UNDERSTANDING *TRICHODERMA* BIO-INOCULANTS IN THE ROOT ECOSYSTEM OF *PINUS RADIATA* SEEDLINGS

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Für Dich, Mama

Abstract

Abstract of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

UNDERSTANDING *TRICHODERMA* BIO-INOCULANTS IN THE ROOT ECOSYSTEM OF *PINUS RADIATA* SEEDLINGS

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The overall aim of this study was to understand better the ecological behaviour of *Trichoderma* bio-inoculants in the root system of commercially grown *Pinus radiata* seedlings. Three *Trichoderma* isolates, representing three different species of the genus, were selected initially to investigate associations between rhizosphere competence and plant growth enhancement parameters. Improvements in various plant health and growth factors were confirmed for *T. hamatum* LU592 in a large-scale experiment under commercial conditions. Application of LU592 increased seedling shoot height and the number of seedlings meeting market specifications by up to 9.5% and 5%, respectively. Strong rhizosphere colonisation and the ability to penetrate the roots were also demonstrated. At the end of the experiment, the *Trichoderma* population was found to be up to 10 times higher compared to the untreated control. LU592 was recovered as the predominant isolate within a diverse indigenous *Trichoderma* population in the root system of 20-week old *P. radiata* seedlings. No stimulation of plant performance was detected for the other two isolates *T. atroviride* LU132 or *T. harzianum* LU686. Both isolates were early rhizosphere colonisers, with populations declining after 12 weeks.

Two different *Trichoderma* application methods were investigated. *Trichoderma* spores were introduced either as a seed coat or a spray application. The inoculum rates between seed coat and spray application differed by a factor of 10, however, no differences in the effect on plant health and growth were observed between application methods. The spray application promoted the establishment of the

isolates in the root zone with increased *Trichoderma* populations for LU132, LU686 and LU592 by 1.4, 2.1 and 3.4 times, respectively, compared with the seed coat.

Two marker genes, expressing the green fluorescent protein (GFP) and the hygromycin B phosphotransferase (hph), were successfully inserted into the genome of *T. hamatum* LU592. Subsequent physiological comparisons with the wild type combined with recovery and visualisation in non-sterile potting mix identified LU592/C as a suitable transformant for ecological studies. Using this marked strain, rhizosphere competence and root penetration ability were confirmed, but LU592/C was shown to perform poorly as a saprophyte. A moderate rather than a high inoculum concentration resulted in strongest establishment of LU592/C in the *P. radiata* root zone. Spatio-temporal population dynamics were directly related to root development. The quantification of total propagules did not always correlate with the biological activity of LU592/C. For instance, low levels of mycelia were detected in the bulk potting mix, despite reasonable total colony forming unit (cfu) concentrations of $>10^3$ cfu/g dry potting mix. By contrast, the proportion of the total cfu as mycelia was 44% around the emerging seedling radicle at a total cfu level of $<10^2$ cfu/g dry potting mix. The results suggest that assessments of root penetration and mycelia proportion should be used as additional indicators of fungal activity to supplement traditional enumeration techniques.

This research highlighted that plant performance assessments need to be associated with comprehensive ecological studies to successfully identify beneficial *Trichoderma*-based bio-inoculants. Implications of the outcomes of this study are discussed and future recommendations made. The research presented here has significantly increased the understanding of the ecological behaviour of *Trichoderma* bio-inoculants in the root ecosystem of *P. radiata* and will contribute to the body of literature on fungal ecology.

Keywords: *Trichoderma*, population dynamics, ecology, rhizosphere competence, root colonisation/penetration, *Pinus radiata*, tree nursery, biological activity, GFP, hph, *Agrobacterium*-mediated transformation, dilution plating, growth promotion, PGS, propagule differentiation.

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Leandro, obrigado por ser você. Eu não vou esquecer!

かわいい祐加、心から深く愛してるよ。

“Only two things are infinite, the universe and human stupidity, and I’m not sure about the former.” Albert Einstein

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Abbreviations and Symbols

ABC	adenosine triphosphate - binding cassette
AMF	arbuscular mycorrhizal fungi
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCA	biological control agent
BDT	Big Dye® Terminator
bp	base pair
BPRC	Bio-Protection Research Centre
cfu	colony forming unit
cm	centimetre
conc.	concentration
d	day
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
DWA	distilled water agar
dpi	days post-inoculation
ECM	ectomycorrhizae
EDTA	ethylenediamine tetraacetic acid
EMF	ectomycorrhizal fungi
F pr	F probability
FSC	Forest Stewardship Council
g	gram
g	relative centrifugal force
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
GUS	β-glucuronidase gene

h	hour
hph	hygromycin B phosphotransferase gene
IMAS	induction medium acetosyringone
IPM	integrated pest management
ITS	internal transcribed spacer
kb	kilobase
L	litre
LB	luria broth
$\log_{10}$	logarithm to the base of 10
LSD	least significant difference
LSE	least significant effect
M	molar
MAF	Ministry of Agriculture and Forestry
MCS	multiple cloning site
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mol	mole
mpi	month post-inoculation
mRNA	messenger ribonucleic acid
ng	nanogram
nm	nanometre
OD	optical density
P	probability
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PEG	polyehylene glycol
pers. comm.	personal communication
PGS	plant growth stimulator
pmol	picomole
psi	pounds per square inch

qPCR	quantitative real-time polymerase chain reaction
®	registered trademark
RNA	ribonucleic acid
rpm	revolution per minute
RSC	rhizosphere study container
RT	root temperature
RT-qPCR	reverse transcriptase quantitative real-time polymerase chain reaction
PC2	Physical Containment 2
s	second
SA	spray application
SC	seed coat
SDW	sterile distilled water
SEA	soil extract agar
spp.	species (plural)
SSA	soil sandwich assay
SSC	sodium chloride sodium citrate
TAE	tris acetate ethylenediamine tetraacetic acid
TBE	tris borate ethylenediamine tetraacetic acid
™	trademark
TSM-LU	*Trichoderma* Selective Medium - Lincoln University
U	unit
UP-PCR	universally primed polymerase chain reaction
UV	ultraviolet
vol%	volume percentage
WHC	water holding capacity
wpi	weeks post-inoculation
ºC	degree celsius
µg	microgram
µL	microlitre
µM	micromolar
µmol	micromole
%	percentage

Chapter One

General Introduction

1.1 Research Background

The forestry industry in New Zealand is one of the most important export sectors worth more than NZ$3.2 billion in 2009, being surpassed only by the dairy products and meat sectors. The total harvest of pine trees in New Zealand in 2009 was about 20 million m^3 contributing 3.8% of the gross domestic product. By 2025, the annual harvest is expected to exceed 40 million m^3 with an estimated rise in exports to NZ$14 billion (NZFOA, 2009). According to the Ministry of Agriculture and Forestry (MAF, 2009), New Zealand will then become one of the top five global suppliers. Furthermore, sustainable forest management is now regarded as one of the major remedies to combat global warming. It is proven that sustainably managed forests, particularly when carbon retained in wood products is included, substantially contribute to carbon sequestration (UNFCCC, 2009; FWPA, 2009). This development is reinforced by the NZ governments involvement in international forestry agreements such as the Montreal Protocol (Anon., 1995), and the forestry sector's adoption of forest certifications such as the Forest Stewardship Council (FSC).

