishes of *Trichoderma* sp. 26, 38 and 101, *G. sepiarium*, and an uninoculated control treatment were incubated for 28 d at 25°C. There were triplicate Petri dishes for each treatment. After the incubation period, the blocks were removed from each Petri dish, the external fungal biomass was carefully brushed off, and each block was weighed and then dried at 80°C to a constant weight. The results were statistically analysed as for Assay 1, but the data did not require transformation.

5.2.2 *Trichoderma* identification

The seven *Trichoderma* cultures selected from Assay 1 (*Trichoderma* sp. 15, 24, 26, 38, 80, 101 and 117) were sent to Dr Gary Samuels (USDA-ARS, U.S.A.) for expert identification.

5.2.3 *Trichoderma* wood decay potential

5.2.3.1 Cellulase, xylanase and mannanase enzymes

The seven identified cultures (*Trichoderma* sp. 15, 24, 26, 38, 80, 101 and 117) were screened for cellulase, xylanase and mannanase activity. The enzyme assays and the standards were the same as those used in Chapter 2 for the basidiomycete fungi (Appendix 2.1). The carbohydrate sources were used at a concentration of 1.0% in glucose-yeast extract nutrient medium (Appendix 1.3). Each duplicate flask was inoculated with a spore suspension of the test *Trichoderma* (21 d old culture). The standard inoculum per flask was 1 mL of 10^6 spores mL^{-1} but for *Trichoderma* sp. 101, 2 mL was required

due to a low spore count. The flasks were incubated at 25°C at 120 rpm. At 8 d, colony morphology and pigmentation of the fungi in each medium was noted but not characterised (Appendix 5).

The results were analysed statistically using analysis-of-variance. The CMCase activity data required logarithmic transformation. Comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level. Standard regression and correlation analyses (Pearson's test) were carried out to determine if there were any significant correlations between enzyme activity and accumulated fungal biomass.

Cellulase was regarded as the most important of the three enzymes because of the focus in the literature on cellulase production by *Trichoderma* species. To gain a greater appreciation of the range in cellulase activity amongst the *Trichoderma* cultures collected in Chapter 4, a further seven cultures from Assay 1 were screened. The unidentified *Trichoderma* sp. 46, 52, 72, 115, 122, 126 and 127 cultures were selected to reflect the range of source and origins.

5.2.3.2 Laccase, peroxidase and proteinase enzymes

The agar-based assays for laccase, peroxidase and proteinase in Chapter 2 were applied to *Trichoderma* sp. 26, 38 and 101 that had been selected for Assay 3.

5.2.3.3 Wood decay

The *Trichoderma* cultures screened for biological control potential in Assays 2 and 3 (including *Trichoderma* sp. 15, 24, 26, 38, 80, 101 and 117) were assessed in control treatments for their ability to cause weight loss of *Pinus radiata* over a period of 12 weeks at 25°C. In Assay 4, *Trichoderma* sp. 26, 38 and 101 were assessed for their ability to cause weight losses over the 21 and 28 d precolonisation periods at 25°C.

5.2.4 Field and controlled temperature room trials

The biological control potential of *Trichoderma* sp. 26, 38 and 101 against *G. sepiarium* was investigated using non-sterile *P. radiata* in field and controlled temperature (CT) room trials. There were two sets of five treatments. In one set of treatments, the blocks were precolonised with the test *Trichoderma* for 3 d prior to inoculation of *G. sepiarium* (*Trichoderma-G. sepiarium*). There was a *G. sepiarium* control treatment which replaced the *Trichoderma* application with distilled water (water-*G. sepiarium*) and an uninoculated control treatment (water-water). In the other set of treatments, the blocks were precolonised with *G. sepiarium* for 3 d prior to inoculation of the test *Trichoderma* (*G. sepiarium-Trichoderma*) and there was a *G. sepiarium* control treatment (*G. sepiarium-water*) and an uninoculated control treatment (water-water).

The ten treatments had four plastic pail replicates and 25 wood blocks per pail. Each plastic pail (4.5 L volume, 180 x 180 x 190 mm high) contained 3 L soil. The soil (from a field site at HortResearch, Hamilton) had been double autoclaved for 60 min at 121°C with a 24 h interval between cycles. The soil WHC was 61.2%. The required water addition to each pail to produce a WHC of 130% was 400 mL. The soil pH was 4.85. Wood blocks (15 x 15 mm cross-cut x 100 mm long) were produced from freshly harvested *P. radiata* sapwood obtained from CHH Timber Putaruru Mill. A sample of 125 untreated blocks were assessed for fresh weight, dry weight and moisture content. The wood blocks were treated green (no initial dried weights) so the final mean weight loss for each treatment was calculated relative to the mean block dry weight of the uninoculated control treatment.

The three test *Trichoderma* were grown on bran-peat moss medium (Appendix 2.6). A 3 L spore suspension of each culture was produced by several successive washes of the medium with Tris buffer (Appendix 1.10). The three spore suspensions were not adjusted to the same concentration but the same spore concentration of each fungus was used for the two sets of treatments. *Gloeophyllum sepiarium* was grown in YM for 10 d at 25°C. The inoculum was harvested from the liquid medium by vacuum filtration, and then blended into a thick slurry in Tris buffer. Inoculation of *G. sepiarium* was carried out in one day using the same batch of inoculum. The wood blocks were treated with the *Trichoderma*, *G. sepiarium* or distilled water solutions (3 L) by immersion for 5 min,

draining until drip-dry on a galvanised metal grid and then storage in covered plastic containers for 3 d at room temperature before subsequent inoculation treatment. After completion of treatment, 25 blocks were inserted 80 mm into the soil of each plastic pail in a 5 x 5 design.

The pails were incubated for 8 months at two sites; in an open field trial site exposed to the weather at HortResearch (Hamilton); and in a dark CT room (25°C) at the University of Waikato (Hamilton). At each site, the pails were arranged randomly within each of the four replicates in a statistical block design. At the field trial site, there was a weather station which collected maximum and minimum air temperature, rainfall and relative humidity data on a daily basis. In the CT room, each pail was covered with muslin cloth to avoid cross-contamination and to maintain relative humidity but after 14 d there was extensive mould and stain present on wood blocks of all treatments. This was thought to have been due to the high humidity and condensation from limited ventilation so the covers were removed and there was no water addition for the remainder of the incubation period.

After 2 months, the central wood block in each pail from the field and the CT room trials, was collected for assessment of the resident microflora. Internal isolations of the 80 blocks were carried out on BA, TSA and yeast extract-malt extract agar (YMA; Appendix 1.14), with triplicate Petri dishes of each medium for each block. The Petri dishes were incubated at 25°C for 7 d. Identification of microorganisms were to the genus level.

After 8 months, each wood block was removed from the pails, the external fungal biomass was carefully brushed off, and then they were dried at 80°C to a constant weight. The wood dry weight data for the field and the CT room trials was analysed statistically using analysis-of-variance, with separate analyses for each set of five treatments. Comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level.

5.3 RESULTS

5.3.1 Wood biological control assays

The wood parameters of the 50 untreated blocks from each assay are given in Appendix 12.1. The mean block fresh weight ranged from 1.047 to 1.346 g, dry weight from 0.443 to 0.558 g and moisture content from 136 to 161%.

Assay 1 *Trichoderma* sp. 1, 15, 23, 24, 26, 33, 38, 46, 52, 72, 80, 99, 101, 115, 116, 117, 118, 119, 120, 121, 122, 126 and 127 were screened for biological control potential against *G. sepiarium*

There were significant differences in mean wood weight loss and moisture content between the 23 *Trichoderma-G. sepiarium* treatments for each block type ($P < 0.001$) (Table 5.1). Regression analyses showed a significant correlation between wood weight loss and moisture content for the three block types. There was a significant correlation between the viability of *G. sepiarium* and wood weight loss but no correlation between viability of the *Trichoderma* fungi and weight loss. There was a significant correlation between the mean weight loss of the blocks used for viability assessment and the blocks used for weight loss assessment.

In 'G. sepiarium precolonised blocks', weight losses of the *Trichoderma-G. sepiarium* treatments ranged from 4.8 to 30.7%. The highest weight loss for each treatment was in this block type. All 23 *Trichoderma* cultures resulted in weight losses that were significantly higher than the uninoculated control treatment but 14 cultures resulted in weight losses that were significantly lower than the *G. sepiarium* control treatment. Of these 14 cultures, *Trichoderma* sp. 26, 38 and 101 resulted in the loss of *G. sepiarium* viability and the lowest weight losses of 6.0, 4.8 and 6.8%, respectively.

In 'sterile blocks', weight losses of the *Trichoderma-G. sepiarium* treatments ranged from 1.7 to 17.6%. Twenty *Trichoderma* cultures resulted in weight losses that were significantly lower than the *G. sepiarium* control treatment and ten cultures resulted in weight losses that were not significantly different to the uninoculated control treatment.

Table 5.1 Assay 1: The biological control potential of 23 *Trichoderma* cultures against *Gloeophyllum sepiarium*, as measured by wood weight loss, moisture content and fungal viability, in three test block types with a precolonisation time of 21 d.

Treatment ^a	<i>G. sepiarium</i> precolonised block			Sterile block			<i>Trichoderma</i> precolonised block		
<i>Trichoderma</i> sp.	Wt loss	MC	V	Wt loss	MC	V	Wt loss	MC	V
1	23.7	69	BT	15.3	61	BT	18.4	64	BT
15	11.6	54	BT	2.8	40	T	2.9	42	T
23	24.0	72	BT	17.6	71	BT	18.2	65	BT
24	10.4	56	BT	2.1	39	T	1.9	41	T
26	6.0	48	T	2.5	39	T	2.5	40	T
33	13.1	57	BT	10.3	61	BT	11.6	58	BT
38	4.8	50	T	1.7	42	T	1.9	44	T
46	10.4	54	BT	2.7	42	BT	1.5	41	T
52	10.8	55	BT	2.4	40	BT	2.2	41	T
72	17.7	65	BT	5.2	45	BT	3.7	42	BT
80	10.6	53	BT	2.7	41	T	2.5	42	T
99	22.5	64	BT	13.9	61	BT	14.8	60	BT
101	6.8	49	T	3.0	41	T	2.8	40	T
115	26.6	73	BT	9.7	52	BT	4.4	45	BT
116	30.7	79	BT	6.7	49	BT	4.2	46	BT
117	9.0	52	BT	2.6	42	T	2.8	42	T
118	18.9	58	B	9.7	54	BT	6.3	47	BT
119	19.2	61	B	12.5	61	BT	12.5	57	BT
120	17.3	57	B	10.8	60	BT	6.8	49	BT
121	17.4	59	B	10.7	59	BT	8.4	48	BT
122	17.7	62	BT	7.7	56	BT	4.6	46	T
126	20.0	66	BT	8.8	52	BT	4.4	45	T
127	10.6	54	BT	6.6	51	T	4.7	45	T
<i>G. sepiarium</i> control	26.2	69	B	21.0	70	B	21.7*	68*	B*
Uninoculated control	1.8*	46*		1.8	51		1.8*	47*	
sed (P<0.05)	2.39	3.5		2.02	3.3		2.11	2.5	

a All treatments except the control treatments were challenged against *G. sepiarium*; * 'Sterile block'

Wt loss= Wood dry weight loss (%); MC= wood moisture content (%); V= fungal viability (B=Viable *G. sepiarium*, T=Viable *Trichoderma*)

Of these ten cultures, *Trichoderma* sp. 15, 24, 26, 38, 80, 101 and 117 prevented *G. sepiarium* colonisation and resulted in weight losses of 1.7 to 3.0%.

In 'Trichoderma precolonised blocks', weight losses of the *Trichoderma-G. sepiarium* treatments ranged from 1.5 to 18.4%. Twenty *Trichoderma* cultures resulted in weight losses that were significantly lower than the *G. sepiarium* control treatment and 15 cultures resulted in weight losses that were not significantly different to the uninoculated control treatment. Of these 15 cultures, *Trichoderma* sp. 15, 24, 26, 38, 46, 52, 80, 101, 117, 122, 126 and 127 prevented *G. sepiarium* colonisation and resulted in weight losses of 1.5 to 4.7%.

Mean wood moisture contents of '*G. sepiarium* precolonised blocks' ranged from 48 to 79%, 'sterile blocks' from 39 to 71% and '*Trichoderma* precolonised blocks' from 40 to 65%. The highest moisture content for 21 of the 23 *Trichoderma-G. sepiarium* treatments was in '*G. sepiarium* precolonised blocks'. The mean moisture content of the *G. sepiarium* control blocks ranged from 68 to 70% and the uninoculated control blocks from 46 to 51%. Wood moisture content was not a limiting factor in the precolonisation stage as the mean moisture content determined for 'sterile blocks' in six precolonisation Petri dishes was 30% after 7 d incubation at 25°C.

Trichoderma sp. 15, 24, 26, 38, 80, 101 and 117 were selected for Assay 2 as they had resulted in mean wood weight losses that were not significantly different to the uninoculated control treatment, and had inhibited *G. sepiarium* colonisation of '*Trichoderma* precolonised blocks' and 'sterile blocks'. *Trichoderma* sp. 26, 38 and 101 had also resulted in the loss of *G. sepiarium* viability in '*G. sepiarium* precolonised blocks'. The mean wood moisture contents of the three block types in these seven *Trichoderma-G. sepiarium* treatments were not significantly different to the uninoculated control treatment and significantly lower than the *G. sepiarium* control treatment.

Assay 2 *Trichoderma* sp. 15, 24, 26, 38, 80, 101 and 117 were screened for biological control potential against *G. sepiarium*

There were significant differences in mean wood weight loss and moisture content between the seven *Trichoderma-G. sepiarium* treatments for each block type ($P < 0.001$) (Table 5.2). The same conclusions as Assay 1 were reached for the regression and

correlation analyses of wood weight loss, moisture content and fungal viability for the three block types.

In '*G. sepiarium* precolonised blocks', weight losses of the seven *Trichoderma-G. sepiarium* treatments ranged from 9.8 to 25.8% and were significantly higher than the uninoculated control and the *Trichoderma* control treatments. Again, the highest weight loss for each treatment was in this block type. *Trichoderma* sp. 26, 38, 101 and 15 resulted in weight losses of 9.8, 12.4, 13.1 and 14.8%, respectively, that were significantly lower than the *G. sepiarium* control treatment, but there was no loss of *G. sepiarium* viability.

In 'sterile blocks', weight losses of the seven *Trichoderma-G. sepiarium* treatments ranged from 0 to 7.3% and were significantly lower than the *G. sepiarium* control treatment. *Trichoderma* sp. 15, 24, 26, 38 and 80 resulted in weight losses of less than 1.0%, that were not significantly different to the uninoculated control or the *Trichoderma* control treatments, and prevented *G. sepiarium* colonisation.

In '*Trichoderma* precolonised blocks', weight losses of the seven *Trichoderma-G. sepiarium* treatments ranged from 0.4 to 7.8% and were significantly lower than the *G. sepiarium* control treatment. *Trichoderma* sp. 15, 24, 26, 38, 80 and 101 resulted in weight losses of less than 1.4%, that were not significantly different to the uninoculated control or the *Trichoderma* control treatments, and prevented *G. sepiarium* colonisation.

Mean wood moisture contents of '*G. sepiarium* precolonised blocks' ranged from 55 to 80%, 'sterile blocks' from 49 to 64% and '*Trichoderma* precolonised blocks' from 44 to 62%. The highest moisture content for the *Trichoderma-G. sepiarium* treatments was in '*G. sepiarium* precolonised blocks'. The mean moisture content of the *Trichoderma* control blocks ranged from 43 to 56% and the *G. sepiarium* control blocks from 83 to 90%. The moisture content of the uninoculated control blocks was 56%.

Table 5.2 Assay 2: The biological control potential of seven *Trichoderma* cultures against *Gloeophyllum sepiarium*, as measured by wood weight loss, moisture content and fungal viability, in three test block types with a precolonisation time of 28 d.

Treatment ^a	<i>G. sepiarium</i> precolonised block			Sterile block			<i>Trichoderma</i> precolonised block		
<i>Trichoderma</i> sp.	Wt loss	MC	V	Wt loss	MC	V	Wt loss	MC	V
15	14.8	64	BT	0.0	55	T	0.4	46	T
24	19.6	64	BT	1.0	51	T	1.0	45	T
26	9.8	55	BT	0.6	49	T	0.6	45	T
38	12.4	63	BT	0.3	55	T	0.4	49	T
80	20.7	65	BT	0.9	49	T	0.9	44	T
101	13.1	58	BT	2.0	50	BT	1.4	45	T
117	25.8	80	BT	7.3	64	BT	7.8	62	BT
<i>G. sepiarium</i> control	26.0	90	B	17.7	83	B	18.6*	84*	B*
<i>Trichoderma</i> sp. 15 control	0.7*	52*	T*	0.8	55	T	1.0	49	T
<i>Trichoderma</i> sp. 24 control	0.5*	45*	T*	0.6	49	T	0.8	43	T
<i>Trichoderma</i> sp. 26 control	1.0*	48*	T*	0.9	49	T	0.9	46	T
<i>Trichoderma</i> sp. 38 control	0.9*	53*	T*	1.0	56	T	0.8	49	T
<i>Trichoderma</i> sp. 80 control	1.2*	49*	T*	0.8	52	T	1.2	47	T
<i>Trichoderma</i> sp. 101 control	0.9*	50*	T*	0.9	50	T	0.8	47	T
<i>Trichoderma</i> sp. 117 control	1.2*	51*	T*	1.2	49	T	1.2	46	T
Uninoculated control	0.9*	56*		0.9	56		0.8*	56*	
sed (P<0.05)	3.82	4.9		2.84	5.5		3.09	4.6	

a All treatments except the control treatments were challenged against *G. sepiarium*; * 'Sterile block'

Wt loss= Wood dry weight loss (%); MC= wood moisture content (%); V= fungal viability (B=Viable *G. sepiarium*, T=Viable *Trichoderma*)

Wood chemical analyses

The wood chemical analyses did not give conclusive evidence of cellulolytic or ligninolytic activity, or the ability to degrade extractives, but there was hemicellulolytic activity observed by *G. sepiarium* and/or the *Trichoderma* cultures (Table 5.3).

DCM extractives were lower in blocks from all *Trichoderma-G. sepiarium* treatments relative to the uninoculated control treatment. Some of the *Trichoderma* treatments e.g. *Trichoderma* sp. 26, 38 and 101 had lower extractives than the *G. sepiarium* control treatment, which indicated that they had the ability to degrade extractives.

G. sepiarium did not degrade Klason lignin as there were no differences between blocks from the uninoculated control and *G. sepiarium* control treatments. Relative to the control treatments, there were losses of Klason lignin in blocks from the *Trichoderma-G. sepiarium* treatments. Klason lignin content was greater in the treatments with the greater wood weight loss and the *Trichoderma* control treatments had the lowest Klason lignin content. This suggested that *G. sepiarium* had little ligninolytic activity relative to the *Trichoderma* cultures. Acid-soluble lignin content was the lowest in the *G. sepiarium* control and *Trichoderma* control treatments which suggested that *G. sepiarium* and the two *Trichoderma* cultures had some ligninolytic activity.

The wood glucose analyses showed that *G. sepiarium* did not degrade cellulose as there were no differences between blocks from the uninoculated control and *G. sepiarium* control treatments. Relative to the control treatments, there were losses of glucose from the *Trichoderma-G. sepiarium* treatments and the *Trichoderma* control treatments, which indicated that the *Trichoderma* cultures had cellulolytic activity. The *Trichoderma* sp. 117-*G. sepiarium* treatment, that resulted in the least control of *G. sepiarium*, had the blocks with the lowest glucose content.

Arabinose, galactose, xylose and mannose content was lower in blocks of all treatments relative to the uninoculated control treatment. Blocks of the *Trichoderma-G. sepiarium* treatments were lower in arabinose, galactose, xylose and mannose content than the *Trichoderma* control treatments which indicated hemicellulolytic activity by *G. sepiarium*. As for glucose, the *Trichoderma* sp. 117-*G. sepiarium* treatment had the blocks with the lowest arabinose, galactose, xylose and mannose content.

Table 5.3 Wood chemical analyses of *Pinus radiata* blocks from *Trichoderma-Gloeophyllum sepiarium* treatments, in comparison to *G. sepiarium* control, *Trichoderma* control and uninoculated control treatments.

Treatment	g per 100 g dry weight wood								
	DCM extractives	Klason lignin	Acid-soluble lignin	Glucose	Arabinose	Galactose	Xylose	Mannose	
<i>Trichoderma</i> sp. 15+ <i>G. sepiarium</i>	0.80	31.5	0.95	43.1	0.77	1.15	3.95	6.85	
<i>Trichoderma</i> sp. 24+ <i>G. sepiarium</i>	0.86	33.3	1.07	42.9	0.74	1.18	3.98	6.23	
<i>Trichoderma</i> sp. 26+ <i>G. sepiarium</i>	0.57	30.0	0.78	43.9	0.83	1.37	4.39	8.17	
<i>Trichoderma</i> sp. 38+ <i>G. sepiarium</i>	0.71	32.0	0.87	42.8	0.81	1.88	4.36	7.12	
<i>Trichoderma</i> sp. 80+ <i>G. sepiarium</i>	0.91	33.8	1.28	41.0	0.75	1.29	3.89	6.93	
<i>Trichoderma</i> sp. 101+ <i>G. sepiarium</i>	0.62	31.5	0.92	43.5	0.82	1.15	4.30	7.54	
<i>Trichoderma</i> sp. 117+ <i>G. sepiarium</i>	0.82	35.8	1.38	39.9	0.67	0.85	3.48	4.85	
<i>G. sepiarium</i> control	0.82	37.2	0.45	53.1	0.68	1.23	3.40	4.76	
<i>Trichoderma</i> sp. 80 control	0.66	27.7	0.49	42.8	1.20	2.45	4.51	9.35	
<i>Trichoderma</i> sp. 101 control	0.59	27.6	0.50	44.2	1.17	2.09	4.49	9.59	
Uninoculated control	1.65	36.5	1.13	50.2	1.25	2.56	6.11	10.10	

Trichoderma sp. 26, 38 and 101 were selected for Assay 3 based on their biological control potential shown in Assay 1 and 2 (Photo 5.2). These cultures had resulted in the lowest mean weight losses in ‘*G. sepiarium* precolonised blocks’. The mean wood moisture contents of the three block types in these three *Trichoderma-G. sepiarium* treatments were not significantly different to the uninoculated control treatment and significantly lower than the *G. sepiarium* control treatment.

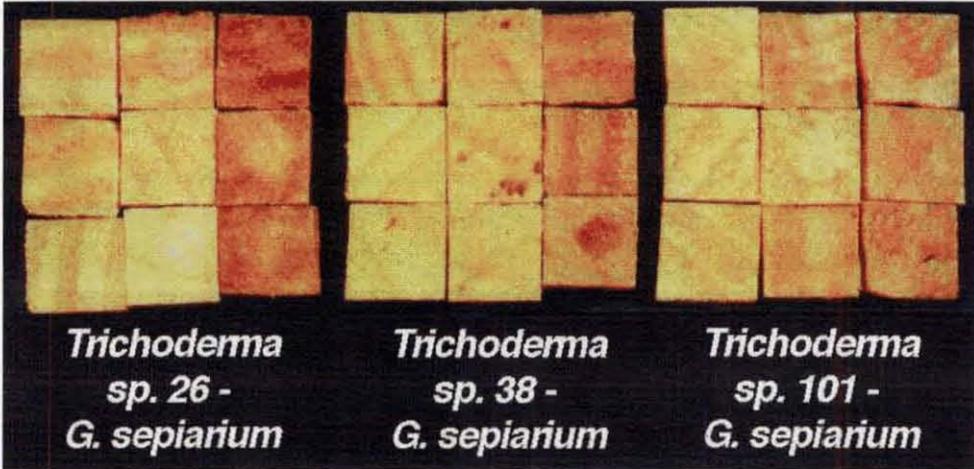


Photo 5.2 The influence of *Trichoderma* sp. 26, 38 and 101 treatments on decay of *Pinus radiata* by *Gloeophyllum sepiarium* in, from left to right for each treatment, the ‘*Trichoderma* precolonised block’, ‘sterile block’ and ‘*G. sepiarium* precolonised block’.

Assay 3 Screen of *Trichoderma* sp. 26, 38 and 101 for biological control potential against *G. sepiarium*, *P. gigantea* and *S. commune*

Regression analyses showed that there was a positive correlation between the viability of *G. sepiarium* or *P. gigantea* and wood weight loss for the three block types. No viable *G. sepiarium* was reisolated from the blocks of the *Trichoderma-G. sepiarium* treatments. There was no correlation between *Trichoderma* viability and weight loss. No analyses were carried out for *S. commune* as no significant weight losses were measured, or viable *S. commune* reisolated, from the *Trichoderma-S. commune* or *S. commune* control treatments.

In the three *Trichoderma-G. sepiarium* treatments, mean wood weight losses were significantly lower than the *G. sepiarium* control treatment ($P < 0.001$) (Table 5.4; Photo 5.3). The highest weight loss for each treatment was in ‘*G. sepiarium* precolonised

blocks'. Weight losses of 'sterile blocks' and '*G. sepiarium* precolonised blocks', and '*G. sepiarium* precolonised blocks' in the *Trichoderma* sp. 38 treatment, were not significantly different to the uninoculated control treatment.

In the *Trichoderma-Phlebiopsis gigantea* treatments, the *Trichoderma* cultures did not have the ability to inhibit weight loss in '*P. gigantea* precolonised blocks' relative to the *P. gigantea* control treatment (Table 5.4). In 'sterile blocks' and '*Trichoderma* precolonised blocks', *Trichoderma* sp. 26 resulted in a significantly lower weight loss than the *P. gigantea* control treatment. No viable *P. gigantea* was reisolated from the *Trichoderma* sp. 26 or 38 treatments but the weight losses indicated that *P. gigantea* had been present prior to harvest.



Photo 5.3 Wood biological control assay treatments, clockwise from top left, *Gloeophyllum sepiarium* control, *Trichoderma* sp. 26-*G. sepiarium*, *Trichoderma* sp. 38-*G. sepiarium* and *Trichoderma* sp. 101-*G. sepiarium*.

Table 5.4 Assay 3: The biological control potential of *Trichoderma* sp. 26, 38 and 101 against *Gloeophyllum sepiarium*, *Phlebiopsis gigantea* and *Schizophyllum commune*, as measured by wood weight loss, moisture content and fungal viability, in three test block types with a precolonisation time of 21 d.

