Lincoln University
Te Whare Wānaka o Aoraki
CHRISTCHURCH • NEW ZEALAND

Lincoln University Digital Thesis

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- you will use the copy only for the purposes of research or private study
- you will recognise the author's right to be identified as the author of the thesis and due acknowledgement will be made to the author where appropriate
- you will obtain the author's permission before publishing any material from the thesis.

Effect of a *Trichoderma* bio-inoculant on ectomycorrhizal colonisation of *Pinus radiata* seedlings

---

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Master of Science

at
Lincoln University
by
R.F Minchin

Lincoln University
2010

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science.

Effect of a *Trichoderma* bio-inoculant on ectomycorrhizal colonisation of *Pinus radiata* seedlings

by

R.F Minchin

Ectomycorrhizal colonisation potential of *Pinus radiata* seedlings inoculated with the commercially available *Trichoderma* species bio-inoculant, Arbor-Guard™, was investigated in a commercial containerised nursery setting and in a separate glasshouse experiment, which included the co-inoculation of specific ectomycorrhizal fungi.

Application of Arbor-Guard™ to *Pinus radiata* seedlings in a containerised commercial nursery had no significant effect on the ability of the naturally occurring ectomycorrhizal (ECM) fungi to colonise the seedlings. *Thelephora terrestris* was the dominant ectomycorrhizal species colonising the *P. radiata* root tips and has been described as a species able to rapidly outcompete other ECM species colonisation, particularly in high organic matter media like that used at the containerised commercial nursery investigated.

In a similar experiment run to augment the commercial experiment, specific ECM fungi identified as *Rhizopogon roseolus*, *Suillus luteus*, and *Rhizopogon villosulus* were co-inoculated with Arbor-Guard™ to investigate the effect on the colonisation potential of the respective ECM species in combination with *Trichoderma*. The treatment effect of the addition of Arbor-Guard™ did not negatively impinge on the ECM species found, or the abundance of ECM root tips colonising the *P. radiata* seedlings. Ectomycorrhizal species in the Thelephoraceae family were the dominant species found colonising the *P. radiata* root tips. Of the inoculated ECM, *S. luteus* was the only detected species colonising the *P. radiata* root tips but was only found in low abundance. Non-conducive abiotic factors for optimum ECM colonisation were considered the most likely reason for the low colonisation of the inoculated ECM species. Any effect of the unintentional co-inoculation of the wood decaying fungi *Hypholoma fasciculare* and *Lentinula edodes*, due to misidentification, with the inoculated ECM species was unable to be resolved in this study. However, it was speculated

that *H. fasciculare* may have had a negative effect on the inoculated ECM species colonisation.

In vitro dual culture assays were initiated to investigate the specific interactions between each of the candidate ECM fungi inoculated in the glasshouse experiment when challenged with each of the six *Trichoderma* isolates in Arbor-Guard™. Both competition for nutrients and/ or space were concluded to be the main antagonistic mechanisms potentially used by five of the *Trichoderma* isolates against all co-inoculated ECM species and *L. edodes*. *Hypholoma fasciculare* was not inhibited by the five *Trichoderma* isolates, however, one *Trichoderma* isolate (LU 663) competitively antagonised all inoculated ectomycorrhizal/ saprophytic species before the mycelial fronts converged. Agar diffusible secondary metabolites were speculated to be potential mechanism of antagonism expressed by LU 663 over volatile antibiotics such as 6-pentyl-α-pyrone. No direct correlation could be dervived from the *in vitro* dual culture assays and what was observed in the containerised *in planta* results.

Overall the results indicated no negative impact of the *Trichoderma* bio-inoculant Arbor-Guard™ on ectomycorrhizal colonisation of *Pinus radiata* seedlings in a containerised nursery system.

Keywords: ectomycorrhiza, *Pinus radiata*, root tip, nursery, containerised, mycorrhization, *Thelephora terrestris*, Thelephoraceae

Acknowledgements

Thankyou to my supervisors Dr Eirian Jones, Professor Leo Condron and Dr Hayley Ridgway for your guidance along the way. In particular I would like to add a special acknowledgement to Dr Jones for her inherent enthusiasm and most importantly not giving up on me, even though that would have been the best option at times!

Special thanks to PF Olsen for the supply of *Pinus radiata* seed and to PF Olsen nurseries Ltd for space within the commercial nursery system to run the experiment.

Thankyou to Dr Robert Hill and Dr Danilo Paderes for your valued input.

A big thankyou to Candice Barclay for your valued assistance of root dissection and processing when time was running short.

A special thankyou to Tanya for your patience and support while I battled on with my own aspirations. Without you I would have not even completed my undergraduate degree let alone this Masters degree. Thankyou for being the rock that kept the gears turning all throughout the years of study and those countless weekends that I spent away.

Table of Contents

Abstract ii

Acknowledgements iv

Table of Contents v

List of Tables ix

List of Figures x

Chapter 1 Introduction 1

1.1 Forestry industry in New Zealand 1
1.1.1 Pinus radiata 1
1.1.2 Pinus radiata seedlings 1
1.2 Mycorrhiza 3
1.2.1 Ectomycorrhiza 4
1.2.2 Physiology of the ECM relationship 4
1.2.3 Nutrient acquisition 5
1.2.4 Pathogen inhibition 6
1.3 Ectomycorrhiza and Pinus radiata 6
1.3.1 Ectomycorrhiza and Pinus radiata seedlings 8
1.3.2 Ectomycorrhizal identification 9
1.4 Trichoderma 10
1.4.1 Mycoparasitism 11
1.4.2 Antibiosis 11
1.4.3 Plant growth promotion and induced resistance 12
1.5 Interactions between ECM and Trichoderma 13
1.6 Research aims and objectives 15
1.6.1 Aim and context 15
1.6.2 Hypothesis 16
1.6.3 Objectives 16

Chapter 2 Effect of *Trichoderma* species inoculation on indigenous ECM colonisation of *Pinus radiata* seedlings in a commercial nursery 17

2.1 Introduction 17
2.1.1 PF Olsen Nursery 17
2.2 Methods 18
2.2.1 Experimental site 18
2.3 Experimental design 18
2.4 Silvicultural management and experimental setup 19
2.5 Trichoderma application and assessement 20
2.6 Seedling harvesting and processing 21
2.7 Ectomycorrhizal analysis 22
2.7.1 Root processing 22
2.7.2 Ectomycorrhizal quantification 22
2.7.3 Ectomycorrhizal morpological characterisation 23
2.8 Molecular methods for ECM identifcation 23
2.8.1 DNA extraction 24
2.8.2 PCR amplification 24
2.8.3 Sequencing reactions 24
2.9 Statistical analysis 25
2.10 Results 26

2.10.1 Total ECM root tips 26
2.10.2 ECM root tips as discriminated by morphotype 26
2.10.3 Non-mycorrhizal root tips 29
2.11 Trichoderma counts (5 weeks) 29
2.12 Trichoderma counts (harvest) 30
2.13 Seedling parameters 30
2.14 Discussion 32

Chapter 3 Effect of *Trichoderma* spp. inoculation on colonisation of *Pinus radiata* by specific ECM species 42

3.1 Introduction 42
3.2 Methods 42
3.2.1 Experimental design 42
3.3 Silvicultural management and experimental setup 43
3.4 ECM inoculation 43
3.4.1 Source and maintenance of ECM fungi 43
3.4.2 Inoculum production 44
3.4.3 Pot inoculation 44
3.4.4 Pure culture species identification 45
3.5 Trichoderma application and assessment 45
3.6 Seedling harvesting and processing 46
3.7 Ectomycorrhizal analysis 46
3.7.1 Root processing 46
3.7.2 Ectomycorrhizal quantification 47
3.7.3 Ectomycorrhizal morphological characterisation 47
3.8 Molecular methods for ECM identification 47
3.9 Statistical analysis 47
3.10 Results 47
3.11 Total ECM root tips 47
3.12 Inoculated ECM species identification 50
3.12.1 Pure species inoculum potential 52
3.13 ECM root tips as discriminated by morphotype 52
3.14 Morphotype change over time 53
3.15 Non-mycorrhizal root tips 54
3.16 Trichoderma counts 55
3.17 Seedling parameters 56
3.18 Discussion 57

Chapter 4 *In vitro* laboratory assays 66

4.1 Methods 66
4.1.1 Co-culture interaction assay 66
4.1.2 Microscope interaction assay 68
4.1.3 Volatile antibiosis 69
4.2 Results 69
4.2.1 Co-culture interaction assay 69
4.2.2 Microscopic assay 72
4.2.3 Antibiosis assay 72
4.3 Discussion 73

Chapter 5 Concluding discussion 77

References 82

Personal communication references 92

Appendix A **92**
A.1 Seedline parameters 92
A.2 Agar recipes 92
A.2.1 Trichoderma selective agar (TSM) 92
A.2.2 Modified Melin Norkrans medium (MMN) 93
Appendix B **94**
B.1 Chapter 2 ANOVA tables 94
B.1.1 Analysis of variance (ANOVA) table for the overall total ECM root tips/ cm root length enumerated at the PF Olsen nursery (Figure 2.2) 94
B.1.2 Analysis of variance (ANOVA) table for the total number of ECM root tips/ cm root length categorised into their respective morphotypes (Figure 2.4) 94
B.1.3 Analysis of variance (ANOVA) table for the total non-mycorrhizal root tips enumerated (Figure 2.5) 94
B.1.4 Analysis of variance (ANOVA) table for the *Trichoderma* cfu populations found 5 weeks after sowing and at harvest for figures 2.6 A & B, respectively. 95
B.1.5 Analysis of variance (ANOVA) table for each of the tree seedling parameters measured (Table 2.4) 95
B.2 Chapter 3 ANOVA tables 96
B.2.1 Analysis of variance (ANOVA) table for the total ECM root tips/ cm root length enumerated in the Lincoln experiment (Figure 3.1) 96
B.2.2 Analysis of variance (ANOVA) table for the total ECM root tips as split by the dominant species enumerated (Figure 3.3) 96
B.2.3 Analysis of variance (ANOVA) table for the relative proportions of morphotypes found, expressed as total ECM root tips/ cm root length (Figure 3.4), and the change in total ECM root tips/ cm root length from the first assessment to the second assessment, also split into each respective morphotype (Figure 3.5). 97
B.2.4 Analysis of variance (ANOVA) table for the total non-mycorrhizal root tips (Figure 3.6) 97
B.2.5 Analysis of variance (ANOVA) table for the total *Trichoderma* cfu population enumerated at harvest (Figure 3.7) 98
B.2.6 Analysis of variance (ANOVA) table for each of the seedling parameters measured (Table 3.4) 98
B.3 Chapter 4 ANOVA tables 99
B.3.1 Analysis of variance (ANOVA) table for the radial growth rates of each of the respective fungal species in the *in vitro* lab assays (Table 4.3) 99
Appendix C **100**
C.1 Table 2.3; Internal transcribed spacer (ITS) region sequences 100
C.1.1 *Thelephora terrestris*; unramified, sequence 1 (640 bp) 100
C.1.2 *Thelephora terrestris*; unramified, sequence 2 (635 bp) 100
C.1.3 *Thelephora terrestris*; dichotomous, sequence 1 (619 bp) 100
C.1.4 *Thelephora terrestris*; dichotomous, sequence 2 (615 bp) 101
C.1.5 *Thelephora terrestris*; multi-dichotomous, sequence 1 (638 bp) 101
C.1.6 *Thelephora terrestris*; multi-dichotomous, sequence 2 (643 bp) 101
C.2 Table 3.2; Internal transcribed spacer (ITS) region sequences 102
C.2.1 Thelephoraceae; unramified (643 bp) 102
C.2.2 Thelephoraceae; dichotomous (644 bp) 102
C.2.3 Thelephoraceae; multi-dichotomous (644 bp) 102
C.2.4 *Suillus luteus*; multi-dichotomous (670 bp) 103
C.3 Table 3.3; Internal transcribed spacer (ITS) region sequences 103
C.3.1 *Rhizopogon roseolus*; (677 bp) 103

C.3.2 *Rhizopogon villosulus*; (675 bp) 103
C.3.3 *Lentinula edodes*; (734 bp) 104
C.3.4 *Suillus luteus*; (669 bp) 104
C.3.5 *Hypholoma fasciculare*; (671 bp) 104
C.3.6 *Scleroderma bovista*; (634 bp) 105

List of Tables

Table 2.1 Outline of the six treatments applied to two *P. radata* seedlines (A + B) in the commercial nursery experiment at PF Olsen nursery. 19
Table 2.2 Macroscopic description of the three morphotypes found. 27
Table 2.3 Sequence results for the GenBank queries of ECM root tips identified as *Thelephora* (*Th.*) *terrestris* classified by levels of ramification at PF Olsen. 28
Table 2.4 Effect of the six treatments consisting of control, Arbor-Guard™ applied at sowing (sow) and Arbor-Guard™ applied after 3 months (3mth), which were further divided by the two seedlines (A and B) on the seedling parameters assessed 5 weeks after seed sowing or at harvest. Treatment means within a row followed by the same letter are not significantly different as analysed by Fisher's Protected LSD p=0.05 32
Table 3.1 Outline of the 4 treatments applied to the *P. radiata* seed (seedline A) in the Lincoln glasshouse experiment. 43
Table 3.2 Sequence results for the GenBank queries of ECM root tips classified by levels of ramification at Lincoln 49
Table 3.3 Fungal species received from Ensis, Rotorua and their actual species identity as identified from pure culture DNA extraction followed by sequencing. 51
Table 3.4 Lincoln seedling parameters (seedline A) measured at final harvest for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. All results within rows were non-significant (NSD) as determined by Fisher's Protected LSD (p=0.05). 57
Table 4.1 Number of days each candidate ECM and saprophytic species respectively were grown on ¼ strength MMN agar before each respective *Trichoderma* isolate was co-inoculated in the co-culture and antibiosis interaction *in vitro* assays 68
Table 4.2 Qualitative interaction observations of each Arbor-Guard™ *Trichoderma* isolate in MMN agar co-culture with each respective candidate fungal species inoculated into the ECM mixture at Lincoln University (n=6). 70
Table 4.3 Radial growth rate (mm day^{-1}) of each candidate fungal species when grown without physical contact in the same atmosphere with each *Trichoderma* isolate in the antibiosis assay. Asterisk within rows indicate significantly different radial growth rate relative to the control as determined using unrestricted LSD p=0.05 72
Table 4.4 Relative amounts of PAP produced (mg/ plate) by each *Trichoderma* isolate from Dodd-Wilson's (1996) work relative to the average level of inhibition found across all fungal species inoculated in the ECM mixture at Lincoln University 76

List of Figures

Figure 2.1 BCC Side-Slit trays used at PF Olsen nursery with *Pinus radiata* seedlings cradled ½ metre above the ground (right picture) 19

Figure 2.2 Number of ECM root tips/ cm root length at harvest for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B, respectively. Treatment means sharing the same letter are not significantly different from each other as determined by Fisher's Protected LSD (*p*= 0.05). 26

Figure 2.3 The three morphotype categories as discriminated by their level of ramification. Unramified (A), Dichotomous (B) and Multi-dichotomous (C). 27

Figure 2.4 Number of ECM root tips/ cm root length for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B respectively categorised in to dichotomous, multi-dichotomous and unramified morphotypes. Means are separated using Fisher's protected LSD (*p*= 0.05). 28

Figure 2.5 Number of non-mycorrhizal root tips found for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B, respectively. Treatment means sharing the same letter are not significantly different from each other as determined by Fisher's Protected LSD (*p*= 0.05). 29

Figure 2.6 *Trichoderma* cfu/ g soil ($\log_{10}$ transformed) five weeks after sowing the *Pinus radiata* seed (graph A) and at final harvest (graph B) for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B, respectively. Treatments sharing the same letter (within graphs) are not significantly different as defined by Fisher's Protected LSD (*p*= 0.05). 30

Figure 2.7 Difference in *Pinus radiata* seedling height due to uneven watering. A; Greener larger seedlings versus B; lighter coloured stunted seedlings (arrows indicate the average height of seedlings) 40

Figure 3.1 Total ECM root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. Means are separated using Fisher's Protected LSD (*p*=0.05). 48

Figure 3.2 Representative samples of the root tips at Lincoln University including the Thelephoid species morhptypes unramified (A); dichotomous (B); and multi-dichotomous (C) mophotypes and *Suillus luteus* multi-dichotomous (D). 49

Figure 3.3 Total ECM root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation, as split by the two dominant detected ECM species in the Thelephoraceae family and *Suillus luteus*. Means are separated by Fisher's Protected LSD (*p*=0.05). 50

Figure 3.4 Relative proportions of dichotomous (D), Multi-dichotomous (MD) and unramified (U) ECM root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation as split by species found in the Thelephoraceae family and *Suillus luteus*. Means are separated using Fisher's Protected LSD (*p*=0.05). 53

Figure 3.5 Relative proportions of total ECM root tips/ cm root length of dichotomous (D), Multi-dichotomous (MD) and unramified (U) morphotypes from the first

set of assessments (Time 1) to the second set of assessments (Time 2). Means separated by Fisher's Protected LSD (*p*=0.05)....... 54
Figure 3.6 Total non-mycorrhizal root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. Means separated by Fisher's Protected LSD (*p*=0.05). 55
Figure 3.7 Log_{10} transformed *Trichoderma* cfu means for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. Treatment means are separated from each other as determined by Fisher's Protected LSD (*p*= 0.05). 56
Figure 4.1 Representative co-culture interaction pictures of the sporulating *Trichoderma* species (right plug) interacting with each candidate ECM species (left plug) (B); Interaction with *Trichoderma* (LU 663) for each of the species included in the ECM mixture (includes saprophytic species) (D); Representative picture of interaction between *Hypholoma fasciculare* and each *Trichoderma* species (C). Letters (B, D and C) represent each category outlined in Table 4.2. 71

Chapter 1
Introduction

1.1 Forestry industry in New Zealand

The forestry industry is the third largest export earner, accounting for 3.1% of New Zealand's gross domestic product (GDP) in 2004. Sustainably harvested wood products in 2004 equated to 21 million m^3 (NZ $3.3 billion), while it is expected to exceed 40 million m^3/ year in 2025, equating to an estimated income of $14 billion, which is >14% of GDP (Anonymous, 2004; MAF, 2004). The forestry industry directly employs 23,000 people with an estimated 100,000 people indirectly employed (Anonymous, 2004).

Globally, NZ is in the top 20 suppliers of timber products, supplying 1.1% of the world's forestry products from just 0.05% of the world's forest resource. By 2025 New Zealand's global position is expected to be in the top 5 (Anonymous, 2004; MAF, 2004).

The main forestry species planted in NZ is *Pinus radiata* D. Don, making up to 90% of the 1.83 million hectares planted in forestry plantations (Burdon, 1992).

1.1.1 Pinus radiata

Pinus radiata D. Don is native to the Californian coast and was introduced to New Zealand in 1859 (Burdon, 1992). Due to the fast growth of radiata pines and ease of silvicultural management from the nursery to the plantation, it has become one of the worlds' major exotic commercial forestry trees of choice (Burdon, 1992). *Pinus radiata's* capacity for fast growth in New Zealand, producing 200-250 m^3 of wood per hectare in 25-30 years, can mainly be attributed to the temperate climate, thereby enabling growth throughout the whole year. *Pinus radiata* also has the capability of growing on a range of soil types and at different altitudes, permitting extensive areas in New Zealand to be planted (Burdon, 1992). Radiata pine can be processed into products such as sawn logs, pulp and paper, plywood, chipboards and particle board (Burdon, 1992). Other uses include erosion control, shelter, municipal firewood and recreation.

1.1.2 Pinus radiata seedlings

Successful forest establishment, when seedlings are planted out into plantations, is a critical phase in commercial forestry. Therefore, the production of high quality *P. radiata* seedlings is important, both for high survival rates and competitive early growth of trees planted into

plantations (Maclaren, 1993). Production of plantable *P. radiata* seedlings (ca. 25-30 cm tall) is done within 9 months from the initial sowing of seed. Seeds are planted from early October – November and harvested for planting out in early July - late August.

The cost of breeding controlled-pollinated seeds is high ($3000-3500/ kg seed) as it is a very labour intensive process and there is a strong market demand for the improved seed (Anonymous, 2003). Coupled to this is the fact that trees are required to be a minimum of 5-10 years old before mature seed is produced, and during this time only a small amount of seed is available (Burdon, 1992). Therefore, the production of genetically improved stock is expensive and takes time before any economic return is realised. For this reason, cuttings are also taken from stoolbed stock, or from field grown trees, in an attempt to supply the demand for genetically improved seedlings. This research, however, is going to focus on *P. radiata* seedlings grown from controlled-pollinated seed.

Traditionally, nurseries were situated in close proximity to the forestry plantation in which they were planted (Chu-Chou and Grace, 1990). This close proximity allowed an ectomycorrhiza (ECM) inoculum bank within the nursery soil to develop as a result of spore dispersal from surrounding forestry plantations.

Bare rooted seedlings, obtained from nurseries described above, are being increasingly replaced by containerised seedling production (Menzies et al., 2001). Seedlings raised in containers have several advantages over bare rooted seedlings, including, an extended planting season, less root disturbance from lifting through to transport and final planting, bestowing a higher establishment percentage due to less transplant shock (Anonymous, 2003). The plug of potting media encapsulating the root system provides this protective function, which increases pre-plant shelf life and reduces the potential of distorting roots while planting into plantations (Anonymous, 2003; Nelson, 1996). Indeed, Chavasse, (1980) stressed that successful seedling establishment depends on careful handling from the initial lifting (de-plugging) to planting. *Pinus radiata* survival in the field is also improved using containerised seedlings as the seedlings have better development of tap roots, in turn reducing the threat of mortality in dry conditions (Anonymous, 2003).

Containers used in propagating seedlings have evolved over the years from tall and thin, to the development of containers with a squat shape (the height of the container is >2.2 times the diameter of the top) that incorporate vertical slots allowing full lateral root-pruning, which is induced by the roots being exposed to the air (Nelson, 1996). Another positive function of the vertical slots is the elimination of roots coiling around the inside surface of the container

forming a “root cage” (Nelson, 1996). Full lateral air root-pruning allows seedlings to develop many new and healthy lateral roots, reducing poor root form that was induced by earlier container designs and bare-rooted seedlings, thus conforming to the trees innate pattern of root morphology (Nelson, 1996). Rapid generation of new roots into the soil after planting out is considered a crucial component of seedling survival and potential growth (Chavasse, 1980). Tap and lateral roots that develop in more than one plane have also been shown to increase the stability of seedlings planted out (Chavasse, 1980). Therefore, containers incorporating full lateral air root-pruning, as a result of side-slits on the wall, have proven to be far superior to traditional growth containers by creating many active root tips throughout the plug. More importantly, modern containers reduce the requirement for harsh root conditioning that bare-rooted seedlings are subjected to in order to generate seedlings that are ready to be transplanted. However, the capital cost of producing containerised seedlings is higher than its bare-root counterparts. Apart from the extra cost of purchasing the trays they are also often suspended above the ground to increase the air flow around and through the trays. This additional cost nevertheless is offset when high value genetic tree stocks are used. Seedlings of this calibre require a high survival and growth rate at planting to ensure an economic return on the initial investment. Containerised seedlings ensure a higher survival, once planted-out, over their bare-rooted counterparts.

1.2 Mycorrhiza

The term mycorrhiza denotes a mutualistic symbiosis between a soil fungus “myco” and the plant root, “rhiza” (Morgan et al., 2005). Mycorrhizal relationships are considered the normal state for the majority (>80%) of terrestrial plants under most ecological conditions, and is characterised by the bi-directional flow of nutrients (Smith and Read, 1997). Photosynthetically derived carbon from the plant is obtained by the mycorrhiza, and in return, nutrients originating from the soil are acquired and transferred by the mycorrhiza to the plant. There is widespread acceptance that the mycorrhizal association is extremely important, without which many plants would not survive, as the mycorrhiza effectively form the interface between plants roots and the soil.

Seven different types of mycorrhizal associations have been defined, including arbuscular mycorrhiza, ectomycorrhiza, ectendomycorrhiza, ericoid, arbutoid, monotropoid, and orchid mycorrhiza. Out of these associations the first two, arbuscular mycorrhiza (AM) and ectomycorrhiza (ECM) form relationships with plants that are most agronomically important. Arbuscular mycorrhiza are the most common mycorrhizal symbiosis, forming in roots of a huge amount of plants, including the taxa, Angiosperms, Gymnosperms, Pteridophytes and

Bryophytes. The obligately symbiotic AM have characteristic structures, arbuscules, that penetrate root cortical cells, and vesicles, which can form within and between the cortical cells. Intracellular arbuscules function to exchange nutrients and carbon between the two symbionts. In contrast, the Hartig net of ECM, which carries out the same function as the arbuscules, is made up of an intercellular labyrinth of hyphae enveloping root cortical cells. In addition, a mantle sheath, which is made up of multiple layers of fungal tissue, encases plant lateral root tips and gives rise to the mycorrhizas "ecto" name, as it is a discernible feature of ECM visible to the naked eye. The fungal taxa that form ECM relationships are highly diverse and include Basidiomycetes (95%) and Ascomycetes (4.8%), with reports of Zygomycetes in rare cases (Taylor and Alexander, 2005). Ectomycorrhizas are mostly associated with woody perennials including members of the Pinaceae, Fagaceae, Betulaceae and Dipterocarpaceae generae. Ectomycorrhizas of the Pinaceae family, in particular *Pinus radiata*, are the focus of this research.

1.2.1 Ectomycorrhiza

A large number of fungal species (between 5000-6000) form ECM relationships, with the majority of them having a broad host range (Smith and Read, 1997). This gives rise to the large diversity found in ECM relationships. However, it has been traditionally thought that the amount of tree species supporting ECM symbiosis is relatively low, with approximately only 3% of plant taxa forming ECM associations (Smith and Read, 1997). The low proportion of plant taxa forming ECM symbiosis, however, is incommensurate compared with the extensive terrestrial land area occupied by these woody perennials. This bestows an enormous ecological and economic importance on the ECM relationship. Nevertheless, the paradigm of thought is evolving, from only a few species of plants thought to form ECM relationships characteristic of the temperate and boreal forest regions, to a much larger appreciation of the previous underestimation of ECM host species in tropical regions (Taylor and Alexander, 2005).

1.2.2 Physiology of the ECM relationship

Perhaps the most fundamentally important constituent of the ECM symbiosis is the extramatrical (extraradical) mycelium extending into the edaphic environment. The function of which is considered to be the primary site of nutrient and water uptake. It is here at the interface between the soil and mycelia where critically important interactions occur with soil micro- and meso-fauna, dubbed the "mycorrhizosphere effect" (Leake et al., 2004;

Linderman, 1988). The intricate interactions occurring in this region are also important in the inhibition of potential pathogens.

1.2.3 Nutrient acquisition

Extramatrical mycelial networks are major components of total soil microbial biomass, with their length and absorptive area very important for nutrient acquisition. For every metre of root length there can be up to 1000-8000 metres of ECM hyphae (Leake et al., 2004). External mycelium in forest humus has been estimated to have seasonal growth rates of 820 kg ha^{-1} yr^{-1} in some cases (Bomberg et al., 2003). Mycelia of many ECM grow in exploratory diffuse fans forming the advancing front. Here, fine hydrophilic hypha, with high surface area to volume ratios, extensively ramify into substrate enabling the efficient uptake of nutrients (Timonen and Sen, 1998). While most research has focused on the upper organic horizon, where root tip density is highest, ECM mycelia is not restricted to these profiles and has been shown to vertically descend into mineral soil (Dickie et al., 2002; Rosling et al., 2003). Overall, the increased surface area and extension of hypha into areas too small for roots, allows exploration of soil outside the nutrient depletive zone surrounding plant roots (Morgan et al., 2005).

Through this extensive network of hyphae, ectomycorrhiza release extracellular enzymes, low molecular weight organic acids and hydroxamate siderophores that in turn acquire essential nutrients such as N, P and Fe from inorganic as well as organic sources (Bending and Read, 1995; Frey-Klett et al., 2005; Landeweert et al., 2001; Leake et al., 2004; Read et al., 2004; Timonen et al., 1998). Another very important function that the extramatrical mycelium performs is the dissemination of photosynthetically derived carbon, and hence energy into the soil environment (Chalot et al., 2002; Timonen et al., 1998). Undeniably this carbon supply to the microbial community is imperative for the energy demands of nutrient acquisition that is required in the multitrophic ectomycorrhizal complex (Morgan et al., 2005). This enables the ectomycorrhiza to structure, and to synergistically interact with the soil microorganism community (Chalot et al., 2002; Frey-Klett et al., 2005; Timonen et al., 1998). Indeed, it has been shown that ectomycorrhiza exert a strong selection pressure on the microbial community associated with the mycorrhizosphere, which in turn is very important in the nutrient acquisition dynamics of plants (Frey-Klett et al., 2005). The role ECM play in the biogeochemistry cycle is more complicated than first thought. Finlay, (2005) commented that there has been a “general evolution in thinking” that mycorrhiza extends, and passively absorbs inorganic nutrients for plants. However, this paradigm of thinking is changing, now acknowledging that mycorrhiza actively influences the abiotic and biotic soil environment,

mainly through mycorrhizosphere exudates, in effect coercing the biotic community structure allowing synergistic interactions to occur that are conducive to plant health and growth.

1.2.4 Pathogen inhibition

In conjunction with increased nutrient acquisition, ectomycorrhizal symbiotic relationships with trees are known to increase resistance to root and shoot pathogens (Duchesne, 1994; Morgan et al., 2005; Whipps, 2004). One of the mechanisms involved to counteract pathogen attack, is simply due to increased vigour of the host plant as a direct result of enhanced nutrient supply (Duchesne, 1994). While the superior nutrient immobilising qualities of ECM can effectively out compete pathogens for essential nutrients, both for propagule germination and growth (Duchesne, 1994). More direct mechanisms include the ECM mantle physically excluding potential pathogen attack as well as inducing the resistance of plants to pathogen attack (Duchesne, 1994; Morgan et al., 2005; Whipps, 2001). Antibiosis is another direct mechanism, whereby ECM are capable of producing both volatile and non-volatile antibiotics, both in the mantle and mycorrhizosphere, in turn antagonising soil pathogens (Duchesne, 1994; Morgan et al., 2005; Slankis, 1974; Whipps, 2001). Niermi et al. (2000) showed the ability of the ectomycorrhizal fungus *Laccaria proxima* (Boud.) Pat. to protect *P. sylvestris* P. containerised seedlings from the infection of two uninucleate *Rhizoctonia* isolates (248 and 264) one year after pathogen inoculation.

1.3 Ectomycorrhiza and Pinus radiata

Ectomycorrhiza species occurring in New Zealand forests of *P. radiata* of varying age (2, 5, 10, and 17 years) of both the North and South islands include *Rhizopogon rubescens* Tul. & Tul., *R. luteolus* Fr., *Suillus luteus* (L.) Roussel., *S. granulatus* (L.) Roussel., *Tuber* sp., *Endogone flammicorona* Trappe & Gerd., *Amanita muscaria* (L.) Lam., *Laccaria laccata* (Scop.) Cooke., *Inocybe* spp., *Tricholoma pessundatum* (Fr.) Quel. and *Thelephora terrestris* Ehrh. (Chu-Chou and Grace, 1983; Chu-Chou and Grace, 1988). Species that are only found in the North Island include *Hebeloma crustuliniforme* (Bull.) Quel. and *Scleroderma* spp. Ectomycorrhizal species most commonly found in both islands were *R. rubescens*, *S. luteus* and *L. laccata* (Chu-Chou and Grace, 1988). While it has been concluded that *Rhizopogon* spp. are the dominant species making up the major component of ECM in most New Zealand forests of all ages (Chu-Chou, 1979). Overall, the ECM diversity of *P. radiata* in NZ is known to be depauperate compared with the potential number of species known to associate with radiata pine overseas (Chu-Chou and Grace, 1988; Walbert, 2008).

Not all species of ECM incur the same benefits to the health and growth of *P. radiata* trees (Dunabeitia et al., 1996; Perry et al., 1987). Significant functional diversity exists within ECM communities as well as habitat related differences in the functioning of ECM communities (Orlovich and Cairney, 2004). Habitat differences have been shown in New Zealand with certain species of ECM affiliated to certain nutrient environments. For example, *R. rubescens* was isolated from 7 year old *P. radiata* trees in a conventional low fertility forestry soil in high proportions (54-79%), relative to very low proportions (0-11%) when grown on farm land with high fertility (Chu-Chou and Grace, 1984; Chu-Chou and Grace, 1987). *Endogone* spp. and *Scleroderma* spp. were not as efficient in promoting nutrient uptake and tree growth as compared with *R. rubescens*, and have been found to mostly colonise trees in high fertility sites (Chu-Chou and Grace, 1984; Chu-Chou and Grace, 1987). *Suillus* spp., and to a lesser extent *Tuber* sp., were also found to be dominant ECM species colonising seedlings in high fertility soils (Chu-Chou and Grace, 1987; Chu-Chou and Grace, 1990). Dynamics of the ECM species colonising *P. radiata* trees have been shown to be complicated, with species colonising *P. radiata* roots seemingly changing to suit the environmental niche. Chu-Chou and Chu-Chou & Grace (1980; 1990) found *S. luteus* to be replaced by *R. rubescens* within 6 months of planting out into low fertility forests, this follows the pattern of colonisation described above. Along with *R. rubescens, R. luteolus* has also been shown to rarely colonise *P. radiata* roots in new nurseries established on former high fertility farm land (Chu-Chou and Grace, 1990). Analogously, *R. luteolus* sporocarps have been associated in large numbers in forestry plantations with soil of low fertility. This led Chu-Chou and Grace, (1990) to conclude that *R. luteolus's* successful colonisation of *P. radiata* is related to nutrient fertility rather than soil type.