The conifer *Pinus radiata*, the world's most widely planted softwood, contributed 89% of New Zealand's entire plantation forestry estate (NZFAO, 2009). The high economic return of *P. radiata* is mainly due to a relatively short cropping rotation (mean of 28 years) and its fast-growing characteristic (FORESTRYinsights, 2009; Palmer *et al.*, 2005). To sustain this major export sector, it is of vital importance to improve the developmental growth of timber and to control disease outbreaks. For instance, *P. radiata* can be susceptible to foliar pathogens such as *Sphaeropsis sapinea* and root pathogens such as *Phytophthora* spp., *Cylindrocarpon destructans* (Poupin & Arce-Johnson, 2005) and *Armillaria* spp. (Hood & Sandberg, 1993).

Traditionally, chemical fungicides have been used to prevent such epidemics. However, both consumers and producers are increasingly favouring the use of biological control agents (BCAs) as valuable alternatives. The major drawbacks of chemical fungicides are environmental pollution, pesticide residues on food, health concerns to growers and pathogen resistance (Harman *et al*., 2004; Hjeljord & Tronsmo, 1998; Mousseaux *et al*., 1998). Furthermore, chemical control is not always cost effective, particularly for the forestry industry, because of the large areas involved and difficulty with access to many hill sites. Most pesticides used in forest plantations are for foliar diseases and pests, and to a lesser extent for soil-borne pathogens (Gibson, 1967; Van der Pas *et al.*, 1984; Woolons & Hayward, 1984, L. Bulman, pers.comm.).

Compared with chemical fungicides, BCAs must grow and propagate to be effective (Lo *et al*., 1998). Some of the advantages of BCAs are that they may provide higher specificity, produce fewer non-target and environmental effects, can be used against fungicide-resistant pathogens and result in reduced probability of resistance development (Brimner & Boland, 2003).

Soil-borne fungi acting as BCAs exhibit beneficial attributes such as mycoparasitism, substrate competition, antibiotic activity and induced resistance through which they inhibit or reduce pathogen infection (Harman *et al*., 2004). BCAs can act as a living barrier to subsequent pathogen invasion and mobilise nutrients for the target plant (Brimner & Boland, 2003). In addition, it is known that fungal biocontrol agents can influence microbial communities and control root pathogens for long periods of time (Harman *et al*., 2004).

Trichoderma spp. have been extensively studied for their use as BCAs against a wide range of soil-borne plant pathogens. Moreover, their direct beneficial effects on plant growth and development have been shown (Brimner & Boland, 2003; Chang *et al*., 1986; Harman *et al*., 2004; Hjeljord & Tronsmo, 1998). *Trichoderma* bio-inoculants have been shown to enhance emergence and vitality of *P. radiata* seedlings when applied at the nursery stage. In addition, mortality from *Armillaria* disease in seedlings once transplanted into the plantation forest was reduced by the bio-inoculant treatment (Hill, 1991; Paderes *et al*., 2005).

There is little knowledge about the relationship between the influence of *Trichoderma* spp. on plant health and growth and their establishment and

proliferation in the root system. It is difficult to predict both factors since efficacy can vary greatly in different systems or under changing conditions (Lo *et al*., 1998). The main focus of research on *Trichoderma* bio-inoculants has been on the interaction between the agent and the pathogen, and to a lesser extent between the agent and the plant. In addition, plant-agent interactions have rarely been connected to beneficial effects on the plant. As a consequence, the routine use of *Trichoderma*-based bio-inoculants for yield improvement in commercial agriculture remains challenging (Verma *et al.*, 2007).

There is, therefore, a need to investigate the relationship between *Trichoderma* spp. and the soil ecosystem of the plant, particularly interactions in the rhizosphere and their ability to colonise and penetrate plant roots. For the agent to effectively control root pathogens and stimulate plant development, it must be able to establish in the spermosphere of germinating seeds, distribute on the emerging radicle and colonise the developing root (Orr & Knudsen, 2004; Tsahouridou & Thanassoulopoulos, 2002). Further, effects on plant performance need to be coupled with information on the ecological dynamics of *Trichoderma* spp. to fully understand their mechanisms of action and improve their efficacy.

1.2 Literature Review

1.2.1 *Trichoderma*

The genus *Trichoderma* is one of the most frequently isolated soil fungi and can be found widely in agricultural, prairie, forest, salt marsh and desert soils of all climatic zones. These general saprophytes are highly interactive in root, soil and foliar environments and have minimal nutritional requirements. They are fast-growing filamentous deuteromycetes which can sporulate profusely and have been reported to constitute up to 3 % of the total fungal propagules in forest soils (Harman *et al*., 2004; Klein & Eveleigh, 1998; Pinto *et al*., 2006).

The first effective classification of the genus *Trichoderma* was attempted in 1969 and was based on species morphology (Rifai, 1969). In 1991, *Trichoderma* spp. were allocated to four sections designated as *Trichoderma*, *Longibrachiatum*, *Pachybasium*, and *Hypocreanum* (Bissett, 1991). *Trichoderma* is a septate fungus that produces highly branched conidiophores that yield numerous phialospores, also called conidia. Less frequently found is the sexual state (teleomorph) belonging to the Hypocreales. Ascospores are formed for sexual reproduction (Kubicek & Harman, 1998). *Trichoderma* spp. are able to grow under a wide range of environmental conditions. The most important abiotic parameters influencing their establishment in soil ecosystems are soil temperature, soil moisture and soil pH (Danielson & Davey, 1973; Tronsmo & Dennis, 1978; Domsch *et al.*, 1980). Depending on the species, hyphal growth was observed at temperatures from 0ºC to 40ºC. Soil moisture can be a limiting factor for most *Trichoderma* spp. as their establishment is optimal in rather moist environments, but growth was observed at soil water potentials as low as -7 MPa. The tolerable soil pH range is between pH 2 and 6 with an optimum of 3.5-5.6.

Besides their saprophytic characteristic, they have developed the ability to interact, both parasitically and symbiotically, with living organisms, including plants and other microbes (Woo *et al*., 2006). *Trichoderma* spp. produce a wide variety of secondary metabolites and assimilate a large diversity of substrates. It has been known for many years that *Trichoderma* spp. are hyper-producers of degradative enzymes, particularly chitinases involved in lysis of fungal mycelia and cellulases

to degrade polysaccharides (Klein & Eveleigh, 1998). In addition, they are able to degrade natural and synthetic chemicals and toxins such as hydrocarbons, chlorophenolic compounds and xenobiotic pesticides (Harman *et al*., 2004a). *Trichoderma* spp. are able to utilise a diverse variety of carbon substrates making them highly competitive. They occur predominantly in acidic soils and are relatively susceptible to low moisture levels (Klein & Eveleigh, 1998). *Trichoderma* spp. are robust and long-lasting colonisers of root ecosystems and for many years have been recognised as BCAs and plant growth stimulators (PGS) (Harman *et al*., 2004). *Trichoderma* spp. are commonly found in the rhizosphere and thus can have biocontrol activity in areas difficult to exploit through chemical control (Hjeljord & Tronsmo, 1998).