Treatment	Basidiomycete precolonised block			Sterile block			<i>Trichoderma</i> precolonised block		
	Wt loss	MC	V	Wt loss	MC	V	Wt loss	MC	V
<i>Trichoderma</i> sp. 26- <i>G. sepiarium</i>	4.8	47	T	2.1	42	T	2.1	41	T
<i>Trichoderma</i> sp. 38- <i>G. sepiarium</i>	1.7	46	T	1.4	45	T	1.5	44	T
<i>Trichoderma</i> sp. 101- <i>G. sepiarium</i>	4.7	46	T	1.7	44	T	1.7	42	T
<i>G. sepiarium</i> control	19.0	66	B	13.2	61	B	13.3*	59*	B*
<i>Trichoderma</i> sp. 26- <i>P. gigantea</i>	10.8	45	T	3.7	46	T	2.3	41	T
<i>Trichoderma</i> sp. 38- <i>P. gigantea</i>	8.1	47	T	6.1	47	T	5.8	46	T
<i>Trichoderma</i> sp. 101- <i>P. gigantea</i>	8.5	52	BT	6.9	52	BT	5.4	46	BT
<i>P. gigantea</i> control	7.7	56	B	5.9	53	B	6.2*	57*	BT*
<i>Trichoderma</i> sp. 26- <i>S. commune</i>	1.9	45	T	1.8	47	T	1.7	44	T
<i>Trichoderma</i> sp. 38- <i>S. commune</i>	1.9	42	T	1.4	44	T	1.2	42	T
<i>Trichoderma</i> sp. 101- <i>S. commune</i>	2.3	41	T	1.9	44	T	1.7	42	T
<i>S. commune</i> control	0.5	50	B	0.6	48	B	0.6*	50*	B*
<i>Trichoderma</i> sp. 26 control	2.1*	47*	T*	2.0	47	T	2.2	43	T
<i>Trichoderma</i> sp. 38 control	1.1*	49*	T*	1.3	49	T	1.2	46	T
<i>Trichoderma</i> sp. 101 control	2.2*	47*	T*	2.0	47	T	2.1	44	T
Uninoculated control	1.1*	52*		1.1	52		0.8*	52*	
sed (P<0.05)	0.81	1.5		1.10	2.0		1.31	1.8	

* 'Sterile block'; Wt loss= Wood dry weight loss (%); MC= wood moisture content (%); V= fungal viability (B=Viable *G. sepiarium*, T=Viable *Trichoderma*)

In the *Trichoderma-Schizophyllum commune* and *S. commune* control treatments, the weight losses (less than 2.3%) were not significantly different to the uninoculated control treatment (Table 5.4). The highest weight loss for each treatment was in 'S. commune precolonised blocks' but differences between the block types were not significant.

For the three *Trichoderma* control treatments, weight losses were less than 2.2%. There were no significant differences in weight loss between the *Trichoderma* control treatments, the uninoculated control treatment, and the *Trichoderma-S. commune* and *S. commune* control treatments.

As reported for the previous assays, there was a significant correlation between wood weight loss and moisture content. The highest moisture contents were in the *G. sepiarium* control treatment and the lowest in the *Trichoderma* control treatments. The moisture contents in the *Trichoderma-G. sepiarium*, *Trichoderma-P. gigantea* and *Trichoderma-S. commune* treatments were not significantly different.

Assay 4 Screen of wood weight losses by *Trichoderma* sp. 26, 38 and 101 and *G. sepiarium*, *P. gigantea* and *S. commune* over the precolonisation times of 21 and 28 d

There were no significant differences in mean wood weight loss between the three *Trichoderma* cultures over the two precolonisation times, as the weight losses were less than 1.1% relative to the uninoculated control treatment (Appendix 12.2). The weight loss by *G. sepiarium* after 28 d was 6.8% compared to 1.7% after 21 d. The weight losses by *G. sepiarium* and *P. gigantea* were not significantly different after 21 d and significantly higher than *S. commune* (0.9%) ($P < 0.001$). There were no significant differences in mean wood moisture content between the treatments after 21 d, but the moisture content of *G. sepiarium* blocks was significantly higher after 28 d.

5.3.2 *Trichoderma* identification

The seven *Trichoderma* cultures were provisionally identified by Dr Gary Samuels as *T. sp. 'long'*, *T. viride*, *T. sp. 'viride'* or *T. crassum* species. The use of 'sp.' indicated that the fungus was closely related to the named species. Herein, the *Trichoderma* cultures were referred to by these given species identifications.

<i>Trichoderma</i> sp. 15	<i>Trichoderma</i> sp. 'long'
<i>Trichoderma</i> sp. 24	<i>Trichoderma</i> sp. 'long'
<i>Trichoderma</i> sp. 26	<i>Trichoderma crassum</i> ined.
<i>Trichoderma</i> sp. 38	<i>Trichoderma</i> sp. 'viride'
<i>Trichoderma</i> sp. 80	<i>Trichoderma</i> sp. 'long'
<i>Trichoderma</i> sp. 101	<i>Trichoderma viride</i> Persoon
<i>Trichoderma</i> sp. 117	<i>Trichoderma</i> sp. 'viride'

5.3.3 *Trichoderma* wood decay potential

5.3.3.1 Cellulase, xylanase and mannanase enzymes

Cellulase

No significant differences ($P < 0.05$) were found between the *Trichoderma* cultures in CMCase activity which ranged from 0.353 to 0.679 micromoles glucose $\text{min}^{-1} \text{mL}^{-1}$ (IU mL^{-1}) (Table 5.5). Maximum activity occurred at 6 or 8 d after inoculation. The highest CMCase activities were produced by *T. sp.* 'viride' 38 and 117, and *T. viride* 101 (0.544 to 0.679 IU mL^{-1}) and the lowest activity was produced by *T. sp.* 'long' 80 (0.353 IU mL^{-1}). No significant differences were found in accumulated fungal biomass which ranged from 8.23 to 10.56 mg mL^{-1} . *Trichoderma viride* and *T. sp.* 'viride' cultures resulted in both the lowest and the highest fungal biomass whereas *T. sp.* 'long' cultures were less variable. There were no significant correlations between CMCase activity and accumulated fungal biomass. For the seven additional *Trichoderma* cultures that were screened, there were significant differences in CMCase activity (0.092 to 1.216 IU mL^{-1}) but no differences in accumulated fungal biomass (7.43 to 9.83 mg mL^{-1}) (Appendix 12.3).

Xylanase

The *Trichoderma* cultures had xylanase activities which ranged from 2.63 to 10.39 IU mL^{-1} (Table 5.5). Maximum activity occurred at 6 or 8 d after inoculation. *T. sp.* 'viride' 38 produced lower xylanase activity than the other cultures which were not significantly different ($P < 0.05$). There were significant differences in accumulated fungal

biomass which ranged from 1.10 to 2.49 mg mL⁻¹. No significant correlations were found between xylanase activity and accumulated fungal biomass. More variation in activity and biomass occurred between *T. viride* and *T. sp. 'viride'* cultures than *T. sp. 'long'* cultures.

Table 5.5 CMCCase, xylanase and mannanase activity and accumulation of fungal biomass by seven *Trichoderma* cultures in liquid medium.

<i>Trichoderma</i> sp.	CMC medium		Xylan medium		Mannan medium	
	CMCCase ¹	BM	Xylanase ¹	BM	Mannanase ¹	BM
<i>T. sp. 'long'</i> 15	0.510	8.41	10.18	1.44	0.811	1.41
<i>T. sp. 'long'</i> 24	0.470	9.53	9.98	1.48	0.281	1.58
<i>T. crassum</i> 26	0.460	8.56	7.04	2.49	0.125	2.41
<i>T. sp. 'viride'</i> 38	0.544	10.56	2.63	1.10	0.492	1.55
<i>T. sp. 'long'</i> 80	0.353	9.30	10.39	1.43	0.423	1.02
<i>T. viride</i> 101	0.660	8.23	6.97	1.54	0.455	1.81
<i>T. sp. 'viride'</i> 117	0.679	8.27	10.23	1.85	0.472	1.30
sed (P<0.05)	0.1043	1.020	1.678	0.083	0.2625	0.276

¹ Micromoles glucose min⁻¹ mL⁻¹ (IU mL⁻¹)

BM= Milligrams dry weight fungal biomass mL⁻¹ after 8 d incubation at 25°C

Mannanase

No significant differences (P<0.05) were found between the *Trichoderma* cultures in mannanase activity which ranged from 0.125 to 0.811 IU mL⁻¹ (Table 5.5). Maximum activity occurred at 1 or 2 d after inoculation. The highest mannanase activity was produced by *T. sp. 'long'* 15 and the lowest activity by *T. crassum* 26. There were significant differences in accumulated fungal biomass which ranged from 1.02 to 2.41 mg mL⁻¹. There was a significant correlation between mannanase activity and accumulated fungal biomass. More variation in activity and biomass occurred between *T. sp. 'long'* cultures than *T. viride* and *T. sp. 'viride'* cultures.

A range in sporulation capability was observed for the seven *Trichoderma* cultures on MA, with spore concentrations ranging from 2.0 x 10⁵ to 1.7 x 10⁸ spores mL⁻¹. Sporulation was most variable for *T. viride* and *T. sp. 'viride'* cultures with *T. viride* 101 having a poor sporulation capability (2.0 x 10⁵ spores mL⁻¹) compared to *T. sp. 'viride'* 38 and 117 (1.0 and 1.7 x 10⁸ spores mL⁻¹, respectively). In comparison, *T. sp. 'long'* cultures produced spore concentrations of 2.7 to 5.5 x 10⁷ spores mL⁻¹ and *T. crassum* 26 had a concentration of 1.9 x 10⁷ spores mL⁻¹.

5.3.3.2 Laccase, peroxidase and proteinase enzymes

The three agar-based enzyme assays were negative for *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101. The laccase assay found no production of purple stain when α -naphthol was applied to the fungal cultures. The peroxidase assay showed no colour change with growth on gSA but on gPDA, sporulation by *T. crassum* 26 and *T. sp. 'viride'* 38 resulted in the production of yellow-brown stain. The proteinase assay showed no clearing zones in the SMA but sporulation by *T. sp. 'viride'* 38 resulted in a change in agar colour from cream to yellow-brown and *T. crassum* 26 and *T. viride* 101 cultures had an appressed dense white growth.

5.3.3.2 Wood decay

As discussed in 5.3.1., the *Trichoderma* cultures did not cause significant weight loss of *Pinus radiata* relative to the uninoculated control treatments. In Assay 2, the weight losses by the seven *Trichoderma* cultures were less than 1.2% (Table 5.2) and in Assay 3, the weight losses by *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 were less than 2.2% (Table 5.4). In Assay 4, the weight losses by *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 were less than 1.1% after the 21 and 28 d precolonisation periods (Appendix 12.2).

5.3.4 Field and controlled temperature room trials

In the field and CT room trials, the uninoculated control treatment had the highest mean wood block dry weight. The mean dry weights of 9.6 and 9.7 g, respectively, were not different to the initial dry weight of 125 untreated blocks (9.7 ± 0.67 g). This sample of pretreatment blocks had a mean fresh weight of 23.1 ± 1.19 g and a mean moisture content of $137 \pm 13.6\%$. In Table 5.6, the mean block dry weight for each treatment has been converted to dry weight loss (%) relative to the mean block dry weight of the uninoculated control treatment. The maximum weight loss for an individual wood block is also given for each treatment to give an indication of the range, as in all treatments there were some blocks that had no weight loss.

Table 5.6 The influence of pre- and post-inoculation of *Trichoderma crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 on mean and maximum weight loss of *Pinus radiata* by *Gloeophyllum sepiarium* in field and controlled temperature room trials.

Treatment	Wood weight loss (%)			
	Field		Controlled temperature room	
	Mean	Maximum	Mean	Maximum
<i>T. crassum</i> 26/ <i>G. sepiarium</i>	8.4	26.5	6.7	24.4
<i>T. sp. 'viride'</i> 38/ <i>G. sepiarium</i>	4.3	25.8	4.4	22.4
<i>T. viride</i> 101/ <i>G. sepiarium</i>	15.3	58.4	7.9	25.5
Water/ <i>G. sepiarium</i>	0.9	26.9	0.0	22.8
sed (P<0.05)	2.20		2.19	
<i>G. sepiarium</i> / <i>T. crassum</i> 26	5.0	18.2	6.5	13.4
<i>G. sepiarium</i> / <i>T. sp. 'viride'</i> 38	5.4	19.2	6.6	15.3
<i>G. sepiarium</i> / <i>T. viride</i> 101	10.0	75.8	5.6	21.3
<i>G. sepiarium</i> /water	5.1	43.6	5.7	20.8
sed (P<0.05)	2.18		2.11	

The maximum weight losses were higher in the field than the CT room trial, but this was not reflected in the mean weight losses except for the *T. viride* 101 treatments. Weight losses for the *G. sepiarium* control treatments were low although there were losses of up to 43.6% in the field and 22.8% in the CT room. The water application was used to simulate application of the *Trichoderma* treatment solutions. Weight losses were lower in the water-*G. sepiarium* treatment than the *G. sepiarium*-water treatment which suggests that *G. sepiarium* had difficulty in colonising wood blocks of a high moisture content. The differences in the *G. sepiarium* control treatments cannot be attributed to differences in inoculum batches as *G. sepiarium* was applied using aliquots of the same batch of inoculum.

The *Trichoderma* spore concentrations produced on bran-peat medium were similar to those produced on MA (in 5.2.3.1). For *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 respectively, the spore production on bran-peat medium was 19, 120 and $0.12 \times 10^6 \text{ mL}^{-1}$ in comparison to 19, 100 and $0.20 \times 10^6 \text{ mL}^{-1}$ on MA.

The *Trichoderma* treatments resulted in similar or higher mean wood weight losses than the *G. sepiarium* control treatments, in both the field and CT room trials. In the field trial, the *T. viride* 101 treatments resulted in a significantly higher weight losses than the other treatments which were not significantly different. Weight losses were higher in the

T. viride 101-*G. sepiarium* treatment, but the maximum weight loss was higher for the *G. sepiarium*-*T. viride* 101 treatment. In the CT room trial, there were no significant differences in mean weight loss between the treatments.

Fruiting bodies of *G. sepiarium* were commonly observed, particularly in the field trial and it was obvious that significant degradation by *G. sepiarium* had occurred in some blocks (Photo 5.4).



Photo 5.4 *Pinus radiata* test blocks in the field trial showing (top) *Gloeophyllum sepiarium* fruiting bodies (note the missing central block used for assessments of the resident microflora) and (bottom) the wood degradation after eight months incubation.

There were a number of problems associated with the field trial. The intention was to leave the pails in the prevailing environmental conditions for the duration of the trial. However, the conditions in the first four weeks after inoculation were not conducive to successful colonisation and growth of *G. sepiarium* or the *Trichoderma* cultures. Although the maximum and minimum air temperatures were suitable for fungal growth,

there was a lack of rainfall. A total of less than 1 mm over the first three weeks resulted in the splitting of some wood blocks and drying out of the soil. After 10 days, it was decided to add distilled water to the pails every second day. The volume of water addition, typically around 400 mL per pail, was determined by the loss in weight of four additional pails with untreated wood blocks. The three weeks of drought were followed by a week with a total of 69 mm, with 35 mm recorded in a single day. This resulted in temporary flooding of the pails despite a number of drainage holes in each pail. Brown et al. (1999) monitored the moisture content of wood blocks in the field and found fluctuations that did not appear to be seasonal but were associated with individual rainfall events. In the CT room, there was no water input which may have caused the low wood moisture contents and weight losses of less than 25.5%. In contrast, the weight losses of blocks in the field trial ranged up to 75.8%.

After two months incubation, there were a range of fungi and bacteria isolated from the sampled wood blocks. These microorganisms may have been resident prior to application of treatments or have colonised the blocks at some stage following. Species of *Trichoderma*, *Penicillium* and sapstain fungi were present in all of the blocks. Visual staining occurred in many blocks within the first week of incubation. There were also *Fusarium*, *Alternaria*, *Acremonium*, fungi from the Mucoraceae, and bacteria and yeast species. There appeared to be a greater range of microorganisms in blocks from the uninoculated control treatment but there were no clear differences in microorganisms from the different treatments or from field and CT room trial blocks.

5.4 DISCUSSION

Wood biological control assays

The wood biological control assay was developed to investigate the concepts of biocontrol and bioprotection (Freitag et al. 1991). Biocontrol was investigated using 'basidiomycete precolonised blocks' and bioprotection was investigated using the '*Trichoderma* precolonised blocks'. The relative colonisation ability of the *Trichoderma* and basidiomycete fungi was investigated using 'sterile blocks'. Wood weight loss was a reflection of the extent of colonisation and decay by the basidiomycete fungus and the degree of biological control by the *Trichoderma* fungi. However, the success of a

biological control treatment cannot be based solely on the basis of low wood weight loss or fungal survival as brown rot fungi can cause extensive reductions in wood strength at low weight losses (Wilcox 1978). The assay of Freitag and Morrell (1990) used soil as a base substrate and assessed the weight loss of only 'sterile blocks', with no fungal viability testing at the conclusion of the assay.

The wood biological control assays showed that the *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 treatments had significant biological control and bioprotection potential against *Gloeophyllum sepiarium*. Weight losses in all three wood block types of these treatments were significantly lower than the *G. sepiarium* control. In 'G. sepiarium precolonised blocks', the mean wood weight losses were higher in Assay 2 for the three treatments and there was viable *G. sepiarium* reisolated from the blocks in comparison to Assay 1 and 3. This may be due to the 28 d precolonisation time in Assay 2 compared to 21 d in Assay 1 and 3. The additional time may have allowed *G. sepiarium* to become more established in the wood blocks and made it more difficult for the *Trichoderma* fungi to colonise, inhibit weight loss and to result in loss of *G. sepiarium* viability. Only *T. sp. 'viride'* 38 in Assay 3 resulted in a mean weight loss that was not significantly different to the uninoculated control treatment. The 'sterile blocks' and '*Trichoderma* precolonised blocks' for the three treatments had low weight losses (less than 1.2 and 1.0%, respectively) that were not significantly different to the uninoculated control treatment. Viable *G. sepiarium* was only reisolated from 'sterile blocks' in Assay 2. The additional precolonisation time in Assay 2 may have allowed the *Trichoderma* to become more established in '*Trichoderma* precolonised blocks' and made it more difficult for *G. sepiarium* to colonise. Also, the *Trichoderma* may have had an advantage in colonisation of the adjacent 'sterile blocks' and thus inhibited *G. sepiarium* colonisation and subsequent weight loss.

The three *Trichoderma* treatments were less inhibitory towards *Phlebiopsis gigantea*, although *T. crassum* 26 showed bioprotection potential. The mechanism of *Trichoderma* inhibition may be basidiomycete species-specific, unlike many wood preservatives which are broad-spectrum antifungal compounds.

The weight loss of blocks in the uninoculated control treatment was likely to be due to handling and processing at harvest and errors from the drying and weighing process. The uninoculated control treatment showed variation in mean weight losses between the

assays but the losses were less than 1.8%. Similarly, the *Trichoderma* control treatments showed variation between the assays but weight losses were less than 2.2%. For the *G. sepiarium* control treatments, there were lower weight losses in Assay 3 (13.3 to 19.0%) compared to Assay 1 (21.0 to 26.2%) or Assay 2 (17.7 to 26.0%). This may be due to the use of different batches of fungal inoculum and differences in wood parameters. In comparison to the decay assays of Chapters 2 and 3 that used 20 mm wood cubes, the smaller 15 x 15 x 5 mm blocks in the biological control assays may allow greater weight losses to occur within a set incubation period, and also allow for an easier trial setup and replication. Blocks of the four biological control assays ranged in mean fresh weight from 1.047 to 1.346 g; dry weight from 0.443 to 0.558 g; and moisture content from 136 to 161%. Blocks used for Assay 2 had the lowest fresh weight, dry weight and moisture content. This may have contributed to the higher weight losses in '*G. sepiarium* precolonised blocks'.

In a screen of 60 *Trichoderma* cultures against *Postia placenta* in *Pseudotsuga menziesii*, Dawson Andoh and Morrell (1991) found 32 cultures showed activity but there was variation in activity between species and between isolates of a species. *Trichoderma* species are often reported to be unable to eliminate established basidiomycete fungi from wood but are generally effective antagonists in a bioprotection capacity (Hulme and Shields 1975; Score et al. 1998; Toole 1971). Tucker et al. (1997) screened two *T. viride* cultures against a range of basidiomycete fungi in three wood block assays (an agar-based assay, a soil-based assay and a soil-contact assay) and found complete protection of *Trichoderma* precolonised blocks irrespective of the assay or whether *Trichoderma* spores or mycelium were used. Ejechi and Obuekwe (1994) found that decay was inhibited in wood blocks simultaneously inoculated with *T. viride* and *G. sepiarium*. Biological control agents (BCA's) may not completely inhibit decay although wood weight losses may be reduced (Canessa and Morrell 1997; Crozier et al. 1999). Highley (1988) found that although *T. virens* could kill *Gloeophyllum trabeum* on agar, it was unable to stop decay in wood precolonised by *G. trabeum* and this was attributed to differences in nutrients between agar and wood.

The sources of the 23 *Trichoderma* cultures screened in Assay 1 were: Tokoroa (8), Rotorua (3), Hamilton (2) and Landcare Research-*Lentinus edodes* medium (8) and *Pleurotus pulmonarius* medium (2). The sources of the seven *Trichoderma* cultures for Assay 2 were Tokoroa (*T. sp.* 'long' 15 and 24, *T. crassum* 26 and *T. sp.* 'viride' 38),

Rotorua (*T. sp.* 80 and *T. viride* 101) and Landcare Research-L. edodes medium (*T. sp.* 'viride' 117). The sources of the three *Trichoderma* cultures for Assay 3 and 4 and the field and CT room trials were Tokoroa (*T. crassum* 26 and *T. sp.* 'viride' 38) and Rotorua (*T. viride* 101). In biological control studies, *T. harzianum* and *T. virens* are the most commonly cited *Trichoderma* species (Chet 1987; Papavizas 1985). Although these species were not represented in the identified cultures, they may have been in the remaining 129 cultures that were studied in Chapter 4.

The wood chemical analyses showed that *G. sepiarium* degraded hemicelluloses in preference to cellulose. The loss of wood components by *G. sepiarium* (as a percentage of that in the uninoculated control blocks) was greatest for mannose, followed by galactose, arabinose and xylose, with no apparent cellulose or lignin degradation. In Chapter 2, *G. sepiarium* had average CMCase activity, insignificant xylanase activity but relatively high mannanase activity, and regression analyses showed that high mannanase activity was indicative of brown rot fungi. Kirk (1973) also observed that brown rot fungi removed mannan faster than cellulose on coniferous wood, and they proposed that degradation of cellulose depended on the prior removal of the hemicellulose component. Further support was given by Eriksson et al. (1990) who found that both brown rot and white rot fungi showed preferential degradation of the hemicellulose wood components. The highest loss was generally of mannose but the higher decaying fungi resulted in the same or slightly greater loss of xylose. *Gloeophyllum sepiarium* tested negative for laccase, peroxidase and proteinase (see Chapter 2) but the wood chemical analyses showed that there was some ligninolytic activity as acid-soluble lignin content was lower in *G. sepiarium* control wood blocks than in uninoculated control blocks.

***Trichoderma* identification**

Rifai (1969) divided *Trichoderma* into nine species aggregates on the basis of morphological features. A revision was carried out by Bisset (1991a) who proposed a sectional classification for *Trichoderma* recognising five sections: *Trichoderma*; *Longibrachiatum*; *Saturnisporum*; *Pachybasium*; and *Hypocreanum*. Bisset (1991b) used conidiophore and conidium morphology to differentiate and describe ten new species and assign them to section *Pachybasium* (Sacc.) Bissett.

Trichoderma crassum 26 was one of the new species, some characteristics of which are: dark green conidia, obovoid to broadly ellipsoid; broad conidiophores sparingly branched aggregated into flat pustules on MA; aerial hyphae more than 1.5 μm diameter; straight smooth sterile apical elongations; large chlamydospores; rapid growth rate of more than 65 mm in 4 d at 20°C; reverse dull yellowish to amber in colour; and indistinct odour.

Trichoderma viride 101 is a culture of the most common mould fungus isolated from *Pinus radiata* by Butcher (1967) and Butcher and Drysdale (1991). *Trichoderma* sp. 'viride' 38 and 117 are cultures that Dr Gary Samuels identified as closely related species. *Trichoderma viride* is the type species in the section *Trichoderma* and some characteristics are: blue-green conidia; globose conidia rough walled; pyramidally branched conidiophores; rapid growth rate of 50 to 90 mm in 4 d at 20°C; reverse colourless to dull yellowish; and some isolates have an odour resembling coconut.

Trichoderma sp. 'long' 15, 24 and 80 cultures refers to a morphology (a long conidiophore) that characterises an unnamed species that Dr Gary Samuels has commonly observed in North America and Europe, the anamorph of a *Hypocrea* species. Some characteristics of this species are: a long conidiophore with a cluster of shorter, fatter phialides at the base and a few slender phialides at the tip; relatively large ellipsoidal conidia; tendency to form 1 to 3 mm diameter pustules; and no odour.

Wood decay potential

Some *Trichoderma* species e.g. *T. reesei*, *T. viride* and *T. koningii* are used for commercial cellulase production (Reese and Mandels 1989). Variation in the production of decay enzymes has been observed between *Trichoderma* species and between isolates of a species (Danielson and Davey 1973b). There has been research to find or produce *Trichoderma* cultures that express high enzyme activity for use in biotechnology. Gadgil et al. (1995) used ultraviolet radiation and sodium nitrite to produce a mutant of *T. reesei* that had CMCCase activity of 6.5 IU mL⁻¹ compared to 3.6 IU mL⁻¹ for the original culture. The carbon source on which the *Trichoderma* species are grown can markedly influence enzyme activity. *Trichoderma* species have been found to produce similar or higher mannanase activities when grown on cellulose than on mannan medium (Haltrich and Steiner 1994; Reese and Shibata 1965). Gashe (1992) found that *Trichoderma* sp. A-001 on CMC had CMCCase activity of 25 IU mL⁻¹ but on filter-paper had 167 IU mL⁻¹.

In fermentor studies, Bailey et al. (1993) found that optimal cellulase production by *T. reesei* was at a minimum pH of 4 compared to pH 6 to 7 for xylanase. The pH of the enzyme liquid medium was 5.6 prior to autoclaving. The glucose-yeast extract medium (Appendix 1.3) was developed by Gashe (1992) who found that KNO_3 as a nitrogen source and the addition of Tween 80 at 0.2% and a pH of 5.5 optimised cellulase production and activity by *Trichoderma* species.