Along with different ECM species suiting particular conditions, there has been speculation of the successional change of ECM species evolving during the life cycle of trees (Smith and Read, 1997). Successional change has been attributed to factors such as the changing quantity and quality of organic matter, and hence nutrient availability, in which the functional characteristics of the ECM community need to change to enable the acquisition of nutrients (Smith and Read, 1997). In *P. radiata* stands older than 5 years of age, both *Suillus* spp. and *Inocybe* spp. were frequently observed but were rarely seen in nurseries or stands younger than 5 years (Chu-Chou, 1979). Further, *Amanita muscaria* and *Scleroderma verrucosum* (Bull.) Pers. were at no time detected in seedlings or trees under 10 years old, however, they were abundant in *P. radiata* stands older than 15 years (Chu-Chou, 1979).

Later research carried out by Walbert, (2008) using a combination of morphological characterisation and molecular techniques of identification found *A. muscaria* a component of both the ECM root tips and above ground sporocarps in an 8 year old *P. radiata* forestry stand in the Kaingaroa forest, located in the North Island of New Zealand.

1.3.1 Ectomycorrhiza and Pinus radiata seedlings

Inoculation of nurseries with efficient ECM fungi, using forest duff originating from forest soils, fungal sporocarps or mycelia (Chu-Chou and Grace, 1990), is increasingly being done (El Karkouri et al., 2005), and considered desirable to do so if a new nursery is established or growing seedlings in potting substrates that are replaced annually (Chu-Chou and Grace, 1990; Theodorou and Benson, 1983; Trappe, 1977). Aside from the selection of potential ECM-seedling associations just for seedling survival and growth, abiotic variables such as temperature ranges of the area that the nursery resides also need to be considered (Theodorou and Bowen, 1971). Nevertheless, the final evaluation of seedling survival and growth performance must be in the forest after planting out (Chavasse, 1980; Chu-Chou and Grace, 1990; Trappe, 1977), thereby matching the ECM species being inoculated with seedlings to the areas where the seedlings will be finally planted. However, ECM species composition has been shown to change once *P. radiata* is planted out in forests from their original composition as seedlings (Chu-Chou and Grace, 1983; Chu-Chou and Grace, 1990; Walbert, 2008), with species diversity in forests generally higher than in nurseries (Chu-Chou and Grace, 1990; Walbert, 2008). Yet, dominant species established in nurseries, such as *R. rubescens* and its respective strains (Chu-Chou and Grace, 1983), and to a lesser extent *R. luteolus*, did not change once planted out into plantations (Chu-Chou and Grace, 1987; Chu-Chou and Grace, 1990). This shows the possible "multi-stage" characteristics (Smith and Read, 1997), of both *R. rubescens* and *R. luteolus*, in that they are present throughout the life cycle of *P. radiata*. From field observations of sporocarps in New Zealand, Chu-Chou, (1979) noted that *R. rubescens*, *R. luteolus*, and *L. laccata* were numerous in nurseries and *P. radiata* stands through the ages of 3-5 years old, but in stands older than 10 years the sporocarps became an infrequent phenomenon. *Hebeloma crustuliniforme* formed abundant sporocarps in nurseries, however, they were not observed as much once planted out (Chu-Chou, 1979). These observations reveal some of the successional dynamics of ECM species emerging from early ECM colonisers of seedlings through to multi-stage or latter ECM colonisers. However, all the descriptions of ECM species colonising seedlings have been done on sporocarp surveys, and there is evidence that sporophore populations may not correlate with ECM species colonising the root tips of seedlings (Smith and Read, 1997; Walbert, 2008). Confounding this

problem is the fact that some species of ECM require special conditions in which to produce sporocarps. In particular, sporocarp production requires enormous amounts of nutrients (Taylor and Alexander, 2005), with nursery conditions precluding the ability for vast nutrient acquisition. While even if the sporocarps are present they can be easily missed, especially hypogeous sporocarps. As a result, ectomycorrhizal species identification and quantification has increasingly focused on directly observing the ECM root tips on seedlings. Unpublished work, looking at ECM root tips on *P. radiata* seedlings, has indicated that *Wilcoxina* sp. is an early coloniser that has not been identified by sporocarps in New Zealand nurseries (Walbert, 2008). Observing root tips will give a better representation of actual ECM species present and offers other advantages such as being able to determine their relative importance.

Root tip observation of ECM species colonising seedlings in containerised systems is required, as sporocarps are extremely rare in these systems. This is mainly due to potting media being replaced each year, therefore not allowing any ECM inoculum bank to accumulate. Further, ECM species colonising *P. radiata* roots in containerised systems in New Zealand have not been investigated.

1.3.2 Ectomycorrhizal identification

Ectomycorrhizal species colonising *P. radiata* seedling root tips can be identified by both morphological and molecular methods. Morphological identification of root tips is the traditional way of identifying ECM, as the mantles and any emanating mycelia and/ or rhizomorphs, colour, structure and surface texture are well conserved features (Agerer, 1991). However, morphological identification is a limited approach in that specific characteristics can change depending on the particular host species, and more importantly in this case, morphology can be expressed differently in contrasting environmental conditions. Coupled to this, is that many species of ECM in association with specific hosts remain morphologically unidentified due to most species being identified only by their fruiting structures alone (Bruns et al., 1998). With the advent of appropriate molecular techniques, such as polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP), many of the identification problems associated with morphotyping alone have been circumvented. As a result, the deduction of ECM in symbiosis with *P. radiata* has been made more accurate. Molecular methods also require less time to perfect as they don't require skilled taxonomic scrutiny. As a result, identification of ECM has taken on a combined approach of the initial screening of root tips using gross morphological characteristics, which are then further analysed with molecular methods to determine the mycorrhizal species. This methodological approach has the advantage of being able to deal with large numbers of samples, as initial

gross morphological characterisation is quick and subsequent samples can be freeze-dried or frozen in liquid nitrogen for further processing at a later stage (Bruns et al., 1998).

The use of PCR to amplify specific regions of deoxyribose nucleic acid (DNA), which is then further digested by restriction endonucleases (PCR-RFLP), is a common method to identify ECM species colonising root tips (Bruns and Gardes, 1993; Gardes and Bruns, 1993). Nuclear encoded ribosomal DNA (rDNA) is the main gene cluster used for phylogenetic analysis of fungi (Bruns and Gardes, 1993; Gardes and Bruns, 1993). The rDNA is used because it is made up of well conserved genes that encode the ribosomal subunits 18S, 5.8S and 28S. While variable regions also exist within this DNA region, which can be used to differentiate between fungal species, and this includes the two non-coding internal transcribed spacer regions (ITS), ITS1 and ITS2, which separate the structural RNA subunits (Bruns and Gardes, 1993; Gardes and Bruns, 1993; White et al., 1990). By designing primers that are complementary to sequences within the rDNA, the species variable non-coding ITS regions are amplified during PCR. The high copy number of rDNA in cells allow for small or dilute DNA samples to be amplified. While the relatively small size, between 600 to 800bp and hence readily amplified ITS regions are convenient for both PCR amplification and further restriction analysis or sequencing for ECM identification.

1.4 Trichoderma

Trichoderma spp. are ubiquitous soil dwellers in temperate and tropical soils. Concentrations of these cosmopolitan soil fungi range from $10^1 – 10^3$ culturable propagules per gram of soil (Klein and Eveleigh, 1998). *Trichoderma* spp. are particularly dominant in the top organically rich soil horizons (F & H) of both deciduous and coniferous forests. Dominance in these soil horizons can mainly be attributed to the aggressive saprophytic nature of *Trichoderma* spp., as they have the ability to compete for and metabolise a wide range of carbon and nitrogen compounds, including some persistent recalcitrant compounds (Kubicek-Pranz, 1998). This innate ability enables some *Trichoderma* spp. to proliferate, compete and survive in complex ecosystems such as the plant rhizosphere (Harman et al., 2004). Strong rhizosphere competency, which is defined as the ability of organisms to colonise and grow rapidly in association with plant roots, is one mechanism employed by particular *Trichoderma* isolates in the bio-control of plant pathogens (Harman et al., 2004). *Trichoderma* hyphae in effect create a “living barrier” along the plant root, thus out competing and preventing pathogen incursion (Brimner and Boland, 2003). *Trichoderma* spp. have been described as avirulent plant symbionts, forming long lasting interrelations with plant roots which are considered *Trichoderma’s* ecological niche (Harman, 2000; Harman et al., 2004).

Trichoderma spp., in addition to competition and rhizosphere competency, also have other mechanisms of bio-control activity that either on their own, or in combination, are antagonistic to potential pathogens.

1.4.1 Mycoparasitism

Mycoparasitism is defined as the direct antagonism of one fungus on another. It is a complex process requiring four successive steps leading to an overall highly species specific mechanism (Chet et al., 1998). The steps include the chemotrophic growth of *Trichoderma* spp. towards the target fungi. A chemical gradient of amino acids and sugars released by the target fungi are thought to be the attractants, but at this stage there is no specific stimulus attracting *Trichoderma* spp. to any one host (Chet et al., 1998). This is followed by the second step, specific recognition, whereby lectins (sugar-binding glycoproteins) on the target fungus agglutinate to specific complementary carbohydrates on the cell wall of *Trichoderma* spp. (Chet et al., 1998; Harman et al., 2004). *Trichoderma* spp. attach themselves in the third step by forming appressorium like structures and coil around the target fungi (Chet et al., 1998; Hjeljord and Tronsmo, 1998). Then finally, the fourth step involves the secretion of specific lytic enzymes including, chitinases, β-glucanases and proteases, which degrade the host cell wall.

1.4.2 Antibiosis

Mycoparasitism by *Trichoderma* spp. is more often than not associated with the concomitant release of secondary metabolites with antifungal and/ or antibacterial activities. *Trichoderma* spp. are prolific producers of volatile or non-volatile secondary metabolites, these metabolites are comprised of a very large and diverse range of compounds in relation to structure and function. Six major groups have been defined including, polyketides, pyrones, terpenoids, isocyano derivatives (isonitiriles), diketopiperazines and peptaibols (Sivasithamparam and Ghisalberti, 1998). The two best known *Trichoderma* antibiotics, gliotoxin and gliovirin, are diketopiperazines, and function to prevent the resynthesis of cell walls (Brimner and Boland, 2003). Antibiotics can also be involved in synergistic mechanisms with hydrolytic enzymes such as chitinases and β-glucanases (Di Pietro et al., 1993; Schirmbock et al., 1994). The concurrent release of antibiotics and hydrolytic enzymes would enable the lowering of the critical concentration of antibiotic required for pathogen inhibition. There is a high ecological relevance of this synergism due to two observations. One, the low effective doses of inhibitory compounds found *in vivo* and, two, the limited amounts of nutrients found in soil for antibiotic production.

1.4.3 Plant growth promotion and induced resistance

Trichoderma spp. have been shown to enhance root growth and development, with the subsequent increase in the uptake of nutrients and overall crop productivity (Harman et al., 2004). Along with the normal NPKS fertilisers, *Trichoderma* spp. can solubilise nutrients such as Cu^{2+}, Mn^{2+} and Zn^{0} that are normally unavailable to plants, and produce siderophores that in turn chelate the very scarce resource iron (Harman et al., 2004; Whipps, 2001). *Trichoderma* spp. also have the ability to help plants tolerate abiotic stresses, such as the inactivation of toxic compounds in the rhizosphere (Harman et al., 2004).

The biochemical elicitors produced by *Trichoderma* spp. and the act of root colonisation itself, is known to induce localised and systemic resistance in plants (Harman et al., 2004). Induced resistance is defined as "the process of active resistance dependant on the host plant's physical or chemical barriers, activated by biotic or abiotic agents" (Whipps, 2001). Plants initiate plant defence responses such as increased peroxidase activity and the secretion of fungitoxic compounds such as chitinases into the rhizosphere once in contact with *Trichoderma* spp. (Howell, 2003). The plants are said to be potentiated, enabling them to react rapidly when attacked by pathogens as a direct result of the *Trichoderma*-plant interaction (Harman et al., 2004).

Mechanisms such as these are the reason behind the use of *Trichoderma* spp. as a bio-control agent. Hence, commercial products have become available on the market for a variety of crops. Included in the commercial products available for pathogen antagonism in forestry trees is Arbor-Guard™. Arbor-Guard™ is a product marketed by PF Olsen and manufactured by Grow-chem NZ Ltd and contains an assortment of 6 different *Trichoderma* isolates found to increase *P. radiata* seedling growth and establishment.

Inoculation of *P. radiata* seedlings with Arbor-Guard™, is currently being studied for control of pine root rot diseases such as *Armillaria* root rot (Hill, R., *per comm.*). Results at present are promising, with *Armillaria* root rot being suppressed at the important transitory stage of seedling planting into plantations, reducing tree mortality in the forest by 35% after two years, and what looks like similar levels of disease suppression up to fours years afterwards (Hill, 2004/2005). Increased growth and establishment of *P. radiata* seedlings after inoculation with Arbor-Guard™ has also been shown in the nursery. Seedling height increases of up to 33%, and stem diameter increases up to 25% over the control have been recorded (Hill, 2004/2005).

1.5 Interactions between ECM and Trichoderma

Biological control organisms have the potential to have negative effects on ECM colonisation and persistence within the rhizosphere (Brimner and Boland, 2003). This is attributed to bio-control agents inherent capacities to control pathogenic organisms, particularly fungal pathogens, and hence the potential to suppress symbiotic mycorrhiza (Brimner and Boland, 2003). As some strains of *Trichoderma* spp. are particularly aggressive rhizosphere competent fungi (Harman, 2000), their potential to out compete ECM for essential resources is highly probable. Also *Trichoderma* spp. are capable of colonising plant rhizospheres long term, so are not just a transitory phenomenon (Harman, 2000). These attributes in effect create a 'living barrier' encapsulating the root, enabling bio-control agents such as *Trichoderma* to disrupt the rhizosphere soil community (Brimner and Boland, 2003). Indeed *Trichoderma* spp. can potentially replace or suppress endogenous fungi that form symbiotic relationships on plant root surfaces (Howell, 2003). However, despite all the potential interactions, from ECM inhibition to synergistic relations, and sometimes ECM antagonism towards bio-control agents, there are conflicting reports on the effect *Trichoderma* spp. have on mycorrhizal fungi (Hjeljord and Tronsmo, 1998).

Both biochemical elicitors and root colonisation by *Trichoderma* spp. are known to induce localised and systemic resistance in plants (Harman et al., 2004). Indeed plants initiate defence responses such as increased peroxidase activity and the production of fungitoxic compounds such as chitinases into the rhizosphere (Howell, 2003). Plants are said to be potentiated, and to react rapidly when attacked by pathogens as a direct result of the *Trichoderma*-plant interaction (Harman et al., 2004). These plant reactions could potentially have an inhibitory effect on ECM colonisation (Brimner and Boland, 2003).

Trichoderma spp. have been shown to enhance root growth and development, with the subsequent increase in the uptake of nutrients (Harman et al., 2004). Increased inorganic nutrient supply to plants by *T. harzianum* Rifai., due to its capacity to solubilize poorly soluble nutrients such as rock phosphate, has been confirmed (Harman, 2000). A *Trichoderma*-plant association such as this may have the potential to inhibit subsequent ECM colonisation due to the plant not requiring nutrition assistance.

Primary ectomycorrhizal development of forest trees is reliant on growth or germination of hyphae and/ or spore propagules stimulated by root exudates (Marx and Krupa, 1978; Smith and Read, 1997). As *Trichoderma* spp. are excellent competitive saprophytes, any specific

seed exudates required for ECM propagule germination or hyphal growth may be metabolised, potentially preventing ECM colonisation (Harman et al., 2004; Howell, 2003).

Werner *et al.*, (2002) questioned the use of *Trichoderma* spp. as bio-control agents in forest nurseries, as a direct result of their antagonism towards ECM colonisation. The authors' cause of concern was due to the heavy re-colonisation of *Trichoderma* spp. observed after soil fumigation, which subsequently eliminated establishment of the inoculated ECM in the *P. sylvestris* root system. Summerbell, (1987) showed the strongly antagonistic effect that *T. viride* Pers. Ex Gray. and to a lesser extent *T. polysporum* (Link) Rifai. had on *Laccaria bicolor* (Maire) P.D. Orton. mycorrhization of Black Spruce (*Picea mariana* (Mill.) Britton.) seedlings. The authors also let *L. bicolor* establish on the root system before the inoculation of *T. viride* and found that a small (4.4%) but significant increase in percentage colonisation could be achieved over the simultaneous inoculation of the two fungal species. Malyshkin, (1951; cited in Summerbell, 2005), showed that what was identified as *Trichoderma lignorum* (Tode) Harz. (probably *T. harzianum* or *T. viride*; (Summerbell, 2005)) stimulated the mycorrhization of oak seedlings in the field. In further work done by Malyshkin, (1955; cited in Summerbell, 2005), the author increased the mycorrhization of oak seedlings once again by approximately 100% using a "biological fertiliser" comprising of three microorganisms, *Azotobacter chroococcum* Beijerinck., *Pseudomonas* sp. and *Trichoderma lignorum* (later identified as *T. viride* (Summerbell, 2005)). Later Shemakhanova, (1962; cited in Summerbell, 2005), using the same three microogansims, this time separately, found that *T. viride* gave the greatest simulative response to ectomycorrhiza colonising pine seedlings in the field. Soil sterilisation has been shown to stunt *P. radiata* seedling growth (Chavasse, 1980). Patchy distribution of ECM in the nursery was attributed to the seedlings growth being stunted, which was a direct result of *Trichoderma* populations successfully out competing the ECM. *Trichoderma* spp., due to their fast saprophytic growth characteristics, may simply out-compete every ECM fungus engaged in the mycorrhization process (Taylor and Alexander, 2005). Taylor and Alexander, (2005) highlighted that there is much to learn about the interactions between ECM and other soil microorganisms, and how these interactions determine the activity and success of the plant-ECM relationship and ultimately the terrestrial ecosystem. Bowen and Theodorou, (1979) suggested that the successful mycorrhization and persistence of ECM could be threatened by bio-control agents and resident soil rhizosphere organisms. The authors also went on to say that enhancing antagonistic interactions of soil pathogens by implementing appropriate management schemes should not be to the detriment of ECM colonisation and persistence.

However, the effect that *Trichoderma* spp. has on ECM colonisation of seedlings is not well understood. Nevertheless microbial interactions in the rhizosphere are very dynamic, with the potential for positive interactions amid *Trichoderma* spp. and ECM. In fact interactions between mycorrhiza and the bio-control species *Trichoderma* could be said to form a continuum, from inhibition of potential mycorrhizal symbiosis, through to mycorrhizal antagonism towards *Trichoderma* spp. Zadworny *et al.* (2004) demonstrated the mycoparasitic abilities of the ECM fungus *L. laccata* towards *T. harzianum* in the rhizosphere of 3-month-old *P. sylvestris* seedlings. Werner *et al.* (2002) in a similar experiment, showed the ability of *L. laccata* to mycoparasitise the bio-control fungus *T. virens* (Mill, Giddens & Foster.) Arx. in the rhizosphere of *P. sylvestris* seedlings. In a more recent study by Zadworny et al. (2007) the authors illustrated the use of cell wall lytic enzymes exuded by the ECM fungus *Laccaria laccata* in association with *P. sylvestris* seedlings when parasitising the cell walls of *T. virens* and *T. harzianum*. The authors showed the dissolution of β-1,3-glucan from the hyphal and spore cell walls of both soil saprophytes. Overall the conflicting results found from different authors confirm the little knowledge researchers have about the interaction between bio-control agents, in particular *Trichoderma* spp., and ECM fungi in the rhizosphere of seedlings (Zadworny et al., 2004). This calls for the much needed research into the rhizosphere interactions of specific *Trichoderma* isolates in bio-control inoculants with ECM colonising *P. radiata* seedlings.

1.6 Research aims and objectives

1.6.1 Aim and context

Trichoderma isolates have been shown to improve *P. radiata* seedlings survival, particularly against Armillaria root rot, once planted into forestry plantations. However, the interactions between *P. radiata* seedlings, ectomycorrhiza and *Trichoderma* spp. are unknown. For seedling health, growth and survival, both in the nursery and after outplanting, the outcome of potential interactions needs to be established in order to produce sustainable commercial tree crops of high quality wood.

The overall aim of this research is to establish the effect of a *Trichoderma* bio-inoculant, applied as the registered commercial product Arbor-Guard™, on the root colonisation of ECM fungi on *Pinus radiata* seedlings.

The framework of this research will follow the commercial production of *Pinus radiata* seedlings at PF Olsen & Co Limited nursery, Waiuku, New Zealand. The research will be comprised of two components, each of which will be separate experiments. The first located

at the PF Olsen commercial nursery, and the second, a glasshouse pot experiment at Lincoln University.

1.6.2 Hypothesis

We premise that the application of *Trichoderma* isolates in the form of a commercial product, Arbor-Guard™, will have an effect on both the overall percentage colonisation and relative species diversity of ECM associated with *P. radiata* seedlings.

1.6.3 Objectives

Experiment 1
To determine what effect *Trichoderma* spp. inoculation, in the form of Arbor-Guard™, has on indigenous ECM colonisation within the PF Olsen commercial nursery setting, the objectives are;

Objective 1
To identify the background ECM species colonising *Pinus radiata* seedlings at the PF Olsen commercial nursery without the application of the *Trichoderma* bio-inoculant, Arbor-Guard™.

Objective 2
To determine the effect of Arbor-Guard™ inoculation at sowing, or after 3 months growth, on ECM species diversity and abundance colonising *Pinus radiata* seedlings in the PF Olsen commercial nursery.

Experiment 2
To determine what effect Arbor-Guard™ inoculation has on the colonisation of specific ECM species inoculated with the *P. radiata* seedlings at sowing at Lincoln University, the objectives are;

Objective 3
To inoculate *Pinus radiata* seedlings with specific ECM species to determine their colonisation potential.

Objective 4
To determine what effect *Trichoderma* spp., inoculated as the commercial product Arbor-Guard™, have on the colonisation of inoculated ECM.

Objective 5

To undertake additional *in vitro* laboratory assays to assess potential interactions between individual *Trichoderma* isolates and the inoculated ectomycorrhizal species.

Chapter 2

Effect of *Trichoderma* species inoculation on indigenous ECM colonisation of *Pinus radiata* seedlings in a commercial nursery

2.1 Introduction

2.1.1 PF Olsen Nursery

Pinus radiata trees are bred to suit a wide range of planting sites and match the particular end use market targeted by the grower. Controlled pollinated seedlots are ranked in a certification system according to their growth and form (GF Plus). Characteristics including wood density, *Dothistroma* resistance, spiral grain, growth, straightness and branching are given individual ratings in a scaling system. High number ratings, of any particular characteristic, correlate to the best genetic potential available at the particular time (Anonymous, 2003). As a result, any two seedlines that express different genetic potentials of specific traits can exude differing qualities and quantities of carbon compounds into the rhizosphere (Morgan et al., 2005). In turn this can have a major influence on the microbial community composition within the rhizosphere. Here lies the potential of genetically distinct trees coercing the rhizosphere environment in different ways, which can have a profound effect on potential symbiotic relationships with ECM species and *Trichoderma* interactions. In light of this, two seedlines with different wood and end use characteristics have been selected for comparative analysis. Their individual GF Plus ratings are outlined in Appendix A.1.

Trichoderma spp. have the ability to out compete or destroy by parasitism/ antibiosis other indigenous soil fungi for resources, this may be true for the ECM/ *Trichoderma* interaction in the rhizosphere of *P. radiata*. Therefore, by staggering the Arbor-Guard™ inoculation, having one treatment inoculated at seed sowing while another treatment is inoculated after 3 months of seedling growth will reveal any potential competitive characteristics expressed by *Trichoderma*. A period of three months should give the ECM enough time to initiate colonisation with the *P. radiata* seedling and therefore not be out-competed by the applied *Trichoderma* spp. in Arbor-Guard™. Indeed, Hall and Perley, (2008) were successful in forming ECM root tips on *Nothofagus* seedlings three months after ECM inoculation, even when *Nothofagus* seedlings were considered a hard species to inoculate artificially.

2.2 Methods

2.2.1 Experimental site

PF Olsen nursery is situated approximately 45 minutes drive SW of Auckland, just outside the town of Waiuku and is owned and operated by P.F Olsen and Company Ltd. The nursery raises containerised seedlings of *P. radiata* either from cuttings or directly from seed in plastic Side-Slit trays (100 mL plug volume) supplied by BCC Sweden, which are designed to incorporate full lateral root pruning. *Pinus radiata* seeds are planted in spring (October-November) in a 50:50 mix of peat and pine bark media and harvested 9 months later in June-July. Seedlings are watered by an automatic irrigation system that aims to keep the potting media at field capacity throughout the 9 month period. Fertiliser is dispensed within the irrigation system (fertigation), along with a slow release fertiliser incorporated in the potting mix at sowing. The overall aim of fertiliser addition is to have a relatively high nitrogen rate after seedlings are planted in spring, followed by autumn applications of fertiliser with a high potassium to nitrogen ratio. Both spring and autumn fertiliser applications are designed to be in harmony with *P. radiata's* natural growth curve. Fertigation is adjusted according to the amount of precipitation, therefore during high rainfall periods the amount of fertiliser applied to the seedlings is reduced.

2.3 Experimental design

A complete randomised block design was incorporated into the existing commercial practice at PF Olsen nursery. Six treatments were set up, including two genetically distinct seed-lines (see Appendix A.1) where each had a control, Arbor-Guard™ inoculation at sowing and Arbor-Guard™ inoculation after 3 months of seedling growth `(Table 2.1). Each treatment was made up of four trays in a row, each of which had 45 seedlings per tray (180 seedlings). Treatment rows were randomly assigned and replicated only once in each block, with five blocks in total. This gave a grand total of four trays per treatment by six treatments per block by five blocks, equalling a total of 120 trays.

Table 2.1 Outline of the six treatments applied to two *P. radata* seedlines (A + B) in the commercial nursery experiment at PF Olsen nursery.

Treatments	Description	Seed line
1	Control	A
2	Arbor-Guard™ at sowing	
3	Arbor-Guard™ at 3 months	
4	Control	B
5	Arbor-Guard™ at sowing	
6	Arbor-Guard™ at 3 months	

2.4 Silvicultural management and experimental setup

As described in Section 1.1.2, trays are designed for lateral root pruning and made by BCC Sweden (http://www.bccab.com). The plastic trays are appropriately named Side-Slit trays and have 63 (arranged in rows of 7 x 9), 100 mL volume plugs for sowing seedlings. Seedlings are planted in two rows adjacent to one another, leaving an empty row before another two rows of seedlings. This configuration gives a plant density of 400 seedlings/ m^2, which has been shown to produce *P. radiata* seedlings of good form and of maximum trunk diameter (5-7 mm) (Benenbroek. M. *pers com.*). As described in Section 1.1.2, the trays are cradled on wires 0.5 m above the ground to increase the efficacy of air pruning and are arranged in rows of four wide across the length of the nursery.

Figure 2.1 BCC Side-Slit trays used at PF Olsen nursery with *Pinus radiata* seedlings cradled ½ metre above the ground (right picture)

The two pine seedlines, GF Plus A and B, obtained from P.F Olsen and Company Ltd, were sown. Prior to sowing the seeds were soaked in cold water for 48 h, drained then left on moist filter paper in a glass Petri dish and stratified at 4ºC for a further 32 days. On the day of sowing the seeds were coated with a bird repellent recipe, made up of 75 g Mezurole, ¾ cup PVA glue and ½ cup red spray indicator in 1 litre of water. Once coated the seeds were spread out over a suspended shade cloth to a depth of approximately 5 mm and dried with a hair dryer before being planted.

Only the five rows to be planted of the seven within the Side-Slit trays were filled with premixed 50:50 peat: bark potting mixture by hand. This was to reduce potential weed pressure due to weeds growing in the non-planted rows at the latter stages of seedling growth. Seeds were then planted to a depth of 5-10 mm by hand after individual indentations in each cell were pressed on the surface of the potting mix by a plate with protruding forks. After sowing the trays were kept in the dark at 23ºC in an incubation room for 3 days before being moved to a glasshouse. Seedlings stayed in the glasshouse (>15ºC) for 3 months to allow protection from wind and birds before being moved outside to their permanent positions. During the seedlings occupancy in the glasshouse and once outside, the potting media was kept at field capacity by an automatic fertigation sprinkler system.

2.5 Trichoderma application and assessement

Trichoderma was applied as a 0.2 g L^{-1} Arbor-Guard™ (5 x 10^9 spores g^{-1}; Arbor-Guard™) solution with a knapsack sprayer at a rate of 250 mL per tray (≈ 5.5 mL/ plug) immediately after sowing to treatments 2 and 5 (Table 2.1). All other non- Arbor-Guard™ treatments (1, 3, 4 and 6) received water at 250 mL per tray immediately after sowing. The aim was to get a *Trichoderma* population base of 5.5 x 10^4 colony forming units (cfu) per gram of potting media (100 g potting media/ cell). This is only an approximate value, as an overall solution (10 L) was made up and applied by hand to each tray (40 trays in total). As a result, an assessment of the actual *Trichoderma* potting mix population was done 5 weeks after application to both seedline controls and the Arbor-Guard™ inoculated seedlings at sowing treatments (1, 2, 4 and 5). Three months after sowing, treatments 3 and 6 (Arbor-Guard™ at 3 months) had the same rate of Arbor-Guard™ applied as described above, with a knapsack sprayer. However, to avoid disturbing the seedlings an assessment to determine the actual *Trichoderma* concentrations in the potting media was not carried out until final seedling harvest.

At the end of the experiment all the treatments were assessed for their *Trichoderma* populations as described below.

For each *Trichoderma* assessment a composite sample of four randomly chosen plugs (one from each of the four trays) of each treatment were collected and stored at 4ºC until processing. Three 10 g sub-samples (field capacity) of each treatment composite sample were weighed out and shaken for 10 minutes in 90 mL of sterile 0.01% water agar. After standing for a further 10 minutes the sample was serially diluted down to 10^{-6}, with 0.1 mL of each dilution factor (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} & 10^{-6}) plated out on three replicate plates of *Trichoderma* selective medium (TSM) (McLean et al., 2005) (Appendix A 2.1). A representative 10 g (fresh weight) sub-sample of each composite sample was dried at 105ºC for 24 hrs to determine water content. Plates were incubated for 10-14 days at 20ºC in the dark, followed by counting the number of *Trichoderma* spp. cfu and expressed relative to soil dry weight.

2.6 Seedling harvesting and processing

Five weeks after sowing a seedling emergence/ survival assessment was conducted on treatments 1, 2, 4 and 5, where each of the respective cells containing no seedlings were recorded. Treatments 3 and 6 were omitted from an emergence/ survival assessment due to these treatments (Arbor-Guard™ applied at 3 months) being the same as the untreated control at this stage.

After nine months, seedlings were harvested by removing the whole seedling from its respective cell with an intact root system and associated potting media. A total of three seedlings per tray were randomly taken from the middle rows of each tray to reduce any boundary effects. Thereby each treatment had twelve seedlings (four trays/ treatment) randomly taken from each block, giving a grand total of 360 seedlings. Seedlings were transferred into pre-labelled plastic bags to reduce moisture loss and stored at 4ºC within 12 hours of harvest.

All of the 360 seedlings were subsequently processed by first measuring and recording the total length of the seedling from the potting media surface to the seedlings tip and measuring the trunk diameter at the root collar with a tape measure and digital callipers, respectively. After which the seedling tops were cut off from the root system approximately 1 cm from the potting media surface and stored in paper bags for drying (oven dried for 48 h at 65ºC), while the root systems were put back in the original plastic bags with enough water added via a spray bottle to soak the potting media and stored at 4ºC until further processing.

2.7 Ectomycorrhizal analysis

A combined approach of separating morphologically discrete ECM under a dissecting microscope, followed by the further identification by molecular methods was the method used in this project (see Section 1.3.2).

2.7.1 Root processing

Individual seedling roots and associated potting mix (50:50 peat and pine bark) were soaked in water overnight. The seedlings root systems were then carefully cut with scissors at 25 mm increments along the plugs length (750 mm) giving a total of three segments. The respective segments were then carefully washed with running tap water over a series of nested sieves ranging in aperture size from 4 mm – 0.85 mm (4, 1.4, 1.18 and 0.85 mm) to remove the majority of adhering potting media. Any roots longer than 25 mm were subsequently cut with a scalpel on a cutting board and transferred to a hyaline plastic tray (36 x 24 cm) filled to 1 cm with water. The base of the plastic tray was divided into a gridline of 100 squares (3.6 x 2.4 cm) for subsequent sub-sampling. Any clumps of potting media/ root system that could not be separated macroscopically were transferred to a Petri dish filled with water for the subsequent removal of the potting media under a dissecting microscope (0.8 – 8× magnification) using fine tweezers, paintbrushes and fine metal needles (Brundrett et al., 1996). Each of the sieves contents were further analysed for root segments and mycorrhizal laterals by suspending the contents in a plastic tray filled to 1 cm with water and any root samples or ECM root tips found were transferred to the root collection tray.

2.7.2 Ectomycorrhizal quantification

A 10% sub-sample of the whole root system was taken by evenly suspending the segmented roots over the whole plastic tray area and transferring the contents of 10 randomly (derived from a random number table) chosen squares to one of two 14 cm round glass Petri dishes that were again divided into squares (1 cm x 1 cm). Total root length was then determined using the gridline intersect method described by (Brundrett et al., 1996). This method entails dispersing roots into a 9 cm Petri dish filled with water that has a 14/11 mm gridline drawn on the base of the dish. By counting all the roots that intersect both the horizontal and vertical lines an estimate of total root length in centimetres can be obtained. Because the Petri dish size and the gridline proportions used in this experiment was different from that of the original a calibration was required. This was done by randomly dispersing a 1 m piece of string cut into 25 mm segments over the Petri dish and counting the intersects as per the original method. A total of 10 individual counts were done after randomly dispersing the

string segments for each count to determine the length of the string, after which a calibration factor (total length x 0.79) was determined enabling the data to be expressed in the required units of centimetres.

After root length was determined each individual ECM root tip was counted and recorded into their respective morphotypes under the dissecting microscope (Nikon SMZ 1000) for both glass Petri dish samples. Any non-mycorrhizal root tips were also recorded.