1.2.1.1 *Trichoderma* Bio-Inoculants

Trichoderma spp. have long been associated with biocontrol activity, due to their presence in suppressive soils or in root ecosystems of disease surviving plants. Their beneficial impacts have been demonstrated in both agricultural and forestry systems (Papavizas, 1985). *Trichoderma* species are able to parasitise other fungi, including important plant pathogens such as *Rhizoctonia solani* and *Fusarium* spp. (Benhamou & Chet, 1993; Elad *et al*., 1983), as well as *Sclerotinia* spp. and *Sclerotium* spp. (Sarrocco *et al*., 2006; Tsahouridou & Thanassoulopoulos, 2001). The main mechanisms by which *Trichoderma* spp. act to suppress plant diseases and promote plant growth are listed below (Harman, 2000; Harman *et al*., 2004; Hjeljord & Tronsmo, 1998):

- Mycoparasitism
- Antibiosis
- Competition for nutrients or space
- Tolerance to biotic and abiotic stresses
- Localised and systemic induced resistance
- Enhanced uptake and use of nutrients
- Inactivation of pathogen's enzymes

Mycoparasitism, antibiosis and competition are categorised as the classical antagonistic mechanisms and are described below in more detail. They are not mutually exclusive, but rather considered to be interconnected as part of the entire control process.

Mycoparasitism is regarded as a direct attack of one fungus on another and can be subdivided into four stages of interaction: **Chemotrophic growth** is the directed growth of the mycoparasite towards the host fungus. *Trichoderma* spp. are attracted by chemical stimuli released from the target fungus, which to some extent is due to the expression of cell wall degrading enzymes. **Specific recognition** occurs by the binding of carbohydrates on the *Trichoderma* cell wall to lectins on the host cell surface. Once both fungi come into contact, *Trichoderma* hyphae **attach and coil** around the host fungus hyphae through formation of hook-like structures and appressorium-like bodies. The **secretion of lytic enzymes** initiates the breakdown of the host cell walls. The subsequent penetration into the lumen of the target fungus together with the production of specific antibiotics causes the death of the host fungus (Chet *et al*., 1998; Harman, 2000; Harman *et al*., 2004). Mycoparasitism is a highly complex mechanism and greater understanding of the 'multigene' interactions is required to identify the species specific process and to develop isolates with improved biocontrol activity (Chet *et al*., 1998).

Trichoderma spp. produce a large number of volatile and non-volatile secondary metabolites, which include several substances which inhibit other microorganisms without the need for physical contact. These inhibitory substances are regarded as antibiotics. The most common antifungal metabolites are viridin (steroid group), gliotoxin and gliovirin (diketopiperazine group) (Howell, 1998). The ability to synthesise antibiotics varies greatly between and within species and is affected, both qualitatively and quantitatively, by environmental conditions. Some of the extracellular lytic enzymes produced by this genus are thought to play a role in mycoparasitism, due to their function in direct physical interactions. For example, *Trichoderma atroviride* has been shown to produce small amounts of an extracellular exochitinase, which cause the release of cell wall oligomers from the target fungus. The oligomers in turn induce the expression of fungitoxic endochitinases (Brunner *et al*., 2003). The research community has not yet

discovered a secondary metabolite to be solely responsible for the success or failure of *Trichoderma*-based biocontrol, rather several antibiotics interact, resulting in antibiosis, and antibiosis in turn is considered to be only one mechanism among the many that comprise a much more complex system (Vinale *et al.*, 2008).

Competition occurs when two or more microorganisms demand the same limited resource, such as nutrients or space. Competition between *Trichoderma* and a plant pathogen obviously leads to disease control if *Trichoderma* shows better saprophytic abilities than its counterpart and consequently reduces the pathogen's growth or propagule production. Whereas, rivalry between the indigenous microflora and an introduced antagonist may impede long-term establishment of the antagonist in the soil system (Hjeljord & Tronsmo, 1998). According to Lockwood (1981, 1992) and Wicklow (1992), competition can be subclassified as "interference competition" and "exploitation competition" (cited in Tuininga, 2005). Interference competition indicates behavioural or chemical mechanisms by which one organism limits another organism's access to the resource due to both inter- and intraspecific mycelial interactions and indirect inhibition. Whereas, during exploitation competition, the antagonist directly competes with the opponent for a resource (Tuininga, 2005).

Trichoderma species, as aggressive soil saprophytes, colonise the substrate rapidly, conidiate prolifically and utilise a wide range of substrates. Along with their ability to produce antibiotics and directly parasitise other organisms, they can successfully conquer the resource, adversely affecting their competitors. Nutrient competition is regarded as the most efficient antagonistic mechanism to prevent pathogenic infection of plants and, thus, is likely to be a key component in biological control systems (Hjeljord & Tronsmo, 1998).

Besides the production of antibiotic metabolites, some *Trichoderma* strains can strongly stimulate plants to synthesise their own antimicrobial compounds. *Trichoderma* strains induce, both locally and systemically, defensive responses in the plant by colonising the root surface and penetrating the superficial root cells. This establishes a zone of interaction in which *Trichoderma* isolates release elicitors of resistance, including peptides, proteins and low-molecular-weight compounds. The release of cell wall fragments enhances plant resistance

responses (Harman *et al*., 2004). The induced change in the metabolic machinery of the plant results in increased levels of pathogenesis-related proteins, the accumulation of phytoalexins and the deposition of structural polymers (Yedidia *et al*., 2000). Due to ATP-binding cassette (ABC) transporters in the cytoplasmic membrane of *Trichoderma*, the fungus is able to decrease the accumulation of toxicants and protect itself against the defence mechanisms of the plant (Harman *et al*., 2004).

Trichoderma spp. cause substantial modifications in the plant proteome and metabolism, which in most cases benefits the plant. They can directly increase root growth, control plant-deleterious root microflora and inactivate their toxic compounds and are able to both directly and indirectly control plant pathogens. A positive development of the root system is associated with improved plant growth and resistance to biotic and abiotic stresses (Bailey & Lumsden, 1998; Harman *et al*., 2004). These beneficial fungi help solubilise and sequestrate inorganic nutrients (Harman, 2000) and also increase the uptake of organic nutrients. *Trichoderma* spp. have been used widely as BCAs and PGSs. However, to improve efficacy, a better understanding of their mechanisms of action is required to enable targeted applications appropriate to the individual situation.

Beneficial impacts of *Trichoderma* on plant performance have been extensively investigated for annual crops with an emphasis on biological control of plant diseases such as *Rhizoctonia solani* on cucumber (Lu *et al.*, 2004) and lettuce (Adesina *et al.*, 2009), *P. ultimum* on cucumber, pea, white clover and perennial ryegrass (Green *et al.*, 2001; Lu *et al.*, 2004), *Phytophthora capsici* on pepper (Ezziyyani *et al.*, 2009), and *Sclerotium cepivorum* on onion (McLean *et al.*, 2005). However, only a few reports demonstrate growth promotion effects by *Trichoderma* spp. on perennial woody plants. Strains of *T. hamatum*, *T. koningii* and *T. viride* were shown to improve morphometric characteristics by 18 - 70% in two different soil systems of *P. sylvestris* (Grodnitskaya & Sorokin, 2007). A *T. harzianum* strain increased shoot height and root dry weight of *Ficus benjamina* grown in a sand:soil:compost mix by 52% and 7.3-fold, respectively (Sinrath *et al.*, 2003). In the same experiment, a synergistic effect was observed when *T. harzianum* was co-inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae*. Adams *et al.* (2007) demonstrated substantial promotion of root and

shoot growth of *Salix fragilis* saplings grown in soil inoculated with the BCA *T. harzianum* T-22. Shoot and root lengths were increased by 40% and root dry weight by 300%.