The presence and activity of decay enzymes could be a biological control screening criterion to select *Trichoderma* cultures with low or no decay potential. *Trichoderma* sp. 'viride' 38 and 117 and *T. viride* 101 were relatively high producers of CMCase, *T. sp.* 'long' 15, 24 and 80 cultures were relatively high producers of xylanase, and *T. sp.* 'long' 15 was the highest producer of mannanase. These enzyme activities were not, however, reflected in significant wood weight losses in *Trichoderma* control treatments in the biological control assays. Eveleigh (1986) also noted that although some *Trichoderma* species had relatively high cellulolytic activity, they had limited wood decay ability and Canessa and Morrell (1997) found that the *Trichoderma* species they studied did not result in significant wood weight losses. The enzyme activities measured in liquid media may or may not be comparable to wood decay but the presence of activity showed that the fungus had the capability to produce the enzyme under some conditions. The wood chemical analyses suggested that the *Trichoderma* cultures had ligninolytic activity but in the agar-based assays, the presence of laccase and peroxidase were not detected. Score et al. (1997) observed peroxidase but not laccase activity in cultures of *T. viride* and *T. harzianum*. They hypothesised that *Trichoderma* species could produce laccase, in the presence of basidiomycete fungi, to degrade defence pigments that are produced by the decay fungus. Assavanig et al. (1992) characterised isoenzymes of laccase and found that they can vary between *Trichoderma* species and between isolates of a species, and have different functions under different environmental conditions.

~~*Trichoderma viride* and *T. sp. viride* cultures had the highest CMCase activities of the seven *Trichoderma* cultures screened (0.544 to 0.679 IU mL⁻¹). Vipan et al. (1994) found *T. viride* had the highest CMCase activity, of the five cellulolytic fungi studied, with 8.56 IU mL⁻¹ under optimised conditions. There is considerable variation within the species as Yamanobe et al. (1987) and Mandels et al. (1974) reported CMCase activities of 89 and 144 IU mL⁻¹, respectively, for *T. viride*.~~

Of the three *T. viride* or *T. sp. 'viride'* cultures, *T. sp. 'viride'* 117 had the highest cellulase, xylanase and mannanase activity and accumulated fungal biomass. This was supported by the wood chemical analyses which showed that blocks from the *T. sp. 'viride'* 117-*G. sepiarium* treatment had the lowest glucose, arabinose, galactose, and xylose contents. The origin of *T. sp. 'viride'* 117 was *L. edodes* medium. Tokimoto (1982) found that *Trichoderma* species from edible fungi growth medium produced chitinolytic enzymes that led to serious losses of edible fungi. A chitinase screen was not carried out as, although *T. sp. 'viride'* 117 was the best sporulator of the seven test *Trichoderma* on MA, the culture showed the lowest biological control potential in Assay 2 and was not selected for further study.

Trichoderma crassum 26, *T. sp. 'viride'* 38 and *T. viride* 101 tested negative for laccase, peroxidase and proteinase but the wood chemical analyses showed that there was some ligninolytic activity as Klason lignin and acid-soluble lignin contents were lower in *Trichoderma*-colonised wood blocks than in uninoculated control blocks. The lowest lignin contents were in blocks of *T. sp. 'long'* 80 and *T. viride* 101 control treatments.

Freitag et al. (1991) studied the interactions between a decay fungus and a potential *Trichoderma* BCA by measuring enzyme activities in mixed liquid cultures of *Trametes versicolor* and *Trichoderma harzianum*. They proposed that the presence of laccase (not produced by *T. harzianum*) indicated the viability of the laccase-producing *Trametes versicolor*. The medium pH adjusted over time to the optimum pH for *Trichoderma harzianum* which suggested physiological dominance over *Trametes versicolor*. The enzyme activities were an indication of the outcome of the interactions but fungal biomasses could not be distinguished, so it was concluded that enzymatic studies on wood were required. Nilsson (1974) developed an assay for the detection of cellulases and hemicellulases in sections of wood blocks inoculated with microfungi but there were problems related to either insufficient enzyme production or binding of enzymes to the wood or mycelium. Freitag and Morrell (1992) found that there were no correlations between activity of any of the enzymes studied, and fungal biomass. No significant correlations were found between CMCase and xylanase activities, and the respective biomasses accumulated, but there was a significant correlation between mannanase activity and biomass. Accumulated fungal biomasses of the *Trichoderma* cultures were comparable to the basidiomycete fungi in xylan and mannan medium but significantly higher in CMC medium.

Field and controlled temperature room trials

There have been relatively few field trials using *Trichoderma* fungi to protect untreated timber, particularly that in soil contact which represents a high decay hazard and a challenge for biological control systems. The earliest biological control field trials were carried out on birch logs (Shields and Atwell 1963) and subsequent studies have concentrated on the biological control of detrimental fungi in utility poles using *Trichoderma* species (Bruce and King 1986b; Ricard et al. 1969). These researchers found variable levels of control that were adversely affected by the resident microbial population.

In microbial succession studies, basidiomycete colonisation of wood is reported to succeed 'pioneer or primary coloniser species' which include sapstain and mould fungi such as *Trichoderma* species (Butcher 1967; Butcher and Drysdale 1991). Hulme and Shields (1972b) noted that if the mould is the primary coloniser, subsequent colonisation and decay by decay fungi could be inhibited. If the mould is the secondary coloniser, the interaction with decay fungi would depend on the growth conditions, the relative colonisation rates and the time period before secondary colonisation. *Trichoderma* fungi are frequently isolated from well-decomposed organic matter in forest soils which indicates that they are secondary colonisers (Danielson and Davey 1973a). Bruce et al. (1989) showed that *Trichoderma* still had biological control activity against decay by *Lentinus lepideus* even when the wood blocks were colonised by mould fungi prior to *Trichoderma* treatment.

Non-sterile test blocks and sources of external contamination were likely to have influenced colonisation, growth and survival of *G. sepiarium* and *Trichoderma* fungi. It was not known whether the microorganisms that were reisolated from the blocks, 2 months into the trial incubation period, had been present before treatment or had colonised at some stage following. Isolations had not been carried out on untreated blocks to determine the initial range of organisms present. Leben (1978) devised a laboratory method for selecting BCA's for field testing, that comprised of dual-inoculation of surface-sterilised wood blocks. Bacteria were commonly observed in these blocks as in this thesis study. Both field and CT room trial sites had been used previously for sapstain studies. The incidence of sapstain discolouration is reported to be greater at temperatures of more than 15°C (Kay and Ah Chee 1999) which was the case during the trial period.

In the field and CT room trials, there was low or no wood weight loss in the *G. sepiarium* control treatment. Ideally, this treatment would have resulted in the highest weight loss and the *Trichoderma* treatments in lesser weight losses depending on their biological control potential. There are a number of possible reasons why little wood degradation had occurred in the *G. sepiarium* control treatments. The physical and environmental conditions may not have favoured *G. sepiarium* colonisation of the wood blocks. *G. sepiarium* may not have been competitive enough to displace the resident microbial population or to prevent subsequent microbial colonisation. There were significant weight losses in the *T. viride* 101 treatments, particularly in the field trial with weight losses of up to 75.8%, which suggested that *G. sepiarium* had the potential for significant wood degradation. *Trichoderma viride* 101 may have inhibited the resident microbial population in the wood blocks or predisposed the wood in some way, to colonisation by *G. sepiarium*. If the relative 'competitiveness' of the *Trichoderma* cultures was assessed, *T. viride* 101 had very low sporulation on MA compared to *T. crassum* 26 or *T. sp. 'viride'* 38 but otherwise had average growth rates on agar and wood, and an average to high ability to accumulate decay enzymes in liquid media. *Gloeophyllum sepiarium* did not show a similar ability to colonise and grow in wood blocks of the *T. crassum* 26 or *T. sp. 'viride'* 38 treatments.

Verrall (1966) found that two isolates of *T. viride* did not prevent decay of pine by *G. sepiarium* and in some cases, decay was greater in the presence of the *Trichoderma* cultures than in the *G. sepiarium* controls. Ejechi (1997) found total inhibition of decay in a field trial using *T. viride* as a bioprotectant applied to wood blocks 24 h before inoculation with *G. sepiarium*. Brown et al. (1999) reported significant biological control potential of a *T. viride* culture against basidiomycete fungi in field and fungus cellar trials.

The ability to sporulate is an important criterion when considering fungal BCA's as it can reflect the ability of the fungus to colonise and survive and also determine the protocol necessary for BCA preparation, storage and application. For the *Trichoderma* cultures, there was no apparent relationship between biological control potential and spore production which was similar on MA and bran-peat medium but other growth medium may give quite different results.

Powell and Faull (1991) estimated that only 5% of BCA's selected through vigorous screening assays were effective in the field situation. From the field and CT room trials,

there were no conclusions drawn on the biological control potential of the three *Trichoderma* cultures. Significant weight losses were shown to be possible in small non-sterile *Pinus radiata* wood blocks over the period of 8 months under field and CT room conditions.

In future trials, there are a number of changes that could be considered i.e. basidiomycete inoculation with precolonised inoculum blocks, the use of surface-sterilised blocks, reduction of airborne contamination, and controlled water input. With different physical and environmental conditions, a repeat field trial may give quite different results. Further trials could investigate: timing of *Trichoderma* inoculation i.e. pre-, dual- or post-inoculation of decay fungus; the form of *Trichoderma* inoculum and concentration; and an inoculum of mixed *Trichoderma* species. The biological control potential of the most promising *Trichoderma* cultures could be tested prior to field trials, under controlled laboratory conditions in high wood decay hazard assays with soil replacing the vermiculite, and the scale of the test blocks could be increased to match those for field trials. A wider screening and evaluation of *Trichoderma* species against basidiomycete fungi could incorporate observations of the interactions between the basidiomycete fungi and other wood fungi in the presence of *Trichoderma* species, but with known fungal species, initially on agar, as for Schoeman et al. (1996), and then using non-sterile or surface-sterilised wood blocks.

In conclusion, the wood biological control assays showed that *Trichoderma crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 had significant biological control potential against *Gloeophyllum sepiarium*, particularly with respect to bioprotection of *Pinus radiata*. *Trichoderma crassum* 26 also showed bioprotection potential against *Phlebiopsis gigantea*. The biological control potential of the *Trichoderma* cultures against *Schizophyllum commune* was not determined due to the insignificant weight loss of *Pinus radiata* by this fungus under the given assay conditions. Concurrent with the field trial, which was the final long-term experiment, were investigations into the modes of antagonism shown by *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 towards *G. sepiarium*, and these are reported on in Chapter 6.

Chapter 6 MODES OF ANTAGONISM OF *TRICHODERMA* CULTURES AGAINST BASIDIOMYCETE FUNGI

6.1 INTRODUCTION

The *Trichoderma* species identified in Chapter 5 as having the greatest biological control potential were *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101. The objective of Chapter 6 was to study the possible modes of their antagonism towards *Gloeophyllum sepiarium*, *Phlebiopsis gigantea* and *Schizophyllum commune* 3. The ability of each *Trichoderma* culture to produce inhibitory volatile compounds during growth on agar and to produce inhibitory non-volatile compounds during growth on agar, *Pinus radiata*, and in liquid medium was assessed in assays with each *Trichoderma* culture alone and in the presence of the basidiomycete fungus. The volatile and non-volatile assays on agar were commonly used in screening microorganisms for the production of inhibitory compounds, and the non-volatile assays on *P. radiata* and in liquid medium were based on techniques used at HortResearch (Hamilton). In addition, investigations for evidence of mycoparasitism between the *Trichoderma* cultures and *G. sepiarium* were carried out using light microscopy.

6.2 MATERIALS AND METHODS

6.2.1 Production of inhibitory volatile compounds on agar

Trichoderma crassum 26, *T. sp. 'viride'* 38 and *T. viride* 101 were screened for the ability to produce inhibitory volatile compounds against growth of *G. sepiarium* on MA and LNA. There were eight treatments: each *Trichoderma* culture alone and in dual inoculation with *G. sepiarium* (*Trichoderma*+*G. sepiarium*); a *G. sepiarium* control treatment; and an uninoculated control treatment. There were triplicate glass Petri dishes (110 mm diameter x 20 mm high) for each treatment. The MA and LNA media were poured to cover both the base and the lid of the Petri dishes. A 7 mm agar core of *G. sepiarium* (6 d old culture) was inoculated in a central position on the agar in the lid of each Petri dish. The base and lid of each Petri dish were reunited and incubated for 4 d (MA) or 6 d (LNA) at 25°C.

After the incubation period, the colony diameter of *G. sepiarium* was measured in two predetermined directions. A 7 mm agar core of *G. sepiarium* or *Trichoderma* culture, alone and in dual inoculation, was inoculated in a central position on the agar in the base of each Petri dish. For the dual inoculation treatments, the two cores were placed 10 mm apart. For the MA assay, the agar cores were taken from a 10 d old *G. sepiarium* culture and 3 d old *Trichoderma* cultures. For the LNA assay, the agar cores were taken from a 12 d old *G. sepiarium* culture and 5 d old *Trichoderma* cultures. Sterile agar cores were used for the uninoculated control treatment. The base and lid of each Petri dish were again reunited and incubated at 25°C.

The colony diameter of *G. sepiarium* on the Petri dish lid was measured in two predetermined directions after 2 and 4 d. At each time, the mean colony diameter of *G. sepiarium* was calculated for each treatment. The results for each agar medium were analysed statistically using analysis-of-variance. The mean colony diameter of *G. sepiarium* prior to application of treatments (day 0) was used as a covariate.

6.2.2 Production of inhibitory non-volatile compounds on agar

Assay 1 Production of inhibitory non-volatile compounds by *T. crassum* 26, *T. sp.* 'viride' 38 and *T. viride* 101 (alone and in dual inoculation with *G. sepiarium*) against *G. sepiarium* after 3 d incubation on MA and LNA

There were eight treatments as described in 6.2.1., with triplicate glass Petri dishes (110 mm diameter x 20 mm high) for each treatment. Cellophane sheets (110 mm diameter) were autoclaved (121°C for 20 min) and placed on the agar surface of each Petri dish. A 7 mm agar core of *G. sepiarium* or *Trichoderma* culture, alone and in dual inoculation, was inoculated in a central position on the cellophane sheet. For the dual inoculation treatments, the two cores were placed 10 mm apart. The agar cores were taken from a 6 d old *G. sepiarium* culture and 8 d old *Trichoderma* cultures. Sterile agar cores were used for the uninoculated control treatment. The Petri dishes were incubated at 25°C.

After 3 d, the cellophane sheet and agar core(s) were removed and a 7 mm agar core of *G. sepiarium* (9 d old culture) was inoculated in a central position on the agar. The Petri dishes were reincubated at 25°C.

The colony diameter of *G. sepiarium* was measured in two predetermined directions after 3, 5, 7, 9, 11 and 14 d (MA assay) and after 5, 7, 9, 11 and 14 d (LNA assay). At each time, the mean colony diameter of *G. sepiarium* was calculated for each treatment. The results for each agar medium were analysed statistically using analysis-of-variance and comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level.

Assay 2 Production of inhibitory non-volatile compounds by *T. crassum* 26, *T. sp.* 'viride' 38 and *T. viride* 101 (alone and in dual inoculation with *G. sepiarium*) against *G. sepiarium* after 2 d incubation on MA and LNA

This assay was a repeat of Assay 1 but the treatments were incubated for 2 d at 25°C before the cellophane sheet and agar core(s) were removed. The agar cores were taken from 8 d old *G. sepiarium* and *Trichoderma* cultures and the *G. sepiarium* culture used for inoculation after cellophane removal was 10 d old.

Assay 3 Production of inhibitory non-volatile compounds by *T. crassum* 26, *T. sp.* 'viride' 38 and *T. viride* 101 against *G. sepiarium*, *P. gigantea* and *S. commune* after 2 d incubation on MA

A 7 mm agar core of the *Trichoderma* fungus (8 d old cultures) was inoculated in a central position on the cellophane sheet. After 2 d incubation at 25°C, the cellophane and agar core(s) were removed and a 7 mm agar core of the basidiomycete fungus (10 d old culture) was inoculated in a central position on the agar. The Petri dishes were incubated at 25°C.

The colony diameter of *G. sepiarium* was measured in two predetermined directions after 3 and 5 d and then daily until 14 d after inoculation; *P. gigantea* at 3, 5 and 6 d after inoculation; and *S. commune* at 3, 5, 6, 7 and 8 d. At each time, the mean colony diameter of the basidiomycete fungus was calculated for each treatment. The results for each basidiomycete fungus were analysed statistically using analysis-of-variance and comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level.

6.2.3 Production of inhibitory non-volatile compounds on *Pinus radiata*

Trichoderma crassum 26, *T. sp. 'viride'* 38 and *T. viride* 101 were screened for the ability to produce inhibitory non-volatile compounds against *G. sepiarium* on *P. radiata*, using the eight treatments described in 6.2.1. Wood blocks (50 x 50 mm cross-cut x 5 mm thick) were produced from freshly harvested *P. radiata* sapwood obtained from CHH Timber Putaruru Mill. The blocks were sterilised by gamma irradiation (56.4 kGy; Schering Plough Animal Health Ltd., Upper Hutt) and then placed in independent sterile Petri dishes. There were ten Petri dish replicates for each treatment.

Assay 1 Agar inoculum

The blocks were inoculated with a 6 mm agar core of *G. sepiarium* or *Trichoderma* culture, alone and in dual inoculation, and the Petri dishes were incubated at 25°C. The agar cores were taken from 6 d old *G. sepiarium* and *Trichoderma* cultures. Sterile agar cores were used for the uninoculated control treatment. After 10 weeks, the Petri dishes and wood blocks were sterilised by gamma irradiation (52.4 kGy). The blocks were then inoculated in a central position with a 6 mm agar core of *G. sepiarium* (11 d old culture). After reincubation at 25°C for 7 d, each block was examined to determine whether growth had occurred from the core. Observations of *G. sepiarium* colony morphology and visual differences in growth on the blocks were noted. Measurements of *G. sepiarium* growth were not possible due to the difficulty in distinguishing *G. sepiarium* and *Trichoderma* mycelia.

Assay 2 Liquid medium inoculum

The blocks were inoculated with 500 µL aliquots of *G. sepiarium* or the test *Trichoderma*, alone and in dual inoculation, and the Petri dishes were incubated at 25°C. The liquid inoculum was taken from 7 d old *G. sepiarium* and *Trichoderma* cultures, both grown in malt extract medium (Appendix 1.7), 100 rpm at 25°C, in the light. Sterile aliquots of medium were used for the uninoculated control treatment. After 3 weeks, the Petri dishes and wood blocks were sterilised by gamma irradiation (54.0 kGy). The blocks were then inoculated in a central position with a 6 mm agar core of *G. sepiarium* (14 d old culture). After reincubation at 25°C for 7 d, each block was examined as for Assay 1.

6.2.4 Production of inhibitory non-volatile compounds in liquid medium

Trichoderma crassum 26, *T. sp. 'viride'* 38 and *T. viride* 101 were screened for the ability to produce inhibitory non-volatile compounds in LNM, using the same eight treatments as 6.2.1, with duplicate flasks for each treatment. The flasks were inoculated with 2 mL aliquots of *G. sepiarium* or the test *Trichoderma*, alone and in dual inoculation. The liquid inoculum was taken from a 7 d old *G. sepiarium* culture and 2 d old *Trichoderma* cultures. All fungi were grown in YM, at 100 rpm and 25°C in the light. Sterile aliquots of medium were used for the uninoculated control treatment.

6.2.4.1 Assay at 7 days

At 7 d after inoculation, each treatment was screened for the production of inhibitory compounds against *G. sepiarium*. A 6 mm agar core of *G. sepiarium* (11 d old culture) was inoculated in a central position on MA. A sample of supernatant from each flask was filter-sterilised (0.2 µM Supor Acrodisc 32 mm syringe filter) and a 10 µL aliquot was placed directly onto each *G. sepiarium* core. There were triplicate Petri dishes for each sample and a sterile distilled water control treatment. The Petri dishes were incubated at 25°C.

The colony diameter of *G. sepiarium* was measured in two predetermined directions after 6 d. The mean colony diameter of *G. sepiarium* was calculated for each treatment. The results were analysed statistically using analysis-of-variance and comparisons between individual means were carried out using Fisher's protected least-significance-difference at the 5% significance level.

6.2.4.2 High-Pressure-Liquid-Chromatography (HPLC) analysis

At 3, 7, 10 and 14 d after inoculation, 400 µL supernatant was sampled from each flask and diluted with 400 µL methanol (Analar grade) for HPLC analysis to detect the production of 6-pentyl- α -pyrone (6PAP) during the incubation period. A Shimadzu SCL-6B high pressure liquid chromatograph was used with a Prodigy 5 ODS-2 column #203339 at 35°C, A=methanol, B=20 methanol:80 water, 45% B at 1 mL min⁻¹. Ten µL of the diluted supernatant sample was run at 300 nm for 16 min isocratically and 6PAP standards (0.519 and 5.19 µg mL⁻¹) used a 30 min programme.

6.2.4.3 Ethyl acetate (EA) extracts

At 14 d after inoculation, the content of each flask was extracted in EA (using three rinses of 100 mL EA). The water fraction was removed using a separating flask and by passing the sample through a sodium sulphate column. The extracts were evaporated to dryness in a Heidolph rota-evaporator at 50°C and reconstituted in 5 mL EA for bioactivity assay.

The EA extract bioactivity assay was based on a filter-paper disc technique used for screening monoterpene compounds against *G. sepiarium* (Appendix 3). A 6 mm agar core of *G. sepiarium* (15 d old culture) was inoculated in a central position on MA and incubated for 3 d at 25°C. Filter-paper discs (6 mm dia. Whatman GF/A filter-paper, autoclaved for 20 min at 121°C) were impregnated by the test sample and two discs were placed 20 mm on either side of the *G. sepiarium* core. There were four replicate Petri dishes for each sample, and EA control and sterile distilled water control treatments. The Petri dishes were incubated at 25°C and the colony diameter of *G. sepiarium* was measured on a daily basis up to 7 d. Two measurements were of growth towards each filter-paper disc (direction 'a') and another two measurements were of growth in the tangential directions (direction 'b').

At each time, the mean colony diameter of *G. sepiarium* in direction 'a' and in direction 'b' was calculated for each treatment. The results for directions 'a' and 'b' were analysed statistically using analysis-of-variance. The mean colony diameter of *G. sepiarium* prior to incubation (day 0) was used as a covariate. Comparisons between individual means were carried out using Tukey's least-significant-difference at the 5% significance level.

Assay 1 Bioactivity of EA extracts (50 µL) against *G. sepiarium*

Filter-paper discs were placed in sterile Petri dishes and impregnated with 50 µL of the test sample, using 10 µL applications with time between applications for the EA to evaporate. The discs were then transferred to the Petri dishes of *G. sepiarium*. There was a problem with the EA causing adhesion of the filter-papers to the Petri dishes. In the following assays, the discs were placed on sterile tinfoil within the Petri dishes for application of the test samples.

Assay 2 Bioactivity of *T. crassum* 26 EA extract (10 to 50 μL) against *G. sepiarium*

Filter-paper discs were impregnated by 10, 20, 30, 40 or 50 μL of *T. crassum* 26 extract, using 10 μL applications with time between applications for the EA to evaporate.

Assay 3 Bioactivity of 6PAP against *G. sepiarium*

There were ten 6PAP concentrations screened against *G. sepiarium*: 0.05, 0.25, 0.5, 1, 1.5, 2, 2.5, 5, 10 and 20% in EA which corresponded to 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2 and 4 mg 6PAP per filter-paper disc, respectively. Each 6PAP concentration was applied at 20 μL , using 10 μL applications with time between applications for the EA to evaporate.

6.2.4.4 Solvent fractions

The EA extracts of the *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 treatments were further solvent extracted using techniques developed at HortResearch, Hamilton (Cooney, pers. comm.). Only one of the duplicate samples of each treatment was used. Each EA extract (1 mL) was placed in a preweighed glass autosampler vial and evaporated to dryness under nitrogen gas. The samples were reweighed and the dry weight was used to calculate the amount of silica for the solvent extraction columns (1 g dry weight of sample:100 g silica). The *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 samples required 1.33, 0.46 and 0.33 g of silica, respectively. The glass columns were plugged with glasswool and the appropriate weight of silica was added. Test columns were set up to determine the volume of solvent application required for the extractions. Hexane was added in aliquots until the silica showed saturation without any runoff. The *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 samples required 2000, 700 and 500 μL solvent applications, respectively. Each sample was dissolved in 100 μL dichloromethane and applied to the silica column in two applications of 50 μL so that there was an even layer of the sample on the silica. An application of hexane was used to saturate the columns prior to application of the solvent series. Each fraction was collected in a preweighed glass autosampler vial until there was no more solvent runoff from the columns, and then the next solvent was applied. The fractions were evaporated to dryness under nitrogen gas, reweighed and then stored at -20°C . The fractions were reconstituted in 0.5 mL EA and their bioactivity was screened using the method described for the EA extracts.

The solvent series:

Fraction 1. hexane

Fraction 2. 5% EA in hexane

Fraction 3. 10% EA in hexane

Fraction 4. 20% EA in hexane

Fraction 5. 50% EA in hexane

Fraction 6. EA

Fraction 7. 10% methanol in EA

Fraction 8. methanol

Fraction 9. methanol

6.2.4.5 Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis

The solvent series fractions were analysed using GC-MS. The chromatograms and spectra of each peak was examined to elucidate the possible structure and identification of the compounds involved in the inhibitory responses shown in the bioactivity assay. Gas Chromatography Flame-Ionisation-Detection (GC-FID) analysis was carried out on a Varian 3400 Gas Chromatograph (GC) fitted with a Hewlett Packard (Avondale, PA) HP-5 capillary MS column, 30 m x 0.20 mm i.d. (0.25 μm film thickness). The GC PTV injector (1078 Varian) temperature was held at 80°C for 0.25 min, then progressed at 180°C per min to 250°C and held for a further 48 min. The sample size injection was 1 μL . The initial GC column temperature was held at 60°C for 4 min, then progressed at 5°C per min to 230°C and held for 1 min, then progressed at 10°C per min to 280°C and held for a further 5 min. A full scan mass spectra (EI, 70 eV, 220°C) was taken at a 1 sec cycle time on a Varian Saturn 2000 Mass Spectrophotometer using ion trap detection.

6.2.5 Mycoparasitism

Glass coverslips (24 x 60 mm) were coated in a thin uniform layer of MA and placed on the surface of water agar (WA; Appendix 1.12), with one coverslip in each Petri dish. One end of the coverslip was inoculated with a 2 mm agar core of the *Trichoderma* species (10 d old cultures) and the other end was inoculated with a 2 mm agar core of *G. sepiarium* (10 d old culture). There were single and dual inoculated control treatments for each *Trichoderma* and *G. sepiarium* culture. There were five replicate coverslips for

each combination of fungi. The Petri dishes were incubated at 25°C. The fungi were left to grow until the leading edges of the colonies were in close proximity to each other. The coverslips were then removed from the Petri dishes and inverted onto glass chambers, sealing the edges with petroleum grease. The chambers were glass slides (25 x 76 mm) on which glass capillary tubing (1.3 mm diameter) was glued to form a frame upon which the coverslips could be placed. The chambers were autoclaved (20 min at 121°C) prior to use. Interactions between the fungi were observed using a light microscope. This experiment was repeated three times to check that the observations were consistent.