2.7.3 Ectomycorrhizal morpological characterisation

Under a dissecting microscope (0.8 – 8× magnification), ECM root tips from each seedlings root system were classified into separate morphotypes following the criteria set out by (Agerer, 1987-2002; Agerer, 1991; Ingleby et al., 1990). Morphotypes were separated into discrete categories according to differences in mantle colour and texture, which can range from smooth surfaces through to a warty texture. The mantles size and extent of ramification, whether being unramified, pinnate, pyramidal, dichotomous, coralloid or had tuberculate structures were included in the overall description (Agerer, 1987-2002). Other features such as emanating rhizomorphs and/ or hyphae were used if necessary to differentiate ECM root tips.

Colour digital photos (Olympus DP12 digital camera) were taken under a dissecting microscope (Olympus SZX12 stereomicroscope) to catalogue discrete ECM morphotypes and allow correlations to be made from subsequent molecular identification. The photos were taken with a black background and the ECM root tip completely submerged in water to eliminate any light reflections (Agerer, 1987-2002).

After morphological identification and cataloguing, a sample of five ECM root tips from each morphologically distinct ECM structure per seedling were dried by pressing between paper towels and quick frozen in a mortar filled with liquid nitrogen, then transferred into 1.5 mL plastic centrifuge tubes and stored at -80°C for DNA extraction.

2.8 Molecular methods for ECM identifcation

Morphological characterisation of root tips followed by RFLP profiling is a known method in the literature to identify ECM fungi. The aim of this experiment was to follow the same methodology. However, after sequencing a sample of root tips previously characterised by their morphological description it was found that there was only one dominant ECM detectable so no further RFLP analysis was undertaken.

2.8.1 DNA extraction

DNA was extracted using the PowerSoil® DNA isolation kit (MoBio laboratories, USA) as per instructions. This kit is designed to extract and purify DNA from environmental samples that are high in PCR inhibitory compounds, such as humic acids, that are commonly found in high organic matter soils, particularly peat, which is paramount in this case. The extreme tip of a liquid nitrogen frozen mycorrhizal root tip (1 mm) was sampled using two sterile 1 mL pipette tips under a dissecting microscope and transferred to the PowerSoil® vial.

Two replicate root tips (frozen with liquid nitrogen, see Section 2.7.3) for each single representative morphotype were randomly taken from the control and 'AborGuard at sowing' treatments for DNA extraction.

2.8.2 PCR amplification

The ITS region of rDNA was amplified using the previously described PCR primers, ITS1F (5` CTTGGTCATTTAGAGGAAGTAA 3`), a fungal-selective primer (Gardes and Bruns, 1993), and ITS4 (5` TCCTCCGCTTATTGATATGC 3`) (White et al., 1990). These primers amplify the ITS region of both basidiomycete and ascomycete fungi from a mixture of other plant and bacterial DNA (Bruns and Gardes, 1993; Gardes and Bruns, 1993; White et al., 1990). To each 25 µl amplification reaction, 10x HotMaster® Taq Buffer (15 mM Mg^{2+}, pH 8.5), 50 mM KCL, 200 µM each of dATP, dTTP, dCTP and dGTP, 5 pmols of each primer and 1 U HotMaster™ Taq DNA polymerase (Eppendorf, Hamburg, Germany) were added. Template DNA (10 ng; quantified using NanoDrop®) was amplified using a BIO-RAD iCycler thermal cycler (96 well x 0.2 mL) with the initial denaturation set at 94°C for 3 min followed by 30 – 35 cycles of 94ºC for 30 s, annealing at 55ºC for 30 s and extension at 68ºC for 30 s, with a final extension of 68ºC for 7 min. Negative controls (template DNA replaced by an equal volume of distilled water) were run for every PCR amplification to test for the presence of DNA contamination from the reaction mixture/ procedure.

Amplified products were separated by electrophoresis in 1.5% agarose gels at 70 V for 1.5 h submerged in 1 ×TAE (Tris-acetate EDTA buffer) with the subsequent staining and washing with ethidium bromide (0.5 µg mL^{-1}) for 30 min and water for 15 min, respectively. Bands were visualised using a VersaDoc™ model 3000 imaging system (BIO-RAD).

2.8.3 Sequencing reactions

Single band PCR products were first purified with BIO-RAD Quantum Prep® PCR Kleen Spin Columns to remove excess dNTPs, primers and primer-dimers. Methodology followed

that of the manufacture apart from the 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 elution buffer being replaced with double distilled water following the manufacturer's instructions. Briefly, three 500 µl aliquots of double distilled water were centrifuged (Eppendorf 5810R centrifuge) separately at 735 × *g* for 30 s with the resultant eluted water/ buffer subsequently discarded in order to wash the original elution buffer out of the Spin Column.

The purified PCR products (20 ng of DNA, as determined by the low molecular weight ladder) were further amplified in a 10 µl sequencing reaction with 1 µl ITS 4 (5 pmol), 2 µl 5× BigDye® Terminator v3.1 5x sequencing buffer and 0.5 µl ABI PRISM® BigDye® Terminator v3.1 (Applied Biosystems, USA). The sequencing reaction was carried out in a BIO-RAD iCycler thermal cycler (96 well x 0.2 mL) with an initial denaturation of 96ºC for 1 min followed by 25 cycles of 96ºC for 10 s, annealing at 50ºC for 5 s and extension at 60ºC for 4 min. After which a post sequencing reaction clean up using an Agencourt CleanSEQ$^+$ Sequencing Reaction Clean-up system (USA) was carried out to remove unincorporated dyes, nucleotides, salts and contaminants. Sequencing products were separated on an ABI Prism 3100-*Avant* Genetic Analyser installed with a 4 capillary 80 cm array using Performance Optimized Polymer 4 (POP4).

DNA sequences were edited and aligned with Sequencher version 4.7 software and ECM identities were determined by the Basic Local Alignment Search Tool (BLAST) using the online nucleotide database, GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

For positive identification to species level a query sequence match of ≥97% was required, while query sequence identities ≤96% were considered only a genus level identification.

2.9 Statistical analysis

Root tips, expressed as root tips/ cm root length for each of the respective ECM species, were subjected to analysis of variance (ANOVA) and analysed as a complete randomised block design with 5 replicates per treatment using Genstat 8.2 (Lawes Agricultural Trust, Rothamsted Experimental Station) software. Log_{10} transformed *Trichoderma* cfu data and the seedling parameters were also analysed using ANOVA. Treatment means for all assessments were separated using Fisher's Protected least significant difference (LSD) tests at the $P<0.05$ level.

2.10 Results

2.10.1 Total ECM root tips

Overall there were no significant differences between treatments or seedlines in the total number of ECM root tips per centimetre root length of the seedling (Figure 2.2). Seedline B, however, tended to have a higher abundance of ECM root tips relative to seedline A throughout the treatments apart from Arbor-Guard™ applied at 3 months (Figure 2.2).

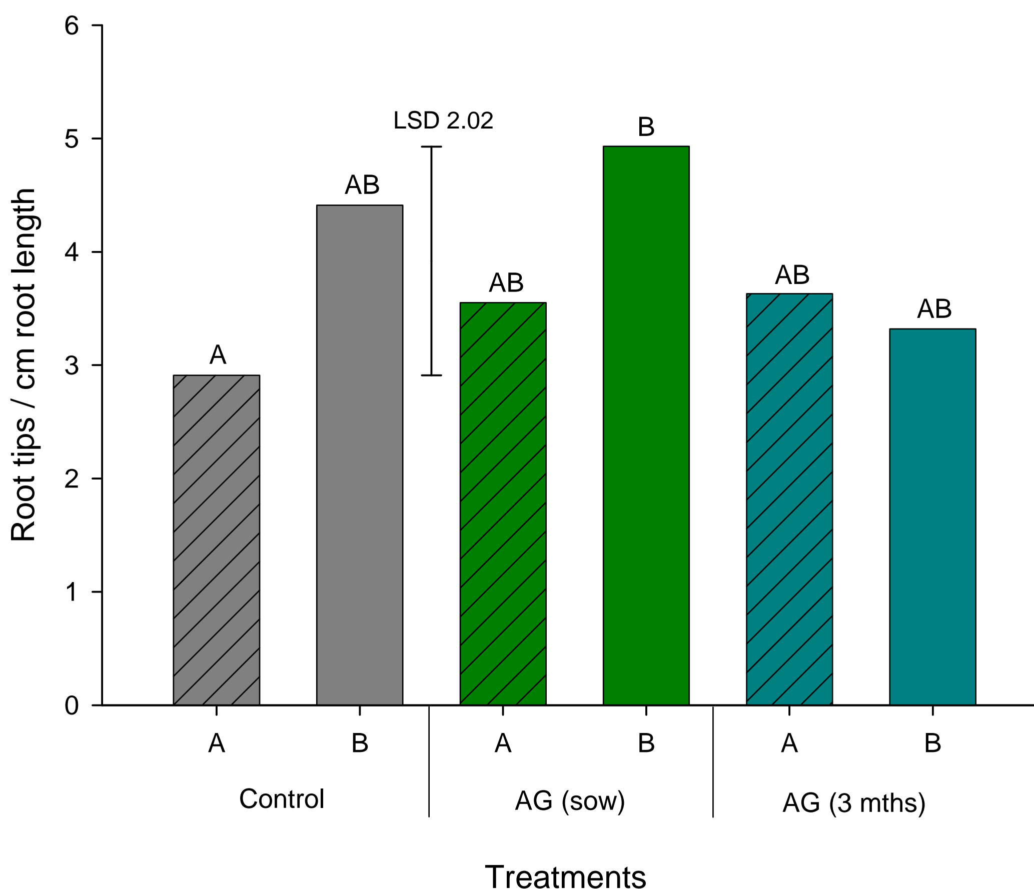

Figure 2.2 Number of ECM root tips/ cm root length at harvest for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B, respectively. Treatment means sharing the same letter are not significantly different from each other as determined by Fisher's Protected LSD (*p*= 0.05).

2.10.2 ECM root tips as discriminated by morphotype

Three broad ECM morphotype categories as discriminated by their level of ramification including unramified, dichotomous and multi-dichotomous, were observed/ classified (Figure

2.3). Apart from the differences in the level of ramification, each morphotype followed a similar description (Table 2.2)

Table 2.2 Macroscopic description of the three morphotypes found.

Morphotype	Description
Unramified	Light brown, smooth, white apex
Dichotomous	Light brown, smooth, white apices
Multi - dichotomous	Light – dark brown, smooth, white apices, tortuous irregular branching

Figure 2.3 The three morphotype categories as discriminated by their level of ramification. Unramified (A), Dichotomous (B) and Multi-dichotomous (C).

No significant differences were observed between either the seedlines or the treatments within each morphotype (Figure 2.4). Overall, there were significantly more dichotomous/ multi - dichotomous morphotypes within each treatments seedline except for Arbor-Guard™ applied at three months within seedline A, where a significantly higher proportion of root tips of the total were made up of multi – dichotomous morphotypes relative to dichotomous morphotypes (Figure 2.4). The abundance of unramified root tips, although not significantly different from multi – dichotomous morphotypes, was very low and only occurred in the control (seedline A) and Arbor-Guard™ applied at three months (seedline A) treatments.

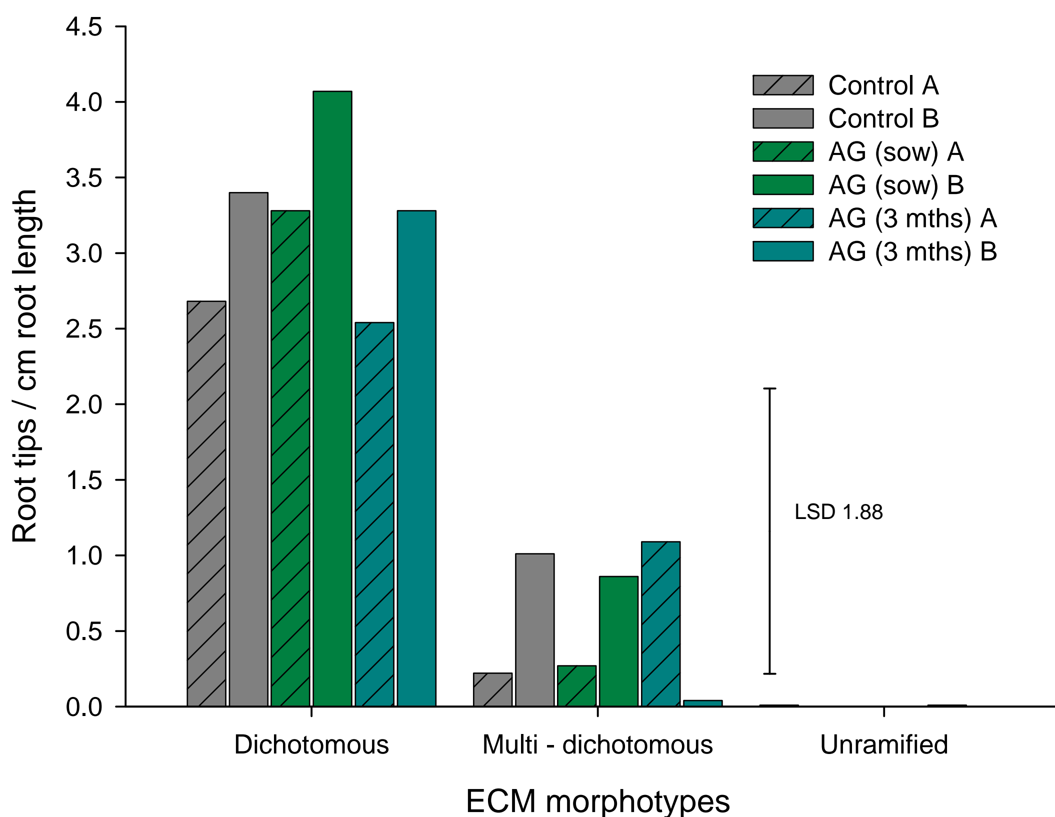

Figure 2.4 Number of ECM root tips/ cm root length for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B, respectively categorised in to dichotomous, multi-dichotomous and unramified morphotypes. Means are separated using Fisher's protected LSD (*p*= 0.05).

After a thorough macroscopic evaluation of the root tips across all treatments and subsequent DNA sequencing analysis of two root tips/ level of ramification it was concluded that *Thelephora terrestris* was the most dominant ECM detectable (Table 2.3; Appendix C.1).

Table 2.3 Sequence results for the GenBank queries of ECM root tips identified as *Thelephora* (*Th.*) *terrestris* classified by levels of ramification at PF Olsen.

Ramification	Sequence #	Sequence length (bp)	GenBank Accession No.	Organism	GenBank score	e - value	Maximum identity (%)
Unramified	1	640	DQ068970.1	*Th. terrestris*	1166	0.00	100
	2	635	DQ068970.1	*Th. terrestris*	1146	0.00	99
Dichotomous	1	619	DQ068970.1	*Th. terrestris*	1118	0.00	99
	2	615	DQ068970.1	*Th. terrestris*	1103	0.00	99
Multi-dichotomous	1	638	DQ068970.1	*Th. terrestris*	1122	0.00	98
	2	643	DQ068970.1	*Th. terrestris*	1171	0.00	100

2.10.3 Non-mycorrhizal root tips

A notable significant difference appears within seedline B between the different treatments, where there is a decrease in non-mycorrhizal root tips relative to both the control and Arbor-Guard™ applied at sowing when Arbor-Guard™ was applied at 3 months (Figure 2.5).

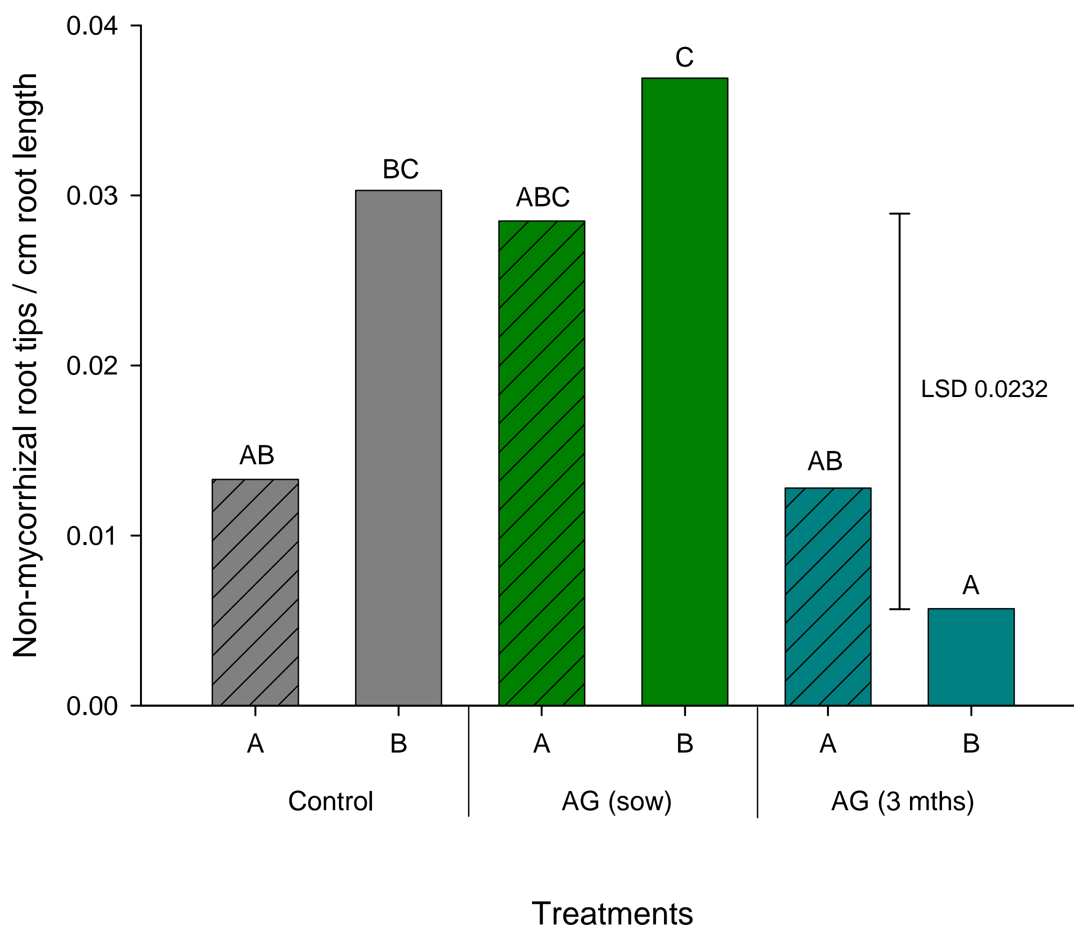

Figure 2.5 Number of non-mycorrhizal root tips found for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B, respectively. Treatment means sharing the same letter are not significantly different from each other as determined by Fisher's Protected LSD (*p*= 0.05).

2.11 Trichoderma counts (5 weeks)

Five weeks after sowing of the seed an assessment of the *Trichoderma* populations in the potting mix for Arbor-Guard™ applied at sowing and the respective controls was conducted (Fig 2.6A). As illustrated in Figure 2.6A, there were no significant differences between the *Trichoderma* populations (cfu g^{-1} soil) between seedline A or B within the control or the

Arbor-Guard™ treatments, however, the application of Arbor-Guard™ significantly increased the *Trichoderma* population within seedline B.

2.12 Trichoderma counts (harvest)

A significant difference, although only small, was observed between seedlines A and B for Arbor-Guard™ applied at sowing (Figure 2.6B). *Trichoderma* cfu was significantly less relative to the control for both Arbor-Guard™ applied at sowing and after 3 months treatments in seedline A. This trend was observed for seedline B, although the significant reduction in cfu numbers was only evident for Arbor-Guard™ applied after 3 months as Arbor-Guard™ applied at sowing was not significantly different from the control.

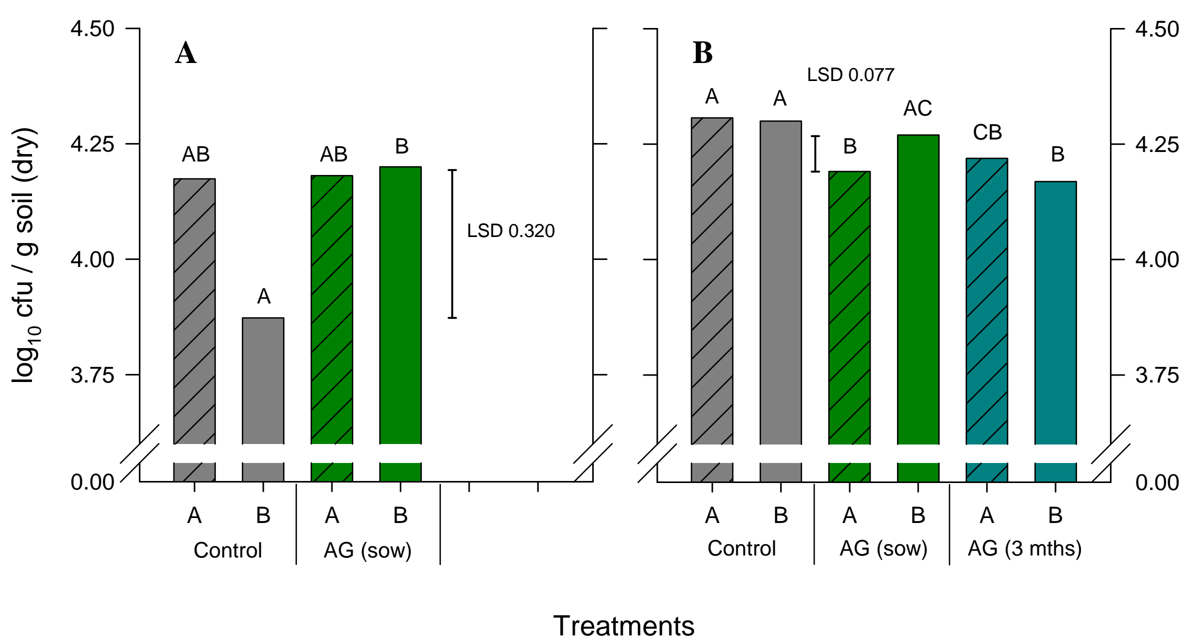

Figure 2.6 ***Trichoderma*** **cfu/ g soil (log_{10} transformed) five weeks after sowing the** ***Pinus radiata*** **seed (graph A) and at final harvest (graph B) for the control, Arbor-Guard™ applied at sowing (AG (sow)) and after 3 months (AG (3 mths)) for both seedlines A and B, respectively. Treatments sharing the same letter (within graphs) are not significantly different as defined by Fisher's Protected LSD (p= 0.05).**

2.13 Seedling parameters

Emergence data were obtained 5 weeks after seed sowing when the seedlings were approximately 5 cm tall. The percentage emergence was significantly higher for seedline A relative to seedline B in the control treatments. While this trend was also shown in the Arbor-Guard™ treatment the values were not significantly different (Table 2.4). No significant

differences were observed within the seedlines between the control and Arbor-Guard™ treatments (Table 2.4). Overall, the Arbor-Guard™ application reduced the differences between seedline A and B, in effect converging the relative emergence profiles of the two seedlines.

Height differences between the control, Arbor-Guard™ applied at sowing and after 3 months (within seedlines) showed no significant differences for both seedlines A and B. While, seedline A overall had significantly taller seedlings relative to seedline B within all treatments apart from when the Arbor-Guard™ was applied at 3 months, where although trending higher, the values were not significant (Table 2.4).

Diameters of the respective seedlings at the root collar of the trunk showed no significant differences between treatments or seedlines. However, the data obtained did show a definite inverse relationship between the height of the seedlings and diameter parameters as illustrated by the height to diameter ratio (Table 2.4). Seedline A had a significantly higher height to diameter ratio than seedline B within treatments for the control and *Trichoderma* at sowing treatments. This trend was observed for the *Trichoderma* applied at 3 months, however, not significant. Overall, there was no significant difference between the treatments within the seedlines.

Above ground dry weight of the seedlings reflected the seedling height data with seedline A tending, however not significant, to have heavier seedlings (Table 2.4).

Table 2.4 **Effect of the six treatments consisting of control, Arbor-Guard™ applied at sowing (sow) and Arbor-Guard™ applied after 3 months (3mth), which were further divided by the two seedlines (A and B) on the seedling parameters assessed 5 weeks after seed sowing or at harvest. Treatment means within a row followed by the same letter are not significantly different as analysed by Fisher's Protected LSD *p*=0.05**

	Treatments						
	Control		Arbor-Guard™ (sow)		Arbor-Guard™ (3mth)		
Seedling parameters	A	B	A	B	A	B	(3) LSD
(1)Emergence (%)	96.4 A	91.8 B	95.1 AB	92.7 B	NA	NA	3.71
(2)Height (cm)	44.5 CD	41.2 A	45.4 D	42.3 AB	43.5 BCD	42.7 ABC	2.12
(2)Diameter (mm)	4.8 A	5.0 A	4.8 A	5.0 A	4.8 A	4.9 A	NSD
(2)Height : diameter	9.5 D	8.4 A	9.7 D	8.7 AB	9.3 CD	8.8 ABC	0.51
(2)Above ground D. wt (g)	4.6 AB	4.1 AB	4.7 AB	4.1 A	4.9 B	4.4 AB	0.79

(1) Assessed after 5 weeks (n=900), (2) Assessed at 9 months (n=60), (3) Fisher's protected LSD (0.05), NA = Not applicable, NSD = no significant difference

2.14 Discussion

Overall the application of *Trichoderma* spp. in the commercially available product Arbor-Guard™ either at sowing or after 3 months did not negatively impact on ECM colonisation of *P. radiata* seedlings. What sets this experiment apart from other work is the observation of what effect the inoculation of six *Trichoderma* species, in the form of a commercially available product, both at seed sowing and after 3 months growth, into a working non-sterilised containerised nursery, has on indigenous ECM colonisation. Some work showing the interaction of *Trichoderma* on ECM colonisation in a field situation was carried out by Malyshkin, (1951) and Shemakhanova, (1962), both cited in (Summerbell, 2005). In the authors' work they found a positive effect of adding one *Trichoderma* species on ECM colonisation of oak seedlings. In contrast, more recent work carried out by Chavasse, (1980) in a nursery situation found that the inoculation of *Trichoderma* was detrimental to ECM colonisation and subsequent growth of *P. radiata* seedlings. However, in this situation the nursery bed had been previously sterilised and as a result it was noted that the *Trichoderma* successfully outcompeted the ectomycorrhizal fungus before mycorrhization was able to take place. Sterilisation of the nursery bed as in the case of Chavasse, (1980) is not considered best

practice and experimental work on the effect of *Trichoderma* on ECM colonisation in un-sterilised potting media is required.

Most other work has focussed on *in vitro* interactions, either looking at interactions between non-mycorrhizal and/ or ectomycorrhizal fungi and *Trichoderma* (Mucha et al., 2007; Mucha et al., 2008; Werner et al., 2002; Zadworny et al., 2004; Zadworny et al., 2007; Zadworny et al., 2008). In these studies it was concluded that *Trichoderma* had no negative impact on the ectomycorrhizal fungal species, instead the *Trichoderma* species tested were themselves antagonised. In contrast to this Summerbell, (1987) found *in vitro* that *Trichoderma* was strongly antagonistic towards mycorrhizal colonisation and growth in the rhizosphere of *Laccaria bicolor* seedlings, even after the mycorrhizal relationship was given time to establish (9 days) before *Trichoderma* inoculation. It is well documented that results of *in vitro* studies, although beneficial in many cases, are not necessarily able to be extrapolated out into field conditions and often results are not repeatable in the field (Whipps, 1987).

Thelephora terrestris was the only predominant ectomycorrhizal fungus detected in association with the *P. radiata* seedlings in this experiment. Nursery systems, especially containerised seedlings, could potentially impose selective pressures on the ECM species able to colonise seedlings, which in turn could decrease the ectomycorrhizal diversity (Karkouri et al., 2005). Other ECM fungal species will be present but their abundance will be low. It could therefore be save to assume that *Th. terrestris* will not only be the dominant species colonising the *P. radiata* root tips but will probably be the most functionally important. If a more intensive molecular analysis was undertaken other ECM species could be detected. However, the quantification of these species would be limited to the morphological characterisation because all the ECM observed, of which on average there were 830 root tips/ seedling analysed, appeared to have the same gross morphological characteristics. Therefore, one would need to do a large number of DNA extractions followed by RFLP profiling to ascertain the proportion of root tips the other species occupy. This was outside the scope of the current experiment.

Thelephora terrestris has been shown to do well in high fertility environments and in high organic matter substrates such as peaty soils (Chu-Chou and Grace, 1990). This experiment is in agreement with Chu-Chou and Grace, (1990) findings where *Th. terrestris* is the dominant ectomycorrhizal fungus found in high fertility/ organic matter sites as a result of the containerised system employed at the nursery. This observation is further backed up by recent work completed by Walbert, (2008) who did not find *Th. terrestris* in a soil bed nursery system where the soil was derived from volcanic parent material. High levels of nutrients by

fertigation as applied to the nursery seedlings in this experiment have been reported to have detrimental impacts on ectomycorrhizal colonisation (Hall and Perley, 2008). However, *Th. terrestris* has been described as tolerant to high levels of nitrogen (Hilszczanska and Sierota, 2006) and as a result would have a competitive advantage in heavily fertilised nurseries as is the case in this nursery. Indeed Aspray et al., (2006) noted that *Thelephora* and *Tomentella* species are common environmental species found in the glasshouse environment in their work looking at mycorrhiza helper bacteria (MHB). While Hall and Perley, (2008) noted that *Th. terrestris* was a common inhabitant in nurseries growing *P. radiata*, and the authors further pointed out that there was no proof that this ECM was beneficial to the seedlings outside the nursery environment. However, *Th. terrestris* has been recently observed in *P. radiata* stands between 8 and 15 years old in the Kaingaroa forest, New Zealand but there was no correlation as to the respective health of the tress (Walbert, 2008).

Fungicide use may also put a selection pressure within the nursery environment conducive to tolerant species of fungi as they too have been shown to be detrimental to ECM colonisation (Hall and Perley, 2008). Although no fungicides were applied to our experiment they were routinely applied at the nursery in the immediate environment surrounding the test seedlings and as a result could lower ectomycorrhizal inoculum levels that otherwise would be present. Indeed Pawuk et al., (1980) found the fungicide "benomyl" to increase the mycorrhizal colonisation of both *Pisolithus tinctorius* (Mont.) Fisch. and *Thelephora terrestris* on container grown *P. palustris* Miller. seedlings. This result could be the result of benomyl inhibiting soil Ascomycetes species that compete against the mycobionts (Summerbell, 2005). Any sterilisation of nursery containers could also lead to a selection pressure conducive to early colonising ECM species (Karkouri et al., 2005). In the case of this experiment it is unknown as to whether the trays that were used had any sterilisation procedure pre seed sowing.

Thelephora terrestris has also been shown to naturally inhabit root systems without the inoculation of any ECM fungi (Bogeat-Triboulot et al., 2004). When Bogeat-Triboulot et al., (2004) inoculated *P. pinaster* Aiton. seedlings with the ectomycorrhizal symbiont *Hebeloma cylindrosporum* Romagn., a specialised ECM that especially increases the root hydraulic conductance of seedlings under high moisture stress, they found *Th. terrestris* was a major contaminant (colonising up to 50% of root tips) of the non-inoculated control seedlings. Further, *Th. terrestris* was much less suited to the health of the seedlings due to the reduced ability of water conductance relative to *Hebeloma cylindrosporum*. This primary colonising

characteristic of *Th. terrestris* has also been found in other work and was even considered a "weed species" in some systems (Hall and Perley, 2008).

It has been hypothesised that the *Th. terrestris* inoculum comes from the surrounding environment via spores being transported in the air currents (Bogeat-Triboulot et al., 2004). Air currents are the likely source of *Th. terrestris* inoculum in our experiment. Hall and Perley, (2008) found that *Th. terrestris* fruited abundantly in late summer through the slots of the side – slit trays growing *Nothofagus* seedlings. If this happened in the PF Olsen nursery this would be inevitability be the source of inoculum that would effectively form an inoculum bank that could further contaminate more seedlings. Another source could be the pine bark that makes up 50% of the potting mix. However, the pine bark is composted before use which should eliminate any ECM inoculum, or again this could exert a selective pressure towards more thermo-tolerant ECM species.

The low diversity of ECM species colonising the seedlings at PF Olsen nursery could be as a direct result of its location as it is not close to any significant forestry stands. As a result the potential of wind dispersed ECM from forestry stands is significantly reduced, which in turn will lower the potential ECM diversity of seedlings. Indeed, Dickie and Reich, (2005) expressed that the lack of an established ectomycorrhizal stand of trees as a source of inoculum would limit the ectomycorrhizal infection of seedlings. Compounding this is the use of a soilless potting mix that is replaced annually in the containerised system at PF Olsen. More traditional soil bed nurseries are based close to the forestry stands where the seedlings will be eventually planted for logistical reasons. So ECM inoculum via wind dispersal in these systems has a higher chance of forming ECM relationships on nursery seedlings. Further to this, and probably more importantly, the soil bed over time will build up a diverse species composition of ECM fungi that are able to readily colonise seedlings (Walbert, 2008).

Three dominant morphotypes (Figure 2.4) of *Th. terrestris* were characterised as described in Table 2.2 into their respective level of ramification. The three morphotypes shared the same GenBank accession number (Table 2.3) and when the sequences were cross compared had 99% similarity (Appendix C.1). This would suggest that the ECM root tips analysed are more than likely to have come from the same origin. Overall the only difference was the root tip morphology, however, this observation was not a treatment effect (Figure 2.4). The obvious reasoning behind this could simply be the age of root tips when assessment took place, after the seedlings were 9 months old. This reasoning explains the pattern of morphotypes found, with the relative abundances of the low levels of unramified root tips followed by the most abundant dichotomous morphotypes and finally the less numerous multi-dichotomous. The

changing colour, from light brown to dark brown, of the multi-dichotomous morphotypes also suggests an aging effect.