T-22 is also well known as a successful BCA against various plant diseases (Harman, 2000). However, antagonism against phytopathogens does not necessarily result in growth promotion as shown with *T. stromaticum* against *Moniliophthora perniciosa* on cacao (De Souza, 2008) and also with T-22 against *Fusarium oxysporum* in container-grown Douglas-fir seedlings.

1.2.1.2 Ecology of *Trichoderma*

Trichoderma spp. are among the most common saprophytes in the rhizosphere. (Yedidia *et al.*, 2000). The rhizosphere is defined as the zone surrounding the roots of plants in which complex relations exist between the plant, the soil microbial community and the soil itself (Dix & Webster, 1994). *Trichoderma* spp. are good ecological competitors and can colonise potential infection areas like growing roots, senescent tissue and wounds (Hjeljord & Tronsmo, 1998). The capability of an organism to establish and grow in the rhizosphere of plants is described as rhizosphere competence and has been identified as a key attribute of both soil-borne pathogens and biocontrol agents resulting in both favourable and deleterious impacts on plants (Bailey & Lumsden, 1998; Tsahouridou & Thanassoulopoulos, 2002).

Researchers have attempted to develop new *Trichoderma* strains with improved rhizosphere competence by genetic alteration, first by mutation (Ahmad & Baker, 1987) and later, even more efficiently, by protoplast fusion (Sivan & Harman, 1991). Many of the early reports of *Trichoderma* not being rhizosphere competent (e.g. Ahmad & Baker, 1987; Chao *et al*., 1986; Papavizas, 1981) have been revised by more recent publications (such as Bailey & Lumsden, 1998; Green *et al*., 2001; Harman *et al*., 2004; Lo *et al*., 1998; Paulitz, 2000; Pinto *et al*., 2006; Tsahouridou & Thanassoulopoulos, 2002; Yedidia *et al*., 2000). For instance, a *Trichoderma koningii* isolate was shown to successfully colonise the roots of tomato plants (Tsahouridou & Thanassoulopoulos, 2002) and *T. harzianum* was detected in the cucumber rhizosphere (Green & Jensen, 1995). The long-term survival of a *T. atroviride* in the rhizosphere of vine was demonstrated in the vineyards of northern Italy (Longa *et al.*, 2008). A *T. hamatum* isolate was found in

the rhizosphere of lettuce and showed biocontrol activity against *Sclerotinia minor* in field experiments (Rabeendran *et al.*, 2006).

Tagu *et al.* (2002) concluded that a finely tuned balance is required between rhizosphere competence and competition with other soil microbes, so that potentially antagonistic interactions with the indigenous microflora such as ectomycorrhizae (ECM) are minimised. ECM represent symbiotic associations between ectomycorrhizal fungi (EMF) and tree roots, whereby, filamentous hyphae interact with the roots to build a new organ. The recognition of diffusible rhizospheric molecules between root and fungal cells results in changes in gene and protein expression. This favourable relationship improves the uptake of water and nutrients by the roots and alteration of root exudates provides a food supply for the EMF (Buee *et al*., 2005; Tagu *et al*., 2002), but also has an effect on other microorganisms in the rhizosphere (Hjeljord & Tronsmo, 1998). This symbiosis plays a decisive role in the stability of the forest ecosystem with EMF also providing increased disease resistance and stress tolerances (Sinclair *et al*., 1982; Smith & Read, 1997; Summerbell, 2005). *Trichoderma* spp., like other BCAs, have been observed to reduce diversity and/or abundance of other fungi, including EMF (Brimner & Boland, 2003). *Trichoderma* colonisation seems to be favoured and beneficial in the rootzone of some soil ecosystems and partially excluded and detrimental in others. *Trichoderma* has been shown not only to mycoparasitise soil saprophytes and plant pathogens, but also EMF and vesicular arbuscular mycorrhizal fungi (AMF) (Brimner & Boland, 2003; Rousseau *et al*., 1996; Summerbell, 1987). In contrast, several *Trichoderma* spp. have shown synergistic interactions with mycorrhizal fungi (EMF and AMF) (Bailey & Lumsden, 1998; Brimner & Boland, 2003; Datnoff *et al*., 1995; Nemec *et al*., 1996; Summerbell, 2005). In addition, recent work has shown no detrimental effect of application of the commercial *Trichoderma* product ArborGuard™ on mycorrhizal colonisation of *P. radiata* seedlings in a nursery system (Minchin, 2010).

Together with rhizosphere competence, direct root colonisation and penetration is recognised as a crucial indicator of biological activity of beneficial microorganisms. *Trichoderma* spp. were reported to penetrate plant roots and induce host defences, including chemical and structural changes (Harman *et al.*, 2004a; Yedidia *et al.*, 1999). Miranda *et al.* (2006) highlighted the importance of ecological

studies for BCA selection and correlated endorhizosphere (inside the roots) colonisation with biological control activity of a *Trichoderma harzianum* isolate. Fahima & Henis (1990) suggested *Trichoderma* was a poor competitor for dead organic matter, but used root exudates as a food source from which to attack pathogens. Similarly, vigorous root colonisation of all root parts (including root hairs, lateral roots and root tips) by a strain of *T. harzianum* was reported in axenically grown cucumber (Yedidia *et al.*, 2000). However, another strain of *T. harzianum* was inoculated in non-sterile potting mix systems of various horticultural crops and root colonisation was only observed on decaying roots, wounded and infected tissue and seed coats (Green *et al.*, 2001). There are no reports of detailed root colonisation by *Trichoderma* for perennial woody plants such as *P. radiata*.

There is little knowledge about *Trichoderma* spp. interacting in the soil ecosystem of *P. radiata*. Only recently, the first peer-reviewed report was published on growth promotion effects of *Trichoderma* on container-grown *P. radiata* seedlings (Donoso *et al.*, 2008). Seedlings were grown in a perlite-compost mix and the addition of a strain of *T. harzianum* improved the stem growth rate and total biomass by 16% and 40%, respectively. Previous studies have been carried out with the isolates chosen for this study. In 1986, a research project was initiated on the biological control of *Armillaria* disease in kiwifruit and *P. radiata* (Paderes *et al.*, 2006b). Initially, microorganisms were isolated from kiwifruit orchards with *Armillaria* infections and tested for activity against *Armillaria*. *Trichoderma* spp. were observed to suppress *Armillaria* disease in some kiwifruit orchards and showed greatest activities in bioassays. Based on these findings, it was decided to develop *Trichoderma*-based formulations to establish *P. radiata* seedlings in plantation forests. A mixture of six *Trichoderma* isolates (including *T. harzianum* LU686) was shown to consistently increase seedling growth by ~20% and reduce mortality rates by up to 50% for up to 4 years after outplanting. This mixture is registered for use in containerised *P. radiata* seedlings and currently used as one of the means to replace pesticide treatments as a requirement for the FSC (Forest Stewardship Council) certification. In 2004, a nursery large-scale experiment was conducted to further evaluate health and growth benefits of *P. radiata* seedlings by *Trichoderma* spp. The selection of treatments included single-isolate applications

of *T. atroviride* LU132 and *T. hamatum* LU592, and *T. harzianum* LU686 within the six-isolate mixture. LU592 proved to be one of the best performing single-isolate applications. Treatment with LU592 increased the shoot height of seedlings by 16% after 9 months (Paderes *et al.*, 2006a). *T. harzianum* LU686, as part of the commercialised mixture, was seen to increase shoot height by 21% and seedling dry weight by 45% (Paderes *et al*., 2005). In addition, the product was shown to protect *P. radiata* from *Armillaria* infection once planted out in the forest plantation. The beneficial effect on *P. radiata* was demonstrated 4 years after outplanting (Hill, 1991). *T. harzianum* LU686, which on its own increased the top dry weight by 12%, was the most bioactive isolate in previous screening experiments (R.A. Hill, unpublished data). In contrast, treatment with *T. atroviride* LU132 showed little biological impact on *P. radiata* growth and health. All three species are commonly found in forest ecosystems. *T. hamatum* was the most commonly isolated *Trichoderma* species in North American forests in all climatic regions investigated (Danielson & Davey, 1973). *T. hamatum*, *T. atroviride* and predominantly *T. harzianum* were found in a mid-European primeval forest area (Wuczkowski *et al.*, 2003).