6.3 RESULTS

6.3.1 Production of inhibitory volatile compounds on agar

There were no consistent significant differences in growth of *G. sepiarium* between the *Trichoderma* treatments and the control treatments on MA or LNA (Appendix 13). Although not significant, the *T. viride* 101 treatments resulted in the greatest inhibition of *G. sepiarium* growth on both agar media. The growth rate of *G. sepiarium* was higher on MA than LNA. At 4 d after inoculation, *G. sepiarium* was still growing in the control treatments but in some of the *Trichoderma* treatments there was contamination by *Trichoderma* spores.

6.3.2 Production of inhibitory non-volatile compounds on agar

Assay 1 Production of inhibitory non-volatile compounds by *T. crassum* 26, *T. sp.* 'viride' 38 and *T. viride* 101 (alone and in dual inoculation with *G. sepiarium*) against *G. sepiarium* after 3 d treatment incubation on MA and LNA

All *Trichoderma* treatments resulted in significant inhibition of *G. sepiarium* growth, relative to the control treatments, but the level of inhibition was different on MA and LNA (Fig. 6.1) ($P < 0.001$). There were no significant differences between the *Trichoderma* alone and *Trichoderma*+*G. sepiarium* treatments so in the figures the treatments are combined for each *Trichoderma* culture.

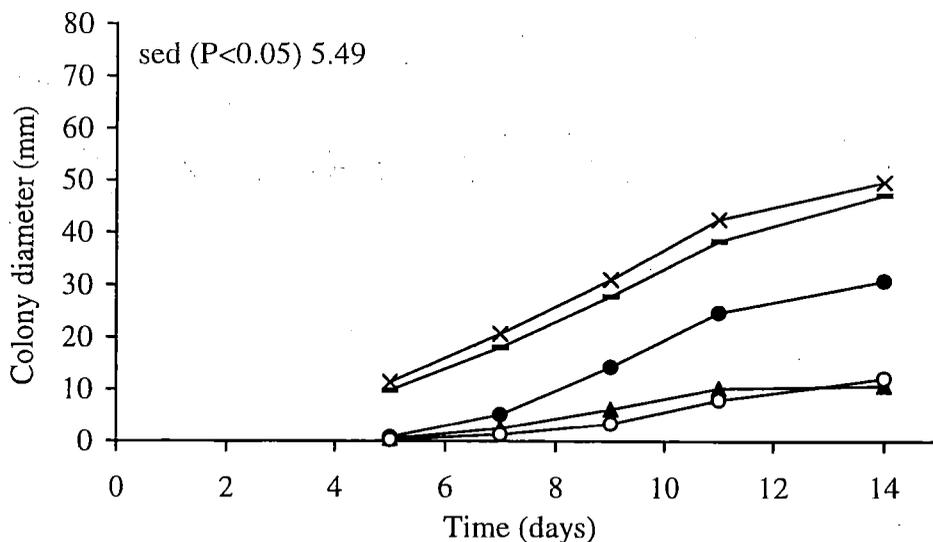
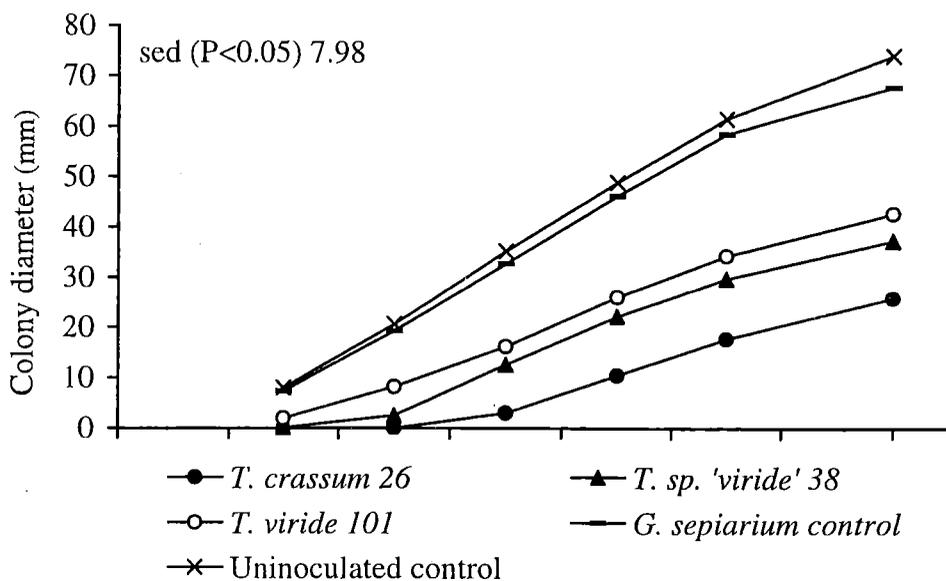


Fig. 6.1 Growth response of *Gloeophyllum sepiarium* to *Trichoderma* non-volatile treatments after 3 d incubation on (top) malt extract agar and (bottom) low nutrient agar.

On MA, the *T. crassum* 26 treatments gave complete inhibition of *G. sepiarium* growth for up to 5 d after inoculation and growth was lower for the remainder of the assay period. The *T. sp. 'viride'* 38 treatments were the next most inhibitive and *T. viride* 101 treatments the least inhibitive, although the differences were not always significant. On LNA, the *T. sp. 'viride'* 38 and *T. viride* 101 treatments (which were not significantly different) gave significantly greater inhibition than the *T. crassum* 26 treatments. Growth of *G. sepiarium* was lower in the *G. sepiarium* control treatment than the uninoculated control treatment, on both MA and LNA, but the differences were not significant.

Assay 2 Production of inhibitory non-volatile compounds by *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 (alone and in dual inoculation with *G. sepiarium*) against *G. sepiarium* after 2 d treatment incubation on MA and LNA

As for Assay 1, the *Trichoderma* alone and *Trichoderma*+*G. sepiarium* treatments were combined for each *Trichoderma* culture and there were no significant differences in growth of *G. sepiarium* in the *G. sepiarium* control treatment and the uninoculated control treatments on MA and LNA.

Trichoderma crassum 26 gave the greatest inhibition of *G. sepiarium* growth on MA and LNA, followed by *T. viride* 101 and then *T. sp. 'viride'* 38 (Fig. 6.2). On MA, the *T. crassum* 26 and *T. viride* 101 treatments (not significantly different) gave complete inhibition of *G. sepiarium* growth for up to 5 d after inoculation. The *T. sp. 'viride'* 38 treatment was the least inhibitive and was not significantly different to the control treatments for some of the assay period. On LNA, the *T. crassum* 26 treatment gave complete inhibition over the 14 d assay. The *T. viride* 101 treatment also gave significant inhibition of *G. sepiarium* growth but the *T. sp. 'viride'* 38 treatment did not.

Assay 3 Production of inhibitory non-volatile compounds by *Trichoderma crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 against *G. sepiarium*, *P. gigantea* and *S. commune* after 2 d treatment incubation on MA

There were different growth responses of the basidiomycete fungi to the *Trichoderma* treatments (Fig. 6.3). Growth of *G. sepiarium* was completely inhibited by *T. crassum* 26 and *T. viride* 101 treatments for up to 8 and 14 d after inoculation, respectively, relative to the control treatment. *Trichoderma sp. 'viride'* 38 initially caused some inhibition but by 7 d after inoculation, *G. sepiarium* growth was not significantly different to the control treatment. Growth of *P. gigantea* was significantly inhibited by the three *Trichoderma* cultures, relative to the control treatment. *Trichoderma viride* 101 gave the greatest inhibition of *P. gigantea* but there were no significant differences between *T. crassum* 26 and *T. sp. 'viride'* 38 treatments. Growth of *S. commune* was significantly inhibited by *T. crassum* 26 and *T. viride* 101 treatments (not significantly different), but not *T. sp. 'viride'* 38, relative to the control treatment.

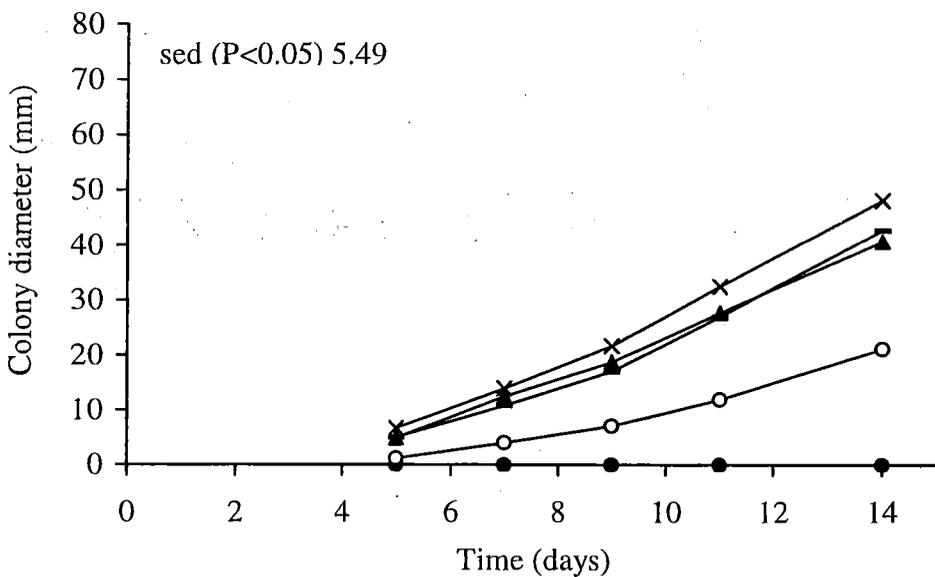
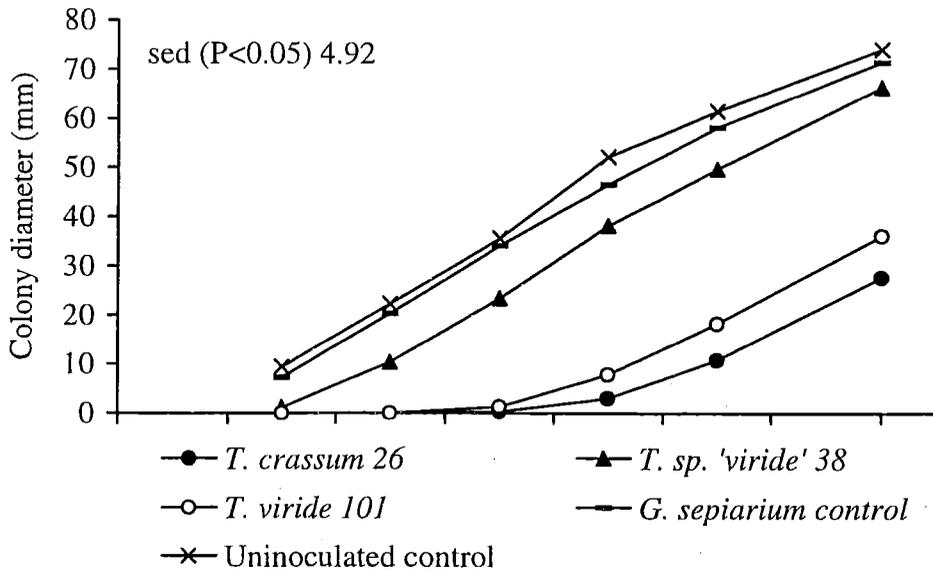


Fig. 6.2 Growth response of *Gloeophyllum sepiarium* to *Trichoderma* non-volatile treatments after 2 d incubation on (top) malt extract agar and (bottom) low nutrient agar.

6.3.3 Production of inhibitory non-volatile compounds on *Pinus radiata*

Growth of *G. sepiarium* was observed on wood-blocks of all *Trichoderma* treatments but growth was slower and sparse in comparison to the control treatments. There was up to 75, 50 and 60% inhibition of *G. sepiarium* growth for the *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 treatments, respectively, relative to the control treatments. There was variability in the initial fungal colonisation of the wood blocks and in the subsequent growth of *G. sepiarium* on the blocks.

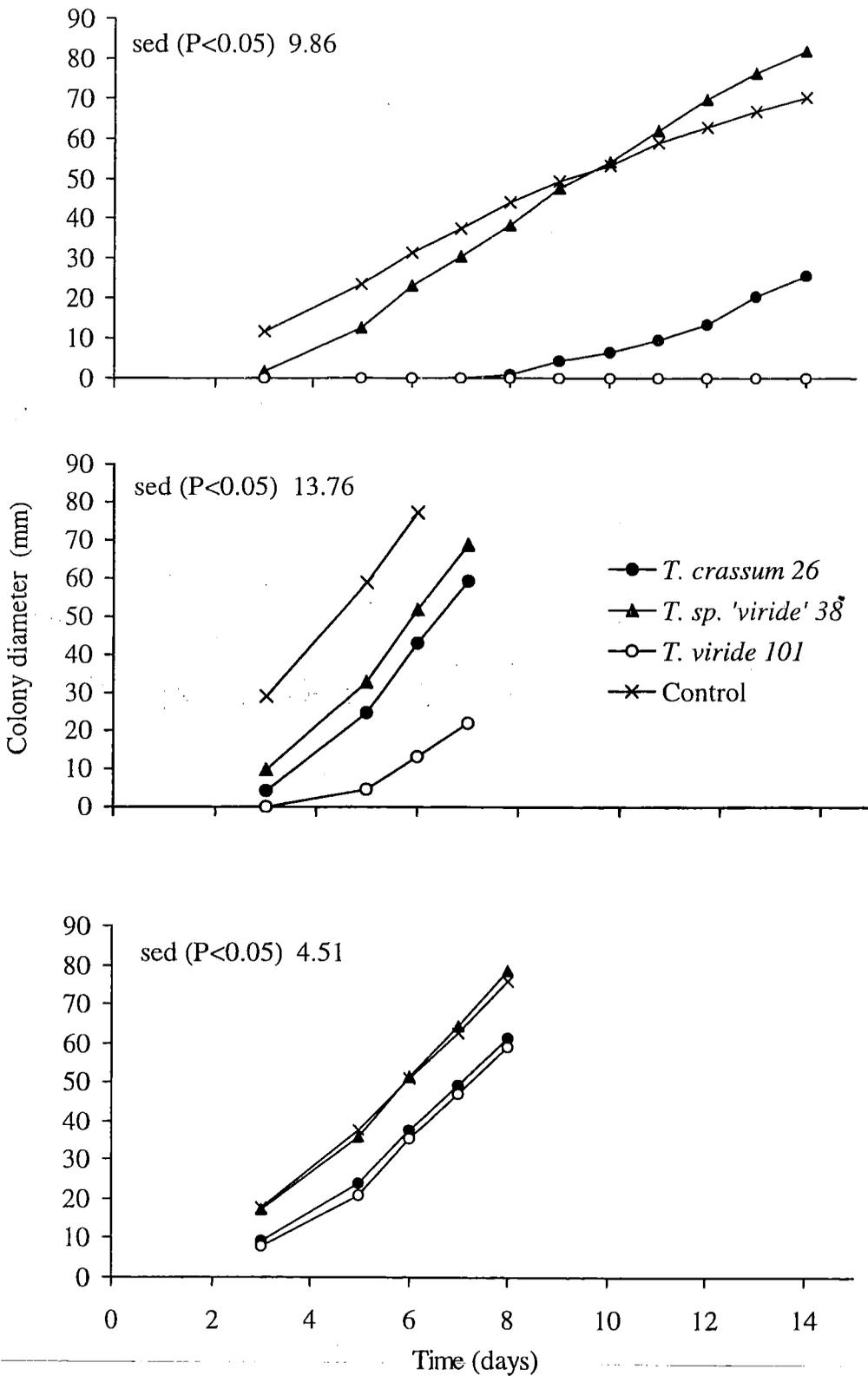


Fig. 6.3 Growth response of (top) *Gloeophyllum sepiarium*, (middle) *Phlebiopsis gigantea* and (bottom) *Schizophyllum commune* to *Trichoderma* non-volatile treatments after 2 d incubation on malt extract agar.

6.3.4 Production of inhibitory non-volatile compounds in liquid medium

6.3.4.1 Assay at 7 days

Trichoderma crassum 26 gave the greatest inhibition of *G. sepiarium* growth followed by *T. viride* 101 and then *T. sp. 'viride'* 38 and the *Trichoderma* alone treatments gave greater inhibition than the *Trichoderma*+*G. sepiarium* treatments (data not shown). No *Trichoderma* treatments resulted in significant inhibition of *G. sepiarium* growth relative to the control treatments.

6.3.4.2 High-Pressure-Liquid-Chromatography (HPLC) analysis

HPLC analysis showed that the *T. viride* 101 alone and *T. viride* 101+*G. sepiarium* treatments had produced 183 and 205 $\mu\text{g 6PAP mL}^{-1}$, respectively, at 14 d after inoculation. The *T. sp. 'viride'* 38 treatments produced less than 4 $\mu\text{g mL}^{-1}$ 6PAP and no 6PAP was detected from the *T. crassum* 26 treatments or the control treatments.

6.3.4.3 Ethyl acetate (EA) extracts

As for the agar assays, there were no significant differences between the *Trichoderma* alone and *Trichoderma*+*G. sepiarium* treatments so in the figures the treatments are combined for each *Trichoderma* culture. For the control treatments, there were no significant differences in growth of *G. sepiarium* in direction 'a' or 'b' and the morphology of *G. sepiarium* was farinaceous with growth over the filter-paper discs.

Assay 1 Bioactivity of EA extracts (50 μL) against *G. sepiarium*

There were significant differences between the *Trichoderma* treatments in inhibition of *G. sepiarium* growth in direction 'a' and 'b' (Fig. 6.4) relative to the control treatments. The *T. crassum* 26 extracts gave almost complete inhibition of *G. sepiarium* growth in direction 'a' and were the only extracts to give significant inhibition in direction 'b' with sparse growth of *G. sepiarium* towards the filter-paper discs. The *T. sp. 'viride'* 38 extracts gave significant inhibition in direction 'a' but not in direction 'b' and *G. sepiarium* morphology was farinaceous and grew around the discs. The *T. viride* 101 extracts gave no significant inhibition in direction 'a' or 'b' and *G. sepiarium* grew over the discs.

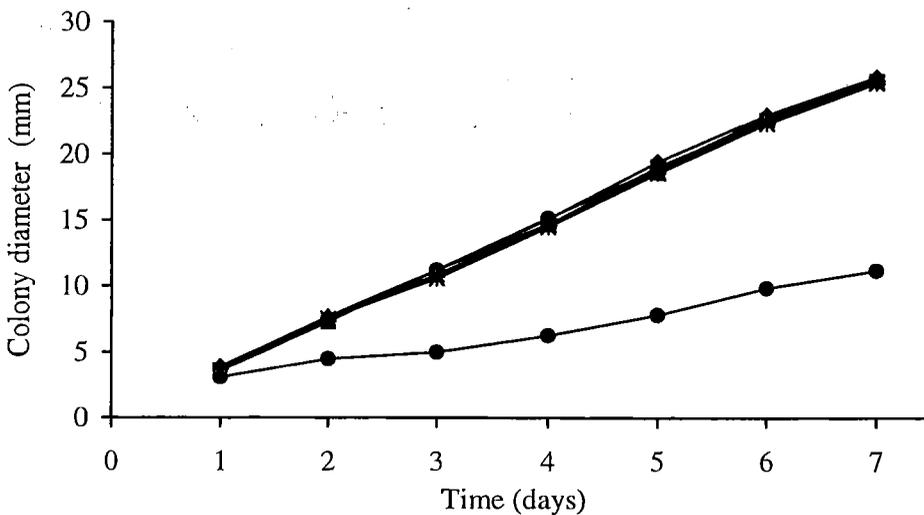
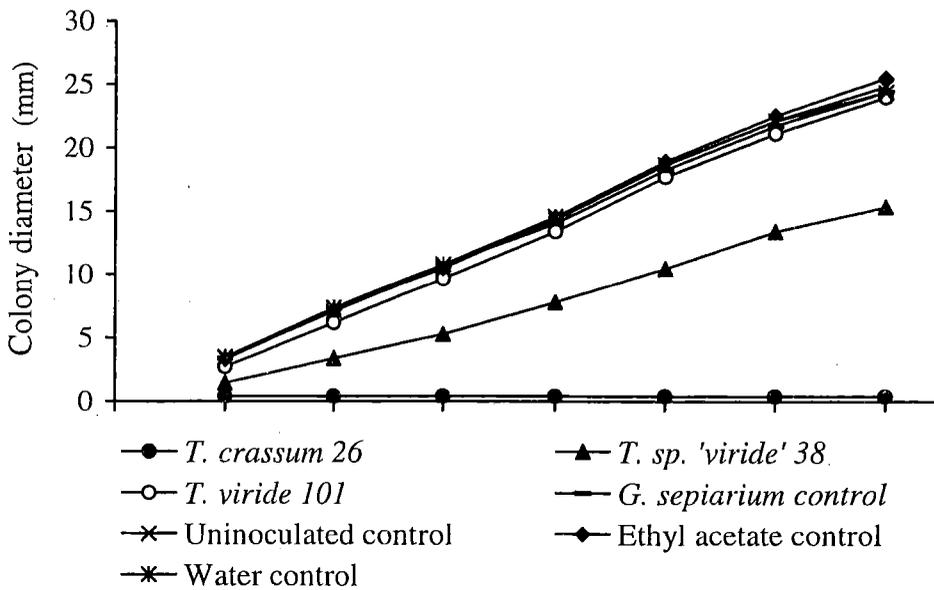


Fig. 6.4 Growth response of *Gloeophyllum sepiarium* to *Trichoderma* ethyl acetate extract treatments, in (top) direction 'a' and (bottom) direction 'b'.

Assay 2 Bioactivity of *T. crassum* 26 EA extract (10 to 50 μ L) against *G. sepiarium*

All application volumes of *T. crassum* 26 extract resulted in significant inhibition of *G. sepiarium* growth in directions 'a' and 'b' relative to control treatments (Appendix 14). In direction 'a', there was almost complete inhibition from the 20 μ L application. Inhibition in direction 'b' was lower but increased with application volume, with no significant differences in inhibition for the 40 and 50 μ L applications.

Assay 3 Bioactivity of 6PAP against *G. sepiarium*

There was a general increase in inhibition of *G. sepiarium* growth with increasing 6PAP concentration in directions 'a' and 'b' (Photo 6.1) relative to the control treatments (Appendix 15). In direction 'a', increasing 6PAP above 0.5% gave increasing inhibition of *G. sepiarium* growth and in direction 'b', increasing 6PAP above 2.5% gave increasing inhibition. Inhibition by 20% 6PAP was significantly higher than the other treatments from 5 d after inoculation.

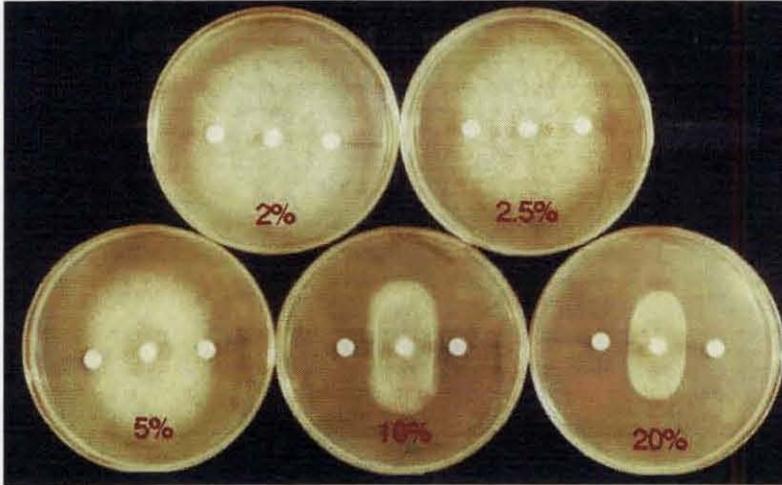


Photo 6.1 Inhibition of *Gloeophyllum sepiarium* growth by 6-pentyl- α -pyrone applied at 2 to 20% to filter-paper discs on malt extract agar.

6.3.4.4 Solvent fractions

The same five solvent fractions of the three *Trichoderma* cultures gave significant inhibition of *G. sepiarium* growth. The fractions were 7, 8, 6, 9 and 5 in order of decreasing inhibition (Photo 6.2). There were no significant differences in growth of *G. sepiarium* in direction 'a' or 'b' between the control treatments.

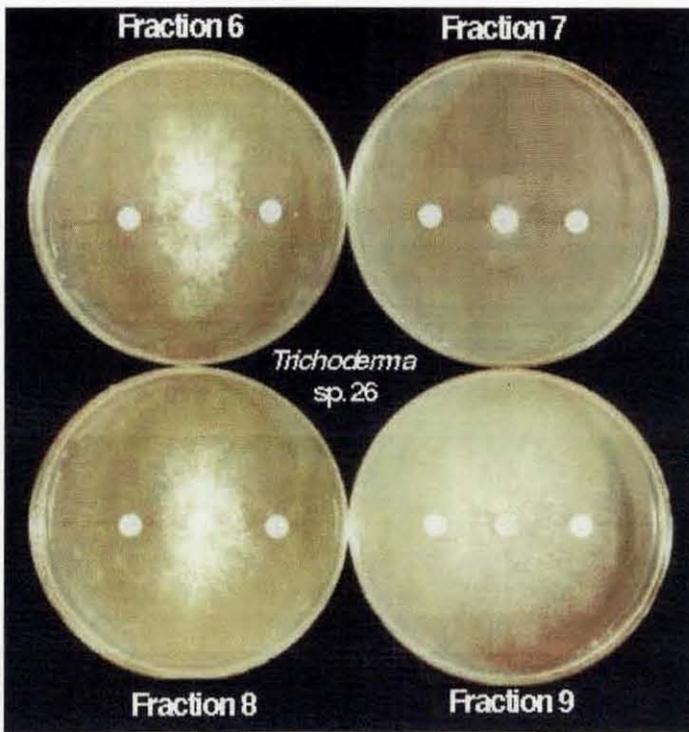


Photo 6.2 Inhibition of *Gloeophyllum sepiarium* growth by *Trichoderma crassum* 26 solvent fractions (clockwise from top left) 6, 7, 9 and 8 on malt extract agar.

The *T. crassum* 26 fractions gave the greatest inhibition of *G. sepiarium* growth in direction 'a' and 'b' (Fig. 6.5) relative to the control treatments. In direction 'a', fraction 7 gave complete inhibition over the assay period. Fractions 8 and 6 were the next most inhibitive in direction 'a', with fraction 8 having significantly higher inhibition than fraction 6 from 3 d after inoculation. Fraction 9 had significant inhibition in direction 'a' at 4 d after inoculation. The remaining five fractions were not significantly different from the control treatments over the assay period. Levels of inhibition in direction 'b' were lower, but fractions 6, 7 and 8 resulted in significant inhibition.