Any indication that the application of Arbor-Guard™ had any effect on ECM morphology is highlighted in the Arbor-Guard™ applied at 3 months (seedline A) treatment. Here, relative to all other treatments, the total proportion of multi – dichotomous to dichotomous root tips was not significantly different (Figure 2.4). This result may indicate that the 3 month delay of Arbor-Guard™ inoculation onto seedline A could have led to stimulatory effects conducive to higher levels of ramification of the root tips. By letting the potential fungal mutualist form a mycorrhizal association before Arbor-Guard™ inoculation this would provide an inoculum base of ECM fungi within the rhizosphere to further colonise new root tips, either by hyphal acropetal growth or from hyphal remnants on the root surface (Marks and Foster, 1973). This inoculum base may be at a threshold level high enough for any antagonistic/ competitive interaction not to impede colonisation. Indeed Marks and Foster, (1973) stated that if there is already mycorrhizal infection the chances of secondary infection are greatly increased. Summerbell, (1987) allowed mycorrhization to take place in a *Laccaria bicolor* – Black spruce complex before the inoculation of *T. viride* and found a small but significant increase of 4.4% ECM colonisation above the simultaneous inoculation of the two fungal species. The *T. viride* isolate used in Summerbell, (1987) experiment was very antagonistic towards *L. bicolor*, reducing percentage mycorrhization by 100% relative to the controls and other co-inoculated indigenous fungal species isolated from the washed mycorrhizal roots of Black spruce. This further shows evidence that the delaying of Arbor-Guard™ inoculation by three months could provide enough time for ECM mycorrhization and therefore increase the abundance of multi – dichotomous morphotypes.

To my knowledge there is no literature on rhizospheric fungi having any effect, either stimulatory or negative, on root tip architecture. Two isolates of mycorrhiza helper bacteria (MHB) *Paenibacillus* sp. and *Burkholderia* sp. were shown to influence, although not quantitatively, the dichotomous root tip architecture of a *Lactarius rufus* (Scop.) Fr. - *Pinus sylvestris* ectomycorrhizal symbiosis (Aspray et al., 2006). The authors acknowledged that changes in root architecture induced by MHB could be an important assessment parameter that is commonly overlooked; however, there was no corresponding discussion on how the change in root architecture influenced seedling growth/ health. Therefore this raises the question of the biological relevance of multiple branching/ ramification in ECM. Zheng and Wu, (2008) found no correlation between the ECM tip morphology of *Rhizopogon luteolus*, *Pisolithus tinctorius* and *Boletus edulis* Bull. with regard to growth of *P. thunbergii* Parl.

seedlings. Vinceti et al., (1998) looked at root tip ramification as one possible factor in the decline in health of a Norway Spruce (*Picea abies* (L.) Karst.) stand of trees in the Italian alps. The authors found a correlation that the less healthy trees tended to have a less complex level of ramification relative to the healthy trees, however, they concluded that the aboveground decline in tree health was not attributed to the below ground conditions. Aspray et al., (2006) noted in their work with MHB that changes in root tip architecture could be just plant host or bacterial/ fungal species specific. It has been well documented in past literature that ECM root architecture can be plant/ ECM species specific and that it can be influenced by environmental conditions such as temperature (Marks and Foster, 1973).

The results indicate that the indigenous population of *Trichoderma* in the potting mix was high; thereby adding more *Trichoderma* in the form of Arbor-Guard™ to the media would not necessarily increase the population size. Instead it is more likely to change the dynamics of the rhizosphere, giving change to the species present and the overall proportion of species occupying the rhizosphere. This study only quantified the total *Trichoderma* population and unfortunately an analysis of the actual *Trichoderma* species present was not undertaken. Therefore, the data does not reveal if the applied Arbor-Guard™ isolates survive or out competed the indigenous *Trichoderma* species present. Hohmann, P. (unpublished) showed that the addition of selected *Trichoderma* isolates can change the *Trichoderma* species composition in the rhizosphere in *P. radiata* seedlings. This was shown by either the total displacement of the indigenous species population or, on the contrary, the inoculated *Trichoderma* species were totally absent after 20 weeks. Further, Hohmann, P. (unpublished) found that particular isolates were dominant in the rhizosphere of *P. radiata* seedlings while others had a preference for the bulk soil environment. These results further illustrate the dynamic interface of the rhizosphere environment. By restricting the assessment of ECM colonisation to only looking at two fungal groups, as was done in this experiment, will not reveal the inherent shift in population dynamics of other rhizosphere species that probably have a significant impact on microbial community structure.

Overall, a more comprehensive assessment of *Trichoderma* cfu numbers to species level is required for any delineation of the potential impacts of Arbor-Guard™ inoculation on ECM colonisation. Further treatments inoculated with the individual Arbor-Guard™ isolates would be helpful in assessing specific ECM/ *Trichoderma* interactions, while also giving an insight in to the dynamics of the indigenous *Trichoderma* community. This would reveal any impact, either as a direct result of a particular isolate or as an indirect influence due to the manipulation of the rhizosphere community, the addition of *Trichoderma* has on the

colonisation of ECM. Another added advantage to approaching the assessment in this way would be the elucidation of what the preferential community structure of *Trichoderma* species is in the rhizosphere for optimum ECM colonisation.

There was a general and sometimes significant decline in *Trichoderma* cfu numbers relative to their respective controls from the initial 5 week assessment to the harvest assessment (Figures 2.6A and 2.6B, respectively). Although the difference between the highest and lowest values (control; seedline A and Arbor-Guard™ applied at 3 months; seedline B respectively) in Figure 2.6B is statistically different, this does not automatically confer that this range is biologically relevant. The species or strain of *Trichoderma* present is more important than the 'total numbers' per se. *Trichoderma* spp. dynamics in the mycorrhizosphere over time is not well understood. One report indicated that both *Trichoderma* spp. and *Fusarium* spp. were characteristic to non-mycorrhizal pine roots and less common in mycorrhizal roots in Russia (Summerbell, 2005). Another report suggested that young germinating seedlings of *Pinus kesiya* Royle. ex Gordon. favoured the growth of *Trichoderma* spp. (including *Verticillium*) in the rhizosphere, however, after mycorrhizal formation they were excluded from the rhizosphere (Summerbell, 2005). Although *Trichoderma* was not totally excluded, our experimental results do follow the same pattern. This pattern could be explained by the successional changes of saprophytic fungi during ectomycorrhzial colonisation. Root exudate quality and quantity is known to change when ectomycorrhiza colonise root tips, which in turn could confer a selection pressure on rhizosphere community structure (Frey-Klett et al., 2005) and in this case prevent *Trichoderma* accessing vital nutrients for rhizosphere colonisation.

An increase in root exudates from the ECM and the ability of ectomycorrhiza to structure, and synergistically interact with the soil microorganism community (Chalot et al., 2002; Frey-Klett et al., 2005; Timonen et al., 1998) could also explain the relative increase in *Trichoderma* cfu numbers from the initial 5 week assessment to the harvest assessment in the controls (Figure 2.6A and 2.6B, respectively).

Another possible reason for the high levels of *Trichoderma* found in the control seedlings could be from cross contamination via water splash, as the first assessment of *Trichoderma* cfu took place 5 weeks after Arbor-Guard™ inoculation. *Trichoderma* species are known to be competitive primary colonisers of potting mix (Leandro et al., 2007), and as a result would rapidly colonise the control potting media substrate if contamination occurred. Contamination could either occur as described above or originate from the high background concentration of Arbor-Guard™ species in the nursery environment due to the routine application of Arbor-

Guard™. The likely hood of cross contamination during the setup of the experiment is small due to the control trays of seeds being planted first and taken out of the general area before the Arbor-Guard™ treatments were inoculated.

Unfortunately there was potential in misdiagnosing between *Trichoderma* and *Penicillium* species on the TSM plates, thereby incorporating *Penicillium* into the cfu counts. This could have been particularly prevalent in the assessment of the control plates due to the dynamics of microbes within the rhizospheres between control seedlings and Arbor-Guard™ treatments potentially being different. Bourguignon, (2008) observed that a decrease in *Trichoderma* numbers in the rhizosphere of vegetable systems was strongly associated with an increase in *Penicillium* numbers. Therefore it is feasible that where there was no addition of *Trichoderma* (i.e. control plants) the population of *Penicillium* could be high relative to where the treatments had Arbor-Guard™ added. Summerbell, (2005) revealed the pre-1985 literature stating that *Penicillium* was a common fungus associated within the ectomycorrhizosphere and it was postulated that the high concentrations of tannins and phenolics exuded in the rhizosphere of woody plants generated a selective pressure towards *Penicillium* inhabitants, which have the ability to degrade these recalcitrant/ refractory molecules. *Penicillium* spp. were also considered to be stimulated by ectomycorrhizal fungal for their ability to dissolve inorganic phosphates (Summerbell, 2005). Indeed, Rambelli, (1973) found the dominating presence of *Penicillium* spp. in the mycorrhizosphere of ECM in association with *P. radiata* throughout four seasons. With the level of mycorrhization in the control seedlings being equivalent to the Arbor-Guard™ treatments in our experiment (Fig 2), and with the potential misdiagnoses of *Penicillium* on the TSM plates, it is highly likely that total *Trichoderma* numbers would be misrepresented in the control plates.

There was no seedling growth promotion of adding Arbor-Guard™ as previously found in other experiments (Hill, 2004/2005) (Table 2.4). Any growth promotion response could however be obscured due to the very uneven watering of seedlings at the early stages of growth within the glasshouse (Figure 2.7). Aside from the watering, any increased growth rates could be masked by leaving the seedlings too long before data collection, as the high application rate of fertiliser to the seedlings over time would counteract the slow start. An intermediate seedling analysis, of around 3 to 4 months of age could have given more robust growth promotion data.

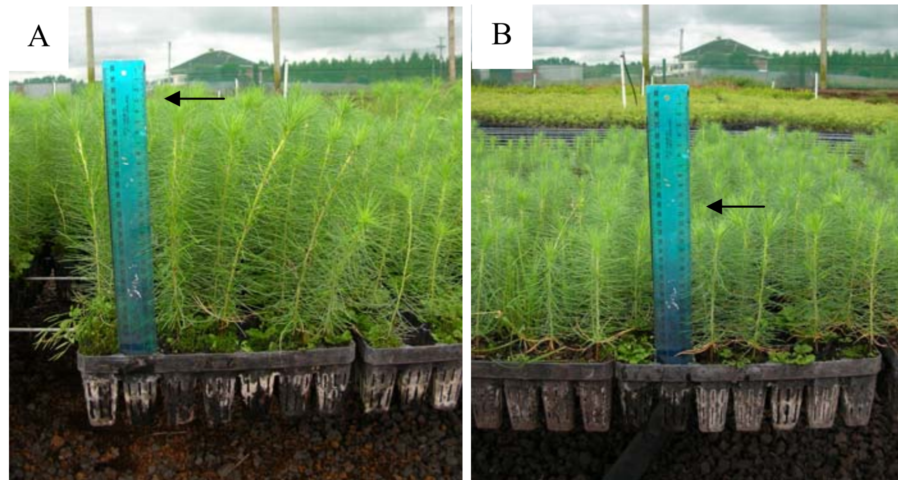

Figure 2.7 Difference in *Pinus radiata* seedling height due to uneven watering. A; Greener larger seedlings versus B; lighter coloured stunted seedlings (arrows indicate the average height of seedlings)

In retrospect, data was required for root dry weights. However, the sampled root length for root tip analysis, which was a 10% sample of the whole root system, should have given an indication of the relative root masses. No significant differences in total root lengths between treatments or seedlines were observed.

The time taken to assess the ECM was initially underestimated with many more replicates being set up in the experiment. The low number of seedlings assessed for each treatment is reflected by the statistical variation in the results. The only way to reduce the variation would be to increase the replicates assessed and this could only be done if a less intensive assessment was undertaken due to time constraints. However, this would reduce the overall resolution, so at the time of sampling the decision was made to intensively record a smaller number of replicates to reduce the overall variation. In hindsight a less intensive and more extensive assessment should have been undertaken to increase the replicate number as it has been shown that there are always inherent variations in results when working with growth-stimulating non-symbiotic microorganisms (Bowen and Rovira, 1961; cited in Summerbell, 2005). An earlier assessment when the seedlings were 4 to 5 months old may have yielded better results. At this stage of growth the seedlings root systems would not have been occupying the whole cell of the Side-Slit tray which would make the sampling, processing and quantification of ECM root tips much easier and quicker. This would have the advantage over leaving the seedlings to their full 9 month term that more seedlings would have been able to be assessed and therefore reduce the variation in results.

An earlier assessment would allow for the differentiation between rhizosphere competent *Trichoderma* spp. inhabitants and species more prevalent in the bulk soil environment due to the root system not taking up the whole cell. Therefore actual *Trichoderma* species colonising the rhizosphere could be elucidated and enumerated.

The PF Olsen commercial experiment was limited at the outset to natural ECM colonisation of *Pinus radiata* seedlings from the surrounding environment. As a result the ECM species diversity was limited to only one predominant species, *Thelephora terrestris* and any effect of Arbor-Guard™ inoculation on ECM colonisation cannot be delineated out of these results alone. Therefore a more comprehensive assessment of how Arbor-Guard™ effects the colonisation of a known species composition of ECM is required to achieve the overall objective of this study and this is investigated in Chapter 3.

Chapter 3
Effect of *Trichoderma* spp. inoculation on colonisation of *Pinus radiata* by specific ECM species

3.1 Introduction

The aim of this experiment was to look at the effect of *Trichoderma* spp. inoculation on the colonisation of known ECM that have been identified as early colonisers of *P. radiata* seedlings. To accommodate for the inoculation of known ECM species into an experimental setup it was necessary to run an additional experiment at Lincoln University. The Lincoln experiment was designed to augment the PF Olsen commercial experiment with the additional ECM inoculation treatments and to address objectives 3 and 4.

Initial results that were obtained from the commercial experiment (PF Olsen experiment) revealed that there was no significant differences between the seedlines A and B, and that Arbor-Guard™ inoculation timing had no significant effect on ECM root tip numbers. As a result the Lincoln experiment was designed to include only seedline A due to its better tree harvesting qualities (see Appendix A.1), while Arbor-Guard™ inoculation at sowing was chosen over the 3 month inoculation as this is the management system used in the commercial nursery. Also, if there is any effect of *Trichoderma* application on ECM colonisation it would be more likely to see an effect when the inoculation was at seed sowing.

3.2 Methods

3.2.1 Experimental design

A glasshouse pot experiment was setup in a split plot design using 200 mL capacity plastic pots with 4 treatments, (Table 3.1) at Lincoln University. Treatments in this experiment included the inoculation of six ECM species both with and without Arbor-Guard™ into the potting media prior to sowing (Table 3.1). The split block design allowed for the inclusion of a time parameter, thus it gave enough time for a subset of each treatment to be harvested and completely analysed, in turn keeping the samples fresh, while any temporal variation could be statistically accounted for. This was achieved by splitting each block, of which there was five, into four split-plots. All 4 treatments were replicated and randomly assigned a position within each of the four split-plots. All four split-plots were also randomly assigned a sampling time within each block. Thereby, at sampling time 1, each of the 4 treatments were replicated five times (1 from each block) giving a final number of 4 treatments x 5 blocks x 4 split-plots

equalling 80 seedlings in total (20 replicates for each treatment). Including a time parameter into the design gave the advantage that the seedlings could be processed immediately after harvesting, thereby ECM were fresh.

Table 3.1 Outline of the 4 treatments applied to the *P. radiata* seed (seedline A) in the Lincoln glasshouse experiment.

Treatment No.	Description
1	Control
2	Arbor-Guard™ at seed sowing
3	ECM inoculation
4	ECM inoculation plus Arbor-Guard™ at seed sowing

3.3 Silvicultural management and experimental setup

Sowing of the *P. radiata* seed (GF Plus A), followed the procedure of experiment 1 (Section 2.4) with the major exception that the potting media mixture was a 50:50 (v/v) mixture of peat and coarse vermiculite. Coarse vermiculite was chosen over finer mixes to give maximum aeration to the root system and to mimic the size of bark material in the commercial experiment. Although the individual 200 mL plastic pots (TEKU square pot 7 x 7x 8 cm) used in the Lincoln experiment were different than the Side-Slit trays in the commercial experiment, they were filled to the same volume of 100 mL. Pots were assigned their respective positions on self draining plastic trays according to the split block design, with the trays placed on tables ¾ metre off the concrete floor of the glasshouse. The application of the bird repellent was omitted in this experiment to reduce factors that could potentially influence ECM colonisation.

Seedlings remained in the glasshouse for the duration of the experiment (9 months) with water applied to keep the potting media at field capacity. No fertiliser was applied to the seedlings at sowing or during the growing period, the reasoning behind this decision was to help the induction of ECM colonisation as it has been well documented that ECM colonisation is inversely related to the nutritional state of the soil (Meyer, 1973).

3.4 ECM inoculation

3.4.1 Source and maintenance of ECM fungi

Pure cultures of the ectomycorrhizal species *Rhizopogon luteolus* (isolate 1812), *R. parksii* Sm. (isolate 246), *Rhizopogon* spp. (isolate 262), *Suillus luteus* (isolate 253), *S. granulatus* (isolate 244), and *Scleroderma bovista* Fr. (isolate 1813) were sourced from Ensis, Rotorua.

These cultures were chosen because of their availability in pure cultures, while being recognised as early ECM colonisers (Walbert K. *pers com.*).

Cultures were isolated from either sporocarp tissue or directly from ECM root tips in pine plantations. Each of the ectomycorrhizal species was stored on Modified Melin Norkrans (MMN, Appendix A2.2) as slopes at 4ºC, and routinely sub-cultured every 6 months onto MMN agar, incubated at 20ºC (24hr dark), before being re-subbed onto new slopes if the cultures were free of contamination.

Malt extract agar (MEA), Difco™ was used for all experimental purposes in the experiment unless stated otherwise.

3.4.2 Inoculum production

All six species of ectomycorrhiza fungi described above were grown individually in a peat and vermiculite mix in 2 L Erlenmeyer flasks in aseptic conditions using a modified method of Aspray *et al* (2006). Two hundred millilitres of a sieved (2 mm) 1:4 (v/v) peat:vermiculite mix, combined with 95 mL of malt extract (20 g L^{-1}) for an energy source, was used as the inoculum substrate. Flasks were autoclaved twice for 15 min at 121ºC, once before the addition of malt extract and again after the addition of malt extract. A foam bung covered with tin foil kept the axenic cultures from contamination and allowed for the passive transfer of respiratory gases. Each flask received 10, 5 mm hyphal plugs that were taken with a cork borer from the outside edge of actively growing ECM fungal colonies on MEA. Flasks were subsequently incubated for a period of 2 months in the dark at 20ºC with periodic mixing by shaking the flasks to help fungal colonisation of the media.

After the incubation period, 20 random samples (≈2 mm diameter) of the inoculum substrate were aseptically removed from each respective ECM flask. Each piece was aseptically plated out on MEA, five pieces per agar plate, and incubated at 20ºC in the dark and assessed after 14 days for hyphal growth of the respective ECM isolates. This allowed the inoculum potential of the substrate to be determined.

3.4.3 Pot inoculation

All six flasks containing the individual ECM species were combined together and thoroughly mixed into 5 L of potting media to give a final inoculum concentration of 19.35% (v/v) (approx 3.2% for each ECM species). To avoid contamination, both the thorough hand mixing of the ECM inoculum substrate in the potting mix prior to sowing and subsequent potting up

into pots was carried out after the non-ECM treatments (treatments 1 and 2, respectively) were potted up.

3.4.4 Pure culture species identification

Extraction of DNA with the subsequent sequencing of the PCR product was required to confirm ECM identities of the pure cultures received from Ensis and to enable the identity of the ECM root tips to be compared to that of the cultures used for inoculation.

This was achieved by inoculating three discs (5 mm) cut from pure cultures of each ECM species growing on MMN agar into separate 100 mL Erlenmeyer flasks containing 20 mL of a static MMN broth. The cultures were incubated at 20ºC for 2 weeks or until the outwardly growing mycelia reached ≈10 mm from each plug. Samples of the mycelia were aseptically taken with disposable 10 µl inoculating loops, dried by pressing between sterile Miracloth and frozen in liquid nitrogen ready for DNA extraction.

DNA was extracted using a BIO-RAD 5% Chelex®100 Molecular Biology Grade Resin (200-400 mesh). A sample of mycelia (approx 4 mm^2) from each ECM fungal isolate was suspended in 100 µl of pre-warmed 5% Chelex®100 resin. The tube was then incubated at 92ºC for 20 minutes in a BIO-RAD iCycler thermal cycler (96 well x 0.2 mL). After which the samples were frozen at -20ºC, thawed at room temperature and then spun at 13,000 rpm (≈7558 x *g*) for 2 minutes in a bench top microcentrifuge. The top aqueous layer was then removed avoiding the pellet and the resulting solution was stored in a -20ºC freezer ready for PCR amplification. All of the respective ECM species DNA was extracted this way except for *Rhizopogon parksii* (isolate 246) DNA where the PowerSoil® extraction kit was used as described in Section 2.8.1. Extraction was done by this method after the repeated failed attempts with the Chelex®100 DNA extraction procedure, probably due to co-extracted products inhibiting the PCR reaction.

DNA was amplified as described in Section 2.8.2 with the subsequent sequencing of the PCR products after purification with the PCR Kleen Spin Column as described in Section 2.8.3.

3.5 Trichoderma application and assessment

An Arbor-Guard™ (5×10^9 spores g^{-1}; Arbor-Guard™) suspension was prepared and applied at the same concentration (0.2 g L^{-1}) as in the commercial experiment (Section 2.5). Although the application of the Arbor-Guard™ solution (5.5 mL/ pot) was done with a 10 mL pipette, instead of a knapsack, with the aim to applying 5.5×10^4 *Trichoderma* cfu g^{-1} potting media

(100 g potting media/ pot). Both the control and ECM treatments (1 and 3, respectively) had 5.5 mL of water applied.

To establish the actual number of *Trichoderma* spp. propagules applied to the treatments at sowing, the Arbor-Guard™ solution just prior to application was serially diluted onto TSM as described in Section 2.5.

At the conclusion of the Lincoln experiment the *Trichoderma* spp. population was again determined by serial dilution of the potting media and subsequent plating out onto TSM, as described in Section 2.5 for all the four treatments (Table 3.1). Briefly, a composite potting mixture sample of four randomly selected seedlings per treatment were processed (removal of seedling root system) and homogenised. From this three 10 g sub-samples were shaken for 10 minutes in 90 ml of sterile 0.01% water agar, left to settle for a further 10 minutes, then serially diluted to 10^{-6} with each dilution factor plated onto TSM. Each dilution factor plated onto TSM was replicated three times.

3.6 Seedling harvesting and processing

All four treatments and their respective replicates were assessed for emergence 4 weeks after seed sowing. Seedlings were collected and processed 9 months after seed sowing as outlined for the commercial experiment in Section 2.6 apart from some exceptions described below.

During harvest one respective replicate for all four treatments was selected from each of the main blocks using the pre-determined random sampling times (split blocks) derived from the split block design. At the time of harvest the seedling root systems were immediately destructively sampled as described for the commercial experiment (Section 2.6) with height and basal trunk diameter recorded.

3.7 Ectomycorrhizal analysis

3.7.1 Root processing

The Lincoln experiment seedlings root systems were easier to process than those of the commercial experiment as there were less overall roots and the majority of the roots/ root tips had accumulated at the base of the pot where there was access to air. Practically this meant that the root system of the Lincoln experiment was not a dense cluster like that of the PF Olsen commercial experiment and could be disassembled and concurrently cut up into 25 mm segments without soaking the root system overnight. Washing of any tightly adhering potting media off the root system was carried out over sieves or teased apart microscopically as described in Section 2.7.1.

3.7.2 Ectomycorrhizal quantification

A 20% sub-sample of the whole root system was taken as described in Section 2.7.2, except that a plastic tray of half the dimensions was used, therefore the tray was dissected into 50 squares and 10 squares were randomly assessed (c.f. 100 for the commercial experiment, Section 2.7.2). Using a smaller plastic tray was done because the total quantity of the Lincoln experiment's roots was approximately half that of the PF Olsen commercial experiment, thus this allowed the sub-sampling to closely resemble that of the commercial experiment's root density.

3.7.3 Ectomycorrhizal morphological characterisation

Morphological characterisation followed the procedure outlined in Section 2.7.3.

3.8 Molecular methods for ECM identification

The molecular methods from DNA extraction through to sequencing were carried out as described in Sections 2.8.1 – 2.8.3, respectively.

3.9 Statistical analysis

Root tips, expressed as root tips/ cm root length for each of the respective ECM species, were subjected to analysis of variance (ANOVA) and analysed as a split block design with a total of 8 replicates per treatment using Genstat 8.2 (Lawes Agricultural Trust, Rothamsted Experimental Station) software. Log_{10} transformed *Trichoderma* cfu data and the seedling parameters were also analysed using ANOVA. Treatment means for all assessments were separated using Fisher's Protected least significant difference (LSD) tests at the $P<0.05$ level.

3.10 Results

A significant majority (66%) of the seedlings in block 5 died due to unforeseen circumstances and as a result block 5 was omitted from analysis. Furthermore, only the first two sampling times (two out of four split-blocks for each of the four main blocks) were assessed due to time constraints, meaning that a total of 32 seedlings were assessed (8 replicates per treatment).

3.11 Total ECM root tips

Overall there were no significant differences between the four treatments with respect to total numbers of ectomycorrhizal root tips per centimetre root length (Figure 3.1). Arbor-Guard™ inoculation tended to decrease the total number of root tips relative to the control, while ectomycorrhizal inoculation on its own tended to increase the total number of root tips.

However, when the ectomycorrhizal symbionts were co-inoculated with Arbor-Guard™ the total number of root tips tended to increase relative to all treatments (Figure 3.1).

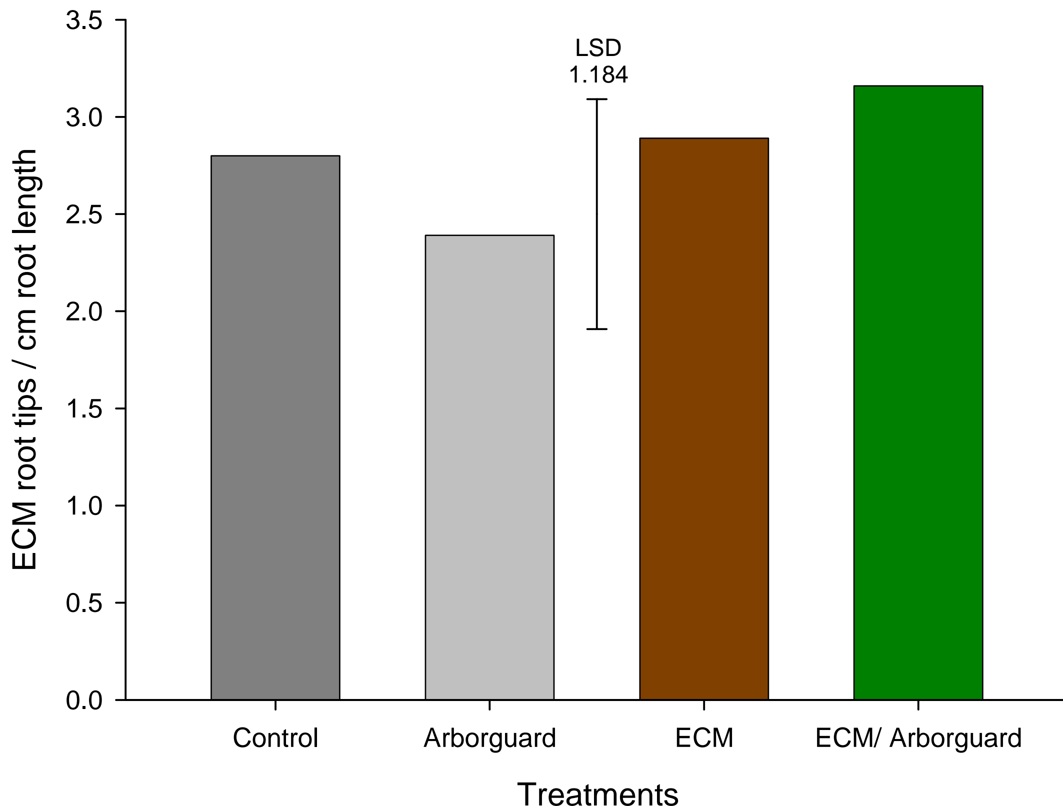

Figure 3.1 Total ECM root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. Means are separated using Fisher's Protected LSD (*p*=0.05).

After a thorough macroscopic evaluation of the total number of root tips across the four treatments, with subsequent DNA sequencing analysis (representative sequences presented in Table 3.2; Appendix C.2), it was concluded that an endogenous ECM species within the Thelephoroid family was the dominant ECM detectable (Table 3.2 & Figure 3.3). *Suillus luteus*, one of the inoculated ECM species was also identified but made up a significantly smaller proportion of the total root tips relative to the Thelephoroid species (Figure 3.3). The *Suillus luteus* species extracted from the root tips (Table 3.2) was confirmed through sequencing to be 100% identical (669 bp) to the inoculated *S. luteus* (Appendix C.2.4 and C.3.4 respectively).

Table 3.2 Sequence results for the GenBank queries of ECM root tips classified by levels of ramification at Lincoln

ECM species	Morphotype	Description	Sequence length	GenBank Accession #	GenBank score	e-value	Maximum Identity (%)
Thelephoraceae	Unramified	Light brown, smooth, white apex	643	AY748885.1	1103	0.00	97
Thelephoraceae	Dichotomous	Light brown, smooth, white apex	644	AY748885.1	1168	0.00	99
Thelephoraceae	Multi–dichotomous	Light – dark brown, smooth, white apices, tortuous irregular branching	644	AY748885.1	1168	0.00	99
Suillus luteus	Multi-dichotomous	Metallic purple, velvety surface, white apices, short dichotomous branching, hyphal fans	670	DQ068969.1	1210	0.00	99

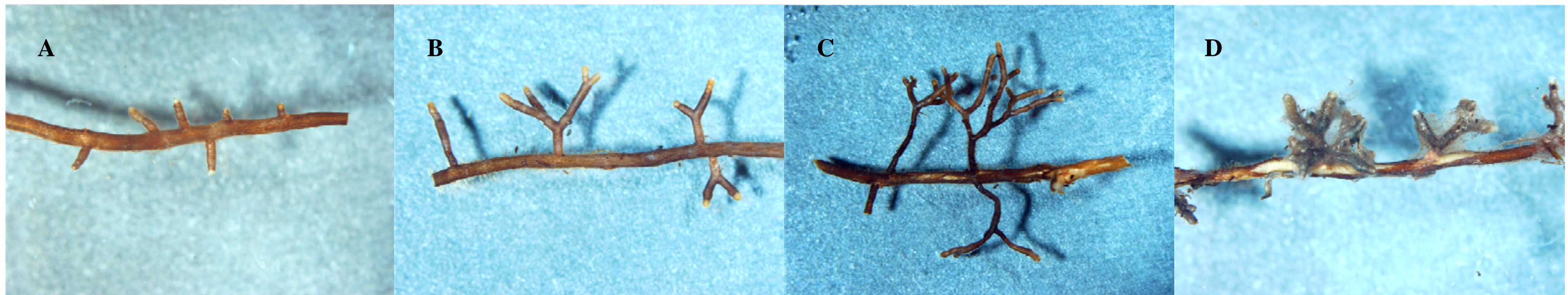

Figure 3.2 Representative samples of the root tips at Lincoln University including the Thelephoid species morphotypes unramified (A); dichotomous (B); and multi-dichotomous (C) morphotypes and *Suillus luteus* multi-dichotomous (D).

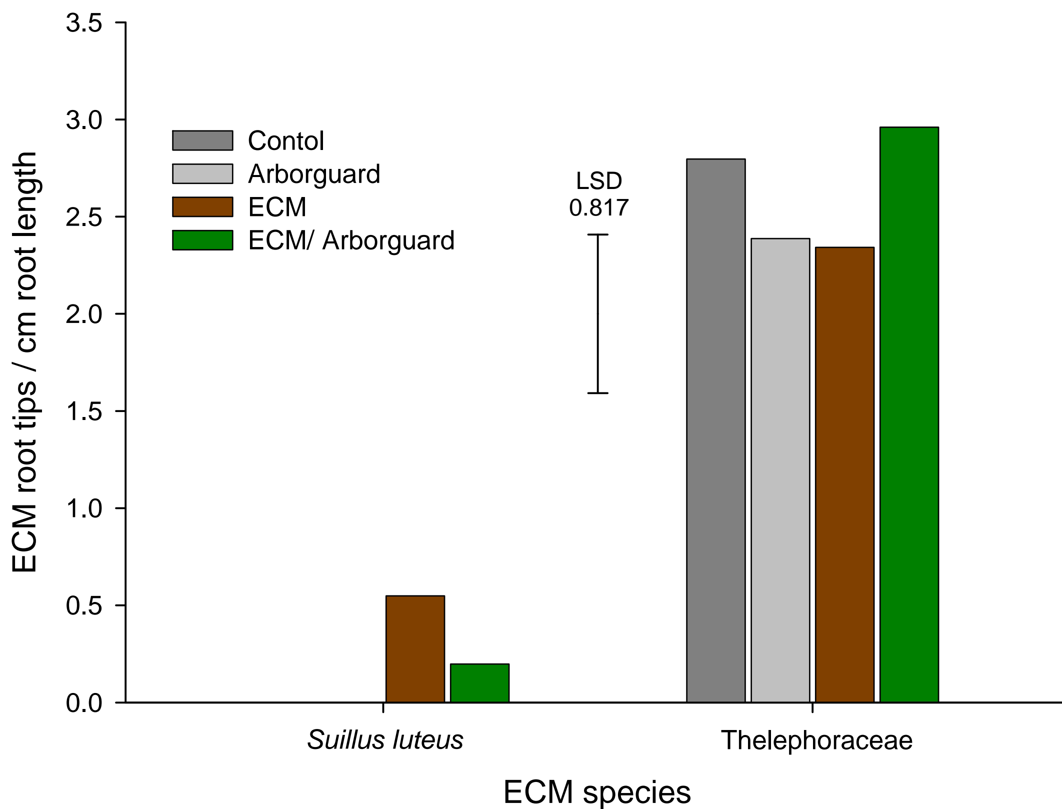

Figure 3.3 Total ECM root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation, as split by the two dominant detected ECM species in the Thelephoraceae family and *Suillus luteus*. Means are separated by Fisher's Protected LSD (*p*=0.05).