Due to varying factors in the different systems influencing the mode of action of *Trichoderma*, more research is needed regarding the ecological and biological behaviour of *Trichoderma* in the soil ecosystem (Thrane *et al.*, 1995). Studies need to determine where on the root/soil system *Trichoderma* is located, and if and how it survives in the different soil-rhizosphere compartments. It is important to understand the dynamics of a beneficial agent in the rhizosphere to effectively predict the success of biological activity. The study of population dynamics can answer basic questions regarding inoculum density and spatio-temporal distribution and is critical to provide more predictable bio-inoculants for use in soil ecosystems (Paulitz, 2000). In addition, a better understanding of the ecological behaviour of different *Trichoderma* isolates is essential to create effective combinations with complementary mechanisms of action to improve biological activity.

1.2.2 The Model Plant *Pinus radiata*

The species *Pinus radiata* (known as Radiata Pine in NZ or Monterey Pine in USA) is native to California, USA, and is the world's most cultivated softwood with plantations in warm temperate climates, mostly in the southern hemisphere including Chile, South Africa, New Zealand and Australia. Selective breeding of *P. radiata* resulted in ideal features of straight trunk, height of up to 60 m under optimum cultivation conditions, and lack of low branches. New Zealand provides ideal climatic conditions for *P. radiata* growth with warm days and cool nights, relatively fertile soil and high rainfall evenly spread through the year (ForestryInsights, 2009; NZFAO, 2009). *P. radiata* timber production in New Zealand contributes 35% of the world's *P. radiata* timber demand. This is second only to Chile's production of 36%. The main characteristics of *P. radiata*'s successful establishment in plantation forests are: easy seed harvest, rapid germination, simple nursery production of seedlings and cuttings, good survival rates, fast growth after thinning, quick healing of pruning wounds, relatively short cropping rotation of 28 years and easy selection and breeding procedure. Further, its timber has excellent properties for general uses such as building and packaging. Therefore, it is not surprising that *P. radiata* contributes ~90% of NZ's entire plantation forestry estate (Palmer, 2005; ForestryInsights, 2009). In 1996, tree stock sales peaked at 100 million with 75% as seedlings and the remaining 25% consisting of cuttings and tissue-cultured plantlets. Traditionally, bare-root planting stock is most commonly used in New Zealand. Bare-root seedlings are grown in raised beds of a soil-based system and require precise spacing and appropriate root conditioning (Menzies *et al.*, 2001; NZFAO, 2005). Seedlings are planted out between May and September at an age of 9 month to 1 year. The disruptive removal of the plants from the soil beds before outplanting can affect subsequent survival and establishment in plantation forests (R.A. Hill, pers. comm.). More expensive container-grown planting stock is increasing in New Zealand. The root-pruning container system improves the development of lateral and vertical roots, shortens the nursery growing time, extends the planting season, and favours the production of vegetative propagules such as cuttings and tissue-cultures plantlets. It also reduces mortality risks in dry conditions and minimises disturbances at lifting, during transport and planting (Menzies *et al.*, 2001; PF

Olsen, 2009). These advantages make it particularly revelant for high-value control-pollinated seedlings.

In both the bare-root and the containerised growing system, fungal and insect pathogen infestations need to be prevented through regular pesticide treatments. The implementation of standard pesticide schemes increased seedling survival rates to >90%. However, the proportion of planting stock meeting market specifications is limited to ~75% (MAF, 2004; K. Haine, pers. comm.). These tree vitality standards are currently challenged by the increasing demand of sustainable forestry management regimes such as the FSC certification. The FSC certificate requires an integrated disease and pest management approach without the use of conventional pesticides (PF Olsen, 2009; FSC, 2009). The application of beneficial microorganisms such as *Trichoderma* spp. have shown promising results to maintain high seedling survival rates and increase the proportion of marketable planting stock in NZ tree nurseries (Hill, 1991; Hill *et al.*, 1999; Paderes, 2005; Paderes *et al.*, 2006b).

1.2.3 Approaches to Study the Ecology of *Trichoderma*

A number of techniques have been developed to estimate fungal biomass in soil, including the quantification of viable fungal propagules and specific biochemical components of fungal cells (e.g. lipids, ergosterol and chitin) as well as direct microscopy (Parkinson & Coleman, 1991). However, a major drawback to being able to evaluate the distribution of an introduced fungus in soil is that the soil ecosystem consists of a complex microbial community. This makes it difficult to observe the growth of a specific fungus within various native fungal and bacterial communities (Lo *et al*., 1998). Methods developed more recently based on marker genes provide opportunities for the selective monitoring and quantification of an introduced fungus in the soil environment (Orr & Knudsen, 2004). In the following section, the advantages and disadvantages of traditional and emerging techniques are compared and the merit of a combination of these techniques to study the ecology and biology of *Trichoderma* in the soil environment is discussed.

The serial dilution plating method is the most widely used method for quantifying soil fungi and has been traditionally used for monitoring *Trichoderma* strains (Elad *et al*., 1981). It is based on serial diluting soil particles in water and spreading onto semi-selective medium followed by morphological examination. Viable propagules

will ideally result in individual colonies on the plate that can be enumerated to provide the concentration of colony forming units (cfu) in the respective system. A medium which favours rapidly growing *Trichoderma* spp. contains specific antibiotics and fungicides to inhibit unwanted non-target organisms (Lübeck & Jensen, 2002; Paulitz, 2000). The dilution plating method, however, does not distinguish between different propagules produced by *Trichoderma* spp. (conidia, chlamydospores, fragments of vegetative hyphae). Therefore, this method on its own does not necessarily correlate to actively growing hyphae (Green *et al*., 2001; Orr & Knudsen, 2004; Parkinson & Coleman, 1991). In addition, although the population number of an introduced *Trichoderma* strain can be distinguished from indigenous strains by determining the background *Trichoderma* levels, this is an indirect estimation and does not consider possible interactions of the introduced agent with indigenous *Trichoderma* species. An advanced approach was demonstrated by Lübeck & Jensen (2002). Inoculated *Trichoderma* BCAs were monitored in the potting mixture of ornamental plants using the dilution plating method in combination with universally primed (UP) polymerase chain reaction (PCR) analysis. The UP-PCR method offers strain specific fingerprints of the introduced agents enabling actual BCA concentration to be determined (Lübeck & Jensen, 2002). However, the technique is reliant on generating those fingerprints for each *Trichoderma* isolate and the indigenous diversity needs to be determined for each soil system individually. Finally, the dilution plating method allows many samples to be processed with a minimum investment in equipment and can be sensitive to 1 cfu/ml substrate, depending on the level of background organisms (Paulitz, 2000). Colony forming unit counts can be correlated to microscopic observations of the predominant morphological state (presence of spores and/or mycelium) of the fungus. This can improve the estimation of the fungal activity. For example, cfu values of *T. harzianum* have been shown to be representative of actual fungal biomass because *T. harzianum* was present primarily as hyphae and not as conidia or other spore forms (Ahmad & Baker, 1987; Green & Jensen, 1995; Lo *et al*., 1998).