Trichoderma sp. 'viride' 38 and *T. viride* 101 fractions 7 and 8 gave inhibition of *G. sepiarium* growth in direction 'a' but not in direction 'b' relative to the control treatments (Appendix 16). Fraction 7 had higher inhibition than fraction 8, but the differences were only significant for the *T. viride* 101 extracts, and the inhibition by fractions 5, 6 and 9 were not significantly different.

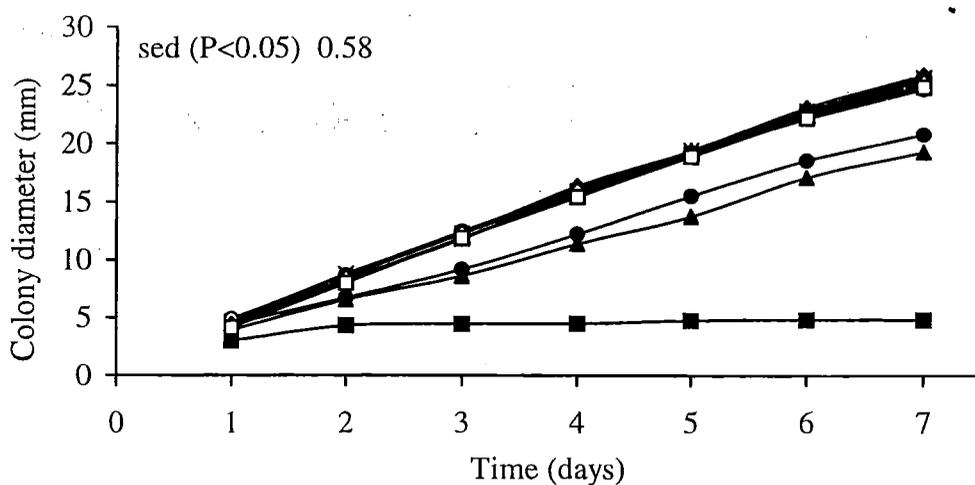
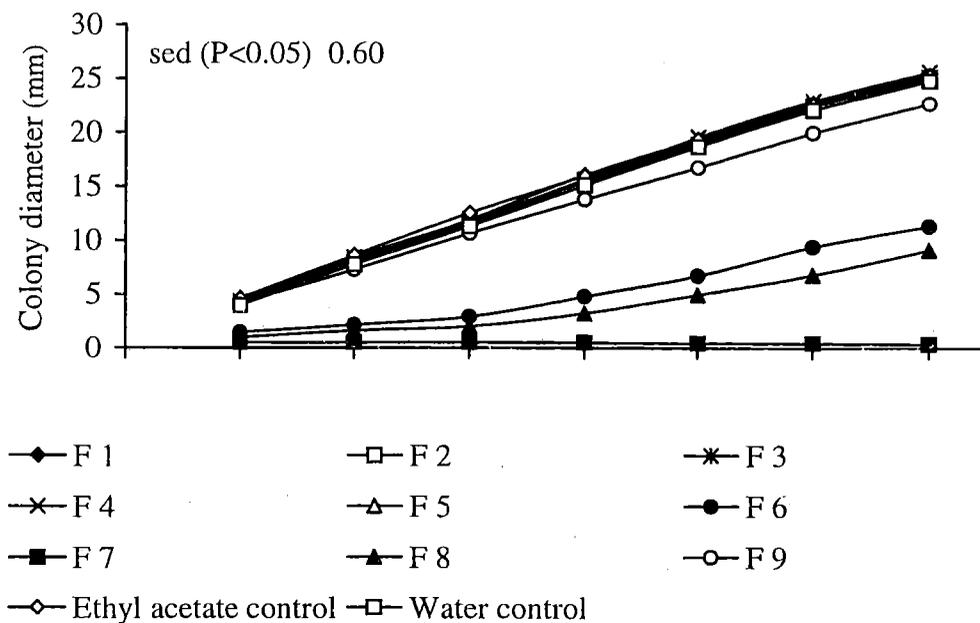


Fig. 6.5 Growth response of *Gloeophyllum sepiarium* to *Trichoderma crassum* 26 solvent extract fractions, in (top) direction 'a' and (bottom) direction 'b'.

6.3.4.5 Gas Chromatography-Mass Spectrophotometry (GC-MS) analysis

A chromatogram was run for each of the nine solvent fractions for each *Trichoderma* culture. From spectral analyses of the chromatogram peaks, there were no conclusive identification of structures or compounds except for 6PAP. There were seven fractions with 6PAP (concentration; kCounts): *T. viride* 101 fraction 6 (9295 k), fraction 7 (3488 k), fraction 8 (143 k), and fraction 9 (23 k); and *T. sp. 'viride'* 38 fraction 6 (25 k) and fraction 9 (24 k). The spectra of each individual peak of the chromatogram for *T. viride* 101 fraction 7 was examined. Only three peaks had identifiable components. The first peak

at the retention time of 18.769 minutes was 6PAP. The second peak at 20.362 minutes and the third peak at 25.519 minutes showed a double bond to be present somewhere in the pentyl or heptyl side chain, respectively.

The chromatograms of the five solvent fractions that showed the highest inhibition in the bioactivity assays (*T. crassum* 26 fractions 6, 7 and 8; *T. sp. 'viride'* 38 fraction 7; and *T. viride* 101 fraction 7 given in Appendix 17) were examined for the occurrence of common peaks. There were 17 peaks in common but no compounds or structures were able to be matched within the mass spectra library available. This was also the case on examination of the common peaks of fraction 7 samples and with *T. crassum* 26 fractions.

6.3.5 Mycoparasitism

Antagonism was observed between hyphae of *T. crassum* 26 and *G. sepiarium*. The *G. sepiarium* hyphae showed a high degree of vacuolation (Photo 6.3) in comparison to *G. sepiarium* control slides. There were no observations of antagonism between *T. sp. 'viride'* 38 and *G. sepiarium*, or *T. viride* 101 and *G. sepiarium*.



Photo 6.3 Vacuolation of *Gloeophyllum sepiarium* hyphae (small diameter) in proximity to *Trichoderma crassum*.26 hyphae (large diameter).

6.4 DISCUSSION

Inhibitory volatile production

Under the given assay conditions, *Trichoderma crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 did not produce volatile compounds that caused significant inhibition of *Gloeophyllum sepiarium* growth. There was some inhibition shown by *T. viride* 101 but the differences were not significant relative to the control treatments. HPLC analyses showed that *T. viride* 101 produced up to $205 \mu\text{g mL}^{-1}$ 6PAP. The inhibition of *G. sepiarium* growth from a 50 μL application of the *T. viride* 101 EA extract was equivalent to that from 2% 6PAP in Assay 3 i.e. 0.4 mg 6PAP per filter-paper or $8000 \mu\text{g mL}^{-1}$ 6PAP. *T. viride* 101 could, however, produce a range of metabolites that are inhibitory to *G. sepiarium* growth, not just 6PAP in isolation.

Bisby (1939) and Rifai (1969) first noted the distinctive aroma, resembling coconut, that was produced by some *T. viride* and *T. harzianum* cultures. *Trichoderma viride* has also been associated with a cheese aroma, identified to be 2-heptanone, but no inhibitory activity has been attributed to this compound (Gervais and Sarrette 1990). Merlier et al. (1984) described 6PAP as a product of the spores of *T. harzianum*. Claydon et al. (1987) proposed that inhibitory *Trichoderma* species were associated with the coconut aroma, which Claydon et al. (1977) had earlier identified as 6PAP, but this was disputed by Bruce et al. (1996) who found no correlation between the ability of *Trichoderma* species to produce this aroma and the ability to inhibit wood decay fungi. The latter researchers also found a *Trichoderma* culture that produced the aroma during growth on agar but 6PAP was unable to be detected.

Volatile compounds from *Trichoderma* species have been shown to inhibit wood decay fungi in assays on MA, but not on a minimal medium similar to LNM (Srinivasan et al. 1992; Wheatley et al. 1997). However, Claydon et al. (1987) found that 6PAP was produced in higher quantities by a *T. harzianum* culture when grown on minimal medium. Bruce et al. (2000) found that subtle changes in the amino acid composition of the growth medium had highly significant effects on the production of inhibitory volatile compounds by *Trichoderma aureoviride* Rifai against decay fungi, and that aldehyde and ketone components were associated with the greatest inhibition.

The ability of *Trichoderma* species to produce inhibitory volatile compounds against basidiomycete fungi is dependent on the *Trichoderma* isolate, and the inhibition of decay fungi may be species-specific with a limited host range (Morrell 1990; Srinivasan et al. 1992). Dennis and Webster (1971b) found that there was no correlation between the production of inhibitory volatile and non-volatile compounds and although some *Trichoderma* species produced highly inhibitory volatiles, no fungicidal activity against wood decay fungi was observed, only reductions in growth. Further assays could study volatile production during growth of *Trichoderma* species in wood and the inhibition of fungal colonisation, growth and decay.

The volatile assay was assessed at 4 d after inoculation but Bruce et al. (1996) observed, for a number of *Trichoderma* cultures, that volatile production occurred between 7 and 14 d and the composition of the volatile profile (alcohols, ketones and various hydrocarbons and esters) changed with time. To validate this, the thesis assay would need to be modified to allow for a greater assessment period, whilst avoiding contamination by *Trichoderma* spores.

Inhibitory non-volatile production

The growth inhibition of basidiomycete fungi by non-volatile compounds produced by *Trichoderma* species generally decreases with time, which is partly due to their diffusion through the medium (Bettucci et al. 1988). Tronsmo and Dennis (1978) found that *Trichoderma* species exhibited greater inhibitory activity by non-volatile compounds at relatively low temperatures around 5°C, and that most of the 15 *T. viride* cultures studied could grow at 2 and 5°C, whereas volatile activity tended to occur only at temperatures above 20°C. The majority of fungal interaction studies are carried out between 20 and 25°C (Dennis and Webster 1971c). The growth medium is important in determining the antagonism of *Trichoderma* species, which has been found to be greater when grown on minimal medium than malt extract medium (Crozier et al. 1999; Ghisalberti and Sivasithamparam 1991; Srinivasan 1993). However, Srinivasan et al. (1992) found that the production of laminarinase and chitinase was greater on malt extract medium than minimal medium and they proposed that antibiotic production may be limited in wood, and to some extent in minimal medium, due to the absence of specific nutrients.

On agar

Trichoderma crassum 26 gave the same levels of inhibition of *G. sepiarium* growth over the three agar assays with complete inhibition for up to 9 d after inoculation, but there was relatively low inhibition of *Phlebiopsis gigantea* and *Schizophyllum commune*. *Trichoderma viride* 101 gave complete inhibition of *G. sepiarium* growth for up to 14 d after inoculation, and the highest levels of inhibition of *P. gigantea* and *S. commune*. Overall, *T. sp.* 'viride' 38 gave relatively low inhibition of *G. sepiarium* and *P. gigantea* growth and no inhibition of *S. commune*.

There were some difficulties experienced in measuring the colony diameter of *G. sepiarium* on LNA as the hyphae were indistinct against the colorless agar. In the second assay, an accurate measurement of colony diameter was only carried out at 7 d when the lid was removed and closer observation was possible. This may partly account for the differences observed in *G. sepiarium* growth in the control treatments on LNA. Variability in the *G. sepiarium* cultures that were used in each assay may also play a part but there were no differences in *G. sepiarium* growth in the control treatments on MA. The differences in inhibition between assays on LNA may be partly attributed to the different fungal cultures used and the shorter treatment incubation time in the second assay. As for the volatile assay, the incubation period of 2 or 3 d may have limited the production of compounds by the *Trichoderma* cultures and also the degree of diffusion through the cellophane. Longer incubation times were not possible due to contamination of the agar when the *Trichoderma* culture grew over the edge of the cellophane sheet. The 3 d incubation used in the first assay was borderline for contamination. A shorter incubation time may give lower levels of inhibition as shown by *T. sp.* 'viride' 38 and to a lesser extent *T. viride* 101, but *T. crassum* 26 gave greater inhibition in the second assay. In similar agar assays, Highley (1997) observed the complete inhibition of a range of decay fungi by the production of antibiotics by *T. virens*. *Trichoderma* antibiotics have been shown to be more stable at acid pH (Dennis and Webster 1971a; Sierota 1977) but the effect of pH on the production and stability of inhibitory non-volatile compounds was not considered in this thesis study.

On *Pinus radiata*

In the wood assays, there were problems associated with the variability in fungal colonisation of the wood blocks. This may partly be attributed to differences between blocks (e.g. in density, moisture content, age) and to the fungal inoculum (e.g. culture age, vigour). The use of liquid inoculum reduced the variability and also the time required for colonisation, in comparison to the use of agar inoculum.

In liquid medium

The bioactivity assays of the EA extracts and solvent fractions showed that the *T. crassum* 26 treatment had the greatest inhibition of *G. sepiarium* growth, followed by the *T. sp.* 'viride' 38 treatment. The *T. viride* 101 treatment did not give the level of inhibition that was shown in the agar assays. This may partly be attributed to the extraction procedure where active inhibitory components were not concentrated, but diluted or lost. For a 50 µL application of the *T. crassum* 26 treatment, the inhibition in direction 'a' was consistent between the two EA extract assays. The inhibition in direction 'b' was higher in the second assay, which is likely to be due to the improved application technique with the filter-papers on tinfoil. In the solvent fraction assays, the active fractions 5 to 9 may have the same inhibitory component in different concentrations.

Bruce and Highley (1991) found that there was no correlation between the results of dual culture and culture filtrate assays of potential BCA's. In the dual culture assays, *T. crassum* 26 and *T. viride* 101 were identified as potential antagonists of *G. sepiarium* in the MA and LNA assays, whereas *T. sp.* 'viride' 38 was only identified in the LNA assay. This may be related to the lower antagonism of *T. sp.* 'viride' 38 relative to the *T. crassum* 26 and *T. viride* 101 treatments. In the culture extract assay, the *Trichoderma* cultures were grown on LNM which may have influenced the antagonism observed. Similar MA and filter-paper assays were used by Quiroga et al. (2001) to screen for growth inhibition of basidiomycete fungi (including *Schizophyllum commune* and *Pycnoporus coccineus*) and mould fungi (including species of *Penicillium* and *Trichoderma*) by ethanol extracts of medicinal plants. Sierota (1977) found that culture filtrates of *T. viride* had greater antagonistic activity when grown on cellulose and xylose. Akpomede and Ejechi (1998) found that culture filtrates of *T. viride* in combination with a high agar pH (pH 8.1) had a fungicidal effect on *G. sepiarium* but the filtrate alone only inhibited growth.

Mycoparasitism

The ability to exhibit mycoparasitism is not present in all *Trichoderma* species and can vary between isolates of a species (Dennis and Webster 1971c). The 'living chamber' technique has been successfully employed in observations of antagonism between *Trichoderma* and wood decay fungi (Doi et al. 1993; Murmanis et al. 1988b). Indicators of mycoparasitism include hyphal hooking and coiling, penetration and growth within the wood decay fungus hyphae, and the production of appressorium-like structures (Croan and Highley 1990). The mycoparasitic process may include chemotropic growth of the *Trichoderma* hyphae towards the wood decay fungus, recognition, production and secretion of extracellular enzymes and lysis of the decay fungus hyphae. Murmanis et al. (1987) observed in SEM studies, the attachment of *Trichoderma* hyphae and spores to decay fungi hyphae and consumption of hyphal contents, but did not find inhibition of the decay fungi by *Trichoderma* culture filtrates or extracts from *Trichoderma*-colonised wood blocks. The *Trichoderma* and wood decay fungi may have different temperature optima for growth and so temperature may influence biological control potential. Ideally in a biological control system, the BCA and the wood decay fungus should have the same optimal growth temperature (Mukherjee and Raghu 1997) but some *Trichoderma* species may produce greater quantities of inhibitory compounds at temperatures higher than those that are optimal for growth. In this thesis study, the only form of antagonism observed was increased vacuolation of *G. sepiarium* hyphae in proximity to *T. crassum* 26 hyphae. This phenomenon has been previously observed and attributed to the action of antibiotics (Dennis and Webster 1971a; Murmanis et al. 1988b). In the agar and liquid medium assays, *T. crassum* 26 had given significant inhibition of *G. sepiarium* growth.

Srinivasan (1993) studied the antagonism of *Trichoderma* species against basidiospores, in contrast to the inhibition of mycelial growth and they found that for brown rot fungi, there was more specific inhibition of basidiospores than mycelium by non-volatile compounds from *Trichoderma* species and that volatile compounds had little effect on basidiospore germination. Kelly et al. (1981) observed that *T. viride* produced toxic secondary metabolites that resulted in the inhibition of spore germination by other fungi.

Further studies could investigate the physiological conditions favoring antagonistic activity of the *Trichoderma* species against the wood decay fungi e.g. light, temperature and pH, carbon and nitrogen sources. The interactions between *Trichoderma* and wood decay fungi

could be observed by growth inhibition on agar or wood blocks, inhibition of wood weight loss, or light microscopy or SEM of dual inoculated blocks. Microscopy could also be used to observe the interactions in the presence of resident microorganisms, by the dual inoculation of non-sterile or surface-sterilised blocks. Other experiments could include sporulation and germination assessments for the *Trichoderma* species and evaluation of fungicide tolerance, for use in integrated control strategies.

Chapter 7 CONCLUSIONS

This thesis research evolved from an original aim to study the biological control of brown rot fungi on *Pinus radiata*. In the first year of study, problems were encountered with the identification and source information of some culture collection basidiomycete fungi, as those most commonly used were up to 60 years old and were originally from overseas collections. A decision was made to obtain a number of fresh isolates of New Zealand basidiomycete fungi from *P. radiata* in the field, to study in addition to culture collection isolates. There were equal numbers of both rot types represented, although it is commonly reported that brown rot fungi prefer softwoods and white rot fungi prefer hardwoods. Brown rot fungi had been originally chosen for study as they can cause the most destructive form of decay with significant changes in wood properties at incipient weight losses. The scope was widened to screen both brown rot and white rot fungi for wood decay potential and the brown rot fungus *Gloeophyllum sepiarium* and the white rot fungi, *Phlebiopsis gigantea* and *Schizophyllum commune* were selected for characterisation in the biology and biological control studies. *Gloeophyllum sepiarium* and *S. commune* are ubiquitous fungi that are studied internationally but there are fewer reports in the literature on *P. gigantea*. A study by Kreber and van der Waals (1999) on the decay potential of *P. gigantea* isolates, including *P. gigantea* 103, 104, 119 and 130, also proposed future decay enzyme studies. Their work acknowledged the relevance of this thesis research, in the stimulation of interest in understanding the significance of basidiomycete fungi in relation to the New Zealand *Pinus radiata* resource.

Trichoderma fungi were investigated as biological control agents against the selected basidiomycete fungi as extensive studies had shown their antagonistic potential in wood systems but no reliable control has been found in a field situation. This study gave a greater understanding of the biology of basidiomycete and *Trichoderma* fungi and their interactions in artificial and wood media. This information can be used to devise future screening criteria for BCA's and also to improve BCA colonisation by inoculum formulation, timing of application or delivery systems. The basidiomycete and *Trichoderma* cultures that were isolated may or may not have been an accurate representation of the species present in the systems sampled but more extensive isolations and ecological studies were not within the scope of this thesis. The fungi were fresh

isolates from known sources and, although not necessarily indicative of different species, there was significant variation between them in the screening assays and in growth morphology. None of the culture collection fungi showed wood decay or biological control potential.

This is the first published report on the wood decay potential of New Zealand isolates of basidiomycete and *Trichoderma* fungi and their interactions in relation to biological control systems. Fungal wood decay potential was defined in relation to the production of decay enzymes, wood weight loss and growth rates on artificial and wood media. These assays were not necessarily correlated as the conditions in agar and liquid media are far removed from those operating in wood. Also, the assay results cannot be extrapolated into the field situation without taking into account the non-sterile wood substrate and the environmental conditions that the fungi would be exposed. This research provided the basis for further ecological studies on New Zealand basidiomycete and *Trichoderma* fungi, their roles and interactions in wood or soil systems, including the potential for biological control, relationships with the natural microbial succession, and the influences of environmental and physical conditions. Chemistry studies could characterise the red-brown or yellow pigment that was produced as a result of some dual culture interactions, and also the pigmentation of *G. sepiarium* grown on agar at temperatures above 30°C and in the light.

There was significant variation in wood decay potential between the basidiomycete fungi and between isolates of the same species. Comparisons to reports in the literature was difficult because of this variation and also the range of assay methods used. The *Trichoderma* cultures that were screened for decay enzymes had far higher CMCase and xylanase activities than *G. sepiarium* or *P. gigantea*, but comparable cellulolytic activities to *S. commune*. Mannanase activity of *G. sepiarium* was greater than the *Trichoderma* cultures, and this was in agreement with the chemical wood analyses which showed preferential degradation of mannan by *G. sepiarium*. *Schizophyllum commune* has been studied extensively because of its high production of decay enzymes, in particular xylanase. However, *S. commune* had a limited ability to cause wood weight losses and was unsuitable for the wood biological control assays unless another parameter such as enzyme activity was used in place of wood weight loss. Similarly, the *Trichoderma* cultures had relatively high enzyme activities but did not cause significant wood weight losses.

The low hazard wood decay assay simulated an above ground situation where biological control systems were more likely to have a chance of success than the soil contact assay that is most commonly used. Wood decay, particularly for the white rot fungi, may have been limited by wood moisture or available nutrients. The relatively low weight losses observed in this assay suggested that, under similar conditions, the visual presence of fungi in *Pinus radiata* did not necessarily indicate significant degradation, although the effects on wood strength parameters were not measured. Under high decay hazard conditions (green wood or blocks in soil contact), *G. sepiarium* and *P. gigantea* resulted in wood weight losses comparable to reports in the literature, where cultures are commonly selected for their virulence.

Fast growing fungi may be more competitive and have an advantage in substrate colonisation. In the dual culture agar assays, most of the *Trichoderma* cultures completely overgrew *G. sepiarium*. The variation in growth rates on agar and *Pinus radiata* was greater for the basidiomycete than the *Trichoderma* fungi. Growth on LNA was correlated to growth on wood for the brown rot and *Trichoderma* fungi. Accumulated biomasses of the *Trichoderma* cultures on CMC medium were significantly higher than for the basidiomycete fungi but the range of biomass on xylan and mannan medium was comparable. Of the three basidiomycete fungi, *P. gigantea* had the fastest growth rates on agar and *Pinus radiata*, and *S. commune* had the fastest growth in liquid media. Although *G. sepiarium* had the slowest growth rates on all three media, there were significant reductions in pH on agar and in liquid media. Growth of *P. gigantea* caused smaller reductions and *S. commune* had little or no effect on pH on either medium. These results suggested that the reduction in pH associated with growth of fungi on agar or in liquid media, may be an indicator of relative wood decay potential.

Regression analyses showed that a number of parameters could be used to predict wood weight loss, ability to colonise *Pinus radiata* and/or the rot type. For brown rot fungi, weight loss was correlated to wood moisture content and external fungal biomass, growth on LNA and the absence of ligninolytic enzymes. Similarly for white rot fungi, weight loss was correlated with wood moisture content and external fungal biomass, and the presence of ligninolytic enzymes. Wood moisture content was an important factor to consider for both brown rot and white rot fungi, but low moisture was more limiting for degradation by white rot fungi. There was significant potential for fungal decay of green wood or wood in

soil contact. Despite reports of fungitoxic compounds in *P. radiata* heartwood, there was no reduction in weight loss compared to sapwood under the same assay conditions. There appeared to be little risk of degradation of *Pseudotsuga menziesii* or *Eucalyptus regnans* sapwood, at least by the three basidiomycete fungi that had been isolated from *Pinus radiata*. Although there were correlations between wood weight loss and whether the fungus was a brown or white rot fungus, and the presence or absence of laccase and peroxidase, there were some exceptions. However, the assay for ligninolytic enzymes was simple and gave a good indication of whether the fungus was a brown or white rot fungus, and by taking into account the decay hazard conditions that the fungus was exposed, an assessment of wood decay potential could be made within a short time period. Further information on individual fungi could be obtained in more detailed assays.

The biological control investigations showed that there was significant antagonism exhibited by the *Trichoderma* against basidiomycete fungi. The dual culture assays identified 95 *Trichoderma* cultures as potential antagonists of *G. sepiarium*. The MA assay identified the greatest number of cultures (86), followed by the LNA assay (29) and the wood-agar assay (12). Only *Trichoderma* sp. 72 showed antagonism in all three assays but 29 cultures showed antagonism in more than one assay. For screening purposes, the literature is divided on the pros and cons of using a high nutrient or a low nutrient minimal medium with a chemical composition closer to wood. This thesis research used MA, LNA and *P. radiata* blocks, where feasible.

The wood biological control assay was developed to screen for three aspects of *Trichoderma-G. sepiarium* interaction within the same assay. Previous reports in the literature have screened for either bioprotection or biological potential within a single treated wood block without considering the protection conferred by BCA's to neighbouring sterile or decay-infected blocks. The 'basidiomycete precolonised block' assessed the ability of the *Trichoderma* fungus to colonise the block, prevent further weight loss and to cause loss of basidiomycete viability. The 'sterile block' assessed the ability of the *Trichoderma* fungus to confer bioprotection by colonising the block and preventing basidiomycete colonisation and decay. The '*Trichoderma* precolonised block' assessed the bioprotection potential of the *Trichoderma* fungus to prevent basidiomycete colonisation and decay. This approach could be applied to other biological control systems and scaled up to closer resemble the field situation.

Results of the biological control assays showed that *T. crassum* 26, *T. sp. 'viride'* 38 and *T. viride* 101 gave significant reductions in wood weight loss and viability of *G. sepiarium* in *Pinus radiata*. These cultures had originated from soils from the CHH Kinleith Forest (*T. crassum* 26), CHH Mill-View Road at Rotorua (*T. viride* 101) and CHH Kinleith Mill at Tokoroa (*T. sp. 'viride'* 38). In the '*G. sepiarium* precolonised block' type, *Trichoderma* treatment could eliminate established *G. sepiarium* from the wood blocks and restrict weight losses to half that found in control treatments. In the 'sterile block' and '*Trichoderma* precolonised block' types, *Trichoderma* treatment could restrict weight losses to less than a quarter of that in control treatments by preventing *G. sepiarium* colonisation and survival. There was less success by the *Trichoderma* cultures in controlling wood weight loss by *P. gigantea*, particularly in the '*P. gigantea* precolonised block' type. However, *T. crassum* 26 caused significant reductions in weight losses of the 'sterile block' and '*Trichoderma* precolonised block' types relative to the *P. gigantea* control. *Phlebiopsis gigantea* was not reisolated from the *T. crassum* 26 or *T. sp. 'viride'* 38 treatments but the weight losses indicated that the fungus had been present for a significant portion of the incubation period.