3.12 Inoculated ECM species identification

Sequencing of the pure ECM species (described in Section 3.4.4) obtained from Ensis, Rotorua (Section 3.4.1) was done post inoculation into each of the respective treatments. The results of the sequencing analysis (Table 3.3; Appendix C.3) revealed that not all species were what they were first identified as. Of the six fungal species received, only four turned out to be actual ECM species after DNA sequencing analysis. The remaining two were the saprophytic fungal species *Hypholoma fasciculare* (Huds.) Kumm. and *Lentinula edodes* (Berk.) Pegler., which were described on inoculum delivery as *Suillus granulatus* and *Rhizopogon* spp. respectively. Of the ECM species *Scleroderma bovista* and *Suillus luteus* were described correctly, while *Rhizopogon luteolus* and *R. parksii* were identified as *R. roseolus* (Corda.) Th. Fr. and *R. villosulus* Zeller. after sequencing, respectively.

Table 3.3 Fungal species received from Ensis, Rotorua and their actual species identity as identified from pure culture DNA extraction followed by sequencing.

ECM species (Ensis)	Sequence length (bp)	GenBank accession #	Actual fungal species	GenBank score	e-value	Maximum identity (%)	Fungal grouping
Rhizopogon luteolus (1812)	677	DQ068965.1	*Rhizopogon roseolus*	1074	0.00	97	ECM
Rhizopogon parksii (246)	675	AF058310	*Rhizopogon villosulus*	1170	0.00	99	ECM
Rhizopogon spp. (262)	734	DQ49707.1	*Lentinula edodes*	1341	0.00	99	Saprophytic (Shiitake mushroom)
Suillus luteus (253)	669	DQ068969.1	*Suillus luteus*	1205	0.00	99	ECM
Suillus granulatus (244)	671	DQ320134.1	*Hypholoma fasciculare*	1164	0.00	98	Saprophytic
Scleroderma bovista (1813)	634	AB19517	*Scleroderma bovista*	1120	0.00	98	ECM

3.12.1 Pure species inoculum potential

Scleroderma bovista (isolate 1813) was the only ECM species that did not have any mycelial growth extending from the peat: vermiculite inoculum substrate after plating onto MEA. For all of the other five fungal species all inoculum samples gave rise to colonies indicating 100% colonisation of the peat : vermiculite inoculum.

3.13 ECM root tips as discriminated by morphotype

Again the dominant ECM species had three distinct morphologies that could be discriminated by their level of ramification as described in Section 2.10.2, while *Suillus luteus* always had a multi-dichotomous morphology (Figure 3.2).

Significantly more multi-dichotomous Thelephoroid morphotypes were observed relative to the less ramified morphotypic structures between all treatments (Figure 3.4). No significant differences were observed between treatments within each of the morphotype descriptions of the Thelephoroid species (Figure 3.4), although the relative proportions of root tips/ cm root length between each treatment was different within the three morphotypes (Figure 3.4). Sequence analysis between the three Thelephoroid morphotypes revealed that they were 99.7% similar (Appendix C.2).

Suillus luteus was only detected in low proportions relative to the Thelephoroid species in the ECM inoculated treatments as a multi-dichotomous morphotype (Figure 3.4).

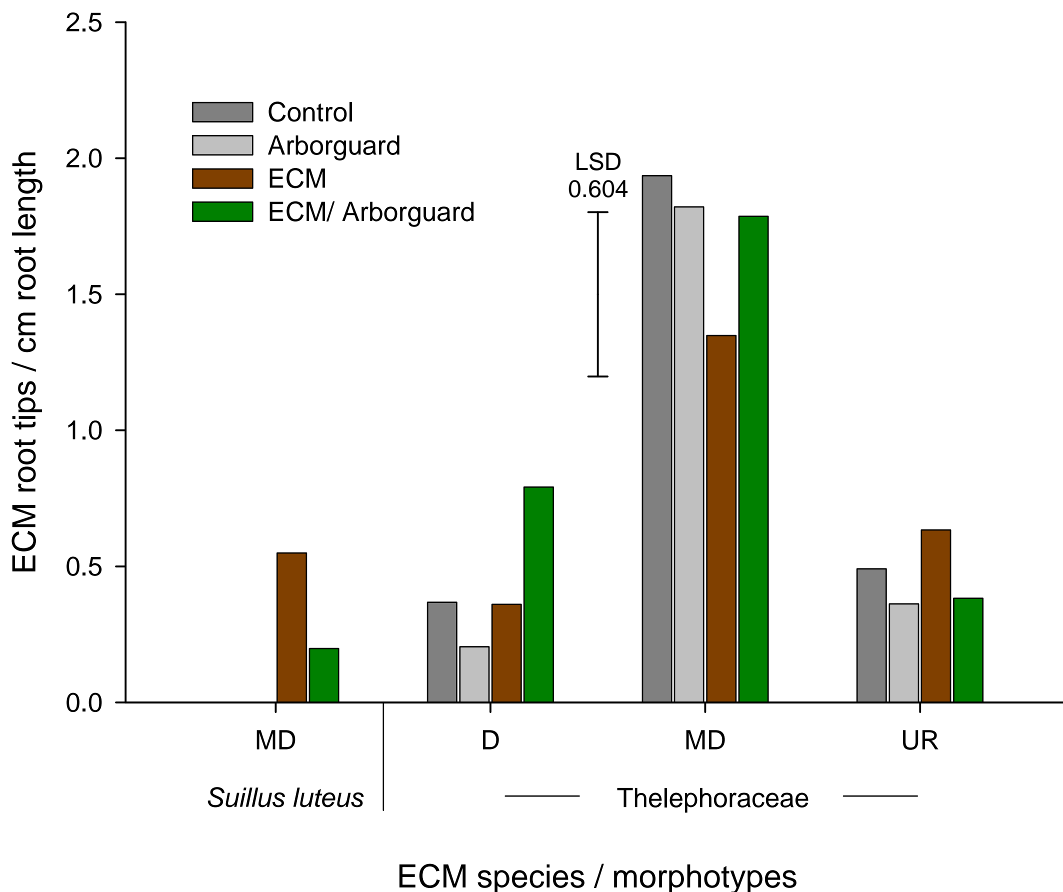

Figure 3.4 Relative proportions of dichotomous (D), Multi-dichotomous (MD) and unramified (U) ECM root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation as split by species found in the Thelephoraceae family and *Suillus luteus*. Means are separated using Fisher's Protected LSD (*p*=0.05).

3.14 Morphotype change over time

A significant increase in the proportion of multi-dichotomous ECM root tips relative to the less ramified morphologies was apparent between sampling time one and two, c.f. 3 weeks (Figure 3.5). Un- ramified root tips significantly dropped between the two sampling times, while dichotomous morphologies decreased in abundance between sampling times but not significantly (Figure 3.5).

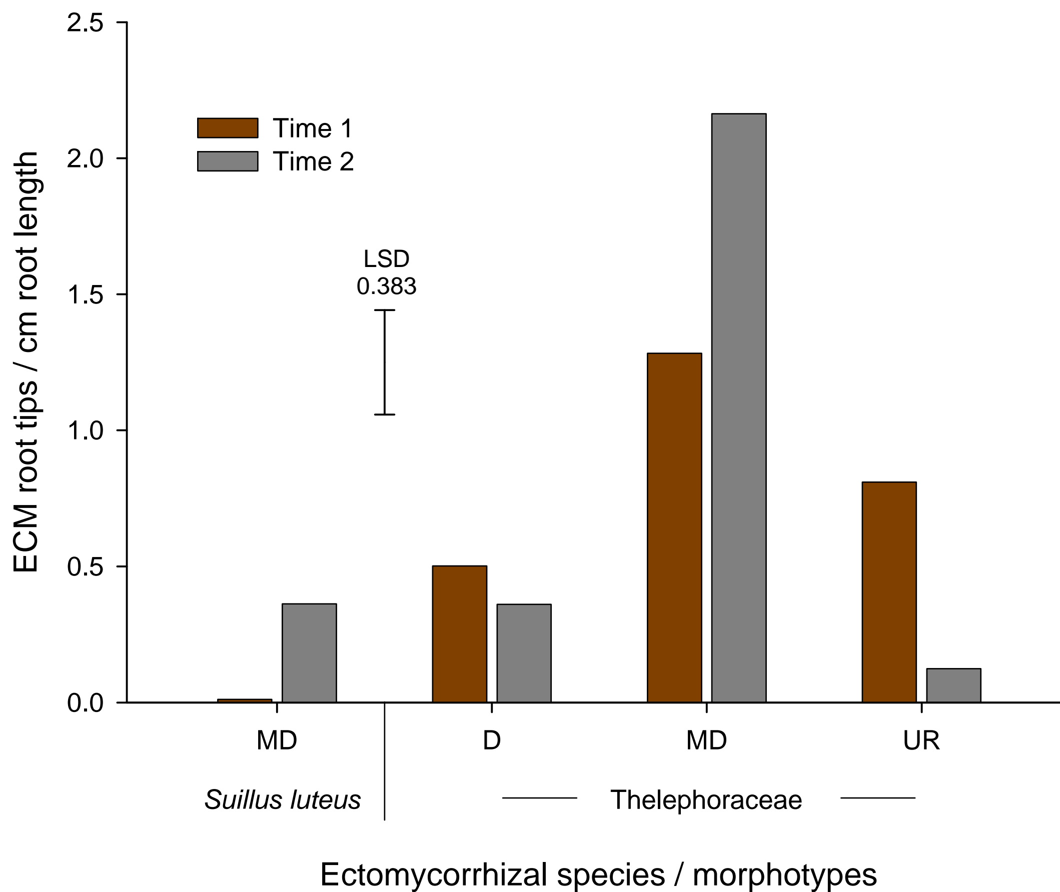

Figure 3.5 Relative proportions of total ECM root tips/ cm root length of dichotomous (D), Multi-dichotomous (MD) and unramified (U) morphotypes from the first set of assessments (Time 1) to the second set of assessments (Time 2). Means separated by Fisher's Protected LSD (*p*=0.05).

3.15 Non-mycorrhizal root tips

No significant differences were observed between any of the treatments with respect to non-mycorrhizal root tips (Figure 3.6). Ectomycorrhiza/ Arbor-Guard™ co-inoculation tended to increase the total number of non-mycorrhizal root tips relative to all the treatments, while both Arbor-Guard™ and ectomycorrhizal inoculation treatments on their own tended to lower the total number of non-mycorrhizal root tips relative to the control, respectively (Figure 3.6).

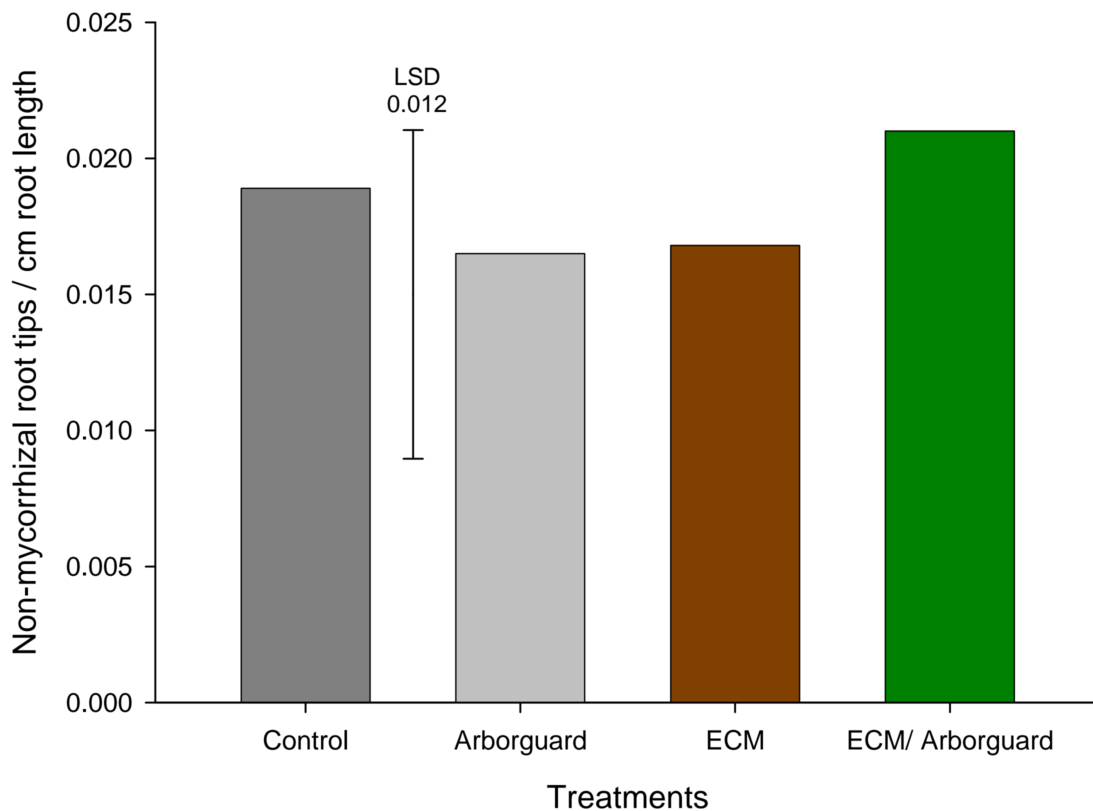

Figure 3.6 Total non-mycorrhizal root tips/ cm root length for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. Means separated by Fisher's Protected LSD (*p*=0.05).

3.16 Trichoderma counts

The Arbor-Guard™ inoculum suspension applied at sowing was assessed for the actual *Trichoderma* cfu by serial dilution onto TSM. After two weeks incubation at 20ºC the total count confirmed that there were 5×10^9 cfu per gram of Arbor-Guard™ applied to the respective treatments.

At harvest, all four treatments (Table 3.1) were analysed for *Trichoderma* cfu after $\log_{10}$ transformation with the results illustrated in Figure 3.7. The Arbor-Guard™ inoculated treatments had significantly higher *Trichoderma* populations by two orders of magnitude than treatments without Arbor-Guard™ applied. ECM inoculated on its own had significantly higher *Trichoderma* cfu counts relative to the untreated control.

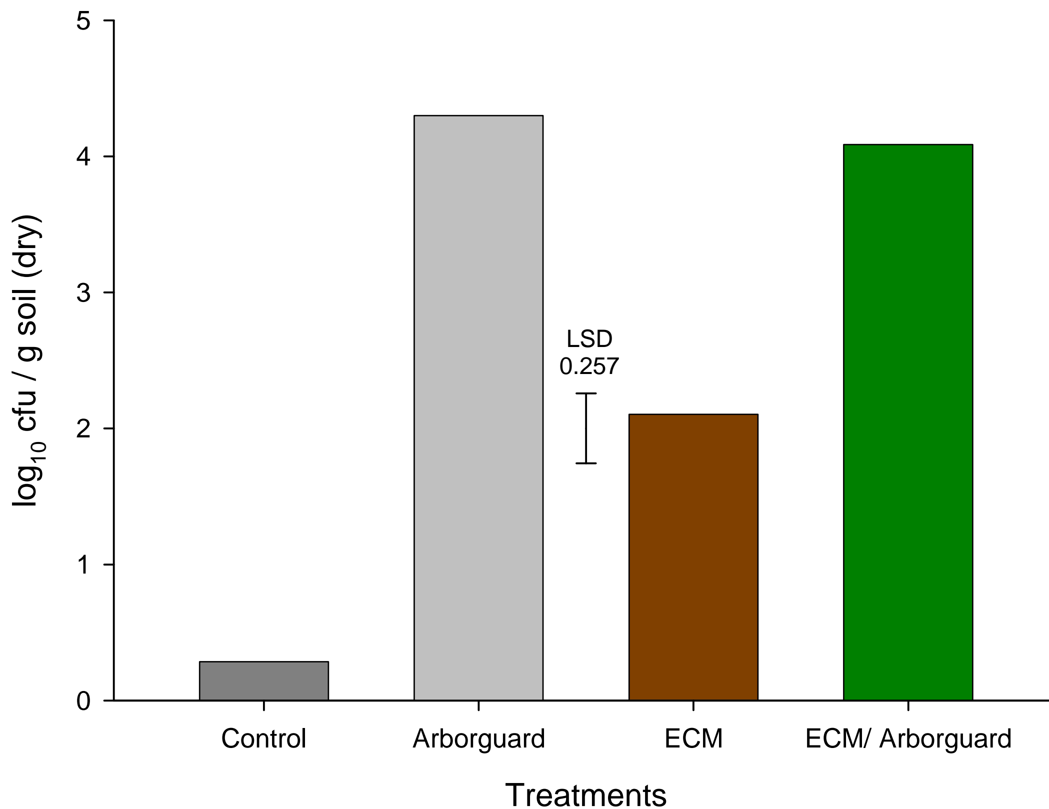

Figure 3.7 Log_{10} transformed *Trichoderma* cfu means for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. Treatment means are separated from each other as determined by Fisher's Protected LSD (*p*= 0.05).

3.17 Seedling parameters

No significant differences were observed between any of the treatments in the seedlings height, diameter or shoot dry weights at harvest (Table 3.4). Similarly no significant differences were observed in the height : diameter ratio. Seedling emergence was 100% across all treatments.

Table 3.4 Lincoln seedling parameters (seedline A) measured at final harvest for the control, Arbor-Guard™ application, ectomycorrhizal (ECM) species inoculation and ECM/ Arbor-Guard™ species co-inoculation. All results within rows were non-significant (NSD) as determined by Fisher's Protected LSD (*p*=0.05).

Seedling parameters	Treatments				LSD
	Control	Arbor-Guard™	ECM	Arbor-Guard™ / ECM	
Height (cm)	11.19	11.81	11.12	10.88	NSD
Diameter (mm)	1.95	2.10	1.94	1.85	NSD
Height : diameter	57.60	57.60	57.60	59.60	NSD
Shoot dry weight (g)	0.492	0.505	0.479	0.417	NSD

3.18 Discussion

Overall the addition of Arbor-Guard™ did not negatively impact on ECM colonisation in glasshouse grown *P. radiata* seedlings. These results confirm the PF Olsen nursery findings. The Lincoln experiment was originally designed to augment the PF Olsen commercial experiment with the co-inoculation of ECM species known to colonise *P. radiata* seedlings in a system designed to be a compromise between an *in vitro* axenic bio-assay and the more realistic nursery conditions.

The dominant ECM fungi colonising the root tips of the *P. radiata* seedlings were identified as belonging to the Thelephoraceae family. Again, as discussed in the PF Olsen experiment, there could be other ECM species colonising the root tips but the overall proportion will be low. On average 780 root tips were analysed per seedling for the Lincoln experiment and of these only three main morphotypes, along with the prominent *S. luteus* morphotype, were characterised. Each of three Thelephoraceae morphotypes characterised (Table 3.2) shared 99.7% sequence similarity between them (Appendix C.2.), which means that is more than likely to be the same species. On average the consensus ITS region sequence of the three Thelephoraceae morphotypes was 85% homologous to the six *Th. terrestris* ITS sequences outlined in Table 2.3 (Appendix C.2 and C.1, respectively for sequences). Therefore, the only conclusion that can be drawn from the sequence results is that the ECM colonising the root tips of the seedlings at Lincoln are not the same species as from PF Olsen but could be the same genera.

The main genera within the Thelephoraceae family that are most likely to form a mycorrhizal relationship with *P. radiata* seedlings and have been shown overseas to be abundant ECM genera include *Tomentella*, *Pseudotomentella* and *Thelephora* (Gardes and Bruns, 1996; Koljalg et al., 2000; Tammi et al., 2001). One could only speculate as to the actual genera observed at Lincoln, however, what is known from our results is that the ECM colonising the seedlings within the Thelephoroid family share very similar morphological and ecological characteristics to *Th. terrestris* observed at the PF Olsen nursery. Similarities such as this have been found else where, Tammi et al., (2001) after sequencing the ITS region of a previously morphotyped *Th. terrestris* ECM found it shared the same sequence as *T. radiosa* (Karst.) Rick. *Tomentella radiosa* has been reported in the literature to be a resupinate form of *Th. terrestris* (Koljalg et al., 2000). Indeed *Th. terrestris* is closely related phylogenetically to the tomentelloid fungal symbionts *Tomentella* and *Pseudotomentella* spp. (Koljalg et al., 2000). Koljalg et al., (2000) went further to suggest that the delimitation between *Tomentella* and *Thelephora* spp. is not as distinct as earlier studies have reported, both in genetic analysis and morphological identification. In fact *T. sublilacina* (Ellis & Holw.) Wakef., a common ECM of pine, is a sister species of *Th. terrestris* (Koljalg et al., 2000).

From an ecological perspective the Thelephoroid species identified in the Lincoln experiment share a similar competitive ability with respect to the early colonisation of peat based environments (Koljalg et al., 2000; Tammi et al., 2001; Taylor and Bruns, 1999) as *Th. terrestris* did in the PF Olsen nursery. One could imply from these observations that the Thelephoroid species could have very similar functional characteristics to *Th. terrestris.* With this in mind the Thelephoroid species could be considered a weed species in nursery environments. Our data is consistent with this hypothesis, as the pattern is similar to the PF Olsen experiment where the total ECM root tip numbers were not significantly different between the control and ECM inoculation. The ability of Thelephoroid species to also out compete other ECM species could be the decisive factor contributing to the failed recovery of the majority of inoculated ECM species in the Lincoln experiment. As found with *Th. terrestris*, the Thelephoroid species could have the ability to out compete other ECM species colonisation by competitive exclusion, which has been documented in the literature in other systems and termed the "priority effect" (Kennedy et al., 2009). Kennedy et al., (2009) found that the first colonising species of *Rhizopogon* was not displaced after the inoculation of a second species of *Rhizopogon* three months later on *P. muricata* D. Don. seedlings. While Marx and Bryan, (1975) observed that the addition of *Pisolithus tinctorius* as basidiospores to 2 month old *P. taeda* L. seedlings could not displace the already colonised *Th. terrestris*. Dunabeitia et al., (2004) attributed the reduction of *Rhizopogon luteolus, R. roseolus* and

Scleroderma citrinum Pers. mycorrhization due to *Th. terrestris* and *Lactaria* sp. having already colonised *P. radiata* seedlings and competitively excluding any other species from colonisation.

The competitive ability of Thelephoroid species to out compete other ECM could be representative of nursery grown seedlings in peat based potting media. Walbert, (2008) in a soil based nursery did not find any Thelephoroid species colonising the root tips of *P. radiata*. Species including *Pseudotomentella tristis* (Karst.) Larsen., *P. sp.*, *Tomentella* sp. and *Th. terrestris* were only recorded by Walbert, (2008) after *P. radiata* trees matured to eight years old in the Kaingaroa forest.

Of the four known ECM species received from Ensis for ECM inoculum (Table 3.3), *Rhizopogon roseolus*, *Suillus luteus* and *Scleroderma bovista* are the only known ECM species able to colonise *P. radiata* seedlings (Chu-Chou, 1979). The remaining potential ECM, *Rhizopogon villosulus*, however, has not been reported to be an ECM on *P. radiata* (Molina and Trappe, 1994). Further, *R. villosulus,* Section *Villosuli,* were only found to colonise Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco.) seedlings (Massicotte et al., 1994) and form non-functional (no Hartig net) ECM symbiosis with species of pine in pure-culture axenic conditions (Molina and Trappe, 1994). Therefore the likelyhood of an ectomycorrhizal symbiosis of *R. villosulus* with the *P. radiata* seedlings in our experiment would be low. Unfortunately the viability test for all the ECM fungal species was done on the morning of inoculation into the potting mix for the *P. radiata* seedlings. This meant that the inoculum from *S. bovista* was included in the ECM mix, however, from the inoculum potential assay performed it was concluded that it was not viable and therefore *Scleroderma bovista* is unlikely to have become mycorrhizal with the *P. radiata* seedlings. In retrospect the viability of the ECM inoculum should have been done earlier. This would have meant that *S. bovista* would not have been included in the ECM mix, which would have been advantageous as it would have reduced any associated variables that could potentially influence treatment effects. Therefore only two respective ECM species, *Rhizopogon roseolus* and *Suillus luteus*, could be counted on as viable inoculum in the Lincoln experiment and go on to form functional ectomycorrhiza with *P. radiata* seedlings. As a result, the low number of total root tips observed in the ECM inoculated treatment relative to the control (Figure 3.1) could be simply attributed to the reduced numbers of ECM propagules actually inoculated (6.45% instead of 19.35% v/v). Also, the total number of ECM species was reduced from six to two; therefore this would have reduced the probability of ECM establishment. Confounding the

problem was the co-inoculation of both *Hypholoma fasciculare* and *Lentinula edodes* into the ECM inoculum mix due to the DNA sequencing analysis carried out post inoculation.

One could premise that the co-inoculation of the two saprophytic fungi could antagonise both *R. roseolus* and *S. luteus* mycorrhization onto the seedlings, which in effect could have resulted in lower overall number of root tips being colonised. Mucha et al., (2008) found that *Hypholoma fasciculare* inhibited *Suillus bovinus* (Pers.) Roussel. in co-culture, however, hyphal damage was not observed. In Vasiliauskas et al., (2007) work they showed that *H. fasciculare* was able to be isolated from the internal root tip tissue of surface sterilised healthy *Picea abies* and *Pinus sylvestris* seedlings. Consequently in the same work, another saprophytic fungus *Phlebiopsis gigantea* (Fr.) Julich., was shown to form a mycelial mantel on *Picea abies* seedlings *in vitro*. This shows the complexity of potential interactions in the rhizosphere between litter decomposing fungi and ectomycorrhizal fungal species and raises the question on how saprophytic fungi compete for space/ nutrients in the rhizosphere (Vasiliauskas et al., 2007). In this experiment we can not rule out the potential negative interaction of *H. fasciculare* on the inoculated ECM species. There is no literature on possible interactions, with respect to ECM colonisation, of *Lentinula edodes* and ectomycorrhizal symbionts but again this cannot be ruled out. The competitive saprophytic ability of both *Hypholoma fasciculare* and *Lentinula edodes* could have detrimentally impacted on the pre-symbiotic ECM fungi. Our results support the potential negative impact of both, or either co-inoculated saprophytic fungi over riding the potential negative impacts of Arbor-Guard™ inoculation. This is illustrated in Figure 3.1, where ECM inoculation on its own was tending to have lower colonisation than the co-inoculation of Arbor-Guard™ and ECM.

The Thelephoroid species was not able to completely inhibit *S. luteus* colonisation of *P. radiata* seedlings, although the abundance of *S. luteus* was low. It could be assumed that *S. luteus* was able to colonise the root tips before the Thelephoroid species, or was more competitive in the rhizosphere environment. Conversely *S. luteus* could have colonised more root tips at the early stages of seedling growth and then replaced by the Thelephoraceae species. However, our trend in data does not show this as there were more root tips colonised by *S. luteus* found on the second analysis (Figure 3.5). A further step to control the potential Thelephoroid dominance over the inoculated ECM species could have been to grow the seedlings in a sterile media. However, keeping the media sterile for the duration of seedling growth before transplanting requires a lot of resources and in this case was not feasible for the size of the experiment. Further, sterile potting media would provide excellent growing conditions, particularly in this case for the high organic layer loving *Trichoderma* and

Thelephoroid species (Leandro et al., 2007; Taylor and Bruns, 1999 respectively) and any other more pathogenic soilborne microorganisms that have the ability to rapidly colonise the sterile media once exposed to the non-sterile glass house environment. Nall, V. (un-published), following similar protocols to the current experiment, successfully inoculated *R. roseolus* via mycelial inoculum onto transplanted *P. radiata* seedlings. However, the seedlings were kept under sterile conditions for the duration of the experiment by growing them individually under cover using transparent plastic bags that were able to allow respiratory gas exchange while stopping any spore contamination.

Rhizopogon is a known to be a good coloniser of *P. radiata* seedlings in nurseries both in New Zealand (Chu-Chou, 1979) and overseas (Molina and Trappe, 1994) but failed to colonise in our experiment irrespective of treatment. Most natural ecosystems inoculum arrives either as spores or from live extramatrical mycelium already colonised to roots. A possible explanation as to the increased colonisation potential of *Suillus luteus* relative to *Rhizopogon* spp. in our experiment could be the type of inoculum. Interestingly Theodorou and Bowen, (1987) found basidiospores of *S. luteus* to be less conducive to germination in the rhizosphere of *P. radiata* relative to basidiospore germination of *R. luteolus*. Theodorou and Bowen, (1987) went on further to say that germination of basidiospores and mycelial growth probably respond to different root exudate compounds. Therefore, one could conclude from our experiment that *S. luteus* is better suited to mycelial inoculum and that the *Rhizopogon* spp. are more suited to basidiospore inoculum as found by Theodorou and Bowen, (1987) and as a result were competitively excluded by the Thelephoroid species. Indeed Molina and Trappe, (1994) in their work had difficulty in obtaining ECM colonised seedlings when they inoculated *Rhizopogon* spp. as mycelial inoculum to *Pinus* spp., instead basidiospores isolated from sporocarp tissue was the preferred method of inoculation. This observation was made even after the authors acknowledged that *Rhizopogon* spp. would be prime candidates, due to their comparative ease of pure culture isolation and fast growth, for mycelial inoculations. Massicotte et al., (1994) also found basidiospores the preferred method of delivery in to a peat/ vermiculite substrate when they inoculated five species of seedlings with eleven different *Rhizopogon* spp. to test host-fungus specificity. Basidiospores were inoculated at least 18 weeks after seedling planting and were found to germinate in the rhizosphere and colonise seedlings well (Massicotte et al., 1994). In more recent work Chavez et al., (2009) measured the mycorrhization potential of three ECM inoculation systems including spore, solid state mycelia and a liquid suspension of mycelia on *P. radiata* seedlings under controlled nursery conditions. After 11 months Chavez et al., (2009) found *R. luteolus* and *S. luteus* gave the best results using the solid and liquid state mycelium inoculum over the spore

suspensions, while *S. bellinii* (Inzenga.) Watling. performed just as well with all three inoculation systems. It was acknowledged that the success of mycorrhization between different species of ECM was dependant on the inoculum system used (Chavez et al., 2009). In our experiment it was assumed that each ECM species had the same potential to form mycorrhizal relationships with the *P. radiata* seedlings irrespective of the type of inoculum used. From Chavez et al., (2009) findings this assumption is likely to be wrong. Hall et al., (2003) expressed the importance of the right abiotic and biotic conditions required to have successful colonisation of root tips from an inoculated ECM species. They went on to say that there is a big difference between simply applying the ECM inoculum and having healthy well colonised root systems of the inoculated species. Not enough attention to detail was administered in our experiment with respect to ECM inoculation and as result could have confounded problems.

Whether or not the inoculated ECM species died before establishment is not known, as apart from analysing ECM root tips at final harvest, no other post inoculation isolations were made. The glasshouse environment was not controlled enough as at times the temperature got up to 30ºC for a number of hours. This could have a detrimental effect on non-symbiotic ECM inoculum prior to colonisation. Confounding this would be the relative humidity of the potting media during pulses of high temperature being too low. Rincon et al., (1999) controlled the glass house temperature between 20 – 25ºC and maintained >40% humidity when they inoculated *P. pinea* L. seedlings in a similar system to our experiment.

The age of seedling roots has been shown to influence the mycorrhization potential with respect to timing of ECM inoculation of different ECM species. Theodorou and Bowen, (1987) found that *R. luteolus* mycorrhization was more rapid on older (21 days old) than younger (4 days old) *P. radiata* seedlings root systems. These results used *R. luteolus* basidiospores, however, in an earlier study Theodorou, (1980) found the same results, irrespective of whether or not basidiospores or mycelial inoculum was used. Application of the ectomycorrhizal inoculum at seed sowing therefore might have been too early. It would have been better to apply the inoculum once secondary laterals had started to form on the roots (Hall and Perley, 2008). Since we didn't have enough time or resources to produce spore suspensions it may have been better to transplant 3 – 4 week old *P. radiata* seedlings into fresh vegetative ECM inoculum rather than incorporating it in at the time of seed sowing. Rincon et al., (1999) successfully formed ectomycorrhizas using a peat/ vermiculite mycelial inoculum with 8 ECM genera, including *Rhizopogon roseolus* and *Suillus* spp., when they

transplanted *P. pinea* emergent seedlings in glasshouse conditions. *Rhizopogon roseolus* had the highest colonisation rates, 63 – 89% of short roots colonised, while *Suillus luteus* failed to colonise the *P. pinea* seedlings (Rincon et al., 1999). However, Rincon et al., (1999), did attribute their success in forming *R. roseolus* ECM seedlings to the high inoculation rate (1:4 v/v) of inoculum to the peat/ vermiculite potting substrate. Bogeat-Triboulot et al., (2004) also had successful mycorrhization after inoculation of *Pinus pinaster* seedlings four weeks after seed germination with a mycelial concentrate of the ECM symbiont *Hebeloma cylindrosporum*. Marx and Bryan, (1975) compared the colonisation potential of *P. taeda* seedlings when inoculated with *Pisolithus tinctorius* either by vegetative mycelium in a peat/ vermiculite media at seed sowing, or by basidiospore application after 2 months growth of the seedlings. After 8 months growth it was found in the vegetative inoculum delivery treatment that 92% of feeder roots were colonised by *P. tinctorius*, whereas in the basidiospore method of application only 23% of the roots formed *P. tinctorius* ectomycorrhiza. Therefore inoculating ECM via vegetative mycelia at the time of seed sowing in this system was markedly better than basidiospore inoculation after plants had established. These results indicate the importance of tailoring a particular inoculum system to the species of both ECM and plant, with respect to timing of ECM inoculation. In retrospect an axenic bio-assay testing the ability of each respective inoculated ECM species to colonise sterile *P. radiata* seedlings would have been helpful to ascertain their colonisation potential. This bio-assay could have helped with identifying reasons as to why all inoculated ECM species but *S. luteus* did not colonise the *P. radiata* seedlings in the glasshouse, while optimising the timing and type of ECM inoculation.