One novel approach to study the ecology of filamentous fungi such as *Trichoderma* is to introduce constitutively expressed genetic markers to give the fungus a unique physiological property. The aim is to transform the fungus to a phenotype enabling selective isolation and visualisation without altering the ability to grow and reproduce, which are basic requirements for biocontrol (Paulitz, 2000). Assuming that the background organisms do not possess the given activity,

the marked strain can be detected and monitored in soil and other non-sterile environments.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was first transformed into a filamentous fungus in 1996 (Spellig *et al*., 1996). The fluorescence of GFP requires only uv, or blue light and oxygen, and, therefore, does not rely on cofactors or substrate activity. In recent years, a plant optimised codon usage has resulted in a marker gene designated as SGFP which is under the control of a strong constitutive promoter for filamentous fungi and can be easily detected by epifluorescence microscopy (Lorang *et al*., 2001). The combination of GFP with a selective marker gene has been shown to be a valuable tool for studying interactions between fungal inoculants and plant root systems *in vivo* (Lorang *et al*., 2001; Maor *et al*., 1998). For example, the colonisation of *T. atroviride* on cucumber roots in sterile soil was clearly visualised by Lu *et al*. (2004). Another BCA, a strain of *Clonostachys rosea* transformed with GFP, has been shown to grow towards and colonise carrot and barley roots (Lübeck *et al*., 2002).

Using this system, a major limitation of the dilution plating method can be reduced. A commonly used selective marker is the hygromycin B phosphotransferase gene (*hph*). This prevents the growth of indigenous strains and unwanted microorganisms and allows the marked strain to be preferentially cultured.

Another widely used genetic marker is the β-glucuronidase gene (GUS) which requires an external substrate for activity. The GFP marker has been shown to be superior because GUS activity was low in resting structures and the addition of the substrate resulted in some sample disruption (Bae & Knudsen, 2000). In addition, both Lübeck *et al*. (2002b) and Orr *et al*. (2004) concluded that GFP shows higher fluorescence activity in the target fungus and less background fluorescence in non-sterile soil.

A GFP homologous gene, designated as DsRed, has been obtained from the reef coral *Discosoma* sp. (Matz *et al*., 1999). The red fluorescence is easily detectable with epifluorescence microscopy and the relatively distant emission maxima to GFP makes DsRed an ideal partner for dual-labelling systems. DsRed, considered as a single colour fluorescent marker, provides a higher signal to noise ratio and is relatively resistant to photobleaching compared with GFP. However, the slow

maturation and the lower solubility of the wild type protein may cause limitations for its use (Mikkelsen *et al*., 2003; Nahalkova & Fatehi, 2003).

Having produced transformed *Trichoderma* strains which constitutively express fluorophores (fluorescent proteins), fungal cells can be visualised and detected by epifluorescence microscopy. The principle is based on the excitation of the fluorophores with light of a specific wavelength causing them to emit light of another, longer wavelength (Bradbury & Evennett, 1996). In contrast to indirect observation methods like the dilution plating method, epifluorescence microscopy affords information on the visible behaviour of the target organism and can provide insights into direct interactions between plant roots, ectomycorrhizal fungi, potential pathogens, the introduced agent and the remaining microflora. The combination of epifluorescence microscopy with quantifying methods such as dilution plating or real-time PCR provide a powerful tool to determine the proliferation and behaviour of the fungal agent in complex systems. For instance, Lu *et al*. (2004) showed mycoparasitic interaction of a fluorescently marked biocontrol strain of *T. atroviride* with *Pythium ultimum* or *Rhizoctonia solani* in co-cultures and on cucumber seeds. Another study, carried out by Bae & Knudsen (2000), demonstrated the successful colonisation of sclerotia of *Sclerotinia sclerotiorum* by a *T. harzianum* strain transformed with a green fluorescent protein in non-sterile soil using epifluorescence microscopy. Preliminary work to combine epifluorescence microscopy with image analysis techniques has shown some promise. For example, Orr & Knudsen (2004) quantified the biomass of *T. harzianum* in non-sterile soil by measuring the radii and lengths of visible hyphae. Consequently, fungal biomass was calculated assuming that hyphal segments were cylindrical in shape with an estimated density reported by Van Veen & Paul (1979). However, some practical obstacles such as observer subjectivity in interpretation of images as well as biological constraints such as long stability of the central fluorescent chromophore still pose challenges. In addition, the use of a constitutive promoter does not necessarily indicate the actual fungal biomass but rather shows the activity of the fungus. Further, improved automated image analysis systems are required to overcome the high labour input (Green & Jensen, 1995; Lorang *et al*., 2001; Orr & Knudsen, 2004).

1.3 Research Aim and Objectives

1.3.1 Aim

The overall aim of this research is to improve the understanding of the ecology and biology of *Trichoderma* bio-inoculants in the soil ecosystem of *P. radiata*. This information will ultimately be used to optimise the use of *Trichoderma* as beneficial bio-inoculants in pine forest systems.

Overall hypothesis

1.3.2 The population dynamics of *Trichoderma* isolates in the root ecosystem of *P. radiata* are related to their beneficial effect on *P. radiata* seedling growth and health.Objectives

This study will be carried out with three *Trichoderma* isolates from different species:

- *T. atroviride* strain LU132
- *T. hamatum* strain LU592
- *T. harzianum* strain LU686

The main objective of this study is to identity a health and growth promoting and rhizosphere competent *Trichoderma* isolate as a bio-inoculant in the root ecosystem of *P. radiata* seedlings. Associations will be made between the beneficial effects and the ecological behaviour of three *Trichoderma* isolates, representing three different species of this genus. Finally, the most promising *Trichoderma* isolate will be genetically marked for isolate-specific root colonisation evaluations. Root penetration assessments and the enumeration of mycelia-derived propagules will elucidate the fungal activity in the root zone of *P. radiata* seedlings.

Research Objectives:

(1) To determine the impact of three different *Trichoderma* isolates on the growth and health of commercially grown *P. radiata* seedlings and their establishment in the different potting mix subsystems (bulk soil, rhizosphere, rhizoplane and endorhizosphere).

(2a) To insert marker genes via *Agrobacterium*-mediated transformation into the genome of the *Trichoderma* isolate showing best rhizosphere competence and growth promoting effects on *P. radiata* seedlings.

(2b) To compare physiological characteristics of the transformed strain with the respective wild type and to visualise and recover this strain in non-sterile potting mix.

(3a) To examine the rhizosphere competence of the selected *Trichoderma* isolate in relation to distance from the root surface of *P. radiata* seedlings at three different inoculum concentrations.

(3b) To examine the *P. radiata* root colonisation behaviour of the selected *Trichoderma* isolate using epifluorescence microscopy and to determine preferred penetration sites of the selected *Trichoderma* isolate in the *P. radiata* root system.

(3c) To determine the spatio-temporal distribution of the selected *Trichoderma* isolate in presence and absence of *P. radiata* seedlings.