The field and controlled temperature room trials showed that significant weight losses can occur in *Pinus radiata* after eight months. The trials were potentially of high decay hazard as the wood blocks were in soil contact. Biological control interactions were not observed as adverse weather conditions and competition with other microorganisms under non-sterile conditions affected colonisation and survival of both basidiomycete and *Trichoderma* fungi. More effective colonisation may be achieved by using precolonised blocks, a higher inoculum load, and restricting contamination by sapstain and mould fungi (e.g. by reducing nutrient addition at inoculation), and competition by resident microorganisms such as bacteria (e.g. by surface sterilisation of test blocks). Intermediate laboratory and field trials with larger wood blocks under semi-controlled conditions may be appropriate or controlled conditions could be undertaken for the initial colonisation period. Different fungal precolonisation times could be investigated. The best form of *Trichoderma* inoculum would have to be established along with an appropriate method of application e.g. pressure impregnation of wood, dipping. Further studies could investigate environmental influences on colonisation, survival on *P. radiata* and the fungi could be screened for resistance to adverse weather conditions such as drying or flooding.

The mode of antagonism studies of *Trichoderma* on agar, wood and in liquid medium showed that, overall, *T. crassum* 26 was the most inhibitive of *G. sepiarium*, followed by *T. viride* 101, and *T. sp.* 'viride' 38. The ethyl acetate extracts and solvent fractions of *T. crassum* 26 gave the greatest inhibition in the bioactivity assays and this fungus was the only one to show antagonism towards *G. sepiarium* in the mycoparasitism assay. The *Trichoderma* cultures did not require the presence of *G. sepiarium* to elicit the production of inhibitory compounds. Further studies could determine the identity of the compound or compounds and the reactions involved in the inhibition that was observed. HPLC analyses confirmed the production of the antifungal compound 6-pentyl- α -pyrone by *T. viride* 101, but significant inhibition of *G. sepiarium* was not observed under the conditions of the volatile assay. *Trichoderma viride* 101 gave the greatest inhibition of *P. gigantea* and *S. commune* in the non-volatile agar assay, but the production of volatile compounds was not screened for.

The screening assays were by no means exhaustive but the development and application of the principles, the approaches taken to characterise the fungi and the interactions between them and the interpretation of the findings met the thesis research objectives. A logical progression from this study would be to obtain *Trichoderma* cultures that had been evaluated for similar systems in overseas studies and assess their biological control performance against New Zealand basidiomycete fungi on *Pinus radiata*, in comparison to the thesis *Trichoderma* cultures, under the same assay conditions.

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APPENDICES

APPENDIX 1 MEDIA

All fungal cultures for inoculum purposes were grown on malt extract agar (Appendix 1.7) unless otherwise stated.

1.1 Benomyl agar (BA)

(Adaskaveg et al. 1993)

Malt extract (DIFCO, U.S.A.)	20 g
Agar (Scientific Supplies Ltd.)	20 g
Benlate (50% benomyl)	0.020 g
Chloramphenicol	0.20 g
Distilled water	1000 mL

The ingredients were mixed together and autoclaved for 20 min at 121°C prior to dispensing into sterile Petri dishes.

1.2 Edible mushroom growth media

Lentinula edodes medium

<i>Beilschmiedia tawa</i> (A. Cunningham) Benth. and Hook.f.ex Kirk sawdust	85%
Cotton bean	10%
Wheat bran	5%

Pleurotus pulmonarius medium

Cotton bean	90%
Wheat bran	10%

1.3 Glucose-yeast extract nutrient medium

(Gashe 1992; Mandels et al. 1974; Penttila et al. 1987)

Carbohydrate source ¹	10 g
Glucose	10 g
Yeast extract (DIFCO, U.S.A.)	2 g
Potassium dihydrogen phosphate	6.2 g
Magnesium sulphate 7 hydrate	0.5 g
Calcium chloride	0.5 g

Ferrous sulphate 7 hydrate	0.01 g
Manganese sulphate 1 hydrate	1.6 mg
Zinc sulphate 7 hydrate	1.4 mg
Cobalt chloride 6 hydrate	3.7 mg
Potassium nitrate	6 g
Tween-80	2 mL
Distilled water	998 mL

¹ carboxymethylcellulose (Aldrich, U.S.A) / oat spelts xylan (Sigma, U.S.A)
/ locust bean galactoglucomannan (Sigma, U.S.A)

The ingredients were mixed together, adjusted to pH 5.6 using 3 M NaOH and then 100 mL aliquots were dispensed into 250 mL conical flasks. The flasks were stoppered with non-absorbant cottonwool and covered with two layers of tinfoil. They were autoclaved for 20 min at 121°C, incubated for 24 h at room temperature and then reautoclaved.

1.4 Guaiacol potato dextrose agar (gPDA)

(Jung et al. 1995)

Potato dextrose agar (DIFCO, U.S.A.)	39 g
Distilled water	1000 mL

The ingredients were mixed together and autoclaved for 20 min at 121°C. Guaiacol (100 µL) was added when the agar had cooled to 65°C and then it was dispensed into sterile Petri dishes.

1.5 Guaiacol sawdust agar (gSA)

(Jung et al. 1995)

<i>Pinus radiata</i> sawdust	2 g
Agar (Scientific Supplies Ltd.)	16 g
Distilled water	1000 mL

The *P. radiata* sawdust was produced by grinding sapwood through a 0.5 mm mesh and dried at 80°C to a constant weight. The ingredients were mixed together and autoclaved for 20 min at 121°C. Guaiacol (100 µL) was added when the agar had cooled to 65°C and then it was dispensed into sterile Petri dishes.

1.6 Low nutrient agar (LNA) and low nutrient medium (LNM)

(Srinivasan et al. 1992)

D-glucose	5 g
L-asparagine	0.013 g
Potassium dihydrogen phosphate	1 g
Magnesium sulphate 7 hydrate	0.6143 g
Potassium chloride	0.5 g
Ferrous sulphate 7 hydrate	0.0183 g
Manganese chloride 4 hydrate	0.01 g
Zinc sulphate 7 hydrate	0.002 g
Calcium nitrate 4 hydrate	0.05 g
Copper sulphate 5 hydrate	0.00313 g
Ammonium nitrate	0.008 g
Distilled water	1000 mL

Agar (Scientific Supplies Ltd.)

10 g –for dual culture *Trichoderma-G. sepiarium* assays

20 g –for fungal growth rate assays

The ingredients for LNA were mixed together and autoclaved for 20 min at 121°C prior to dispensing into sterile Petri dishes.

LNM was made up omitting the agar component. The ingredients for LNM were mixed together and 100 mL aliquots were dispensed into 250 mL conical flasks. The flasks were stoppered with non-absorbant cottonwool and covered with two layers of tinfoil. They were autoclaved for 20 min at 121°C, incubated for 24 h at room temperature and then reautoclaved.

1.7 Malt extract agar (MA) and malt extract medium

Malt extract (DIFCO, U.S.A.)	20 g
Agar (Scientific Supplies Ltd.)	20 g
Distilled water	1000 mL

The ingredients were mixed together and autoclaved for 20 min at 121°C prior to dispensing into sterile Petri dishes.

Malt extract medium was made up omitting the agar component. Aliquots (100 mL) were dispensed into 250 mL conical flasks. The flasks were stoppered with non-absorbant cottonwool and covered with two layers of tinfoil. They were autoclaved for 20 min at 121°C, incubated for 24 h at room temperature and then reautoclaved.

1.8 Skim milk agar (SMA)

Skim milk powder	15%
Agar (Scientific Supplies Ltd.)	3%

A solution of 300 g skim milk powder in 1 L distilled water was autoclaved for 15 min at 113°C. A solution of 60 g agar in 1 L distilled water was autoclaved for 20 min at 121°C. The two solutions were mixed together and then dispensed into sterile Petri dishes.

1.9 *Trichoderma*-selective agar (TSA)

(Askew and Laing 1993; Elad et al. 1993)

Glucose	3 g
Ammonium nitrate	1 g
Dipotassium hydrogen phosphate	0.9 g
Magnesium sulphate 7 hydrate	0.2 g
F.C.M.B. (Terraclor)	0.2 g
Rose Bengal	0.15 g
Chloramphenicol	0.25 g
Potassium chloride	0.15 g
Agar (Scientific Supplies Ltd.)	20 g
Micronutrient solution (see below)	1 mL
Distilled water	1000 mL

The ingredients were mixed together and autoclaved for 20 min at 121°C prior to dispensing into sterile Petri dishes.

Micronutrient solution

Ferrous sulphate 7 hydrate	1 g
Manganese sulphate 1 hydrate	0.65 g
Zinc sulphate 1 hydrate	0.90 g

Distilled water 1000 mL

The ingredients were mixed together and the solution was stored at 4°C.

1.10 Tris buffer

Trizma Base (Sigma, U.S.A.)

Tris [hydroxyl methyl] amino methane

A 1 M solution was prepared and HCl (conc.) was used to adjust the pH to 7 and Tween 80 was added at 0.05%. The solution was autoclaved for 20 min at 121°C, incubated for 24 h at room temperature and then reautoclaved. When required, the solution was diluted to 100 mM.

1.11 Tryptone-yeast extract nutrient medium

(Rainey 1992)

Carbohydrate source ¹	2 g
Tryptone	2 g
Yeast extract (DIFCO, U.S.A.)	1 g
Dipotassium hydrogen phosphate	1.5 g
Potassium dihydrogen phosphate	0.75 g
Ammonium chloride	0.9 g
Sodium chloride	0.9 g
Magnesium chloride 6 hydrate	0.2 g
Ferric chloride solution (see below recipe)	1 mL
Selenium / Tungstate solution (see below recipe)	1 mL
Trace element solution (see below recipe)	1 mL
Distilled water	1000 mL

¹ carboxymethylcellulose (Aldrich, U.S.A) / oatspelts xylan (Sigma, U.S.A)
/ locust bean galactoglucomannan (Sigma, U.S.A)

The ingredients were mixed together and 100 mL aliquots were dispensed into 250 mL conical flasks. The flasks were stoppered with non-absorbant cottonwool and covered with two layers of tinfoil. They were autoclaved for 20 min at 121°C, incubated for 24 h at room temperature and then reautoclaved.

The following solutions were prepared and stored at room temperature until use.

Ferric chloride solution

Ferric chloride 6 hydrate	0.28 g
Distilled water	1000 mL

Selenium / Tungstate solution

Sodium hydroxide	0.5 g
Sodium selenite 5 hydrate	3 mg
Sodium tungstate 2 hydrate	4 mg
Distilled water	1000 mL

Trace element solution

Zinc chloride	70 mg
Manganese chloride 4 hydrate	100 mg
Boric acid	6 mg
Cobalt chloride 6 hydrate	130 mg
Copper chloride 2 hydrate	2 mg
Nickel chloride 2 hydrate	24 mg
Sodium molybdate 2 hydrate	36 mg
Hydrochloric acid 5 M	15 mL
Distilled water	985 mL

1.12 Water agar (WA)

Agar (Scientific Supplies Ltd.)	20 g
Distilled water	1000 mL

The ingredients were mixed together and autoclaved for 20 min at 121°C prior to use.

1.13 Wood fixative

(for scanning electron microscope work)

Formalin (40%)	100 mL
Ethanol (80%)	850 mL
Acetic acid	50 mL

The ingredients were mixed together. The wood samples were stored submerged in the fixative solution, at room temperature.

1.14 Yeast extract-malt extract agar (YMA) and yeast extract-malt extract medium (YM)

Yeast extract (DIFCO, U.S.A.)	2 g
Malt extract (DIFCO, U.S.A.)	15 g
Agar (Scientific Supplies Ltd.)	20 g
Distilled water	1000 mL

The ingredients were mixed together and autoclaved for 20 min at 121°C prior to dispensing into sterile Petri dishes.

Yeast extract-malt extract medium was made up omitting the agar component. Aliquots (100 mL) were dispensed into 250 mL conical flasks or 10 mL aliquots into 25 mL Universals. The flasks were stoppered with non-absorbant cottonwool and covered with two layers of tinfoil. The flasks or Universals were autoclaved for 20 min at 121°C, incubated for 24 h at room temperature and then reautoclaved.

APPENDIX 2 METHODS

2.1 Cellulolytic enzyme assays

Cellulase

Cellulolytic activity was determined by measuring the release of glucose using a p-hydroxybenzoic acid hydrazide (PAHBAH; Sigma, U.S.A.) method (Lever 1973). Tryptone-yeast extract nutrient medium (Appendix 1.11) was used with carboxymethylcellulose (CMC) as a carbon substrate. There were duplicate flasks for each test fungus. The flasks were inoculated with 5 mL of culture (3 d old cultures grown in YM at 25°C). The flasks were incubated in a rotary shaker set at 140 revolutions per min (rpm) at 25°C. Samples of culture supernatant (1 mL) were taken at 1, 2, 4, 6 and 8 d after inoculation. The highest enzyme activity over the five sampling times was averaged over the replicates and used for comparative purposes.

Cellulase activity was measured in a reaction mixture containing 0.02 to 0.10 mL of culture supernatant and 0.30 mL of 1% (w/v) CMC in 100 mM citrate buffer, pH 6. The mixture was incubated for 15 min at 50°C before the reaction was stopped by addition of 1 mL PAHBAH reagent and boiling for 6 min. Samples were cooled in ice water and then centrifuged at 10 000 k for 5 min prior to reading of absorbance at 420 nm. A dilution series of standards were included with each assay series. Cellulase activity was expressed as micromoles glucose $\text{min}^{-1} \text{mL}^{-1}$ or International Units mL^{-1} (IU mL^{-1}) against reagent blanks and in relation to standard curves of glucose at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 nmoles. Samples and standards were run in duplicate with one reagent blank.

PAHBAH reagent:

Stock solutions

0.5 M Sodium citrate dihydrate	36.76 g per 250 mL
1.0 M Sodium sulphite 7-hydrate	63.05 g per 250 mL
0.2 M Calcium chloride dihydrate	7.35 g per 250 mL
5.0 M Sodium hydroxide	50.00 g per 250 mL

Working solution

In the order given above, 10 mL of each stock solution was added to 50 mL distilled water, mixing between additions. 1.52 g PAHBAH was added, dissolved and the volume was made up to 100 mL. The solution was used within 24 h.

Xylanase

Xylanase activity was determined by measuring the release of glucose using a dinitrosalicylic acid (DNSA; Sigma, U.S.A.) method (Lever 1973). The same culture medium and procedure was used as for the PAHBAH method, with oat spelts xylan as a carbon substrate. The reaction mixture contained 0.02 to 0.10 mL of culture supernatant and 0.30 mL of 4.0% (w/v) oat spelts xylan in 100 mM citrate buffer, pH 6. The xylan was sonicated in the buffer at high intensity for 10 min on ice, boiled for 1 min, cooled and then centrifuged at 10 000 k for 10 min. The reaction mixture was analysed as for CMCase except the reaction was stopped by the addition of 1 mL DNSA reagent and absorbance was read at 575 nm. Xylanase activity was expressed as for CMCase in relation to standard curves of xylose at 0, 100, 200, 300, 400, 600, 700, 800, 900 and 1000 nmoles.

DNSA reagent

Dinitrosalicylic acid	13.6 g
Sodium sulphite (anhydrous)	0.68 g
Sodium hydroxide	13.6 g
Potassium sodium tartrate	273 g
Phenol	2.70 g

The first four ingredients were dissolved in 1000 mL distilled water and passed through a 0.8 µm filter prior to addition of phenol.

Mannanase

Mannanase activity was determined by the DNSA method (as for xylanase) with locust bean galactoglucomannan as a carbon substrate. The reaction mixture contained 0.05 to 0.10 mL of culture supernatant and 0.25 mL of 0.5% (w/v) locust bean galactoglucomannan in 100 mM citrate buffer, pH 6. The mannan was dissolved in the buffer by heating at 50°C and, after cooling, centrifuging at 10 000 rpm for 10 min. The reaction mixture was analysed as for xylanase and absorbance was read at 540 nm.

Mannanase activity was expressed as for CMCase in relation to standard curves of mannose at the same concentrations as the glucose standards.

2.2 Glycerol storage of basidiomycete cultures

Universals of YM were inoculated with the fungal cultures and incubated at 25°C for 5 d. Aliquots (800 µL) of inoculum were placed in 1.5 mL Eppendorfs with 200 µL glycerol (sterilised by autoclaving for 20 min at 121°C), vortexed to distribute the glycerol and then stored at -80°C.

2.3 Freeze-dried storage of *Trichoderma* cultures

Spore suspensions of *Trichoderma* cultures, in 1.5% skim milk (sterilised by autoclaving for 15 min at 113°C), were frozen at -80°C and dried under vacuum in a freeze-dryer (Dura-dry MP, FTS Systems, U.S.A.).

2.4 Soil pH

The soil was passed through a 3.5 mm sieve and air dried for 24 h at room temperature. A suspension was made with 12 mL soil and 25 mL distilled water and left to stand for a minimum of 4 h or overnight at room temperature. The soil suspension was stirred and the pH read using an electrode (Mettler Delta 350) when a steady value was obtained.

2.5 Soil water-holding-capacity (WHC)

The soil was passed through a 3.5 mm sieve and air dried for 24 h at room temperature. A 45 mm Whatman #2 filter-paper was placed into a Buchner funnel (50 mm diameter x 25 mm deep) which was then filled with the soil. The soil was compacted by dropping the funnel five times onto a firm surface from a height of 10 mm and then the soil surface was leveled. The funnel was then placed into a 400 mL beaker and held upright using tinfoil wedges. Distilled water was added to the beaker to a depth slightly above the filter-paper. The soil was allowed to become wet by capillary action to avoid entrapping air in the soil or funnel. When the upper soil surface showed signs of wetting, more distilled water was added to a depth close to the top of the funnel. The soil was left to soak for a minimum of 12 h or overnight at room temperature. The funnel was placed in a suction flask, a moist cloth was placed over the funnel and a vacuum pump was applied for 15 min. The soil was removed, weighed and dried at 80°C to a constant weight for moisture content determination.

2.6 *Trichoderma* inoculum production

(Sale et al. 1997)

Bran	250 mL
Peat moss	250 mL
Distilled water	250 mL

The ingredients were mixed together and placed in a 20 cm diameter glass Petri dish. The Petri dishes were autoclaved for 30 min at 121°C, incubated for 24 h at room temperature and then reautoclaved. The *Trichoderma* cultures were grown for 10 to 14 d on MA. Spore suspensions were produced and 2 mL was used to inoculate the dishes. Parafilm was used to seal the dishes for the first 24 h and then removed for the remaining 9 d incubation. Spore suspensions were produced by successive washings of the medium.

APPENDIX 3 BIOLOGICAL CONTROL POTENTIAL OF MONOTERPENE COMPOUNDS AGAINST GLOEOPHYLLUM SEPIARIUM

INTRODUCTION

Six oxygenated monoterpenes (alpha-terpineol, terpinen-4-ol, limonene, borneol, p-ethyl phenol, and carvacrol) were obtained from Pat Holland (HortResearch, Hamilton) and assessed for inhibition of *G. sepiarium* growth on agar. The terpenoids were in solvent solution (85% methanol: 15% water) and diluted with water to give the treatment concentrations. There were two assays, both using filter-paper discs that were impregnated with the test compound. In Assay 1, four treated discs were placed on the agar around the *G. sepiarium* colony. This assay aimed to measure the effect of direct contact of each test compound (i.e. its solubility and diffusibility) and the effect of volatile compounds on *G. sepiarium* growth. In Assay 2, a treated disc was adhered to the lid of each inverted Petri dish directly below the *G. sepiarium* colony and aimed to measure the effect of volatile compounds only.

This work was carried out to satisfy the requirements of Objective 2, in FRST programme (96-HRT-05-5441), titled "Terpenoids as natural fungal control agents in timber".

MATERIALS AND METHODS

Assay 1

The treatments were alpha-terpineol, terpinen-4-ol, borneol, limonene, and p-ethyl phenol at 10, 20 and 30%; carvacrol at 3, 6, and 10%; and solvent control and sterile distilled water control treatments. Six mm agar cores of *G. sepiarium* (14 d old culture) were placed in a central position on MA and incubated at 25°C for 3 d. There were five replicate Petri dishes for each treatment. The filter-paper discs (Whatman GF/A filter-paper; 6 mm diameter) were autoclaved for 20 min at 121°C. Each treatment was applied at 20 µL, using 10 µL applications with time between applications for the solvent to evaporate. Four treated discs were placed equidistant apart on the perimeter of each Petri dish around the *G. sepiarium* colony. The Petri dishes were incubated at 25°C and growth of *G. sepiarium* towards each disc was measured on a daily basis up to 10 d and then at 12 and 14 d. The measurements at each time were averaged for each treatment and results

were expressed as percent inhibition of *G. sepiarium* growth relative to the control treatments. Each *G. sepiarium* colony was assessed for viability by reisolation onto MA and incubation at 25°C for up to 21 d.

Assay 2

The *G. sepiarium* colonies and treated filter-paper discs were prepared as for Assay 1. The treatments were alpha-terpineol and terpinen-4-ol, both at 10 and 30%, and solvent control and sterile distilled water control treatments. Each Petri dish was inverted and a treated disc was adhered with WA in a central position on each lid directly below the *G. sepiarium* colony. The Petri dishes were incubated at 25°C and the colony diameter of *G. sepiarium* was measured in two predetermined directions on a daily basis up to 7 d. The measurements at each time were averaged for each treatment and results were expressed as percent inhibition of *G. sepiarium* growth relative to the control treatments. Each *G. sepiarium* colony was assessed for viability by reisolation onto MA and incubation at 25°C for up to 21 d.

RESULTS

Assay 1

Growth of *G. sepiarium* in the solvent control and water control treatments was not significantly different and steady growth occurred up to 12 d when the Petri dish was fully covered.

Complete inhibition of growth over the assay period was caused by p-ethyl phenol (10, 20 and 30%) and by carvacrol (3, 6 and 10%) (Photo 1 a), with no significant difference between the two compounds or any effect of concentration. Alpha-terpineol at 20 and 30% also caused complete inhibition, but the 10% treatment only gave initial inhibition (Photo 1 b).

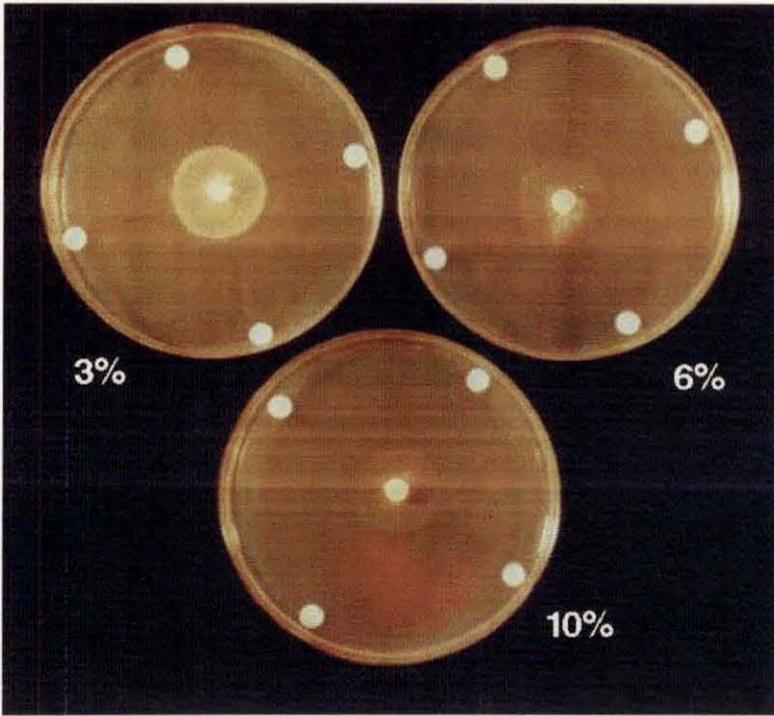


Photo 1 a *Gloeophyllum sepiarium* grown on malt extract agar at 25°C in the presence of 3, 6 and 10% carvacrol.

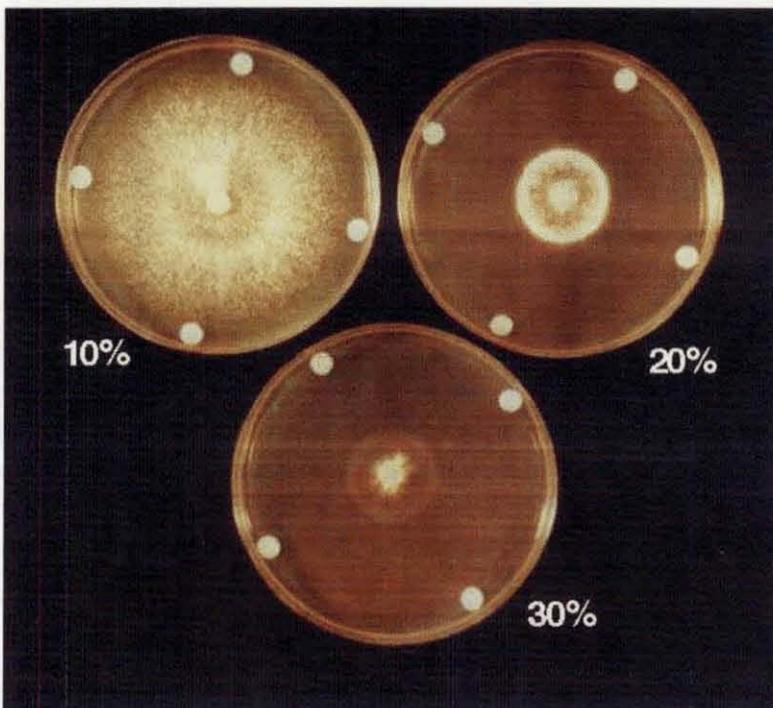


Photo 1 b *Gloeophyllum sepiarium* grown on malt extract agar at 25°C in the presence of 10, 20 and 30% alpha-terpineol.

Terpinen-4-ol at 30% completely inhibited growth of *G. sepiarium* up to 10 d, the 20% treatment caused inhibition only up to 4 d and the 10% treatment caused insignificant inhibition relative to the control treatments. Borneol at 20 and 30%, but not 10%, caused significant inhibition of *G. sepiarium*. Limonene did not cause significant inhibition at any concentration. At 21 d from reisolation, *G. sepiarium* showed viability from all treatments except the 20 and 30% p-ethyl phenol and the 6 and 10% carvacrol treatments.

Assay 2

The 30% alpha-terpineol volatile treatment caused the greatest inhibition of *G. sepiarium* growth relative to the control treatments, followed by the 30% terpinen-4-ol treatment. There was no significant inhibition by the 10% alpha-terpineol and terpinen-4-ol treatments which were not significantly different. *Gloeophyllum sepiarium* showed viability from all treatments, with growth at 3 d from reisolation.