With respect to timing of ECM inoculation, different types of ECM inoculum types have their own advantages and disadvantages. Basidiospore inoculation has the advantage of being able to be inoculated at any stage of seedling establishment. Seed inoculation of *Rhizopogon luteolus* by coating basidiospores onto *P. radiata* has been successfully done in large scale nursery systems (Theodorou and Bowen, 1973; Theodorou and Benson, 1983). The inherent ability of basidiospores to survive adverse conditions and germinate only when specific root exudates from the host are present probably allows this system of delivery (Bowen, 1994; Theodorou and Bowen, 1987). A disadvantage to basidiospore inoculation is the germination rates can be determinant on whether or not the particular ECM species is a primary (pioneer) or later coloniser of roots (secondary or third stage colonisers) (Bowen, 1994; Ishida et al., 2008). However, the major disadvantage of basidiospore inoculum based delivery systems, particularly in the case of this experiment, is basidiospores can not be cultured *in vitro*. Instead gathering inoculum is dependent on retrieving fresh sporocarps from mature stands of

trees. This is dependant on the time of year for sporocarp production, which is often erratic and sometimes requires collection and storage when the sporocarps are abundant (Rossi et al., 2007). This was outside the scope of this experiment so basidiospore inoculation could not be employed as a deliverly system.

Withholding ECM inoculation until the seedlings root system is established would also increase the chances of successful ECM colonisation as the roots would have a receptive mycorrhizal infection zone (Marks and Foster, 1973). Both basidiospore inoculation and/ or administering the ECM inoculum via a mycelial slurry are options. Flores et al., (2005) using a mycelial slurry obtained mycorrhizal colonisation 25 days post inoculation when they inoculated five Neotropical species of pine seedlings with two species of *Lactarius indigo* (Schwein.) Fr. Both a mycelial slurry and basidiospore inoculation also has the added benefit of multiple inoculations over time to be administered to increase the chances of ECM colonisation. In the case of this experiment a mycelial slurry would be a feasible option and in hindsight a better delivery system of ECM.

It could be concluded that ectomycorrhizal inoculation in this experiment does not significantly alter the Arbor-Guard™ species populations within the rhizosphere of *P. radiata* (Figure 3.7). This result follows that of the literature with respect to ectomycorrhizal species generally having no direct influence on *Trichoderma*. Only a few reports have indicated ECM species having antagonistic impacts on *Trichoderma*. Zadworny et al., (2007) concluded that the ECM fungus *Laccaria laccata* could parasitise hypha and condida of *T. virens* and *T. harzianium* in co-culture and in the rhizosphere of Scots pine when the authors assessed the role of cell wall lytic enzymes in mycoparasitism. Zadworny et al., (2008) showed the increased translocation of ^{32}P from conidia of *T. virens* to *P. sylvestris* seedlings colonised either by *Suillus bovinus* or *Laccaria laccata* compared to non-mycorrhizal control seedlings of 4.15 and 15.57%, respectively.

An underlying parameter with the inoculation of ECM species was the co-inoculation of the two saprophytic fungi *Lentinula edodes* (Shiitake mushroom) and *Hypholoma fasciculare*, which in turn could influence *Trichoderma* populations. The probability that *Lentinula edodes* would have any detrimental effect on *Trichoderma* survival is very low due to the well documented negative effects that *Trichoderma* spp. (green mould disease) have on *Lentinula edodes* in co-culture (Miyazaki et al., 2009). *Hypholoma fasciculare* on the other hand is known to be an aggressive cord forming soil saprophyte (Boddy, 2000), and as with *Trichoderma* spp. have the potential to be bio-control agents against wood-decaying fungi in forestry (Boddy, 2000; Nicolotti and Varese, 1996). *Hypholoma fasciculare*, when challenged

with *T. harzianum*, *T. pseudokoningii* Rifai. and *T. viride* isolates in dual agar – based culture experiments, was shown to have the highest competitive ability relative to 16 other wood-decaying mushroom species (Badalyan et al., 2004). However, our results reveal that any potential negative interaction between *H. fasciculare* and *Trichoderma* spp. are not being expressed as there is not any significant difference between the *Trichoderma* populations between the Arbor-Guard™ applied on its own and when Arbor-Guard™ is co-inoculated with the mycorrhizal species mix (containing *H. fasciculare*).

From the results it could be concluded that the addition of the ECM inoculum had a direct impact on the indigenous *Trichoderma* population as there is a significant increase in *Trichoderma* cfu relative to the control (Figure 3.7). This effect could be attributed to the fact that the control did not receive any peat:vermiculite mix, which would contain, along with the respective fungal species, all their corresponding metabolites. Not to add a peat: vermiculate mix to the control in hindsight was probably a mistake, however, the only way for this to happen would be to add an extra control comprising of a sterilised mix of the ECM inoculum. This in itself would not be a perfect control because there is an extra source of energy introduced in the form of dead/ lysed fungal mycelia from the ECM cultures which isn't present in the treatment mix. Indeed, the addition of fungal structures has shown to be a nutrient source for ECM (Zadworny et al., 2008). Mucha et al., (2007) also showed the increased proteolytic activity of several ectomycorrhizal fungi when associated with mycelia of autoclaved saprotrophic fungi.

Seedling growth was not influenced by the addition of Arbor-Guard™, however, no nutrients were applied to the seedlings as the experiment was set up to induce ECM colonisation. This was considered to be an important factor, as supported by previously stated literature, to support the mycorrhization of the inoculated ECM species. Further, the aim of this work was not to measure the impacts of Arbor-Guard™ addition on seedling growth/ health, as this has already been described elsewhere (Hill, 2004/2005). As a result the seedlings growth was stunted due to nutrient limitation, which would completely take out any positive effects of seedling growth induced by *Trichoderma* addition as found by (Hill, 2004/2005).

Chapter 4
In vitro laboratory assays

In order to enumerate any potential interactions between the inoculated ECM species in Chapter three and the *Trichoderma* spp. in the Arbor-Guard™ formulation, additional *in vitro* dual co-culture laboratory assays were conducted. These controlled dual culture assays allowed work to be done in axenic conditions to permit potential interactions between specific fungal species to be recorded. Three *in vitro* assays were run to address objective five, including a co-culture assay observing the physical interaction at the macro level of each individual ECM species inoculated in the Lincoln mixture with each *Trichoderma* isolate in Arbor-Guard™. A similar second co-culture assay was also run, this time on glass slides to observe any potential antagonistic interactions, for instance mycoparasitism, at the microscopic level. While a third *in vitro* assay, to assess whether there is ECM inhibition due to the potential liberation of inhibitive volatile organic compounds (VOC) from each of the *Trichoderma* isolates, was undertaken. Dual culture assays have been used widely in the literature as they are useful in the detection of specific mechanisms (detailed in Section 1.4) enabling antagonists such as *Trichoderma* spp. to be understood, which may relate to the mode(s) of action operating in field conditions (Whipps, 1997).

As this experiment was initated and completed before the formal identification of each of the fungal species inoculated in the Lincoln ECM mixture, all five (not including *Scleroderma bovista* due to it not being inoculated) fungal isolates were tested due to the assumption that they were ECM.

4.1 Methods

4.1.1 Co-culture interaction assay

Individual isolates of the *Trichoderma* species in Arbor-Guard™, the three asymbiotically growing ectomycorrhizal species (*R. roseolus*, *S. luteus* and *R. villosulus*) and the two saprophytic fungal species (*H. fasciculare* and *L. edodes*) used in the Lincoln experiment ECM mixture were grown in co-culture plate assays to determine macro hyphal interactions. The six *Trichoderma* isolates used were *T. harzianum* LU686 and the *T. atroviride* Bissett. isolates LU655, LU659, LU660, LU661 and LU663. Each of the five fungal species in the ECM mixture were co-inoculated with each one of the six *Trichoderma* isolates *via* a 5 mm MMN (¼ strength; Appendix A.2.2) agar plug taken from the actively growing edge of the

respective colonies growing on MMN (¼ strength) agar plates. Each 5 mm plug of inoculum was then placed opposite to one another, 2 cm from the edge of a 9.0 cm Petri dish containing MMN (¼ strength) agar. Control plates consisted of each ectomycorrhizal species being co-inoculated together on one plate containing MMN agar (¼ strength). Six replicate plates for each interaction was set up. The plates were incubated in the dark at 20ºC for around 10 days, or until no further interaction was observed. Growth rates of both *Trichoderma* and ectomycorrhizal fungi were assessed daily by measuring the distance travelled of the respective hyphal fronts along a previously drawn transect line on the reverse side of the 9.0 cm Petri dish.

Any inhibition, over growth of colonies or colour change was noted and described by the following critera adapted from (McLean, 2001):

A Hyphae of the two colonies intermingle but remain clearly distinguishable.

B Advancing mycelial fronts meet; the candidate fungi are inhibited and overgrown by the *Trichoderma* followed by sporulation.

C The mycelial fronts of the two fungi approach one another and stop growing.

D Growth of the candidate fungi is inhibited at a distance with a clear zone of inhibition between the co-inoculated species.

Due to the known relatively fast *in vitro* growth of *Trichoderma*, each ectomycorrhizal species was allowed to establish a hyphal front before one of the six *Trichoderma* isolates was inoculated on the opposite side of the agar dish. The aim was to have the colonies of both species of fungi meet in the middle of the agar plate at the same time, thus having equal hyphal mass. The relative growth rates of each respective *Trichoderma* and ectomycorrhizal/ saprophytic species/ isolates were predetermined on MMN (¼ strength) agar prior to the commencement of the experiment, thereby allowing the approximate inoculation time to be known. Actual inoculation timing of each *Trichoderma* isolate relative to the candidate fungal species inoculation timing is outlined in Table 4.1.

Table 4.1 Number of days each candidate ECM and saprophytic species respectively were grown on ¼ strength MMN agar before each respective *Trichoderma* isolate was co-inoculated in the co-culture and antibiosis interaction *in vitro* assays

Trichoderma spp. LU No.	ECM species			Saprophytic species	
	Rhizopogon roseolus	*Suillus luteus*	*Rhizopogon villosulus*	*Hypholoma fasciculare*	*Lentinula edodes*
655	26	28	28	13	39
659	26	28	28	13	39
660	26	28	28	13	39
661	26	28	28	13	39
663	9	7	7	9*	15
686	26	28	28	13	39

* LU 663 was inoculated 9 days before *Hypholoma fasciculare*

4.1.2 Microscope interaction assay

To determine the interactions at the advancing hyphal front at the microscopic scale, another experiment was conducted, this time on microscope slides using a modified method of Berry et al., (1993). Sterile cover slips (22 x 50 mm) were dipped into molten MMN agar (¼ strength) and then put on the surface of a Petri dish with ¼ strength MMN agar to dry. Each of the ectomycorrhizal/ saprophytic species in the Lincoln ECM mixture was co-inoculated with one of the six *Trichoderma* isolates *via* a 5 mm hyphal plug taken from the actively growing edge of an axenic culture growing on ¼ strength MMN agar. The plugs were put at opposite ends of the cover slip, allowing only 2 mm in from the cover slip edge to the edge of the 5 mm plug. Again as described in Section 4.1.1, species of fungi in the ECM mixture were inoculated first until they had a sufficient hyphal front to meet the relatively fast growing *Trichoderma* species in the middle of the cover slip. As the two fungal species began to converge, the cover slip was aseptically removed by tweezers from the Petri dish and placed up-side down on a sterile standard glass slide (26 x 76 mm). The depth of the two 5 mm agar plugs separated the glass slide and cover slip from one another and allowed room for un-hindered hyphal growth. The cover slip was subsequently sealed to the glass slide by squeezing pre-warmed petroleum jelly from a 25 mL syringe around the cover slip/ glass slide edge. This reduced moisture loss from the system and stopped any contamination. Three replicate slides for each interaction were set up, with the control slides being two ectomycorrhizal/ saprophytic 5 mm plugs of the same species co-inoculated onto one cover

slip. The respective hyphal interactions were observed under a compound microscope (40× magnification) within a day of inverting the cover slip onto the glass slide, this in turn limited the time that the two fungal species remained between the glass slides before any observations. If more time was required for the interactions to proceed the same slides were incubated in the dark at 20ºC with subsequent observations of the interactions being made as soon as the hyphae met, which was usually the following day.

4.1.3 Volatile antibiosis

To test for the potential production of inhibitory volatile antibiotics being released by the *Trichoderma* isolates, a dual plate bio-assay was conducted on MMN (¼ strength) agar. Both fungal species were kept physically separated by inoculating the 5 mm plugs obtained from actively growing fronts of the respective fungal species cultures onto individual 9.0 cm Petri plate bottoms. Each plug of agar was placed 2 cm from the edge of its respective plate. A previously drawn transect line on the reverse side of the plates allowed for agar plug placement and hyphal growth measurements to be made during the experiment. Joining of the two plates and inoculation of the *Trichoderma* plugs was done after the ectomycorrhizal/ saprophytic species had grown to approximately 25 mm radius, again allowing for the relatively slow growth of the species in the ECM mixture (see Table 4.1 for inoculation timing). The plates with the ectomycorrhizal and saprophytic species were inverted and sealed to the *Trichoderma* plates using cling wrap and incubated in the dark at 20ºC. Each treatment was replicated four times and the *Trichoderma* plates were always in the upright position to reduce contamination from any condensation dropping *Trichoderma* spores onto the ectomycorrhizal/ saprophytic cultures.

4.2 Results

4.2.1 Co-culture interaction assay

Each of the five candidate fungal species interaction with the co-inoculated *Trichoderma* isolates was qualitatively characterised into one of the four categories outlined in Section 4.1.1 with the results outlined in Table 4.2. All three positively identified ECM species were consistently overgrown by five of the *Trichoderma* isolates followed by sporulation (category B; Table 4.2, Figure 4.1). *Trichoderma* isolate LU 663 was the exception, as this isolate inhibited the radial growth to a larger extent relative to the other *Trichoderma* isolates towards both the ECM and saprophytic species. However, LU 663 rarely grew or sporulated over the co-inoculated ectomycorrhizal or saprophytic fungal cultures (category D; Table 4.2, Figure 4.1). *Suillus luteus* in co-culture with all six *Trichoderma* isolates had a yellow

discolouration on the advancing mycelial front that was not apparent in the respective control plates (discolouration most apparent in the category B picture, Figure 4.1). The radial growth of most of the *Trichoderma* isolates, with the exception of LU 663, in co-culture with *Hypholoma fasciculare* stopped with no clear indication of any positive or negative interaction (category C; Table 4.2, Figure 4.1). *Trichoderma* isolate LU 663 clearly inhibited the radial growth of *H. fasciculare*. None of the *Trichoderma* isolates sporulated over *H. fasciculare* (category C; Table 4.2, Figure 4.1).

Lentinula edodes did not grow well (slow diffuse hyaline hyphal growth) on MMN agar and as a result the qualitative assessment was omitted because no conclusions could be dervived with respect to the interaction.

Table 4.2 Qualitative interaction observations of each Arbor-Guard™ *Trichoderma* isolate in MMN agar co-culture with each respective candidate fungal species inoculated into the ECM mixture at Lincoln University (n=6).

Trichoderma spp. LU No.	ECM species			Saprophytic species	
	Rhizopogon roseolus	*Suillus luteus* *	*Rhizopogon villosulus*	*Hypholoma fasciculare*	*Lentinula edodes*
655	B	B	B	C	n/a
659	B	B	B	C	n/a
660	B	B	B	C	n/a
661	B	B	B	C	n/a
663	D	D	D	D	D
686	B	B	B	C	n/a

B Advancing mycelial fronts meet; the candidate fungi are inhibited and overgrown by the *Trichoderma* followed by sporulation.
C The mycelial fronts of the two fungi approach one another and stop growing.
D Growth of the candidate fungi is inhibited at a distance with a clear zone of inhibition between the co-inoculated species.
* *Suillus luteus* had a yellowing mycelial front on hyphal contact with each *Trichoderma* species that was not seen in the control plates.
n/a Not assessed

	ECM species			Saprophytic species	
Category	*Rhizopogon roseolus*	*Suillus luteus*	*Rhizopogon villosulus*	*Hypholoma fasciculare*	*Lentinula edodes*
B					
D					
C					

Figure 4.1 **Representative co-culture interaction pictures of the sporulating *Trichoderma* species (right plug) interacting with each candidate ECM species (left plug) (B); Interaction with *Trichoderma* (LU 663) for each of the species included in the ECM mixture (includes saprophytic species) (D); Representative picture of interaction between *Hypholoma fasciculare* and each *Trichoderma* species (C). Letters (B, D and C) represent each category outlined in Table 4.2.**

4.2.2 Microscopic assay

Hyphae of all co-inoculated species grew into each other and intermingled, however, no *Trichoderma* hyphal coiling, hyphal penetration or degradation of the respective candidate species in the ECM mixture was observed in the microscopic slide interaction assay.

4.2.3 Antibiosis assay

Radial growth measurements were ceased after 7 days due to each of the *Trichoderma* isolates hyphal growth reaching the outer edge of the Petri dish and extending up the walls of the Petri dish, in turn contaminating each of the test fungal species. Most fungal species radial growth rate was inhibited to a certain extent by each *Trichoderma* isolate relative to their respective control plates radial growth (Table 4.3). Between the ECM species, *Rhizopogon roseolus* on average tended to be inhibited the most relative to it respective control (19% growth rate inhibition) than *S. luteus* (11%) and *R. villosulus* (1%), respectively across all *Trichoderma* isolates. Radial growth rates of *Hypholoma fasciculare* were inhibited by the *Trichoderma* isolates to the same extent (12% inhibition) relative to its respective control as the ECM growth rates. However, no statistical analysis was conducted between each ectomycorrhizal/ saprophytic species, only within a species, so no robust conclusions can be drawn from this observation.

Table 4.3 Radial growth rate (mm day^{-1}) of each candidate fungal species when grown without physical contact in the same atmosphere with each *Trichoderma* isolate in the antibiosis assay. Asterisk within rows indicate significantly different radial growth rate relative to the control as determined using unrestricted LSD *p*=0.05

	Mean radial growth rate (mm day^{-1}) n=4								
	Trichoderma LU No.								
Fungal species	655	659	660	661	663	686	Control	LSD	F. pr
R. roseolus	0.61	0.57	0.61	0.50	0.75	0.61	0.75	0.35	0.70
S. luteus	1.09	1.07	1.02*	1.09	1.02*	1.11	1.20	0.13	0.15
R.villosulus	0.80	0.72	0.84	0.65	0.79	0.79	0.76	0.27	0.84
H. fasciculare	1.16*	1.31*	1.38	1.44	1.48	1.31*	1.53	0.19	0.01

4.3 Discussion

Due to the relatively fast growth rates of the *Trichoderma* isolates in the co-culture assay, no growth measurement analysis of the respective candidate fungal species in the Lincoln mixture was able to be carried out as was originally planned. On average the three ECM cultures only grew 4 mm from the time each *Trichoderma* isolate was inoculated to the time the mycelial fronts converged. This made any quantitative delineation of the growth rates relative to the controls unable to be statistically derived. Therefore only qualitative observations could be assessed with any certainty (Table 4.2 and Figure 4.1).

Qualitatively, the co-inoculation interaction assay across all *Trichoderma* species, apart from LU 663, yielded similar results. Each of the five *Trichoderma* isolates (LU 655, 659, 660, 661 and 686) overgrew and sporulated over each of the three ECM species (category B; Table 4.2, Figure 4.1). Summerbell, (1987) also had a similar result when *T. viride* came into contact with *Laccaria bicolor* in agar co-culture. Nutrient competition was probably the cause of the reduction in growth of the ECM species at the zone of interaction, while the concurrent competition for space illustrates the aggressive nature of the *Trichoderma* isolates and is probably the main mechanism of antagonism in the current experiment. It was also observed that the abundance of sporulating areas covering the mycelia of *R. roseolus* was more relative to the amount over *S. luteus*. Further, it was noted that *R. roseolus* grew at a slower rate than *S. luteus* in the antibiosis assay. These results were consistent for each of the *Trichoderma* isolates apart from LU 663 and could be a reason as to why *R. roseolus* ECM root tips were not recovered in the Lincoln experiment whereas *S. luteus* ECM root tips were recovered all be it in low numbers. Mycoparasitism was not observed in the microscope interaction assay in the current experiment. Summerbell, (1987) also observed no evidence of *T. viride* mycoparasitising colonies of *Laccaria bicolor* growing on agar even though the ectomycorrhizal colony stopped growing after initial hyphal contact and was overgrown by the *T. viride* isolate. However, these results are not uncommon as it is well known that parasitism is a highly species specific mechanism (Chet et al., 1998; Harman et al., 2004). Mycoparasitism involves a number of steps from the initial recognition of specific lectins on the target host through to appressoria formation and finally lytic enzymes being released for cell penetration (Chet et al., 1998; Harman et al., 2004). Further, agar media containing normal nitrogen levels tend to suppress mycoparasitism therefore making observations in artificial media hard to conduct (Summerbell, 1987).

From the clear zone of inhibition observed on the co-culture plates it is unlikely that *Trichoderma* isolate LU 663 suppressed the growth of any candidate fungi it came up against

using mycoparasitism as the mode of antagonism. Although the growth rate of LU 663 was on average four times slower than the other *Trichoderma* isolates, it showed consistent inhibitive interactions across all three ECM species and the two saprophytic species (category D; Table 4.2, Figure 4.1). Interestingly LU 663 inhibited the growth of *Hypholoma fascicular* in particular relative to all other candidate species in the ECM mixture. This observation is augmented by the observation that all the other five *Trichoderma* isolates did not overgrow or sporulate over *Hypholoma fascicular* (category C; Table 4.2, Figure 4.1). It has been documented that antibiotics can inhibit the growth of fungi, however, this antagonistic effect can be very *Trichoderma* species specific (Howell, 1998). If antibiosis is responsible for the decline in fungal growth then one would assume that LU 663 would show the greatest reduction in growth for all the candidate fungal species concerned due to the consistent inhibitory interactions (category D) observed in Figure 4.1. All of the candidate fungal species in category D had their growth inhibited before the hyphal fronts converged. However, the results indicate (Table 4.3) that relative to the other *Trichoderma* isolates LU 663 did not suppress the growth of the fungal species considerably more, with the exception of *S. luteus*. These observations are in favour of agar diffusible secondary metabolites inhibiting the respective fungal species and not VOC compounds. Further, *H. fascicular* was least affected by LU 663 relative to the other five *Trichoderma* isolates in the antibiosis assay (Table 4.3). Yet *H. fascicular* was inhibited in the co-inoculated interaction assay to the same extent, although not significant, as the other fungal species (Figure 4.1). So one could deduce from these results that volatile antibiosis is not the mechanism of antagonism observed with LU 663 in the co-inoculation interaction assay.

Due to cross-contamination issues the antibiosis assay was restricted to the amount of time the *Trichoderma* isolates took to reach the outside margin of the Petri plate. No absolute conclusions can be drawn from the data in Table 4.3 as the growth rates of the *Trichoderma* isolates were too fast. This indicates that there was no nutrient limitation and therefore the likelihood of secondary metabolite production would be low due to most antibiotics being produced only when *Trichoderma* species are under stress or growing in media with a high carbon to nitrogen ratio (Howell, 1998; Howell and Stipanovic, 1984). On average the total growth of each fungal species in the ECM mixture was only 6.6 mm over the seven days of measurements. This in turn reduced the resolution of the data making it hard to deduce any differences, which is reflected in the high error values associated with each candidate species. Therefore, drawing the conclusion from the data to confidently state that antibiosis is a mechanism of antagonism used against the fungal species in the current experiment should be approached with caution.

Further, the reduced radial growth rates of the fungal species in the ECM mixture can only be speculated to be caused by inhibitive VOC's as there was no analysis of the head space gas composition. Reduced growth rates relative to each respective candidate species control could just be the result of the higher respiration rate of the actively growing *Trichoderma* species relative to the slow growth of each candidate fungal species, in turn depleting the oxygen and increasing the partial pressure of carbon dioxide. The closed chamber method used in the current experiment will also tend to build up any respiratory gases or VOC's in the head space so any effect could be actually a false positive not able to be transferred to the field situation.

Of the abundant array of potential antagonistic volatile antifungal compounds *Trichoderma* species are known to produce, quantification of the relative amounts of a pyrone, 6-pentyl-α-pyrone (PAP), has been previously described for all of the six isolates in the present study (Dodd-Wilson, 1996). It is unkown whether PAP has any activity against any of the ECM/ saprophytic species used in this study, however, previous work has shown PAP to have antifungal activity against a wide distribution of fungal plant pathogens (reviewed in Dodd-Wilson, 1996). However, from the relative levels of PAP produced in pure culture analysis on MEA plates from Dodd-Wilson's, (1996) results (Table 4.4), one could safely conclude that PAP is not produced by LU 663, the most probable isolate to use antibiosis as a potential mechanism in our results. One could also conclude from our results that none of the other five *Trichoderma* isolates were using PAP as a volatile antibiotic, even though LU 660 was found to be the highest producer of PAP (8 mg plate^{-1}) out of all 50 isolates tested in Dodd-Wilson's, (1996) work. However, this is only one secondary metabolite out of the myriad of other antagonistic metabolites known to be produced by *Trichoderma* spp. So the only conclusion that can be drawn from our results is that PAP is most probably not an influencing volatile antibiotic produced by any of the six *Trichoderma* isolates under the conditions in our experimental design. This is not to say that PAP will not be produced, particularly by *Trichoderma* isolate LU 660, as an antagonistic secondary metabolite against any of the candidate fungal species in the ECM mixture under different conditions.

Table 4.4 Relative amounts of PAP produced (mg/ plate) by each *Trichoderma* isolate from Dodd-Wilson's (1996) work relative to the average level of inhibition found across all fungal species inoculated in the ECM mixture at Lincoln University

	Trichoderma LU No.					
	655	659	660	661	663	686
Inhibition (%)[A]	12	13	8	16	4	9
PAP: mg/ plate (±S.E.)[B]	3.6 (±0.8)	4.6 (±1.1)	8.0 (±3.4)	4.1 (±1.3)	<0.01	<0.01

[A] Mean overall percentage inhibition that each *Trichoderma* isolate had over all 4 fungal species tested in the antibiosis assay in Table 4.3

[B] Values taken directly from the PhD thesis written by Dodd-Wilson, (1996)

From our results one could conclude that nutrient and/ or space competition is the most probable mode of any antagonism expressed by the *Trichoderma* isolates, except LU 663, against the fungal candidates inoculated in the ECM mixture. The evidence suggests that LU 663 could produce other agar diffusible secondary metabolites that in turn could inhibit the growth of all the co-inoculated fungal species. Although *in vitro* dual culture assays have been extensively used in the literature, especially in biological control, to test for potential antagonistic interactions between candidate microorganisms the results need to be approached with caution (Merriman and Russell, 1990). Whipps, (1997) went further to add that *in vitro* agar plate studies do not resemble the environmental or microbiological dynamics experienced in the field. Indeed, results obtained from any laboratory based media assays should be approached with caution and will never replace studying *in vivo* rhizosphere interactions (Bowen and Theodorou, 1979). However, Whipps, (1997) did state that if one understands the limitations of *in vitro* assays the results can still be an important indication of potential interactions in the field.

Unfortunately the saprophytic species *H. fasciculare* and *L. edodes* were not challenged with the ECM species in the Lincoln mixture due to the assumption that they were in fact ECM species at the time the assays were carried out. Dual co-culture assays of this kind may have given an insight into the low recovery of the inoculated ECM species in the Lincoln experiment (Chapter 3). While also given an insight into the relative dynamics observed with respect to the total number of root tips recovered tending to be lower in the ECM mixture on its own than the ECM/ Arbor-Guard™ treatment (Figure 3.1). In particular the interaction between *H. fasciculare* and the ECM species *R. roseolus* and *S. luteus*, respectively would have been pertinent to deduce any potential antagonistic interactions.

Chapter 5
Concluding discussion

Results from the current work do not support our original hypothesis (Section 1.6.2) of Arbor-Guard™ having an effect on the overall colonisation of ectomycorrhiza on containerised *Pinus radiata* seedlings, as there was no measured impact of the addition of Arbor-Guard™ on ECM colonisation in either the commercial or Lincoln experiments. However, this statement can only be qualified under the provision that the *Trichoderma* isolates in Arbor-Guard™ were viable at the time of inoculation in the commercial experiment, which was not determined at the time. However, the same Arbor-Guard™ formulation was used later to inoculate the Lincoln experiment and it was shown that *Trichoderma* spp. viability was at 10^9 cfu g^{-1}, which was the concentration stipulated by the manufacturer. While the unfortunate inoculation of saprophytic and un-viable ECM fungi in the Lincoln experiment makes it hard to delineate any potential effect, whether positive or negative, that Arbor-Guard™ had on ECM colonisation.

Dual culture *in vitro* assays conducted showed a level of inhibition towards the inoculated ECM species in the Lincoln experiment when challenged with the *Trichoderma* isolates in Arbor-Guard™. *Trichoderma* isolate LU 663 was the most antagonistic *Trichoderma* isolate in the *in vitro* assays towards all candidate fungi challenged. However, the antagonistic relationships expressed *in vitro* were not reflected in the *in planta* Lincoln experiment where there was not any real correlation of the total number of ECM root tips found in the control relative to the Arbor-Guard™ inoculated treatments. These results could be a simple case of the *Trichoderma* isolates not surviving during the course of the experiment as the actual determination of *Trichoderma* spp. to isolate level was not done.

Overall the results are restricted to the finding that the ECM diversity was low with members of the Thelephoroid family being the dominant species family found. The dominance of the ECM within the Thelephoroid family is not surprising as these species have been recorded elsewhere in the literature as ubiquitous ECM genera in the *Pinus* family (Taylor and Bruns, 1999), particularly in high organic matter environments (Koljalg et al., 2000). For instance *Thelephora terrestris* has been well documented to be an ECM of *P. radiata* (Bowen and Theodorou, 1979), being prevalent in *P. radiata* nurseries (Chu-Chou, 1979) and mature forests (Dunstan et al., 1998). These findings confirm the multi-stage characteristics of *Th. terrestris*, which has also been documented with *Tomentella* spp., another genus within the

Thelephoroid family (Taylor and Bruns, 1999). However, the sheer abundance of Thelephoroid species found colonising *P. radiata* does not necessary confirm that this association is beneficial to the seedlings. For instance *Pinus taeda* (Loblolly pine) seedlings colonised by the naturally occurring ECM *Th. terrestris* only produced half the amount of biomass as seedlings colonised by the introduced ECM *Pisolithus tinctorius* (Marx and Bryan, 1975).

It has been successfully shown that the "controlled" inoculation of selected ECM species that provide benefits tailored to the specific environmental conditions and species of tree into nurseries can perform better after transplanting than leaving the seedlings to be naturally colonised by the native ECM (Garbaye, 1990; cited in Rossi et al., 2007) . Although in the current experiment the "controlled mycorrhization" (Rossi et al., 2007) did not work as well as anticipated this does not immediately confer that members of the Thelephoroid family will always outcompete any other ECM species colonisation of *P. radiata* seedlings. Hall and Perley, (2008) showed that the inoculation of ECM fungi, of which *Coenococcum* sp. was documented, onto *Nothofagus menziesii* (Hook.) Oerst. (Silver beech) seedlings were able to successfully outcompete *Thelephora* colonisation even though the latter ECM was dominant in the uninoculated seedlings. In Hall and Perley's, (2008) experiment the successful colonisation of inoculated ECM was probably due to the "priority effect" discussed in Chapter 3 as the authors noted that there was extensive mycorrhization on the inoculated seedlings after 6 months, whereas the uninoculated seedlings only became mycorrhizal with the *Thelephora* species two months later. Indeed the success of any controlled mycorrhization of ECM fungi is not an easy task and is probably one of the reasons why there are not many commercial ECM inoculum products on the market (Rossi et al., 2007). Hall et al., (2003) stressed the importance of the correct ecological conditions required for successful ECM colonisation to proceed and continue. Low colonisation of the inoculated ECM in our experiment at Lincoln University is most probably as of a consequence of the abiotic conditions in the glasshouse not being conducive to ECM colonisation and/ or growth. This, and the fact that the commercial experiment was also not appropriately tuned for conditions conducive to ECM colonisation, for instance using a soilless potting media that most probably was depauparate in both the abundance of mycorrhizal propagules and species diversity (Hall and Perley, 2008), means that drawing any conclusions from our work on the effect of *Trichoderma* spp. inoculation on ECM mycorrhization is difficult. One can only speculate the effect of *Trichoderma* spp. inoculation, in the form of Arbor-Guard™, will have on the establishment of other species of ECM fungi either specifically selected for their beneficial characteristics or innately found in bare rooted nursery systems.

Our findings are not able to be necessarily translated into other nursery systems, such as bare rooted propagation, used to grow *P. radiata* seedlings. Further, our experiment was a relatively simplistic model, with respect to using a containerised system, in comparison to bare rooted nursery systems. Containerised systems are more easily manipulated compared to bare root systems where edaphic conditions will be a major influencing factor outside the control of the nursery manager. However, this opens up a valued opportunity with respect to the relative ease of being able to artificially inoculate beneficial ECM over the bare rooted system. Further, the ECM species inoculated not only could be selected specifically for the containerised system but more importantly could be tailored to the specific site where the seedlings will be eventually planted out (Trappe, 1977). So the ability to segregate areas of seedlings inoculated with different ECM fungi is another major advantage of containerised systems besides their added value to the seedlings already described in Section 1.1.2. Therefore the artificial inoculation of appropriate ECM into containerised propagation systems, as successfully described by Hall and Perley, (2008) above, is definitely warranted and should be integrated into all nursery systems as a standard practise (Hall and Perley, 2008; Trappe, 1977). With the change in focus from raising bare rooted seedlings to more seedlings being propagated in containerised systems (Menzies et al., 2001), there should be a concurrent paradigm shift to artificially inoculate specific ECM fungal species. Therefore, this calls for more research to be conducted on the potential effects of *Trichoderma* species bio-inoculants on ECM colonisation inoculated into commercial nurseries.