Hypothesis – Objective 1:

Rhizosphere competence and the ability of *Trichoderma* isolates to penetrate the roots are associated with their beneficial effects on plant performance.

Hypothesis – Objective 2:

The integration of the green fluorescent protein and hygromycin B phosphotransferase genes enable isolate-specific visualisation in and

recovery from non-sterile potting mix without compromising phenotypic characteristics.

Hypothesis – Objective 3:

Marker genes are a useful tool to study the ecology of *T. hamatum* LU592 in a non-sterile root ecosystem.

Inoculum concentration affects the colonisation of *P. radiata* roots and rhizosphere by *T. hamatum* LU592 and the relative proportion of spores to mycelium.

1.3.3 Thesis Format

This thesis consists of five chapters including three experimental chapters. The chapters are structured to be able to easily adapt them for submission for publications. Each experimental chapter includes an abstract, introduction, materials and methods, results, and discussion section. The complete list of references is presented at the end of the thesis to keep repetitions to a minimum.

Chapter Two

Identification of a Rhizosphere Competent and Growth Promoting *Trichoderma* Isolate for Commercially Grown *Pinus Radiata* Seedlings

2.1 Introduction

Rhizosphere competence, the ability to proliferate and function in association with plant roots, has been identified as one of the most important factors in determining the biological control potential of microorganisms such as *Trichoderma* spp. (Ahmad & Baker 1987, Harman 2004). Some of the relationships between root colonisation and biological control have been established. Lewis & Papavizas (1984) suggested that a successful biological control agent (BCA) first of all needs to survive, grow and proliferate in the soil and rhizosphere and also be able to overcome fungistasis, a state of exogenous dormancy, which is mostly dependent on the soil microbial community composition and nutrient availability (Lockwood, 1977). Improved biocontrol activity against *Pythium ultimum* was shown with rhizosphere competent mutants of *Trichoderma* on cucumber seedlings (Ahmad & Baker, 1988). However, the relationship between the establishment of *Trichoderma* spp. on plant roots and plant growth has had limited investigation. Chang *et al.* (1986) demonstrated increased plant growth when a strain of *T. harzianum* established in the soil ecosystem of *Petunia hybrida* and more recently, Srinath *et al.* (2003) reported the first association between *Trichoderma* root colonisation and plant growth stimulation in a perennial plant, *Ficus benjamina*.

An effective bio-inoculant should also preferably colonise the root surface and penetrate the first layers of the roots to not only be able to directly antagonise root pathogens, but also benefit plant growth and vigour through various mechanisms such as solubilisation and concentration of soil nutrients and induction of host

defences (Vinale *et al.*, 2008). Several species of *Trichoderma* (including *T. hamatum* and *T. harzianum*) were shown to induce such growth promoting effects on different perennial woody plants (including *P. radiata* and *P. sylvestris*) (Srinath *et al.*, 2003; Paderes *et al.*, 2005; Grodnitskaya & Sorokin, 2007; and Adams *et al.*, 2007). However, the population dynamics of these *Trichoderma* spp. were not examined and, to the best of our knowledge, there have been no reports on the association between *Trichoderma* root colonisation and plant growth performance for *P. radiata*.

The economic impact of improved seedling vitality is immense for tree nurseries worldwide. In New Zealand alone, ~40 million *P. radiata* seedlings were sold in 2009 with an estimated value of ~NZ$16M (W.Y. Wang, pers. comm.). *Pinus radiata* tree stock sales in NZ are expected to increase 2- to 5-fold within the next 10 years. For large-scale tree nurseries even small improvements in plant growth and health can result in a significant increase in profit margins. There are two main growing systems for *P. radiata* in New Zealand. *P. radiata* seedlings, or, at times of seed shortage, cuttings, may be grown in a bare-root system where seeds/cuttings are sown directly into soil in raised nursery beds. The use of more expensive container-grown systems are increasing which allow quicker production of planting stock and an extention of the planting season (Menzies *et al.*, 2001). For the containerised system, there is also less root disruption when outplanted and an increase in lateral root development when root-pruning containers are used (R.A. Hill, pers. comm.).

A frequent approach to applying *Trichoderma* spp. in tree nursery situations is via a spray application of a *Trichoderma* spore suspension. This application method is widely accepted due to its convenience in introducing reasonable concentrations of viable spores. The application of a coating of *Trichoderma* spores onto the seed surface is also commonly used. Besides its cost-effectiveness (lower application rates required) the seed coat application was shown to be a valuable strategy to establish *Trichoderma* spp. in the spermosphere and fill critical niches to effectively antagonise aggressive, rapidly growing soil-borne pathogens such as *P. ultimum* and *Fusarium oxysporum* (Mousseaux *et al.*, 1998; Bell *et al.*, 2000).

This study focused on the container-grown system simulating the conditions of commercially grown *P. radiata* seedlings in root-pruning pots. To understand the

differences in ecological behaviour of *Trichoderma* spp., three isolates representating three different species were chosen. *Trichoderma atroviride* LU132 (previously designated as C52) is a highly successful bio-inoculant against *Sclerotium cepivorum* in onion and *Botrytis cinerea* in grapevine (McLean & Stewart, 2000; Card *et al.*, 2009), but showed little biological impact on *P. radiata* in a previous growth promotion screening experiment in 2004 (Paderes *et al.*, 2006a). In the same screening trial, *T. hamatum* LU592 consistently enhanced various seedling growth factors (16% increase in shoot height and 31% root dry weight increase) and *T. harzianum* LU686 was part of a mixture of six *Trichoderma* isolates that resulted in shoot height and dry weight increases of 21% and 62%, respectively. Of these isolates, *T. harzianum* LU686 was considered the most bioactive strain in preceding bioassays (Paderes *et al.*, 2006a). *T. atroviride* LU132 was expected to show a clear contrast in colonisation behaviour compared to the growth promoting isolates LU592 and LU686.

The objective of this study was to identify a health and growth promoting *Trichoderma* isolate for commercially grown *P. radiata* seedlings and to determine rhizosphere competence of these strains in the root ecosystem of *P. radiata*. The relationship between plant growth performance and *Trichoderma* population dynamics of the three representative *Trichoderma* species was also investigated.

2.2 Materials and Methods

2.2.1 Origin and Maintenance of *Trichoderma* isolates

Three *Trichoderma* isolates were used in this study. *Trichoderma atroviride* LU132 was originally isolated from soil, Pukekohe, Auckland, NZ in 1991 (designated as C52 in Kay & Stewart, 1994). *T. hamatum* LU592 from soil, Christchurch, NZ in 1997 (Rabeendran *et al.*, 1998) and *T. harzianum* LU686 from a kiwifruit orchard, Te Puna, NZ in 1989 (GrowChem NZ Ltd). Cultures were stored at -80ºC in glycerol and routinely cultured on potato dextrose agar (PDA; Difco™ Laboratories, USA) at 20ºC in darkness.

2.2.2 Experiment 1: *P. radiata* Growth Promotion and Rhizosphere Competence by *Trichoderma* spp.

Three *Trichoderma* isolates, *T. atroviride* LU132, *T. hamatum* LU592 and *T. harzianum* LU686 applied as both a seed coat and a spray application were tested for their ability to promote growth and health of *P. radiata* seedlings and to colonise the rhizosphere, rhizoplane and endorhizosphere.