CONCLUSIONS

Of the six monoterpene compounds that were screened, p-ethyl phenol and carvacrol were the most effective at inhibiting *G. sepiarium* growth and fungitoxicity was observed at the two higher concentrations used. The other compounds, excluding limonene, also gave inhibition with the higher concentration treatments but there was no fungitoxicity. Higher concentrations of alpha-terpineol and terpinen-4-ol gave volatile inhibition of *G. sepiarium* growth but no fungitoxicity was observed.

APPENDIX 4 BASIDIOMYCETE CULTURE RECORD SHEET

BASIDIOMYCETE CULTURE RECORDS

Isolate No.	Origin (basidiocarp, decayed wood, etc):
Species (if known)	Source of Collection:
Remarks:	Date Collected:
.....	Host (if known):

KEY CHARACTERS ON 3% MALT AGAR

(from Nobles, 1965; cf. Stalpers, 1978).

Check numbers applicable

LACCASE (extracellular oxidase) using γ naphthol:

1. Negative
2. Positive

TYROSINASE, using p-cresol:

Negative/Positive

PEROXIDASE, using pyrogallol H₂O₂:

Negative/Positive

CLAMPS (On thin-walled, generative hyphae)

3. Present at all septa (figs. 1, 2)
4. Absent or rare at colony margin; present elsewhere (except, possibly, on wider hyphae; Figs. 4, 5)
5. Present, but rare; mostly colony margin (may be multiple and/or on wider hyphae; Figs. 6, 7)
6. Absent (Fig. 3)

PRESENCE OF HYPHAL STRUCTURES

7. None; hyphae all thin walled
8. Skeletals, branched or unbranched (Figs. 8-12)
9. Clamped "skeletals" (Figs 13-16)
10. Cuticular cells (Figs. 48-55)
11. Interlocking hyphae plectenchyma (Figs. 56-63)
12. Encrusted hyphal tips (Figs. 42-44, 91)
13. Cystidia on vegetative mycelium (Figs. 17-23)
14. Cystidia in hymenium where fruiting (Figs. 24-31) includes "metuloids" of Cunninghamham
15. Gloeocystidia - hymenial or vegetative - contain resinous material (Figs. 32-41)
16. Rhizomorphs
17. Setae, brown; includes asterosetae (not a consistent feature; Figs. 74-77)
18. Setal hyphae (only in Hymenochaetaceae; Figs. 78, 95, 98)
20. Minute projections on hyphae (Fig. 47)
21. Resinous masses on hyphae (Fig. 46)
22. Hyphal knots (includes "staghorn" and "witches broom" hyphae; Figs. 69-70)
23. Sclerotia
26. Hyphal swellings; includes allocysts e.g. *Chondrostereum purpureum* (Figs. 71-73, 94)

Additional (Stalpers):

Acanthophyses/Inequivalent branching/Stephanocysts. Skeletoid hyphae (thick walled, septate, generative hyphae)

Hyphal Morphology

Drawings

(include dimensions and culture age)

PRESENCE OF PROPOGATIVE STRUCTURES

32. No conidia, oidia, chlamydospores
33. Conidia (Figs. 79-83, 85)
34. Chlamydospores, terminal or intercalary, with thickened walls (Figs. 84, 87-89, 97)
35. Oidia (arthrospores; Figs. 64-66)

COLONY COLOUR

36. Hyphae hyaline, mats white or pale
37. Hyphae yellow or brown, especially in KOH; mats yellow or brown, in part at least

RECORD MAT COLOUR IF DISTINCTIVE

38. Reverse unchanged in colour
39. Reverse brown, at least in part
40. Reverse bleached, at least in part

COLONY GROWTH: 9cm plate covered in:

41. 1 week
42. 2 week
43. 3 week
44. 4 week
45. 5 week
46. 6 week
47. Not covered in 6 weeks

FRUCTIFICATION PRESENT

48. Fruiting body produced in culture before 6 weeks

COLONY ODOUR

50. Fragrant
51. Earthy/musty
52. Antiseptic
53. Otherwise distinctive

HOST

54. Hardwood decay
55. Softwood decay
56. Other habitat, e.g. soil

Note whether host tree is standing dead; standing live; windthrown; timber, or branch slash/litter

COLONY TEXTURE

Cottony - long, erect, irregularly arranged hyphae

Woolly - long hyphae, matted like woollen cloth

Felty - Cottony or woolly, but an interwoven mat

Sub felty - an appressed felt in a thin mat; pellicular

Sub felty - an appressed felt in a thin mat; pellicular

Velvety - Straight, erect hyphae in a velvet "pile"

Downy - short, fine, erect hyphae; a translucent mat

Silky - long, parallel, prostrate hyphae

Plumose - radiating fans

Floccose - small, erect, cottony/woolly tufts

Crustose - dark or light crust

Lacunose - pits, depressions

Farinaceous - powdery, mealy

Chamois - smooth, no aerial hyphae

APPENDIX 5 MORPHOLOGY AND PIGMENTATION OF BASIDIOMYCETE AND TRICHODERMA FUNGI IN LIQUID MEDIA

The basidiomycete and *Trichoderma* fungi were assessed for the production of CMCase, xylanase and mannanase in CMC, xylan and mannan liquid medium. After 8 d incubation, morphology and pigmentation of the fungi in each medium was noted but not characterised.

Basidiomycete cultures

In CMC medium, most of the basidiomycete fungi grew as gold globules (ranging in relative size from very small to medium) in a clear gold medium. There were a few exceptions where the medium was pigmented specifically. Growth of *Gloeophyllum sepiarium* 15, *Gloeocystidiellum sacratum* 118, and BR 6, 112 and 113 resulted in an orange pigmentation and *Gloeophyllum sepiarium* 14, *Phlebiopsis gigantea* 104 and 119, *Oligoporus* sp. 117, and BR 7 and 10 in a brown-black pigmentation.

In xylan medium, most of the fungi grew as dull gold globules (ranging in relative size from very small to small) in a clear gold medium. Growth of BR 113 resulted in an orange pigmentation, *Antrodia serialis* 1 in brown, BR 9, 10 and 12 and *G. sepiarium* 4 in brown-grey, and *P. gigantea* 104 and 130 in a brown-black pigmentation.

In mannan medium, most of the fungi grew as gold globules (ranging in relative size from very small to medium) in a clear gold medium. Growth of BR 113 resulted in an orange pigmentation, BR 9, 10, 12 and 109, *A. serialis* 1, *Pycnoporus coccineus* 101 and *Hapalopilus nidulans* 122 in brown, and *Phlebiopsis gigantea* 104 and 130 in a brown-black pigmentation.

The pigmentation of the medium did not appear to be related to enzyme activity or to rot classification. It was more likely to be isolate-related but with the three enzyme media this was only consistent for *P. gigantea* 104 and BR 113.

Trichoderma cultures

The *Trichoderma* cultures showed differences in growth morphology on the liquid medium. *Trichoderma* sp. 'long' 15, 24 and 80 cultures grew as fine to medium clumps on CMC medium, clear large globules on xylan medium, and fluffy growth on mannan medium with some sporulation occurring. *Trichoderma crassum* 26 grew as medium sized clumps on CMC medium and as a thin slurry on xylan and mannan medium. *Trichoderma viride* 101 and *T.* sp. 'viride' 38 and 117 cultures grew as a slurry with a brown tinge on CMC medium, on xylan medium *T. viride* 101 and *T.* sp. 'viride' 117 had some sporulation occurring but *T.* sp. 'viride' 38 grew as a slurry, and on mannan medium there was fluffy growth with some sporulation occurring.

APPENDIX 6 CORRELATIONS BETWEEN ASSAY PARAMETERS FOR BROWN ROT (B) AND WHITE ROT FUNGI (W)
AT $P < 0.001$ (1), $P < 0.01$ (2) AND $P < 0.05$ (3)

Assay	Medium	Parameter	Wood decay assay			Enzyme assays				Laccase	Peroxidase gSA gPDA	Proteinase	Growth assays			P. radiata
			Wt loss	MC	BM	CMC medium CMCase ¹ BM	Xylan medium xylanase ¹ BM	Mannan medium mannanase ¹ BM	MA				LNA	BA		
Wood decay		Wt loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		MC	1 b 3 w	-	-	-	-	-	-	-	-	-	-	-	-	-
		BM	1 b 3 w	3 b	-	-	-	-	-	-	-	-	-	-	-	-
Enzyme	CMC	CMCase ¹														
		BM				3 w	-	-	-	-	-	-	-	-	-	-
	Xylan	Xylanase ¹														
		BM														
	Mannan	Mannanase ¹		3 b												
		BM						3 b 1 w								
		Laccase	1 b 1 w		2 w			3 w	3 b	3 b						
		Peroxidase (gSA)	2 b 1 w	3 w	3 b 3 w				2 b		1 b 1 w	-	-	-	-	-
		Peroxidase (gPDA)	2 b	3 b	3 b			3 w	1 b		1 b 2 w	1 b	-	-	-	-
		Proteinase	3 b							3 w	2 b	3 b	-	-	-	-
Growth		MA									3 b	2 b	2 b			
		LNA	2 b	3 b	3 b		3 w			3 w	2 b	2 b	2 b	1 b 1 w	-	-
		BA					3 w			3 w				2 b 1 w	2 b 1 w	-
		<i>P. radiata</i>												2 b	2 b	3 b

1 Micromoles glucose min⁻¹ mL⁻¹ (IU mL⁻¹)

Wt loss= Wood weight loss (%)

MC= Wood moisture content (%)

BM= Milligrams dry weight fungal biomass after 8 d incubation at 25°C.

gPDA= Guaiacol potato dextrose agar

gSA= Guaiacol sawdust agar

Appendix 7.1 *Gloeophyllum sepiarium*

Gloeophyllum sepiarium is a common brown rot decay fungus found in standing trees, stumps, stored logs, and in timber structures exposed to full sunlight. In dark, damp situations various abnormal forms of fruiting bodies may be produced. In this thesis research, fruiting forms were commonly observed on wood blocks in the decay assays. Normal basidiocarps are laterally attached, leathery to corky brackets of 20 to 100 mm diameter. The colour is initially yellowish becoming rusty brown to dark brown, with brighter orange at the edges. The hymenium on the lower surface has a maze of gill-like folds that are a rusty to ochre-brown colour. This fungus attacks both sapwood and heartwood of mainly softwoods. As well as New Zealand, *G. sepiarium* has been observed in Australia, Asia, Europe, North America, South Africa, Iran and less commonly in the United Kingdom. On agar, cultures have an appressed, downy to felty colony morphology that develops a yellow to light brown color with age. The hyphae have numerous clamps and arthroconidia are usually abundant.



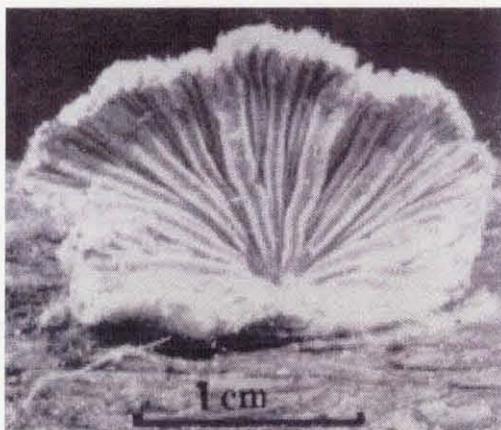
Appendix 7.2 *Phlebiopsis gigantea*

Phlebiopsis gigantea is the main white rot decay fungus that invades *Pinus radiata* in standing trees and logs where there is localised loss of wood moisture (Butcher and Drysdale 1991). They found that *P. gigantea* can infect logs within a few weeks of felling as it is tolerant of up to 70% moisture content and can spread rapidly due to the production of copious airborne asexual spores and oidia. Basidiomycete fungi are generally considered as secondary colonisers in the microbial succession in wood but *P. gigantea* preferentially colonises freshly cut sapwood (Behrendt and Blanchette 1997). This common saprophyte is also a successful coloniser of aged wood including wood debris and the rhizophore in conifer forests (Asiegbu et al. 1996). Butcher (1967) found that *P. gigantea* was the main cause of a yellow-brown discolouration in *Pinus radiata* logs during seasoning. The presence of fruiting bodies was correlated to internal decay. *Phlebiopsis gigantea* fruiting bodies are smooth, white, leathery sheets when dry, and like candlewax when wet. On agar, culture have a white farinaceous colony morphology. *Phlebiopsis gigantea* has been used as a BCA against sapstain fungi and has shown potential applications to the pulp and paper industry in biological processing (Behrendt and Blanchette 1997) and for the biological control of *Heterobasidium annosum* (Rishbeth 1963).

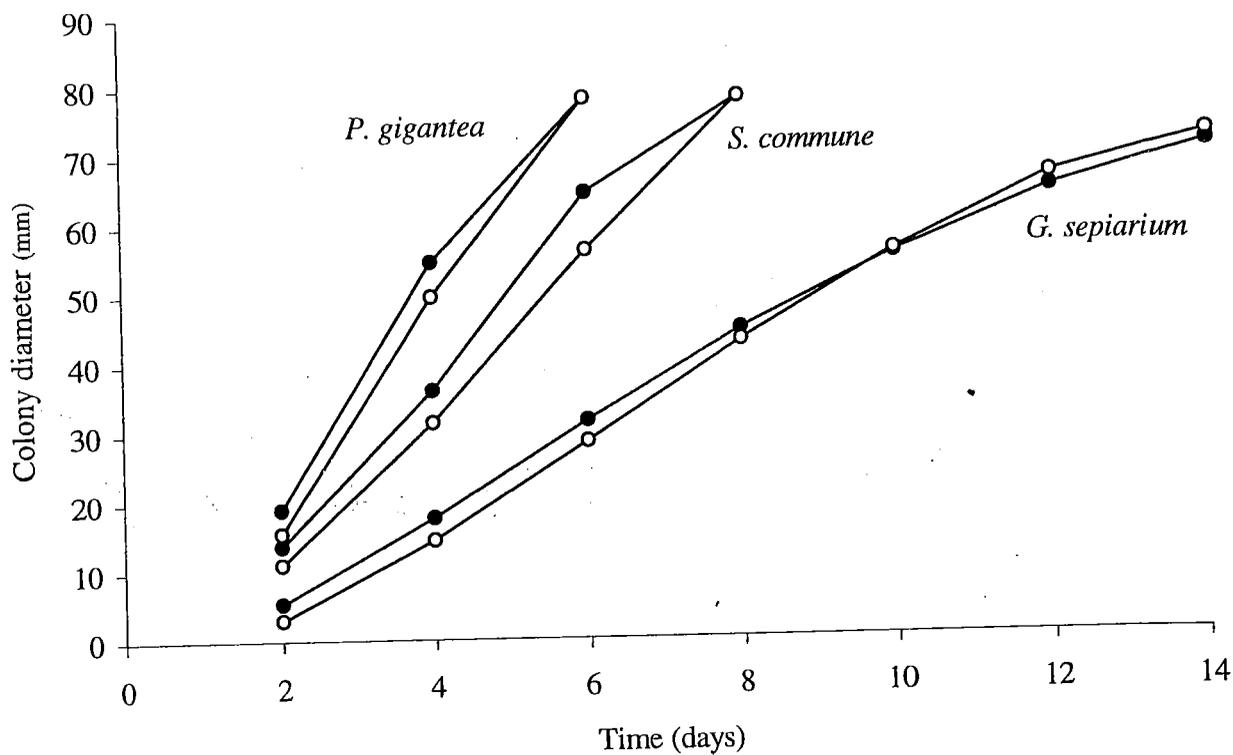


Appendix 7.3 *Schizophyllum commune*

Schizophyllum commune is a very common white rot decay fungus that is distributed worldwide in living plants as well as decaying wood (Schmidt and Liese 1980). In New Zealand, it is often found fruiting on felled trees or logs that are being seasoned (Butcher and Drysdale 1991). This fungus attacks species of both softwoods and hardwoods but is usually limited to the sapwood. It is easy to recognise as it has thin fan-shaped basidiocarps, up to 4 cm in diameter, that are laterally attached, with a hairy upper surface and gill-like folds on the lower surface that characteristically have a split edge and hence the common name of 'split-gill fungus'. In the thesis research, fruiting forms were observed on wood blocks in the decay assays. The basidiocarps are grey-brown to flesh coloured when wet and whitish grey when dry. The split gills may fold over to cover to protect the hymenium during periods of desiccation. *Schizophyllum commune* is tolerant to low water potential and is adapted to high salinity, where growth rates are still 50% of the optimum at salinities higher than seawater (Castillo and Demoulin 1997). On agar, cultures have a white woolly colony morphology. The presence of *S. commune* fruiting bodies on *Pinus radiata* did not indicate incipient decay (Butcher 1967). As in this thesis research, Butcher (1967) found *S. commune* resulted in low or no wood weight losses in *P. radiata* although variation between cultures has been reported (Nilsson and Daniel 1983). Crozier et al. (1999) found *S. commune* resulted in a 30% weight loss in three species of Malaysian hardwood and 9% in rubberwood. Using light microscopy and transmission electron microscopy, Nilsson and Daniel (1983) observed unusual decay patterns by *S. commune* in *Pinus silvestris*. Fungal hyphae were present in the cell lumina and enzymes diffused into the cell wall resulting in a loosening of the S3 layer and degradation of the S2-S1 interface and within the S2 layer. Safo-Sampah and Graham (1976) measured a 3.2% weight loss in hemlock by *S. commune* along with a significant decrease in bending tolerance. Human infections by *S. commune* have been reported (Rihs et al. 1996).

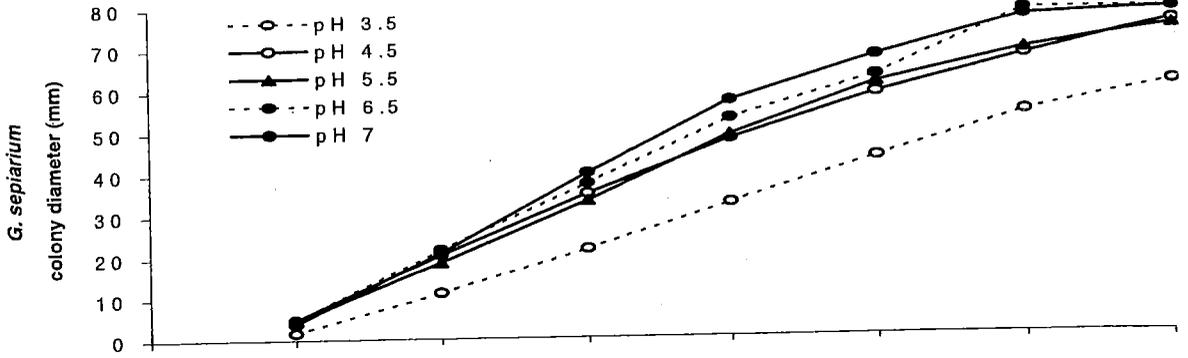


APPENDIX 8 GROWTH RESPONSES OF GLOEOPHYLLUM SEPIARIUM, PHLEBIOPSIS GIGANTEA AND SCHIZOPHYLLUM COMMUNE TO LIGHT (○) AND DARK (●) ON MALT EXTRACT AGAR (pH 5.5) AT 25°C SED (P<0.05) 1.01

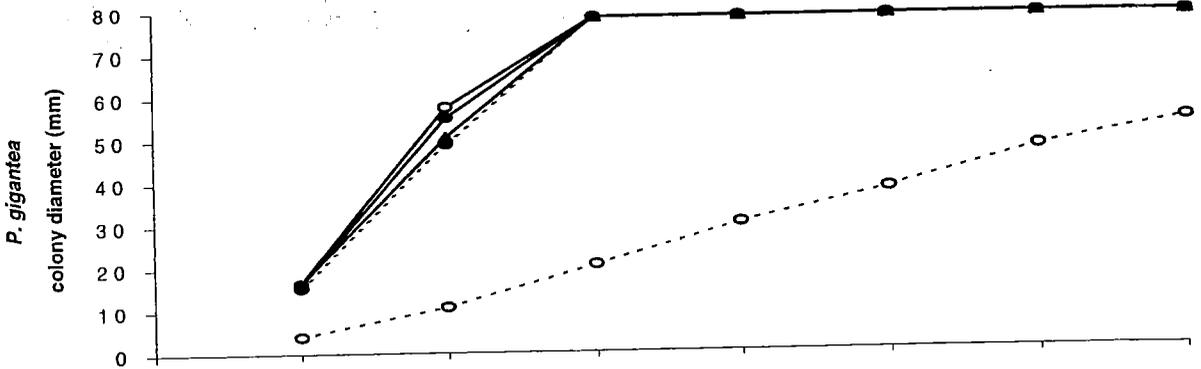


APPENDIX 9 GROWTH RESPONSES OF (A) GLOEOPHYLLUM SEPIARIUM, (B) PHLEBIOPSIS GIGANTEA AND (C) SCHIZOPHYLLUM COMMUNE TO pH ON MALT EXTRACT AGAR AT 30°C IN THE DARK

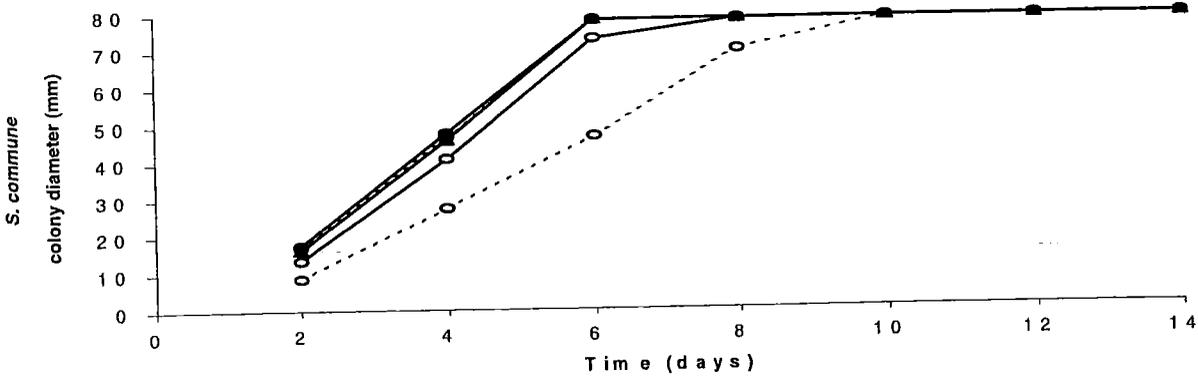
a sed ($P < 0.05$) 2.55



b sed ($P < 0.05$) 1.38



c sed ($P < 0.05$) 2.19



APPENDIX 10 THE INFLUENCE OF (a) ASSAY pH AND (b) TEMPERATURE ON ENZYME ACTIVITY OF *GLOEOPHYLLUM SEPIARIUM*, *PHLEBIOPSIS GIGANTEA* AND *SCHIZOPHYLLUM COMMUNE*

a

Enzyme	Assay pH	Enzyme activity (micromoles glucose min ⁻¹ mL ⁻¹)		
		<i>G. sepiarium</i>	<i>P. gigantea</i>	<i>S. commune</i>
CMCase	4	0.198	0.121	0.246
	5	0.148	0.094	0.290
	6	0.039	0.049	0.318
	6*	0.349	0.096	0.095
	7	0.002	0.018	0.147
Xylanase	4	-	-	8.626
	5	-	-	8.856
	6	-	-	8.569
	6*	0.095	0.667	0.081
	7	-	-	8.795
Mannanase	4	2.485	-	0.011
	5	2.198	-	0.010
	6	1.745	-	0.007
	6*	5.882	0.156	0.288
	7	1.002	-	0.004

* Results from Chapter 2 assays; mean enzyme activity from duplicate flasks.

- No detectable enzyme activity.

b

Enzyme	Assay Temperature (°C)	Enzyme activity (micromoles glucose min ⁻¹ mL ⁻¹)		
		<i>G. sepiarium</i>	<i>P. gigantea</i>	<i>S. commune</i>
CMCase ¹	25	0.08	0.094	0.229
	37	0.105	0.231	0.308
	50	0.299	0.361	0.350
Xylanase ²	25	-	-	3.768
	37	0.015	-	7.683
	50	-	-	8.899
Mannanase ³	25	0.510	-	0.005
	37	1.681	-	0.003
	50	2.997	-	0.005

1 At pH 4 for *G. sepiarium* and *P. gigantea* and at pH 6 for *S. commune*.

2 At pH 5.

3 At pH 4.

- No detectable enzyme activity.