Although there was no impact of Arbor-Guard™ inoculation on the mycorrhization of containerised *P. radiata* seedlings, further research of the on going interactions after the seedlings are transplanted out into forestry stands is necessary. Summerbell, (1987) made the point in their research that any microorganism that may impede mycorrhization may also influence the absorptive capacity of the extramatrical mycelium, in turn reducing the functional characteristics of the mycorrhizal relationship with regard to nutrient and water uptake. Although our research did not find any negative effects of adding Arbor-Guard™ to the potential mycorrhization of containerised *P. radiata* seedlings, the ongoing interactions with respect to the colonised ectomycorrhizas functional ability to increase seedling health and productivity once transplanted needs to be investigated. What is known is that the *Trichoderma* spp. inoculated in Arbor-Guard™ do have a residual effect on the seedlings after transplanting, as Arbor-Guard™ inoculation has been shown to reduce the incidence of *Armillaria novae-zelandiae* (Stev.) Boesew. infection after the first eight years of seedling transplant (Hill, R., *per comm.*). From these results one can assume that some or all of the *Trichoderma* isolates in Arbor-Guard™ are active within the rhizosphere or are endophytic

within the root. In fact, it has been repeatedly shown that *Trichoderma* spp. are avirulent plant symbionts that are able to induce localised systemic responses and increase nutrient uptake (Harman et al., 2004). These results indicate that what could be considered as a positive effect of increased seedling growth and health induced by the ECM fungi in the field could actually be as a result of the *Trichoderma* spp. Which raises the question; how long will this positive interaction last or is it just transient phenomenon? We know that species within the *Pinus* family are particularly dependant on ECM colonisation for optimum growth and development under natural conditions (Smith and Read, 1997). So if the *Trichoderma* isolates inoculated in Arbor-Guard™ effectively fill the ECM niche or coerce what species of ECM colonise the seedling during the early stages of seedling development after transplanting how will this impinge on ECM functionality in the long term? However, the long term effects could be negligible due to the known successional dynamics of ECM colonisation of *P. radiata* trees in North Island forestry plantations in New Zealand (Walbert, 2008). Indeed Walbert, (2008) showed the dramatic succession of ECM root tip species within the first few years of transplanting out into a plantation forest. What is not known, however, is what effect the inoculation of Arbor-Guard™ has on ECM succession. This is another area of research in need of attention to fully delineate the effect of *Trichoderma* species bio-inoculants on ECM dynamics.

The current experiment only looked at two fungal groups and did not take into account the myriad of other potential synergistic, neutral or antagonistic interactions of the rhizospheric microbial community on mycorrhization. A review written by Summerbell, (2005) on the pre-1980 literature summarised the large variation in results, either synergistic or antagonistic, found from a bulk of work completed looking at mycorrhizosphere microorganisms. In the authors review he pointed out, for instance, that fluorescent pseudomonads were natural antagonists of any root-colonising fungi. Indeed Bowen and Theodorou, (1973; cited in Summerbell, 2005) attributed a 20-50% reduction of mycorrhizal formation in part to the presence of pseudomonad species. Yet the closely related *Pseudomonas* spp. are also known to be synergistic to the mycorrhization process and dubbed mycorrhiza helper bacteria (MHB) (Bowen and Theodorou, 1979; Garbaye and Bowen, 1987). Indeed the mycorrhizosphere is made up of a complex diversity of organisms each functioning at different tropic levels that either directly or indirectly influence the dynamics of mycorrhization. As this experiment didn't quantify any other microorganisms their presence goes un-detected and therefore any potential antagonistic impacts can only be assumed to be from the inoculated *Trichoderma* isolates, or in the case of the Lincoln experiment, from the co-inoculated saprophytic fungi *Hypholoma fasciculare* and *Lentinula edodes*. On the contrary any microorganisms such as

MHB will not be recognised as the potential controlling microorganisms synergistic to the mycorrhization process.

Future research priorities

Future work needs to address the potential effect of Arbor-Guard™ inoculation on specific ECM species colonisation potential of *P. radiata* seedlings in more detail. In particular the focus should be directed on ECM species such as *Rhizopogon* and *Suillus* spp. that could potentially be commercially inoculated into containerised and bare rooted nursery systems.

A more methodological approach into what effect each particular *Trichoderma* species/ isolate in Arbor-Guard™ has on each individual ECM species colonisation potential needs to be conducted. This research needs each of the *Trichoderma* species to be able to be traced *in planta* to assess both the survival of *Trichoderma* and the corresponding functionality that each species expresses upon ECM colonisation.

Research needs to be directed into what effect Arbor-Guard™ inoculation into nurseries has on any potential successional changes of ECM species colonising the *P. radiata* seedlings after out planting into commercial forestry stands. This research will give valuable information into whether or not a change, if any, in ECM diversity has a negative impact on functionality or whether or not there is a level of functional redundancy within ECM species.

References

Agerer, R. 1987-2002. Colour atlas of ectomycorrhizae Einhorn-Verlag, Munich.

Agerer, R. 1991. Characterization of ectomycorrhiza, p. 25-73, *In* J. R. Norris, et al., eds. Methods in microbiology. Techniques for the study of mycorrhiza, Vol. 23. Academic Press Limited, London.

Anonymous. 2003. Selecting treestocks for your forest [Online]. Available by PF Olsen and Company www.pfolsen.com (posted 2004; verified 10/11/2005).

Anonymous. 2004. Forestry Insights [Online] www.insights.co.nz (verified 01 January 2006).

Aspray, T.J., P. Frey-Klett, J.E. Jones, J.M. Whipps, J. Garbaye, and G.D. Bending. 2006. Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation. Mycorrhiza 16:533-541.

Badalyan, S.M., G. Innocenti, and N.G. Garibyan. 2004. Interactions between xylotrophic mushrooms and mycoparasitic fungi in dual-culture experiments. Phytopathologia Mediterranea 43:44-48.

Bending, G.D., and D.J. Read. 1995. The structure and function of the vegetative mycelium of ectomycorrhizal plants. V. Foraging behaviour and translocation of nutrients from exploiting litter. New Phytologist 130:401-409.

Berry, L.A., E.E. Jones, and J.W. Deacon. 1993. Interaction of the mycoparasite *Pythium oligandrum* with other *Pythium* species. Biocontrol Science and Technology 3:247 - 260.

Boddy, L. 2000. Interspecific combative interactions between wood-decaying basidiomycetes. FEMS Microbiology Ecology 31:185-194.

Bogeat-Triboulot, M.B., F. Bartoli, J. Garbaye, R. Marmeisse, and D. Tagu. 2004. Fungal ectomycorrhizal community and drought affect root hydraulic properties and soil adherence to roots of *Pinus pinaster* seedlings. Plant and Soil 267:213-223.

Bomberg, M., G. Jurgens, A. Saano, R. Sen, and S. Timonen. 2003. Nested PCR detection of Archaea in defined compartments of pine mycorrhizospheres developed in boreal forest humus microcosms. FEMS Microbiology Ecology 43:163-171.

Bourguignon, E.T. 2008. Ecology and diversity of indigenous *Trichoderma* species in vegetable cropping systems. PhD, Lincoln University, Canterbury, New Zealand.

Bowen, G.D. 1994. The ecology of ectomycorrhiza formation and functioning. Plant and Soil 159:61 - 67.

Bowen, G.D., and A.D. Rovira. 1961. The effects of microorgansims on plant growth. I. Development of roots and root hairs in sand and agar. Plant and Soil 15:166 - 188.

Bowen, G.D., and C. Theodorou. 1973. Growth of ectomycorrhizal fungi around seeds and roots, p. 107-150, *In* G. C. Marks and T. T. Kozlowski, eds. Ectomycorrhizae, Their ecology and physiology. Academic Press, Inc., New York and London.

Bowen, G.D., and C. Theodorou. 1979. Interactions between bacteria and ectomycorrhizal fungi. Soil Biology and Biochemistry 11:119-126.

Brimner, T.A., and G.J. Boland. 2003. A review of the non-target effects of fungi used to biologically control plant diseases. Ecosystems and Environment 100:3-16.

Brundrett, M., N. Bougher, B. Dell, T. Grove, and N. Malajczuk. 1996. Working with mycorrhizas in forestry and agriculture Australian Center for International Agricultural Research, Canberra Australia.

Bruns, T.D., and M. Gardes. 1993. Molecular tools for the identification of ectomycorrhizal fungi - taxon-specific oligonucleotide probes for suilloid fungi. Molecular Ecology 2:233-242.

Bruns, T.D., T.M. Szaro, M. Gardes, K.W. Cullings, J.J. Pan, D.L. Taylor, T.R. Horton, A. Kretzer, M. Garbelotto, and Y. Li. 1998. A sequence database for the identification of ectomycorrhizal basidiomycetes by phylogenetic analysis. Molecular Ecology 7:257-272.

Burdon, R.D. 1992. Introduced forest trees in New Zealand: Recognition, role, and seed source. 12. Radiata pine (*Pinus radiata* D. Don). FRI Bulletin 124:1-59.

Chalot, M., A. Javelle, D. Blaudez, R. Lambilliote, R. Cooke, H. Sentenac, D. Wipf, and B. Botton. 2002. An update on nutrient transport processes in ectomycorrhizas. Plant and Soil 244:165-175.

Chavasse, C.G.R. 1980. Planting stock quality: A review of factors affecting performance. New Zealand Journal of Forestry 25:144-171.

Chavez, D., G. Pereira, and A. Machuca. 2009. Effect of inoculum type of three fungal species on the controlled mycorrhization of *Pinus radiata* seedlings. Bosque 30:4-9.

Chet, I., N. Benhamou, and S. Haran. 1998. Mycoparasitism and lytic enzymes, p. 153-172, *In* C. P. Kubicek and G. E. Harman, eds. *Trichoderma* and *Gliocladium*, Vol. Volume 2. Taylor & Francis, London.

Chu-Chou, M. 1979. Mycorrhizal fungi of *Pinus radiata* in New Zealand. Soil Biology and Biochemistry 11:557-562.

Chu-Chou, M. 1980. Mycorrhizal fungi of Radiata pine in New Zealand. What's New in Forest Research 89:1-4.

Chu-Chou, M., and L.Y. Grace. 1983. Characterization and identification of mycorrhizas of Radiata pine in New Zealand. Australian Forestry Research 13:121-132.

Chu-Chou, M., and L.Y. Grace. 1984. *Endogone flammicorona* and *Tuber* sp. as mycorrhizal fungi of *Pinus radiata* in New Zealand. New Zealand Journal of Botany 22:525-531.

Chu-Chou, M., and L.Y. Grace. 1987. Mycorrhizal fungi of *Pinus radiata* planted on farmland in New Zealand. New Zealand Journal of Forestry Science 17:76-82.

Chu-Chou, M., and L.Y. Grace. 1988. Mycorrhizal fungi of Radiata pine in different forests of the North and South islands in New Zealand. Soil Biology and Biochemistry 20:883-886.

Chu-Chou, M., and L.Y. Grace. 1990. Mycorrhizal fungi of Radiata pine seedlings in nurseries and trees in forests. Soil Biology and Biochemistry 22:959-966.

Di Pietro, A., M. Lorito, C.K. Hayes, R.M. Broadway, and G.E. Harman. 1993. Endochitinase from *Gliocladium virens*: Isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. Phytopathology 83:308-313.

Dickie, I.A., and P.B. Reich. 2005. Ectomycorrhizal fungal communities at forest edges. Journal of Ecology 93:244 - 255.

Dickie, I.A., B. Xu, and R.T. Koide. 2002. Vertical niche differentiation of etomycorrhizal hyphae in soil as shown by T-RFLP analysis. New Phytologist 156:527-535.

Dodd-Wilson, S.L. 1996. Biochemical and molecular characterisation of *Trichoderma* species. PhD, University of Auckland, Auckland.

Duchesne, L.C. 1994. Role of ectomycorrhizal fungi in biocontrol, p. 27-45, *In* F. L. Pfleger and R. G. Linderman, eds. Mycorrhiza and plant health. APS Press, Minnesota.

Dunabeitia, M.K., S. Hormilla, I. Salcedo, and J.I. Pena. 1996. Ectomycorrhizae synthesized between *Pinus radiata* and eight fungi associated with *Pinus* spp. Mycologia 88:897-908.

Dunabeitia, M.K., S. Hormilla, J.I. Garcia-Plazaola, K. Txarterina, U. Arteche, and J.M. Becerril. 2004. Differential responses of three fungal species to environmental factors and their role in the mycorrhization of *Pinus radiata* D. Don. Mycorrhiza 14:11-18.

Dunstan, W.A., B. Dell, and N. Malajczuk. 1998. The diversity of ectomycorrhzial fungi associated with introduced *Pinus* spp. in the Southern hemisphere, with particular reference to Western Australia. Mycorrhiza 8:71 - 79.

El Karkouri, K., F. Martin, E.J.P. Douzery, and D. Mousain. 2005. Diversity of ectomycorrhizal fungi naturally established on containerised *Pinus* seedlings in nursery conditions. Microbiological Research 160:47-52.

Finlay, R.D. 2005. Mycorrhizal symbiosis: myths, misconceptions, new perspectives and future research priorities. Mycologist 19:90-95.

Flores, R., G. Diaz, and M. Honrubia. 2005. Mycorrhizal synthesis of *Lactarius indigo* (Schw.) Fr. with five Neotropical pine species. Mycorrhiza 15:563-570.

Frey-Klett, P., M. Chavatte, M. Clausse, S. Courrier, C. Le Roux, J. Raaijmakers, M.G. Martinotti, C. Pierrat, and J. Garbaye. 2005. Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. New Phytologist 165:317.

Garbaye, J. 1990. Use of mycorhizas in forestry, p. 197 - 248, *In* D. G. Strullu, ed. Les Mycorhizes Des Arbres et Plantes Cutivees. Lavoisier, Paris.

Garbaye, J., and D.J. Bowen. 1987. Effect of different microflora on the success of ectomycorrhizal inoculation of *Pinus radiata*. Canadian Journal of Forest Research 17:941 - 943.

Gardes, M., and T.D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2:113-118.

Gardes, M., and T.D. Bruns. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above and below-ground views. Canadian journal of Botony 74:1572-1583.

Hall, I.R., and C. Perley. 2008. Removing a significant constraint limiting the diversity of the forestry estate [Online] http://www.maf.govt.nz/sff/about-projects/search/05-142/05-142-final-report.pdf.

Hall, I.R., W. Yun, and A. Amicucci. 2003. Cultivation of edible ectomycorrhizal mushrooms. TRENDS in Biotechnology 21:433 - 438.

Harman, G.E. 2000. Myths and dogmas of biocontrol: changes in perceptions derived from research on *Trichoderma harzianum* T-22. Plant disease 84:377-393.

Harman, G.E., C.R. Howell, A. Viterbo, I. Chet, and M. Lorito. 2004. *Trichoderma* species - opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2:43-56.

Hill, R. 2004/2005. Beneficial organisms to enhance tree health. Forest Biosecurity Research Council.

Hilszczanska, D., and Z. Sierota. 2006. Persistence of ectomycorrhizas by *Thelephora terrestris* on outplanted Scots pine seedlings. Acta Mycologica 41:313-318.

Hjeljord, L., and A. Tronsmo. 1998. *Trichoderma* and *Gliocladium* in biological control: an overview, p. 131-151, *In* C. P. Kubicek and G. E. Harman, eds. *Trichoderma* and *Gliocladium*, Vol. Volume 2. Taylor & Francis, London.

Howell, C.R. 1998. The role of antibiosis in biocontrol., p. 173-184, *In* C. P. Kubicek and G. E. Harman, eds. *Trichoderma* and *Gliocladium*., Vol. Volume 2. Taylor & Francis, London.

Howell, C.R. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Disease 87:4-10.

Howell, C.R., and R.D. Stipanovic. 1984. Phytotoxicity to crop plants and herbicidal effects on weeds of viridiol produced by *Gliocladium virens.* Phytopathology 74:1346 - 1349.

Ingleby, K., P.A. Mason, F.T. Last, and L.V. Fleming. 1990. Identification of ectomycorrhizas Institute of Terrestrial Ecology. Natural Environment Research Council, London.

Ishida, T.A., K. Nara, M. Tanaka, A. Kinoshita, and T. Hogetsu. 2008. Germination and infectivity of ectomycorrhizal fungal spores in relation to their ecological traits during primary succession. New Phytologist 180:491-500.

Karkouri, K.E., F. Martin, E.J.P. Douzery, and D. Mousain. 2005. Diversity of ectomycorrhizal fungi naturally established on containerised *Pinus* seedlings in nursery conditions. Microbiological Research 160:47 - 52.

Kennedy, P.G., K.G. Peay, and T.D. Bruns. 2009. Root tip competition among ectomycorrhizal fungi: Are priority effects a rule or an exception? Ecology 90:2098-2107.

Klein, D., and D.E. Eveleigh. 1998. Ecology of *Trichoderma*, p. 57-74, *In* C. P. Kubicek and G. E. Harmen, eds. *Trichoerma* and *Gliocladium*. Basic biology, taxonomy and genetics, Vol. Volume 1. Taylor & Francis, London.

Koljalg, U., A. Dahlberg, A.F.S. Taylor, E. Larsson, N. Hallenberg, J. Stenlid, K.H. Larsson, P.M. Fransson, O. Karen, and L. Jonsson. 2000. Diversity and abundance of resupinate thelephoroid fungi as ectomycorrhizal symbionts in Swedish boreal forests. Molecular Ecology 9:1985-1996.

Kubicek-Pranz, E.M. 1998. Nutrition, cellular structure and basic metabolic pathways in *Trichoderma* and *Gliocladium.*, p. 95-119, *In* C. P. Kubicek and G. E. Harman, eds. *Trichoderma* and *Gliocladium*, Vol. Volume 1. Taylor & Francis, London.

Landeweert, R., E. Hoffland, R.D. Finlay, T.W. Kuyper, and N. Van Breemen. 2001. Linking plants to rocks: ectomycorrhizal fungi mobilize from minerals. Trends in Ecology & Evolution 16:248-254.

Leake, J., D. Johnson, D. Donnelly, G. Muckle, L. Boddy, and D.J. Read. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Canadian Journal of Botany 82:1016-1045.

Leandro, L.F.S., T. Guzman, L.M. Ferguson, and F.G. Louws. 2007. Population dynamics of *Trichoderma* in fumigated and compost - amended soil and on strawberry roots. Applied Soil Ecology 35:237 - 246.

Linderman, R.G. 1988. Mycorrhizal interactions with the rhizosphere microflora: The Mycorrhizosphere Effect. Phytopathology 78:366-371.

Maclaren, J.P. 1993. Radiata pine growers' manual New Zealand Forest Research Institute Ltd, Rotorua.

MAF. 2004. The forestry sector in New Zealand [Online] http://www.maf.govt.nz/forestry/.

Malyshkin, P.E. 1951. Influence of soil microorganisms on the growth of oak [Vliyanie pochvennykh mikroorganizmov na rost duba]. Pochvovedenie 5:280-288.

Malyshkin, P.E. 1955. Stimulation of tree growth by microorganisms, p. 211 - 220, *In* A. A. Imshenetskii, ed. Mycotrophy of plants, Israel.

Marks, G.C., and R.C. Foster. 1973. Structure, Morphogensis, and Ultrastructure of Ectomycorrhizae, p. 1-41, *In* G. C. Marks and T. T. Kozlowski, eds. Ectomycorrhizae their ecology and physiology. Academic Press, London.

Marx, D.H., and W.C. Bryan. 1975. Growth and ectomycorrhizal development of Loblolly Pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. Forest Science 21:245-254.

Marx, D.H., and S.V. Krupa. 1978. Ectomycorrhiza, p. 373-400, *In* Y. R. Dommergues and S. V. Krupa, eds. Inteactions between non-pathogenic soil microorganisms and plants. Elsevier Scientific Publishing Company, New York.

Massicotte, H.B., R. Molina, D.L. Luoma, and J.E. Smith. 1994. Biology of the ectomycorrhizal genus, *Rhizopogon.* II. Patterns of host-fungus specificity following spore inoculation of diverse hosts grown in monoculture and dual culture. New Phytologist 126:677 - 690.

McLean, K.L. 2001. Biological control of onion white rot using *Trichoderma harzianum*. PhD, Lincoln University, Christchurch.

McLean, K.L., J. Swaminathan, C.M. Frampton, J.S. Hunt, H.J. Ridgway, and A. Stewart. 2005. Effect of formulation on the rhizosphere competence and biocontrol ability of *Trichoderma atroviride* C52. Plant Pathology 54:212-218.

Menzies, M.I., D.G. Holden, and B.K. Klomp. 2001. Recent trends in nursery practice in New Zealand. New Forests 22:3-17.

Merriman, P., and K. Russell. 1990. Screening strategies for biological control, p. 427 - 435, *In* D. Hornby, ed. Biological control of soil - borne plant pathogens. C.A.B International, Wallingford.

Meyer, F.H. 1973. Distribution of ectomycorrhizae in native and man-made forests, p. 79 - 105, *In* G. C. Marks and T. T. Kozlowski, eds. Ectomycorrhizae. Their ecology and physiology. Academic Press, London.

Miyazaki, K., Y. Tsuchiya, and T. Okuda. 2009. Specific PCR assays for the detection of *Trichoderma harzianum* causing green mold disease during mushroom cultivation. Mycoscience 50:94-99.

Molina, R., and J.M. Trappe. 1994. Biology of the ectomycorrhizal genus, *Rhizopogon*. I. Host associations, host-specificity and pure culture syntheses. New Phytologist 126:653-675.

Morgan, J.A.W., G.D. Bending, and P.J. White. 2005. Biological costs and benefits to plant-microbe interactions in the rhizosphere. Journal of Experimental Botany 56:1729-1739.

Mucha, J., H. Dahm, and A. Werner. 2007. Influence of autoclaved saprotrophic fungal mycelia on proteolytic activity in ectomycorrhizal fungi. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 92:137-142.

Mucha, J., M. Zadworny, A. Werner, A. Napierala-Filipiak, and P. Lakomy. 2008. Antagonistic activity of the ectomycorrhizal fungus *Suillus bovinus* challenged by saprotrophic fungi from different soils. Nova Hedwigia 87:373-385.

Nelson, W. 1996. Container types and containerised stock for New Zealand afforestation. New Zealand Journal of Forestry Science 26:184-190.

Nicolotti, G., and G.C. Varese. 1996. Screening of antagonistic fungi against air-borne infection by *Heterobasidion annosum* on Norway spruce. Forest Ecology and Management 88:249-257.

Niermi, K., A. Lilja, A. Kasurinen, and H. Heinonen-Tanski. 2000. Ectomycorrhizal fungi as biological control agents against Rhizoctonia root infection of container-grown Scots pine seedlings. Proceedings of the 4th Meeting of Iufro Working Party 7.03.04 - Disease and Insects in Forest Nurseries 781:151-162.

Orlovich, D.A., and J.W.G. Cairney. 2004. Ectomycorrhizal fungi in New Zealand: current perspectives and future directions. New Zealand Journal of Botany 42:721-738.

Pawuk, W.H., J.L. Ruehle, and D.H. Marx. 1980. Fungicide drenches affect ectomycorrhizal development of container-grown *Pinus palustris* seedlings. Canadian Journal of Forest Research 10.

Perry, D.A., R. Molina, and M.P. Amaranthus. 1987. Mycorrhizae, mycorrhizospheres, and reforestation: current knowledge and research needs. Canadian Journal of Forest Research 17:929-940.

Rambelli, A. 1973. The rhizosphere of mycorrhizae, p. 299 - 349, *In* G. C. Marks and T. T. Kozlowski, eds. Ectomycorrhizae. Their ecology and physiology. Academic Press, London.

Read, D.J., J.R. Leake, and J. Perez-Moreno. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Canadian journal of Botony 82:1243-1263.

Rincon, A., I.F. Alvarez, and J. Pera. 1999. Ectomycorrhizal fungi of *Pinus pinea* L. in northeastern Spain. Mycorrhiza 8:271-276.

Rosling, A., R. Landeweert, B.D. Lindahl, K.H. Larsson, T.W. Kuyper, A.F.S. Taylor, and R.D. Finlay. 2003. Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. New Phytologist 159:775-783.

Rossi, M.J., A. Furigo, and V.L. Oliveira. 2007. Inoculant production of ectomycorrhizal fungi by solid and submerged Fermentations. Food Technology and Biotechnology 45:277-286.

Schirmbock, M., M. Lorito, Y.L. Wang, C.K. Hayes, I. Arisan-Atac, F. Scala, G.E. Harman, and C.P. Kubicek. 1994. Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Applied and Environmental Microbiology 60:4364-4370.

Shemakhanova, N.M. 1962. Mycotrophy of woody plants Translated by US Dept. of Agriculture and the National Science Foundation, Washington DC. (orig. *Mikotrofiya drevesnykh porod. Institut Mikrobiologii, Akademiya Nauk SSSR, Moscow*).

Sivasithamparam, K., and E.L. Ghisalberti. 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*, p. 139-191, *In* C. P. Kubicek and G. E. Harman, eds. Trichoderma & Gliocladium, Vol. vol 1. Taylor & Francis, London.

Slankis, V. 1974. Soil factors influencing formation of mycorrhiza. Annual Review of Phytopathology 12:437-457.

Smith, S.E., and D.J. Read. 1997. Mycorrhizal symbiosis. Second edition ed. Academic Press, London.

Summerbell, R.C. 1987. The Inhibitory Effect of Trichoderma Species and Other Soil Microfungi on Formation of Mycorrhiza by *Laccaria bicolor* In vitro. New Phytologist 105:437-448.

Summerbell, R.C. 2005. From Lamarckian fertilizers to fungal castles: recapturing the pre-1985 literature on endophytic and saprotropic fungi associated with ectomycorrhizal root systems. Studies in Mycology 53:191-256.

Tammi, H., S. Timonen, and R. Sen. 2001. Spatiotemporal colonization of Scots pine roots by introduced and indigenous ectomycorrhizal fungi in forest humus and nursery

Sphagnum peat microcosms. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere 31:746-756.

Taylor, A.F.S., and I. Alexander. 2005. The ectomycorrhizal symbiosis: Life in the real world. Mycologist 19:102-112.

Taylor, D.L., and T.D. Bruns. 1999. Communtiy structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant porpagule communities. Molecular Ecology 8:1837 - 1850.

Theodorou, C. 1980. The sequence of mycorrhizal infection of *Pinus radiata* D. Don following inoculation with *Rhizopogon luteolus* Fr. and Nordh. Australian Forest Research 10:381 - 387.

Theodorou, C., and G.D. Bowen. 1971. Influence of temperature on the mycorrhizal associations of *Pinus radiata* D. Don. Australian Journal of Botany 19:13-20.

Theodorou, C., and G.D. Bowen. 1973. Inoculation of seeds and soil with basidiospores of mycorrhizal fungi. Soil Biology and Biochemistry 5:765-771.

Theodorou, C., and A.D. Benson. 1983. Operational mycorrhizal inoculation of nursery beds with seed-borne fungal spores. Australian Forestry 46:43-47.

Theodorou, C., and G.D. Bowen. 1987. Germination of basidiodpores of mycorrhzial fungi in the rhizosphere of *Pinus radiata* D Don. New Phytologist 106:217-223.

Timonen, S., and R. Sen. 1998. Heterogeneity of fungal and plant enzyme expression in intact Scots pine *Suillus bovinus* and *Paxillus involutus* mycorrhizospheres developed in natural forest humus. New Phytologist 138:355-366.

Timonen, S., K.S. Jorgensen, K. Haahtela, and R. Sen. 1998. Bacterial community structure at defined locations of *Pinus sylvestris* - *Suillus bovinus* and *Pinus sylvestris* - *Paxillus involutus* mycorrhizospheres in dry pine forest humus and nursery peat. Canadian journal of Microbiology 44:499-513.

Trappe, J.M. 1977. Selection of fungi for ectomycorrhizal inoculation in nurseries. Annual Review of Phytopathology 15:203-222.

Vasiliauskas, R., A. Menkis, R.D. Finlay, and J. Stenlid. 2007. Wood-decay fungi in fine living roots of conifer seedlings. New Phytologist 174:441 - 446.

Vinceti, B., E. Paoletti, and U. Wolf. 1998. Analysis of soil, roots and mycorrhizae in a Norway Spruce declining forest. Chemosphere 36:937 - 942.

Walbert, K. 2008. Ectomycorrhizal communities associated with a *Pinus radiata* plantation in the North Island, New Zealand. PhD, Lincoln University, Christchurch.

Werner, A., M. Zadworny, and K. Idzikowska. 2002. Interaction between *Laccaria laccata* and *Trichoderma virens* in co-culture and in the rhizosphere of *Pinus sylvestris* grown in vitro. Mycorrhiza 12:139-145.

Whipps, J.M. 1987. Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. New Phytologist 107:127 - 142.

Whipps, J.M. 1997. Developments in the biological control of soil-borne plant pathogens. Advances in Botanical Research 26:1 - 134.

Whipps, J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany 52:487-511.

Whipps, J.M. 2004. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Canadian journal of Botony 82:1198-1227.

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322, *In* M. A. Innis, et al., eds. PCR protocols: A guide to methods and applications. Academic Press, London.

Zadworny, M., A. Werner, and K. Idzikowska. 2004. Behaviour of the hyphae of *Laccaria laccata* in the presence of *Trichoderma harzianum* in vitro. Mycorrhiza 14:401-405.

Zadworny, M., D.J. Smolinski, K. Idzikowska, and A. Werner. 2007. Ultrastructural and cytochemical aspects of the interaction between the ectomycorrhizal fungus *Laccaria laccata* and the saprotrophic fungi, *Trichoderma harzianum* and *T. virens*. Biocontrol Science and Technology 17:921-932.

Zadworny, M., Z. Gorski, E. Koczorowska, and A. Werner. 2008. Conidia of *Trichoderma virens* as a phosphorus source for mycorrhizal *Pinus sylvestris* seedlings. Mycorrhiza 19:61-66.

Zheng, L., and X. Wu. 2008. Ectomycorrhizal morphology and root hair growth and their relationships with the growth of *Pinus thunbergii* seedlings. Journal of Nanjing Forestry University (Natural Sciences Edition) 32:107-111.

Personal communication references

Bennenbroek, M. PF Olsen Ltd Nursery

Hill, R. Bio-Protection Research Centre, Lincoln University

Appendix A

A.1 Seedline parameters

Seedline description/ No.	Seedling GF plus ratings					
	Growth	Straightness	Branching	Dothistroma resistance	Wood density	Spiral grain
A (268.323 x 875.066)	21.2	23.5	23.6	17.6	21.1	21.4
B (268.539 x 875.242)	26.0	21.1	23.4	20.3	16.2	18.2

A.2 Agar recipes

A.2.1 Trichoderma selective agar (TSM)

To make 1 L:

20.0 g agar
3.0 g glucose
1.0 g ammonium nitrate
0.9 g dipotassium hydrogen orthophosphate trihydrate ($K_2HPO_4.3H_2O$)
0.2 g magnesium sulphate 7 hydrate ($MgSO_4.7H_2O$)
0.15 g potassium chloride (KCl)
0.2 g Terrachlor 75WP fungicide* (quintozene 750 g/kg a.i.)
0.15 g Rose Bengal

1 mL chloramphenicol stock solution:
- 250 mg of chloramphenicol in 100 mL of absolute (96%) ethanol.

1 mL salt stock solution:
1.0 g iron sulphate (Ferrous sulphate) 7 hydrate ($FeSO_4.7H_2O$)
0.65 g manganous sulphate tetrahydrate ($MnSO_4.4H_2O$)
0.9 g zinc sulphate ($ZnSO_4.7H_2O$)
→ preparation: dissolve all three ingredients in 1 L distilled water

Make up to 1 L and autoclave at 121 °C for 15 minutes.

A.2.2 Modified Melin Norkrans medium (MMN)

(Marx, D. H. (1969), *Phytopathology* 59 153 – 163.)

Ingedients:	Full strength:	¼ strength (for experiments):
$CaCl_2\ 2H_2O$	0.05 g (1 mL stock)	0.05 g (0.25 mL stock)
NaCl	0.025 g (1 mL stock)	0.025 g (0.25 mL stock)
$(NH_4)_2HPO_4$	0.25 g (1 mL stock)	0.25 g (0.25 mL stock)
$MgSO_4.7H_2O$	0.15 g (1 mL stock)	0.15 g (0.25 mL stock)
KH_2PO_4	0.50 g (8 mL stock)	0.50 g (2.0 mL stock)
FeNaEDTA (2% soln)	1.2 mL	0.30 mL
Thiamine HCL (1% soln)	1.0 mL	0.25 mL
Glucose	2.5 g	0.625 g
Malt extract	10 g	Omitted as it is undefined
Agar	15 g	15 g
Distilled water	1 L	1 L

Agar suspension adjusted to pH 4.7 using 1 M HCl

Stocks (full strength):

$CaCl_2\ 2H_2O$ (0.05 g L^{-1} agar)
5 g in 100 mL distilled water (add 1 mL L^{-1} agar)

NaCl (0.025 g L^{-1} agar)
2.5 g in 100 mL distilled water (add 1 mL L^{-1} agar)

$(NH_4)_2HPO_4$ (0.25 g L^{-1} agar)
25 g in 100 mL distilled water (add 1 mL L^{-1} agar)

$MgSO_4.7H_2O$ (0.15 g L^{-1} agar)
15 g in 100 mL distilled water (add 1 mL L^{-1} agar)

KH_2PO_4 (0.5 g L^{-1} agar)
12.5 g in 200 mL distilled water (add 8 mL L^{-1} agar)

FeNaEDTA (2% solution)
2 g in 100 mL distilled water (add 1.2 mL L^{-1} agar)

Thiamine HCl (1% v/v solution)
1 g in 100 mL distilled water (add 1 mL L^{-1} agar)

Appendix B

B.1 Chapter 2 ANOVA tables

B.1.1 Analysis of variance (ANOVA) table for the overall total ECM root tips/ cm root length enumerated at the PF Olsen nursery (Figure 2.2)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Total ECM root tips	Block stratum	4	11.344	2.836	1.21	
	Treatment	5	13.824	2.765	1.18	0.353
	Residual	20	46.811	2.341		
	Total	29	71.979			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.1.2 Analysis of variance (ANOVA) table for the total number of ECM root tips/ cm root length categorised into their respective morphotypes (Figure 2.4)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ECM root tips/ cm root length split into morphotypes	Block stratum	4	3.781	0.945	0.42	
	Treatment	5	4.608	0.922	0.41	0.839
	ECM	2	174.925	87.463	39.08	<.001
	Treatment.ECM	10	8.127	0.813	0.36	0.958
	Residual	68	152.206	2.238		
	Total	89	343.648			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.1.3 Analysis of variance (ANOVA) table for the total non-mycorrhizal root tips enumerated (Figure 2.5)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Non-mycorrhizal root tips	Block stratum	4	0.002556	0.000639	2.06	
	Treatment	5	0.003776	0.000755	2.43	0.071
	Residual	20	0.006209	0.000311		
	Total	29	0.012541			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.1.4 Analysis of variance (ANOVA) table for the *Trichoderma* cfu populations found 5 weeks after sowing and at harvest for figures 2.6 A & B, respectively.

Analysis	Assessment	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Log_{10} *Trichoderma* cfu	Seed sowing	Treatment	3	1.9786	0.6595	1.83	0.146
		Residual	104	37.4578	0.3602		
		Total	107	39.4365			
	Harvest	Treatment	5	0.45262	0.09052	3.9	0.002
		Residual	156	3.62468	0.02324		
		Total	161	4.0773			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.1.5 Analysis of variance (ANOVA) table for each of the tree seedling parameters measured (Table 2.4)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Seedling emergence	Block stratum	4	91.6	22.9	0.66	
	treatment	3	278.52	92.84	2.69	0.053
	Residual	72	2488.15	34.56		
	Total	79	2858.27			
Seedling height	Block stratum	4	986.22	246.55	7.1	
	Treatment	5	692.52	138.5	3.99	0.002
	Residual	342	11881.27	34.74		
	Total	351	13551.59			
Seedling diameter	Block stratum	4	10.6826	2.6707	3.47	
	Treatment	5	2.7937	0.5587	0.73	0.605
	Residual	342	263.5625	0.7707		
	Total	351	276.8977			
Seedling height : diameter ratio	Block stratum	4	34.761	8.69	4.36	
	Treatment	5	74.841	14.968	7.52	<.001
	Residual	342	680.961	1.991		
	Total	351	789.861			
Seedling above ground D. wt	Block stratum	4	45.437	11.359	3.52	
	Treatment	5	20.408	4.082	1.26	0.28
	Residual	230	742.74	3.229		
	Total	239	808.585			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.2 Chapter 3 ANOVA tables

B.2.1 Analysis of variance (ANOVA) table for the total ECM root tips/ cm root length enumerated in the Lincoln experiment (Figure 3.1)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Total ECM root tips	Block stratum	3	2.818	0.939	0.71	
	Treatment	3	2.457	0.819	0.62	0.609
	Residual	25	33.071	1.323		
	Total	31	38.345			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.2.2 Analysis of variance (ANOVA) table for the total ECM root tips as split by the dominant species enumerated (Figure 3.3)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Total ECM root tips split by ECM species	Block stratum	3	1.4089	0.4696	2.15	
	Time	1	0.651	0.651	2.98	0.183
	Residual	3	0.6545	0.2182	0.27	
	Treatment	3	1.2284	0.4095	0.51	0.683
	Time.Treatment	3	0.6864	0.2288	0.28	0.837
	Residual	18	14.5436	0.808	1.65	
	ECM	1	94.8234	94.8234	193.26	<.001
	Time.ECM	1	0.3553	0.3553	0.72	0.403
	Treatment.ECM	3	2.6054	0.8685	1.77	0.18
	Time.Treatment.ECM	3	1.3109	0.437	0.89	0.46
	Residual	24	11.7758	0.4907		
	Total	63	130.0435			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.2.3 Analysis of variance (ANOVA) table for the relative proportions of morphotypes found, expressed as total ECM root tips/ cm root length (Figure 3.4), and the change in total ECM root tips/ cm root length from the first assessment to the second assessment, also split into each respective morphotype (Figure 3.5).

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
ECM root tips split by ECM morphotypes and time	Block stratum	3	0.7045	0.2348	2.15	
	Time	1	0.3255	0.3255	2.98	0.183
	Residual	3	0.3272	0.1091	0.27	
	Treatment	3	0.6142	0.2047	0.51	0.683
	Time.Treatment	3	0.3432	0.1144	0.28	0.837
	Residual	18	7.2718	0.404	1.13	
	ECM	3	45.9648	15.3216	42.82	<.001
	Time.ECM	3	10.7783	3.5928	10.04	<.001
	Treatment.ECM	9	4.4821	0.498	1.39	0.208
	Time.Treatment.ECM	9	3.4661	0.3851	1.08	0.391
	Residual	72	25.7625	0.3578		
	Total	127	100.0402			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.2.4 Analysis of variance (ANOVA) table for the total non-mycorrhizal root tips (Figure 3.6)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Non-mycorrhizal root tips	Block stratum	3	5.43E-05	1.81E-05	0.12	
	Time	1	3.61E-05	3.61E-05	0.23	0.662
	Residual	3	0.000465	0.000155	1.18	
	Treatment	3	0.000104	3.46E-05	0.26	0.851
	Time.Treatment	3	0.000355	0.000119	0.9	0.46
	Residual	18	0.002367	0.000132		
	Total	31	0.003382			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.2.5 Analysis of variance (ANOVA) table for the total *Trichoderma* cfu population enumerated at harvest (Figure 3.7)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Log_{10} *Trichoderma* cfu at harvest	Block stratum	3	15.469	5.156	4.24	
	Treatment	3	383.728	127.909	105.18	<.001
	Residual	137	166.61	1.216		
	Total	143	565.808			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.2.6 Analysis of variance (ANOVA) table for each of the seedling parameters measured (Table 3.4)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Seedling height	Block stratum	3	5.688	1.896	0.72	
	Treatment	3	3.812	1.271	0.49	0.696
	Residual	25	65.5	2.62		
	Total	31	75			
Seedling diameter	Block stratum	3	1.22594	0.40865	6.26	
	Treatment	3	0.25844	0.08615	1.32	0.29
	Residual	25	1.63281	0.06531		
	Total	31	3.11719			
Height : diameter	Block stratum	3	489.26	163.09	3.47	
	Treatment	3	25.71	8.57	0.18	0.908
	Residual	25	1176.33	47.05		
	Total	31	1691.3			
Shoot dry weight	Block stratum	3	0.05041	0.0168	0.91	
	Treatment	3	0.03613	0.01204	0.65	0.59
	Residual	25	0.46238	0.0185		
	Total	31	0.54892			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

B.3 Chapter 4 ANOVA tables

B.3.1 Analysis of variance (ANOVA) table for the radial growth rates of each of the respective fungal species in the *in vitro* lab assays (Table 4.3)

Analysis	Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
R. roseolus	Trich_spp	6	0.203	0.034	0.63	0.701
	Residual	21	1.117	0.053		
	Total	27	1.32			
S. luteus	Trich_spp	6	0.085316	0.014219	1.81	0.145
	Residual	21	0.164731	0.007844		
	Total	27	0.250047			
R. villosulus	Trich_spp	6	0.087	0.015	0.44	0.844
	Residual	21	0.694	0.033		
	Total	27	0.782			
H. fasciculare	Trich_spp	6	0.375	0.062	3.82	0.01
	Residual	21	0.343	0.016		
	Total	27	0.718			

NB: d.f = degrees of freedom; s.s =sums of squares; m.s = mean square; v.r = variance ratio; F pr. = F probability

Appendix C

C.1 Table 2.3; Internal transcribed spacer (ITS) region sequences

C.1.1 *Thelephora terrestris*; unramified, sequence 1 (640 bp)

```
1       CTACCTGATT TGAGATCGAA CGTTAAAAAA GCTGTCCTCG CTGAGGAGAG
51      ACATCTGTGA GCTCCAGCAA ACCTTTGTGA CCAAAGGTTA CCTGGCAGAC
101     AACAGCGAGC GTAGATATTT ATCACACCCG TGATGCCACC AAACACTGGG
151     AGGCTGATTA ATTTGAGAGG AGCCGACCAC AGGCCAGCAA AACCCCCAGA
201     GTCCAACTCA TCATGGCAAA CCATGAGAGT TGAGGTGTTC ATGATACTCA
251     AACAGGCATG CCCCTCGGAA TAGCCAAGGG GCGCAAGGTG CGTTCAAAGA
301     TTCGATGATT CACTGAATTC TGCAATTCAC ATTACTTATC GCATTTCGCT
351     GCGTTCTTCA TCGATGCGAG AGCCAAGAGA TCCGTTGCTG AAAGTTGTAT
401     TGTATTGCGT TAGACGCGAT GTACATTCCA TAAAACTTTA TTACAGTGTG
451     TGTGTAAAGA CGTAGAACCA CAGAAGGAAG ACAGGGTCCC CCAGACCATA
501     GAACTACAGA GGGTGCACAG GTGTGAGTGG ATGTGTAAAC AGAGCGTGCA
551     CATGCCCCCT ATGAGGGCCA GCAACAACCC GTTTGACAAT TCAGTAATGA
601     TCCTTCCGCA GGTTCACCTA CGGAAACCTT GTTACGACTT
```

C.1.2 *Thelephora terrestris*; unramified, sequence 2 (635 bp)

```
1       AGTCCTACCT GATTTGAGAT CGAACATTAA AAAAGCTGTC CTCGCTGAGG
51      AGAGACATCT GTGAGCTCCA GCAAACCTTT GTGACCAAAG GTTACCTGGC
101     AGACAACAGC GAGCGTAGAT ATTTATCACA CCCGTGATGC CACCAAACAC
151     TGGGAGGCTG ATTAATTTAA GAGGAGCCGA CCACAGGCCA GCAAAACCCC
201     CAGAGTCCAA CTCATCATGG CAAACCATGA GAGTTGAGGT GTTCATGATA
251     CTCAAACAGG CATGCCCCTC GGAATAGCCA AGGGGCGCAA GGTGCGTTCA
301     AAGATTCGAT GATTCACTGA ATTCTGCAAT TCACATTACT TATCGCATTT
351     CGCTGCGTTC TTCATCGATG CGAGAGCCAA GAGATCCGTT GCTGAAAGTT
401     GTATTGTATT GCGTTAGACG CGATGTACAT TCCATAAAAC TTTATTACAG
451     TGTGTGTGTA AAGACGTAGA ACCACAGAGG GAAGACAGGG TCCCCCAGAC
501     CATAGAACTA CAGAGGGTGC ACAGGTGTGA GTGGATGTGT AAACAGAGCG
551     TGCACATGCC CCCTATGAGG GCCAGCAACA ACCCGTTTGA CAATTCAGTA
601     ATGATCCTTC CGCAGGAGTC ACCTACGGAA ACCTT
```

C.1.3 *Thelephora terrestris*; dichotomous, sequence 1 (619 bp)

```
1       ACGTTAAAAA GCTGTCCTCG CTGAGGAGAG ACATCTGTGA GCTCCAGCAA
51      ACCTTTGTGA CCAAAGGTTA CCTGGCAGAC AACAGCGAGC GTAGATATTT
101     ATCACACCCG TGATGCCACC AAACACTGGG AGGCTGATTA ATTTGAGAGG
151     AGCCGACCAC AGGCCAGCAA AACCCCCAGA GTCCAACTCA TCATGGCAAA
201     CCATGAGAGT TGAGGTGTTC ATGATACTCA AACAGGCATG CCCCTCGGAA
251     TAGCCAAGGG GCGCAAGGTG CGTTCAAAGA TTCGATGATT CACTGAATTC
301     TGCAATTCAC ATTACTTATC GCATTTCGCT GCGTTCTTCA TCGATGCGAG
351     AGCCAAGAGA TCCGTTGCTG AAAGTTGTAT TGTATTGCGT TAGACGCGAT
401     GTACATTCCA TAAAACTTTA TTACAGTGTG TGTGTAAAGA CGTAGAACCA
451     CAGAAGGAAG ACAGGGTCCC CCAGACCATA GAACTACAGA GGGTGCACAG
501     GTGTGAGTGG ATGTGTAAAC AGAGCGTGCA CATGCCCCCT ATGAGGGCCA
551     GCAACAACCC GTTTGACAAT TCAGTAATGA TCCTTCCGCA GGTTCACCTA
601     CGGAAACCTT GTTACGACT
```

C.1.4 *Thelephora terrestris*; dichotomous, sequence 2 (615 bp)

```
1       AAAAAGCTGT CCTCGCTGAG GAGAGACATC TGTGAGCTCC AGCAAACCTT
51      TGTGACCAAA GGTTACCTGG CAGACAACAG CGAGCGTAGA TATTTATCAC
101     ACCCGTGATG CCACCAAACA CTGGGAGGCT GATTAATTTG AGAGGAGCCG
151     ACCACAGGCC AGCAAAACCC CCAGAGTCCA ACTCATCATG GCAAACCATG
201     AGAGTTGAGG TGTTCATGAT ACTCAAACAG GCATGCCCCT CGGAATAGCC
251     AAGGGGCGCA AGGTGCGTTC AAAGATTCGA TGATTCACTG AATTCTGCAA
301     TTCACATTAC TTATCGCATT TCGCTGCGTT CTTCATCGAT GCGAGAGCCA
351     AGAGATCCGT TGCTGAAAGT TGTATTGTAT TGCGTTAGAC GCGATGTACA
401     TTCCATAAAA CTTTATTACA GTGTGTGTGT AAAGACGTAG AACCACAGAA
451     GGAAGACAGG GTCCCCCAGA CCATAGAACT ACAGAGGGTG CACAGGTGTG
501     AGTGGATGTG TAAACAGAGC GTGCACATGC CCCCTATGAG GGCCAGCAAC
551     AACCCGTTTG ACAATTCAGT AATGATCCTT CCGCAGGTTC ACCTACGGAA
601     ACCTTGTTAC GACTT
```

C.1.5 *Thelephora terrestris*; multi-dichotomous, sequence 1 (638 bp)

```
1       CCTGATTTGA GATCGAACGT TAAAAATGCT GTCCTCGCTG AGGAGAGACA
51      TCTGTGAGCT CCAGCAAACC TTTGTGACCA AAGGTTACCT GGCAGACAAC
101     AGCGAGCGTA GATATTTATC ACACCCGTGA TGCCACCAAA CACTGGGAGG
151     CTGATTAATT TGAGAGGAGC CGACCACAGG CCAGCAAAAC CCCCAGAGTC
201     CAACTCATCA TGGCAAACCA TGAGAGTTGA GGTGTTCATG ATACTCAAAC
251     AGGCATGCCC CTCGGAATAG CCAAGGGGCG CAAGGTGCGT TCAAAGATTC
301     GATGATTCAC TGAATTCTGC AATTCACATT ACTTATCGCA TTTCGCTGCG
351     TTCTTCATCG ATGCGAGAGC CAAGAGATCC GTTGCTGAAA GTTGTATTGT
401     ATTGCGTTAG ACGCGATGTA CATTCCATAA AACTTTATTA CAGTGTGTGT
451     GTAAAGACGT AGAACCACAG AAGGAAGACA GGGTCCCCCA GACCATAGAA
501     CTACAGAGGG TGCACAGGTG TGAGTGGATG TGTAAACAGA GCGTGCACAT
551     GCCCCCTATG AGGGCCAGCA ACAACCCGTT TGACAATTCA GTAATGATCC
601     TTCCGCAGGT TCACCTACGG AAACCTTGTT ACGACTTT
```

C.1.6 *Thelephora terrestris*; multi-dichotomous, sequence 2 (643 bp)

```
1       GTCCTACCTG ATTTGAGATC GAACGTTAAA AAAGCTGTCC TCGCTGAGGA
51      GAGACATCTG TGAGCTCCAG CAAACCTTTG TGACCAAAGG TTACCTGGCA
101     GACAACAGCG AGCGTAGATA TTTATCACAC CCGTGATGCC ACCAAACACT
151     GGGAGGCTGA TTAATTTGAG AGGAGCCGAC CACAGGCCAG CAAAACCCCC
201     AGAGTCCAAC TCATCATGGC AAACCATGAG AGTTGAGGTG TTCATGATAC
251     TCAAACAGGC ATGCCCCTCG GAATAGCCAA GGGGCGCAAG GTGCGTTCAA
301     AGATTCGATG ATTCACTGAA TTCTGCAATT CACATTACTT ATCGCATTTC
351     GCTGCGTTCT TCATCGATGC GAGAGCCAAG AGATCCGTTG CTGAAAGTTG
401     TATTGTATTG CGTTAGACGC GATGTACATT CCATAAAACT TTATTACAGT
451     GTGTGTGTAA AGACGTAGAA CCACAGAAGG AAGACAGGGT CCCCCAGACC
501     ATAGAACTAC AGAGGGTGCA CAGGTGTGAG TGGATGTGTA AACAGAGCGT
551     GCACATGCCC CCTATGAGGG CCAGCAACAA CCCGTTTGAC AATTCAGTAA
601     TGATCCTTCC GCAGGTTCAC CTACGGAAAC CTTGTTACGA CTT
```

C.2 Table 3.2; Internal transcribed spacer (ITS) region sequences

C.2.1 Thelephoraceae; unramified (643 bp)

```
1       TCCTACCTGA TTTGAGATCG AACGTTCAAA GTTGTCCTCG CCGAGGAGAG
51      ACGTTTATGA GCTCCAATGA ACCTTCATTG CTGAGGGTTA CCTGGCAGAC
101     GACCGCGAGC GTAGATAGTT ATCACACCCG TGATGCCACC AAACACTGGC
151     AAGCTGATTC ATTTGAGAGG AGCCGGCCGC AGGGCCAGCA AACCCCCAAA
201     GTCCAAGCTC ATCACGGAAG ACCGTGAGAG TTGAGGTGTT CATGATACTC
251     AAACAGGCAT GCTCCAAGGA ATAACCAAGG GGCGCAAGGT GCGTTCAAAG
301     ATTCGATGAT TCACTGAATT CTGCAATTCA CATTACTTAT CGCATTTCGC
351     TGCGTTCTTC ATCGATGCGA GAGCCAAGAG ATCCGTTGCT GAAAGTTGTA
401     TTGTATCGCG TTAAACGCAT AAAAACATTC CATGAGACAT CGCTACGGCG
451     TGTGTGTAAA GACGTAGAGC TACAGAAGGA AGACGGGGTC TTCCGAACCA
501     TAGGACTACA GAGGGTGCAC AGGTGTGAGT GGATGCGTAA ACAGAGCGTG
551     CACATGCCCC GTTCGGGAGG CCAGCAACAA CCCGTGTTTG ACGATTCGGT
601     AATGATCCTT CCGCAGGTTC ACCTACGGAA ACCTTGTACG ACT
```

C.2.2 Thelephoraceae; dichotomous (644 bp)

```
1       CCTACCTGAT TTGAGATCGA ACGTTCAAAG TTGTCCTCGC CGAGGAGAGA
51      CGTTTATGAG CTCCAATGAA CCTTCATTGC TGAGGGTTAC CTGGCAGACG
101     ACCGCGAGCG TAGATAGTTA TCACACCCGT GATGCCACCA AACACTGGCA
151     AGCTGATTCA TTTGAGAGGA GCCGGCCGCA GGGCCAGCAA ACCCCCAAAG
201     TCCAAGCTCA TCACGGAAGA CCGTGAGAGT TGAGGTGTTC ATGATACTCA
251     AACAGGCATG CTCCAAGGAA TAACCAAGGG GCGCAAGGTG CGTTCAAAGA
301     TTCGATGATT CACTGAATTC TGCAATTCAC ATTACTTATC GCATTTCGCT
351     GCGTTCTTCA TCGATGCGAG AGCCAAGAGA TCCGTTGCTG AAAGTTGTAT
401     TGTATCGCGT TAAACGCATA AAAACATTCC ATGAGACATC GCTACGGCGT
451     GTGTGTAAAG ACGTAGAGCT ACAGAAGGAA GACGGGGTCT TCCGAACCAT
501     AGGACTACAG AGGGTGCACA GGTGTGAGTG GATGCGTAAA CAGAGCGTGC
551     ACATGCCCCG TTCGGGAGGC CAGCAACAAC CCGTGTTTGA CGATTCGGTA
601     ATGATCCTTC CGCAGGTTCA CCTACGGAAA CCTTGTTACG ACTT
```

C.2.3 Thelephoraceae; multi-dichotomous (644 bp)

```
1       CCTACCTGAT TTGAGATCGA ACGTTCAAAG TTGTCCTCGC CGAGGAGAGA
51      CGTTTATGAG CTCCAATGAA CCTTCATTGC TGAGGGTTAC CTGGCAGACG
101     ACCGCGAGCG TAGATAGTTA TCACACCCGT GATGCCACCA AACACTGGCA
151     AGCTGATTCA TTTGAGAGGA GCCGGCCGCA GGGCCAGCAA ACCCCCAAAG
201     TCCAAGCTCA TCACGGAAGA CCGTGAGAGT TGAGGTGTTC ATGATACTCA
251     AACAGGCATG CTCCAAGGAA TAACCAAGGG GCGCAAGGTG CGTTCAAAGA
301     TTCGATGATT CACTGAATTC TGCAATTCAC ATTACTTATC GCATTTCGCT
351     GCGTTCTTCA TCGATGCGAG AGCCAAGAGA TCCGTTGCTG AAAGTTGTAT
401     TGTATCGCGT TAAACGCATA AAAACATTCC ATGAGACATC GCTACGGCGT
451     GTGTGTAAAG ACGTAGAGCT ACAGAAGGAA GACGGGGTCT TCCGAACCAT
501     AGGACTACAG AGGGTGCACA GGTGTGAGTG GATGCGTAAA CAGAGCGTGC
551     ACATGCCCCG TTCGGGAGGC CAGCAACAAC CCGTGTTTGA CGATTCGGTA
601     ATGATCCTTC CGCAGGTTCA CCTACGGAAA CCTTGTTACG ACTT
```

C.2.4 *Suillus luteus*; multi-dichotomous (670 bp)

```
1       CCTACCTGAT TTGAGGTCAA CGTCAATGAG GAAGACGCCC CTAGACGGCG
51      TCGACGCATT AGAGGCACGG GACCATTCTG TCTTGCACTT CGGCGAACGG
101     CGATCATTAT CACGCCAAAG GCCTTGTCAT GCAAAGTCGA AAGTCGACCG
151     CGAGCCGATT CATTTAAGAG GAGCCCGAGT CCTGGACGAA TCCAGTGTCT
201     CCGGCAGCCC CCAACATCCA AGCACCCGCT CGAAGCAAAT CGAGAGGGGT
251     TGAGAATTTA CTGACACTCA AACAGGCATG CTCCTCGGAA CACCGAGGAG
301     CGCAAGGTGC GTTCAAAGAT TCGATGATTC ACTGTAGATC TGCAATTCAC
351     ATTACATATC GCGATTCGCT GCGTTCTTCA TCGATGCGAG AGCCAAGAGA
401     TCCGTTGCTG AAAGTTGTAA TAACTTTTTT CTCAAAGAAT CGCGTCTCCT
451     AGAAGTCGCG ACTCGATGAT GGTAAAACAT TCAAAGACTT TCTACACGAA
501     GAGGTATATG AAGACGCGGG TCGCCCCGCG CCCATACGGC GAAAGGTCCG
551     GAAGAGAGCG TGCACATGCC CCTGGAGGCC AGCTACAACT CTCCGCCTTT
601     CCCCTCGCCG GATTATAATT TCATTAATGA TCCTTCCGCA GGTTCACCTA
651     CGGAAACCTT GTTACGACTT
```

C.3 Table 3.3; Internal transcribed spacer (ITS) region sequences

C.3.1 *Rhizopogon roseolus*; (677 bp)

```
1       CCTACCTGAT TTGAGGTCAA AGTCAATAAG GAAGACCGTT AAGTCGACGC
51      ATTAGAGGCA CGGAACCTTC ATTCATGCGC TTCAGCGAAC GGCGATCATT
101     ATCACGCCGA AAGCCTTGTC GCGCAAAGTC GAAAGTCGAC CGCAAGCCGA
151     TGCATTTAAG GAGAGCCCGA GTCCAGGACG AGTCCTAGTC TCCGGCAGCC
201     CCCACCATCC AAGCTCCCCC TCGAAACAAA TCGAGAGGGG TTGAGAATTT
251     ACTGACACTC AAACAGGCAT GCTCCTCGGA ACACCGAGGA GCGCAAGGTG
301     CGTTCAAAGA TTCGATGATT CACTGTAGAT CTGCAATTCA CATTACATAT
351     CGCTTTTCGC TGCGTTCTTC ATCGATGCGA GAGCCAAGAG ATCCATTGCT
401     GAAAGTTGTA ATTAACTTTT ATCTCAAAAG ATTCGCGTCT CCTAGAAGTC
451     GCGACTCTCT GATAGTAAAC ATTCTAAGAC TTTCTACACG AAGAGGTATA
501     TGAAGACATA GGTCCCCCCT CCCGAAAGAG GAGCATCCTA CATTAGGTGC
551     ACGGGTGAGT TGTGAAAAAC AGAAGAGCGT GCACATGCGT CGTTTCCGAA
601     GCCAGCTACA ACCCCTCCGA ATTATATTCG TTAATGATCC TTCCGCAGGT
651     TCACCTACGG AAACCTTGTT ACGACTT
```

C.3.2 *Rhizopogon villosulus*; (675 bp)

```
1       CCTACCTGAT TTGAGGTCAA AGTCAATAAA GAAGACCTTT TCTCTCCTAA
51      GAGATAATAA GAGTCGACGT ATTAGAGGCG CGTAACCTTC ATTCATGCAC
101     TTCAGCGAAC GGCGATCATT ATCACGCCGA AAGCCTTGTC GCGCATAGTC
151     GAAAGTCGAC CGCAAGCCGA TGCATTTCAG GAGAGCCCGA GTCAAAAAGT
201     CTCCGGCAAA CCCCCACTAT CCAAACTCCC TCAATCAAGA GGGGTTGAGA
251     ATTTACTGAC ACTCAAACAG GCATGCTCCT CGGAACACCG AGGAGCGCAA
301     GGTGCGTTCA AAGATTCGAT GATTCACTGT AGATCTGCAA TTCACATTAC
351     ATATCGCTTT TCGCTGCGTT CTTCATCGAT GCGAGAGCCA AGAGATCCAT
401     TGCTGAAAGT TGTAATAACT TTTATCTCAC AGATTCGCGT CTCCTAGAAG
451     TCGCGACTCT ATGATAGTAA ACATTCTAAG ACTTTCTACA CGGAGATGTA
501     TATGAAGACA TAGGTCCCCT CTCCCTAAGG AAAGGCATCC TACATTAGGT
551     TCACAGGTGA GAAATTTATG AAACACGGTC GGCGTGCACA TGCCCGAAGG
601     CCAGCGACAG CTTTCCCGAT TTATATTCGT TAATGATCCT TCCGCAGGTT
651     CACCTACGGA AACCTTGTTA CGACT
```

C.3.3 *Lentinula edodes*; (734 bp)

```
1       TCCTACCTGA TTTGAGGTCA GCAAATAAGT TATATATAGT CAATCAAGAC
51      AGTTAGAAAG CAGAACTTCC CTTTTTCTCC AATGAATAGA ACAGATTGAG
101     CAAACTAAAT GCAACAACCC AAACCAATAG AGCTTTATTA TTGTAAGGTT
151     CCACCAAAAT GTAGATAATT ATCACACCAA GGTTAGAACT AACAAAACAG
201     GGTTCCCACT AATAAATTTA AGAGGAGCTG ACAAACGCCT GCAAGCCTCC
251     AACATCCAAG CTTTAATAAG TAAAAACTTA TAAAGTTGAG AATTTAATGA
301     CACTCAAACA GGCATGCCCT CCGGAATACC AGAGGGCGCA AGGTGCGTTC
351     AAAGATTCGA TGATTCACTG AATTCTGCAA TTCACATTAC TTATCGCATT
401     TCGCTGCGTT CTTCATCGAT GGGAGAGCCA AGAGATCCGT TGCTGAAAGT
451     TGTATTAAGT TTAAAGGGTC AATAAAGTCC CAATAACAAG ATCATTCTAT
501     AACATACTTC AATGGTTTAT AAGAACATAG AAGCCTTGTC AACTAGTCTT
551     TTCAAGTAAC TCATAATGAG CACCTTCAAA AACCCGATGA AAGAACTCCT
601     ACAAAAAGTG CACAGGTGGA TGAATAGAAA TCGGAGGAGG ATGTGCACAT
651     ACCCAAAGGC CAGCAACAAT CCACCACCAA AAAATTCAAT AATGATCCTT
701     CCGCAGTTCA CCTACGGAAA CCTTGTTACG ACTT
```

C.3.4 *Suillus luteus*; (669 bp)

```
1       CTACCTGATT TGAGGTCAAC GTCAATGAGG AAGACGCCCC TAGACGGCGT
51      CGACGCATTA GAGGCACGGG ACCATTCTGT CTTGCACTTC GGCGAACGGC
101     GATCATTATC ACGCCAAAGG CCTTGTCATG CAAAGTCGAA AGTCGACCGC
151     GAGCCGATTC ATTTAAGAGG AGCCCGAGTC CTGGACGAAT CCAGTGTCTC
201     CGGCAGCCCC CAACATCCAA GCACCCGCTC GAAGCAAATC GAGAGGGGTT
251     GAGAATTTAC TGACACTCAA ACAGGCATGC TCCTCGGAAC ACCGAGGAGC
301     GCAAGGTGCG TTCAAAGATT CGATGATTCA CTGTAGATCT GCAATTCACA
351     TTACATATCG CGATTCGCTG CGTTCTTCAT CGATGCGAGA GCCAAGAGAT
401     CCGTTGCTGA AAGTTGTAAT AACTTTTTTC TCAAAGAATC GCGTCTCCTA
451     GAAGTCGCGA CTCGATGATG GTAAAACATT CAAAGACTTT CTACACGAAG
501     AGGTATATGA AGACGCGGGT CGCCCCGCGC CCATACGGCG AAAGGTCCGG
551     AAGAGAGCGT GCACATGCCC CTGGAGGCCA GCTACAACTC TCCGCCTTTC
601     CCCTCGCCGG ATTATAATTT CATTAATGAT CCTTCCGCAG GTTCACCTAC
651     GGAAACCTTG TTACGACTT
```

C.3.5 *Hypholoma fasciculare*; (671 bp)

```
1       CTACCTGATT TGAGGTCAAT TGTCATATAT TGTCTGAATG AACAGACGAT
51      TATAAGCAGT GCTATAAACG GCAAGTAGCC CACGGCGTAG ATAATTATCA
101     CACCAATAGA CATGTTTGCA CAAGGCAACC AGCTAATGCA TTTCAGGGGA
151     GTTTATTTCA ATGAAGAAAC CAACATGCCC CCACTTCCAA TCCACTTACT
201     AACCAAAAAG TTAATAAAGG TTGAGAATTT AATGACACTC AAACAGGCAT
251     GCTCCTCGGA ATACCAAGGA GCGCAAGGTG CGTTCAAAGA TTCGATGATT
301     CACTGAATTC TGCAATTCAC ATTACTTATC GCATTTCGCT GCGTTCTTCA
351     TCGATGCGAG AGCCAAGAGA TCCGTTGCTG AAAGTTGTAT ATAGTTTATA
401     AGGCAATTAA GCCTAATAAT GACATTCTGT TACATTCGTA AGGTGTATAT
451     GAAAACATAG CCCTGGAAAC GAACAAGGAA AGCCTATTAA GCAACTCCTC
501     ACAACCGAGT TTCCTCGGAA AGTTGAATCC AGGTCTACAA AAGGTGCACA
551     GGTGGAGATA TAAAGATGAC CAGGTGTGCA CATGTCTCCG AAAAGACCAG
601     CATCAACCAA GCCAGATTTA TTCAATAATG ATCCTTCCGC AGGTTCACCT
651     ACGGAAACCT TGTTACGACT T
```

C.3.6 *Scleroderma bovista*; (634 bp)

```
1       CCTACCTGAT TTGAGGTCAG CTTCGATAAC ACGCGGCCGG ACGGACCGGG
51      CTCGCAGAGT TGGAGAGCGA CGGGCATCTA CGATCCACGC ACTTCCAGCC
101     CACGACGGTC ATTATGACGT CGAAGAGGCC GTGCCACGCG AGGCTCGCAC
151     CCAACGCTAA TGCTTTTGAG GAGAGCCGAC GTCCCCCCGA CGGGAGGTTC
201     GCCCGCAGAC TCCCATAAGT CCAAACCGAG CTCCGACGAG GTCGAAAGCT
251     TCGATCTGAT GTTTCGATGA CACTCAAACA GGCATGCTCC TCGGAATACC
301     AAGGAGCGCA AGGTGCGTTC AAAGATTCGA TGATTCACGG AAAATCTGCA
351     ATTCACATTA CTTATCGCGA TTCGCTGCGT CCTTCATCGA TGCGAGAGCC
401     AAGAGATCCA TTGCTGAAAG TTGTATTAGG TTTCCTGTGA CCGAGGTCAC
451     GGACGACATT CTGTAGACAT GCGAGTTCGA AGAAGACATA GGTCCCTAAG
501     GACCTACAGT GGGTGCACAC AGGTGTTAGA GGGCTGAAGC CTCGAAAGGG
551     TTCGGGAAGC CCTCCCCCTC CCAGAGGTTC GATCTCGATA ATGATCCTTC
601     CGCAGGTTCA CCTACGGAAA CCTTGTTACG ACTT
```