APPENDIX 11 ORIGINS OF 41 TRICHODERMA CULTURES NOT IDENTIFIED AS POTENTIAL ANTAGONISTS OF GLOEOPHYLLUM SEPIARIUM

<i>Trichoderma</i> sp.	Origin
27	CHH Kaingaroa Mill
28	CHH Kaingaroa Mill
32	Hamilton-HR trial site
40	CHH Kinleith log yard
43	CHH Kinleith Forest-Osprey Road litter
44	CHH Kinleith Forest-Osprey Road litter
51	Hamilton-HR trial site
53	Hamilton-HR trial site
54	CHH Kinleith forest-Osprey Road young <i>Pinus radiata</i>
56	Hamilton-HR trial site
57	Hamilton-HR trial site
60	Rotorua-Mamuku cutover
62	Rotorua-Mamuku-mature <i>P. radiata</i>
71	Rotorua scenic reserve
73	Rotorua Redwood Grove-State Forest Park
74	Rotorua Redwood Grove-State Forest Park
76	FR- <i>Eucalyptus</i> species
77	FR- <i>Eucalyptus</i> species
84	FR-mature <i>P. radiata</i>
85	FR-mature <i>P. radiata</i>
86	FR-mature <i>P. radiata</i>
87	FR-mature <i>P. radiata</i>
93	CHH Kinleith log yard
97	CHH Kinleith Mill
100	CHH Kinleith Mill
103	LR- <i>Lentinula edodes medium</i>
104	LR- <i>L. edodes medium</i>
105	LR- <i>L. edodes medium</i>
106	LR- <i>L. edodes medium</i>
107	LR- <i>L. edodes medium</i>
108	LR- <i>L. edodes medium</i>
109	LR- <i>L. edodes medium</i>
110	LR- <i>L. edodes medium</i>
112	LR- <i>L. edodes medium</i>
114	LR- <i>L. edodes medium</i>
125	LR- <i>Pleurotus pulmonarius medium</i>
130	CHH Kinleith Forest-Loop Road mature <i>P. radiata</i>
131	HR culture collection Hend
132	HR culture collection D
136	HR culture collection MI057
137	HR culture collection HR1

CHH = Carter Holt Harvey

FR= Forest Research; LR= Landcare Research; HR= HortResearch

APPENDIX 12.1 WOOD BLOCK PARAMETERS¹ FOR THE BIOLOGICAL CONTROL ASSAYS

Assay	Fresh weight (g)		Dry weight (g)		Moisture content (%)	
	mean	sd	mean	sd	mean	sd
1	1.305	0.0782	0.503	0.0544	161	15.4
2	1.047	0.0917	0.443	0.0368	136	10.0
3	1.346	0.0615	0.558	0.0351	142	11.5
4	1.307	0.0914	0.510	0.0470	157	12.4

¹ Mean of 50 untreated *Pinus radiata* blocks
sd= Standard deviation

APPENDIX 12.2 WEIGHT LOSS AND MOISTURE CONTENT OF PINUS RADIATA BLOCKS OVER 21 AND 28 d PRECOLONISATION TIMES FOR GLOEOPHYLLUM SEPIARIUM, PHLEBIOPSIS GIGANTEA AND SCHIZOPHYLLUM COMMUNE, AND TRICHODERMA SP. 26, 38 AND 101

Treatment	21 d		28 d	
	Wt loss	MC	Wt loss	MC
<i>G. sepiarium</i>	1.7	51	6.8	65
<i>P. gigantea</i>	1.7	49		
<i>S. commune</i>	0.9	50		
<i>T. sp. 26</i>	0.8	46	0.0	47
<i>T. sp. 38</i>	0.8	51	0.1	48
<i>T. sp. 101</i>	1.1	49	0.4	51
Uninoculated contro	0	48	0.3	49
sed (P<0.05)	0.33	1.8	0.42	2.1

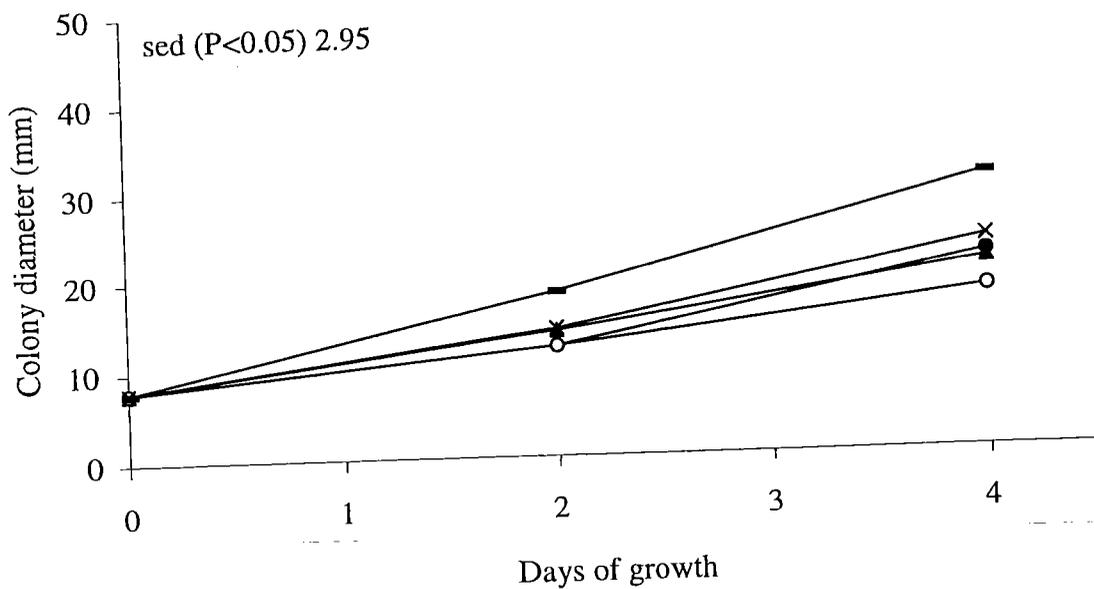
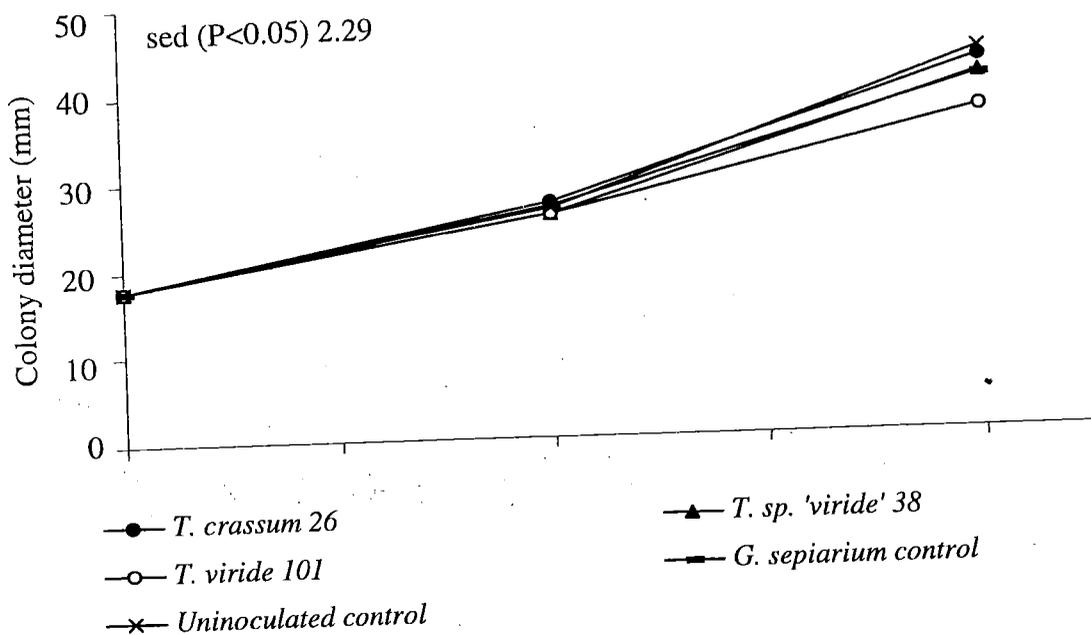
Wt loss= Wood weight loss (%)
MC= Wood moisture content (%)

APPENDIX 12.3 CMCASE ACTIVITY AND FUNGAL BIOMASS IN CMC MEDIUM OF SEVEN UNIDENTIFIED TRICHODERMA CULTURES

<i>Trichoderma</i> sp.	CMCase ¹	BM
46	0.098	7.58
52	0.819	8.49
72	0.246	9.53
115	0.092	9.83
122	0.554	7.77
126	1.141	8.12
127	1.216	7.43
sed (P<0.05)	0.2594	1.600

¹ Micromoles glucose min⁻¹ mL⁻¹
BM= Milligrams dry weight fungal biomass mL⁻¹ after 8 d incubation at 25°C

APPENDIX 13 GROWTH RESPONSE OF GLOEOPHYLLUM SEPIARIUM TO TRICHODERMA VOLATILE ASSAY TREATMENTS ON (A) MALT EXTRACT AGAR AND (B) LOW NUTRIENT AGAR.



APPENDIX 14 GROWTH RESPONSE OF GLOEOPHYLLUM SEPIARIUM TO DIFFERENT APPLICATION VOLUMES OF TRICHODERMA CRASSUM 26 ETHYL ACETATE EXTRACT, IN (TOP) DIRECTION 'A' AND (BOTTOM) DIRECTION 'B'

Application (μL)	Colony diameter (mm) in direction 'a'						
	Days of growth						
	1	2	3	4	5	6	7
10	0.16	0.21	0.37	0.36	0.36	0.37	4.36
20	0.18	0.19	0.18	0.18	0.19	0.21	0.64
30	0.21	0.24	0.23	0.22	0.21	0.22	0.23
40	0.16	0.18	0.17	0.16	0.15	0.16	0.18
50	0.21	0.24	0.23	0.21	0.21	0.22	0.24
<i>G. sepiarium</i> control	4.40	8.00	11.82	16.21	19.53	22.77	25.19
Uninoculated control	4.28	8.00	12.07	16.21	19.47	23.27	25.75
Ethyl acetate control	4.32	8.17	11.86	15.82	19.64	23.25	25.42
Water control	4.44	8.20	11.91	16.12	19.95	22.73	25.77

Application (μL)	Colony diameter (mm) in direction 'b'						
	Days of growth						
	1	2	3	4	5	6	7
10	3.13	4.56	5.34	6.71	7.92	9.96	13.15
20	2.74	3.69	4.11	4.65	5.15	5.66	8.79
30	2.59	3.23	3.67	4.12	4.29	4.59	6.27
40	2.23	2.91	3.37	3.62	3.78	4.01	4.73
50	2.09	2.58	3.15	3.34	3.44	3.53	3.75
<i>G. sepiarium</i> control	3.89	8.01	11.94	16.07	20.39	23.82	26.02
Uninoculated control	4.62	8.22	12.14	16.37	19.74	23.24	25.51
Ethyl acetate control	4.47	8.21	12.12	16.41	19.47	22.91	25.10
Water control	4.48	8.71	12.44	16.16	19.91	23.23	25.61

APPENDIX 15 GROWTH RESPONSE OF GLOEOPHYLLUM SEPIARIUM TO DIFFERENT CONCENTRATIONS OF 6 PENTYL-ALPHA-PYRONE, IN (TOP) DIRECTION 'A' AND (BOTTOM) DIRECTION 'B'

6 PAP concentration	Colony diameter (mm) in direction 'a'						
	Days of growth						
	1	2	3	4	5	6	7
0.05	3.99	7.80	11.24	15.42	18.80	21.86	24.67
0.25	3.95	7.82	11.19	15.51	18.71	21.95	25.00
0.5	4.00	7.29	10.92	16.25	18.31	21.56	24.23
1	3.33	6.57	10.19	13.89	17.14	20.39	22.82
1.5	3.01	6.29	9.99	13.82	17.14	20.75	23.04
2	3.06	5.83	9.03	12.87	15.82	18.73	21.46
2.5	2.36	4.86	7.92	11.55	14.61	17.61	20.23
5	2.05	3.11	4.92	7.17	9.68	12.49	14.11
10	1.28	1.76	1.76	2.27	2.53	3.27	4.34
20	1.45	1.60	1.61	1.63	1.64	1.62	1.60
<i>G. sepiarium</i> control	4.40	8.00	11.82	16.21	19.53	22.77	25.19
Uninoculated control	4.28	8.00	12.07	16.21	19.47	23.27	25.75
Ethyl acetate control	4.32	8.17	11.86	15.82	19.64	23.25	25.42
Water control	4.44	8.20	11.91	16.12	19.95	22.73	25.77

6 PAP concentration	Colony diameter (mm) in direction 'b'						
	Days of growth						
	1	2	3	4	5	6	7
0.05	4.26	8.10	12.03	16.26	19.32	22.51	25.10
0.25	4.25	8.31	11.88	16.19	19.25	22.69	25.07
0.5	4.32	7.88	11.57	15.32	19.14	22.14	24.58
1	4.24	7.56	11.24	15.30	18.61	21.99	24.67
1.5	4.13	7.75	11.31	15.31	18.69	22.31	25.13
2	4.62	7.78	11.52	15.49	18.93	21.93	24.64
2.5	4.07	7.03	10.61	14.32	17.44	20.88	23.98
5	4.07	6.94	10.07	13.39	16.51	19.51	22.33
10	3.97	6.74	8.98	10.56	12.47	13.89	15.54
20	4.07	6.72	8.90	10.38	11.13	11.39	11.60
<i>G. sepiarium</i> control	3.89	8.01	11.94	16.07	20.39	23.82	26.02
Uninoculated control	4.62	8.22	12.14	16.37	19.74	23.24	25.51
Ethyl acetate control	4.47	8.21	12.12	16.41	19.47	22.91	25.10
Water control	4.48	8.71	12.44	16.16	19.91	23.23	25.61

**APPENDIX 16 GROWTH RESPONSE OF GLOEOPHYLLUM SEPIARIUM
IN DIRECTION 'A', TO SOLVENT EXTRACT FRACTIONS OF
(TOP) TRICHODERMA SP. 'VIRIDE' 38 AND (BOTTOM) T. VIRIDE 101**

	Colony diameter (mm)						
	Days of growth						
<i>T. sp 'viride' 38</i>	1	2	3	4	5	6	7
Fraction 1	4.03	7.73	11.36	15.11	18.52	21.97	24.41
Fraction 2	4.43	8.24	12.12	15.87	19.51	23.08	25.85
Fraction 3	4.19	7.94	11.31	15.19	18.38	21.63	24.32
Fraction 4	4.18	8.02	11.58	15.02	17.99	21.45	24.33
Fraction 5	3.79	6.96	10.40	13.90	16.85	20.15	22.90
Fraction 6	3.11	6.18	9.49	12.92	15.83	19.34	22.13
Fraction 7	2.65	5.33	8.39	11.27	13.92	17.10	19.86
Fraction 8	3.22	5.96	8.96	11.83	14.48	17.65	20.08
Fraction 9	3.62	6.94	10.25	13.12	16.00	19.32	21.63
Ethyl acetate control	4.64	8.64	12.57	16.01	19.43	22.61	25.33
Water control	3.97	7.77	11.33	15.14	18.73	22.10	24.86

	Colony diameter (mm)						
	Days of growth						
<i>T. viride 101</i>	1	2	3	4	5	6	7
Fraction 1	4.39	8.14	11.82	15.57	19.18	22.73	25.02
Fraction 2	4.47	8.30	11.79	15.36	18.71	21.85	24.72
Fraction 3	4.15	8.27	11.64	15.70	18.92	22.41	24.60
Fraction 4	4.28	8.08	11.71	15.45	18.92	22.29	25.05
Fraction 5	4.45	7.88	11.51	14.69	18.12	21.31	23.61
Fraction 6	3.51	7.13	10.82	14.63	17.81	21.06	23.86
Fraction 7	1.22	3.65	6.65	10.15	12.85	15.78	18.46
Fraction 8	2.97	6.27	9.83	13.20	16.48	19.72	22.30
Fraction 9	3.90	7.70	11.27	14.70	18.11	21.54	23.92
Ethyl acetate control	4.64	8.64	12.57	16.01	19.43	22.61	25.32
Water control	3.97	7.77	11.33	15.14	18.73	22.10	24.86

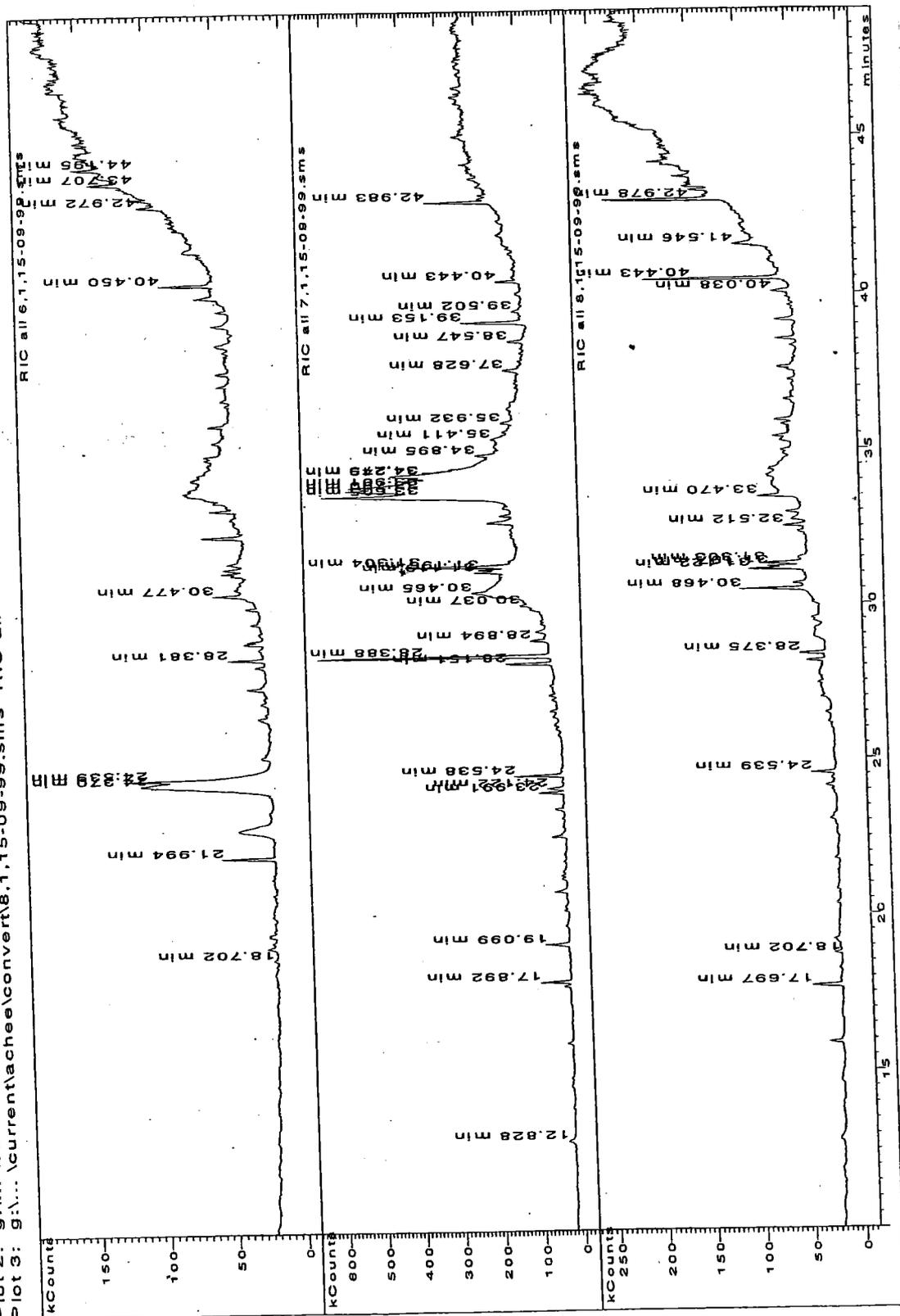
APPENDIX 17 GAS CHROMATOGRAPHY-MASS SPECTROPHOTOMETRY CHROMATOGRAMS OF TRICHODERMA SOLVENT FRACTIONS

Trichoderma crassum 26 fractions 6 (Plot 1), 7 (Plot 2) and 8 (Plot 3)

Print Date: 04 May 2000 10:41:19

Chromatogram Plots

Plot 1: g:\... \current\achee\convert\6,1,15-09-99.sms RIC all
 Plot 2: g:\... \current\achee\convert\7,1,15-09-99.sms RIC all
 Plot 3: g:\... \current\achee\convert\8,1,15-09-99.sms RIC all

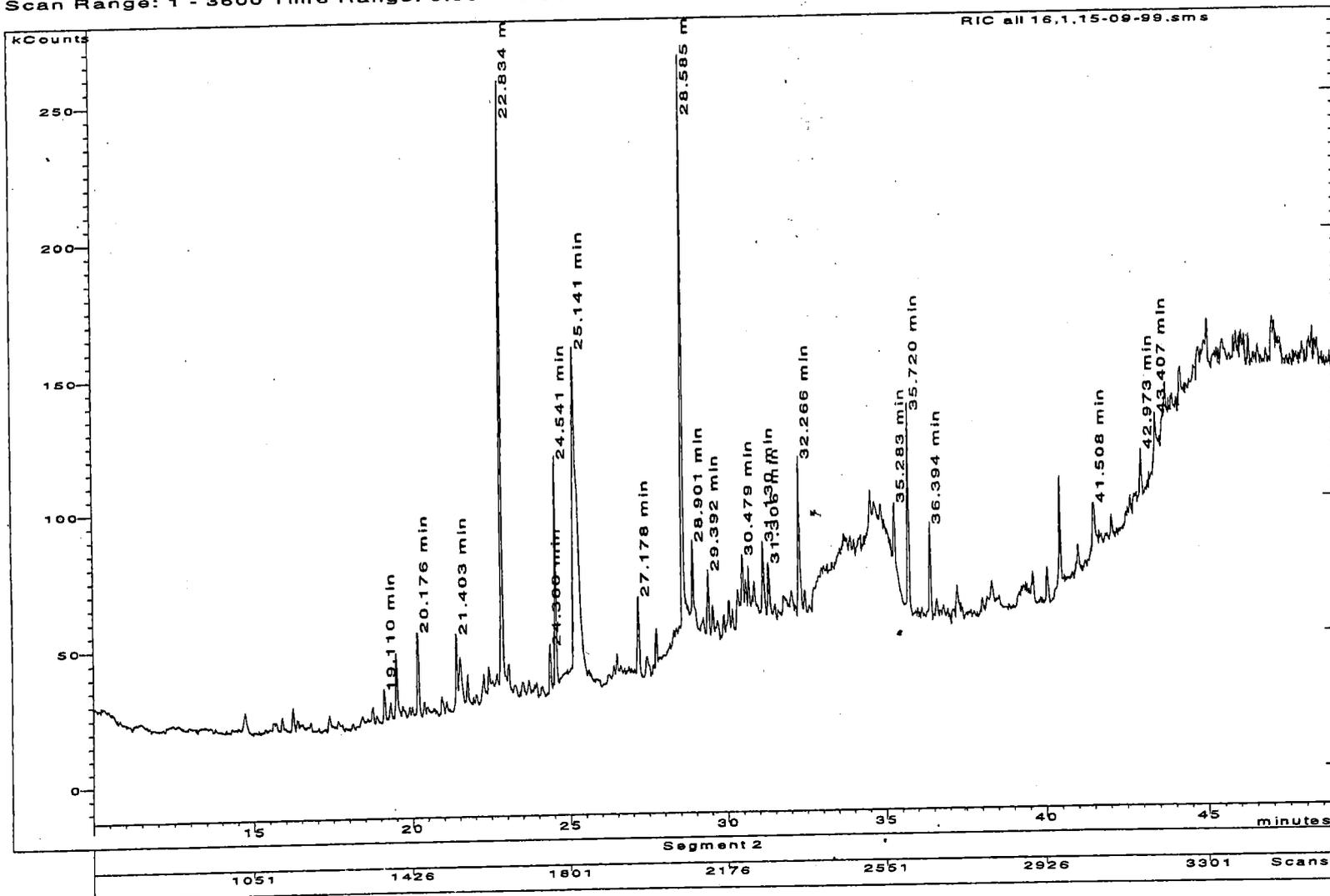


Print Date: 19 May 2000 14:50:49

Chromatogram Plot

File: g:\data\tmt\current\achee\convert\16.1.15-09-99.sms
Sample: 16
Scan Range: 1 - 3600 Time Range: 0.00 - 48.98 min.

Operator: TMT
Date: 15/09/99 19:27



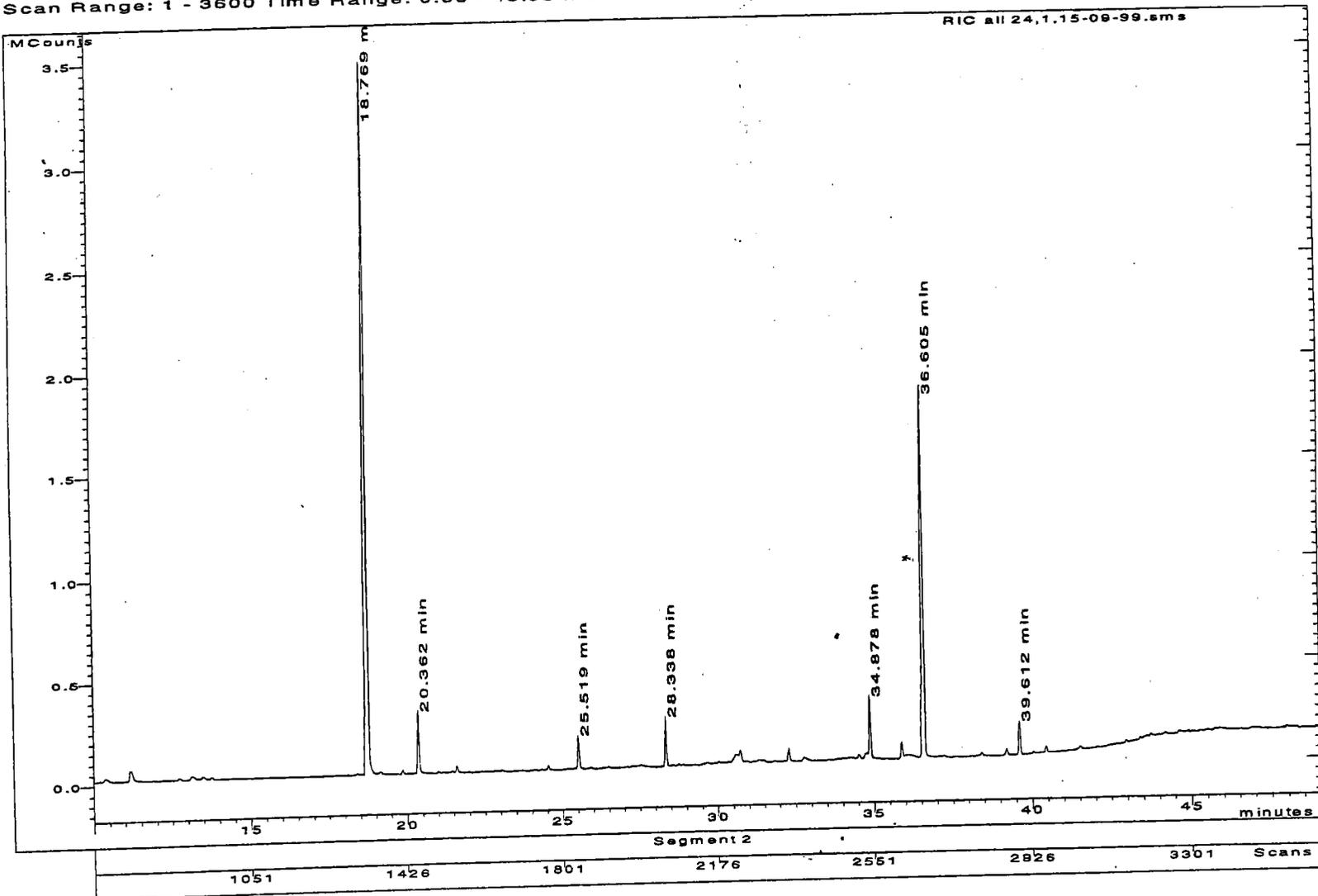
Trichoderma sp. 'viride' 38 fraction 7

Print Date: 19 May 2000 14:07:33

Chromatogram Plot

File: g:\data\tmt\current\achee\convert\24.1.15-09-99.sms
Sample: 24
Scan Range: 1 - 3600 Time Range: 0.00 - 48.98 min.

Operator: TMT
Date: 15/09/99 18:30



Trichoderma viride 101 fraction 7

Results from Chapter Two of this thesis were presented in a paper given by the author to the New Zealand Plant Protection Society Conference held in Hamilton in August 1998.

Ah Chee, A., Farrell, R.A., Stewart, A. and Hill, R.A. (1999). "Decay potential of basidiomycete fungi from *Pinus radiata*." Proceedings of the 51st New Zealand Plant Protection Conference, 235-240.