4.2.1.1 *Pinus radiata* Seeds

Seeds of the *P. radiata* seedline AO 880.692 x 268.539, obtained from PF Olsen Nursery Ltd, New Zealand, were used for this experiment. The seeds were stratified prior to use to enhance germination. The seeds were soaked in sterile distilled water (SDW) for 1 day after which the excess water was drained off. The seeds were then stored in a closed container for 1 month at 0-2°C before sowing.

2.2.2.1 *Trichoderma* Inoculum Preparation and Application

Each *Trichoderma* isolate, LU132, LU592 and LU686, was applied either as a seed coat preparation or spore suspension sprayed directly after sowing of the *P. radiata* seeds into the potting mixture. Concentrations for both application methods were as used at the PF Olsen nursery site (R.A. Hill, pers. comm.). The spore suspension was prepared as described in Rabeendran *et al*. (2006). Spore suspensions of each isolate were prepared by flooding plates of 21 day old PDA

cultures with SDW, and then spores were scraped off with a sterile hockey stick and filtered through a double layer of sterile lens tissue (Whatman; W and R Balston Ltd, New Zealand). Spore concentrations were adjusted with SDW to 10^6 spores/mL based on haemocytometer counts. Five mL of this suspension was then applied to each planting cell to achieve a spore concentration of 5 x 10^6 spores/cell. For the untreated control treatments and the seed coat application, 5 mL of SDW was applied to each cell. Spore viability was verified as follows: the spore suspension (diluted to 10^6 spores/mL) was mixed with potato dextrose broth (PDB; Difco™ Laboratories, USA) (1:1; V:V) transferred onto microscope slides and incubated in the dark at 20ºC. Three replicates per isolate were set up. After 24 h, two separate counts of 50 spores were observed per replicate (300 in total per isolate) and the number of germinated spores counted under 400x magnification using a compound microscope and the percentage of germinated spores was then calculated. A spore was considered germinated if the germ tube length was greater than the diameter of the spore.

The procedure for the seed coat preparation was supplied by the PF Olsen nursery Ltd and is confidential. The formulation consisted of inert substances (including natural glue) and did not provide any nutritional sources for the plant or microbes. A spore concentration of 4 x 10^5 spores/seed was applied. Spore concentrations on the seed surface were verified as follows: three seeds plus five small glass beads were vortexed for 5 min in 1 mL SDW (amended with 0.005% Tween80) in a 2 mL centrifuge tube. Serial dilutions were plated out onto *Trichoderma* Selective Medium – Lincoln University (TSM-LU, **Appendix 1**) which is a modification of the recipe used by Askew & Laing (1993) and is described in detail by McLean *et al*. (2005). The plates were incubated in the dark at 20ºC for 7 days and characteristic *Trichoderma* colonies were recorded and spores per seed calculated. Seeds were also coated with the coating formulation without *Trichoderma* spores to act as controls.

2.2.2.2 Experimental Setup

The experiment was set up at the Lincoln University nursery site. Eight treatments were applied to *P. radiata* seeds: (i) *T. atroviride* LU132 seed coat; (ii) *T. atroviride* LU132 spore suspension; (iii) *T. hamatum* LU592 seed coat; (iv) *T. hamatum* LU592 spore suspension; (v) *T. harzianum* LU686 seed coat; (vi) *T. harzianum*

LU686 spore suspension; (vii) untreated seed coat control and (viii) water control as described previously.

P. radiata seeds were sown in trays (BCC Sweden; **Figure 2.1**) with each tray containing 63 cells (arranged in rows of 7 x 9) with a volume of 100 mL per cell (depth: 8.5 cm, diameter: ~3.85 cm). The trays are designed for lateral root-pruning which increases the development of new and healthy lateral roots, promoting the stability of seedlings once planted out.

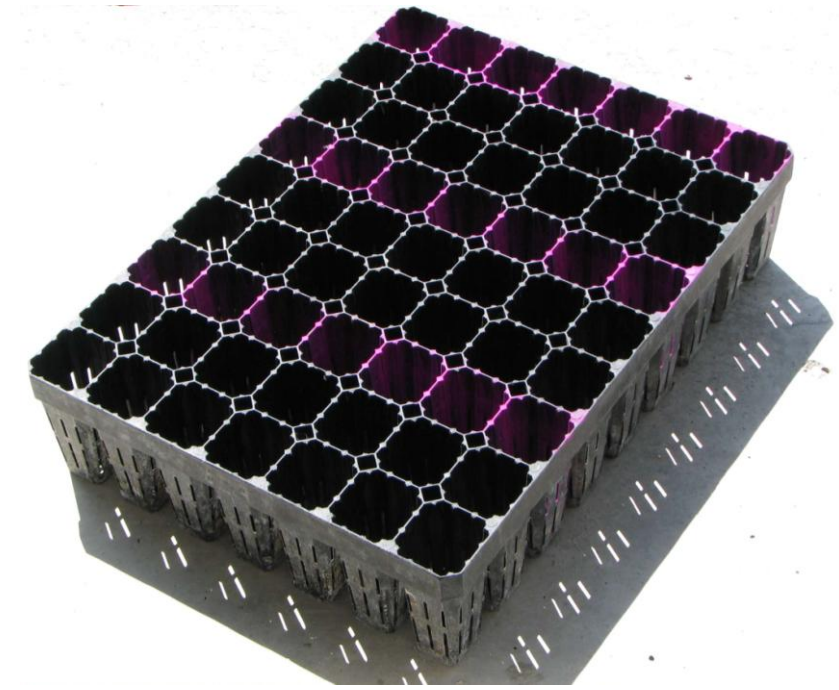


Figure 2.1 The photograph illustrates a tray with 63 cells in which *P. radiata* seedlings were grown. The cells have openings on each side and the bottom to allow root-pruning.

The trays were filled with premixed potting mixture. The potting mixture consisted of 50% composted pine bark, 25% peat and 25% pumice with the addition of 12.5 kg/m^3 slow-release fertiliser (1.5 kg/m^3 dolomite, 2 kg/m^3 gypsum coarse, 2 kg/m^3 gypsum fine, 1 kg/m^3 hydraflo II G wetting agent, 6 kg/m^3 osmocote). Seeds were sown to a depth of 5-10 mm by hand. In each tray, seeds were sown in parallel rows (two), each pair of rows separated from the next pair by an empty buffer row,

resulting in 45 seedlings per tray (180 seedlings per treatment). This ensured sufficient space and light for the seedlings. Each treatment was applied to four trays. The experiment was set up with a completely randomised treatment structure.

P. radiata seeds were sown at the end of September 2007 and maintained for 7 months. The growing conditions reflected those used in the commercial PF Olsen nursery site at Waiuku. The seedlings were first grown in the glasshouse (average T_{max} = 25ºC (day), average T_{min} = 16ºC (night); 65% shading effect) for 6 weeks, before being transferred to the shade house. Half a teaspoon of the solid fertiliser Osmoform® (Scotts, The Netherlands) was added to each cell after 10 and 20 weeks. An automatic irrigation system was set up during the crucial seedling emergence period (6 weeks) to maintain a constant potting mix water content of ~45%. The irrigation system, consisting of six spray nozzles, was placed around the set up. A fine water spray was set to turn on for 30 s/h between 8am and 5pm and 10 s/h between 5pm and 8am (with a flow of 0.1 mL/cm^2/min). A heating mat underneath the trays ensured the potting mix temperature was above 16ºC.

2.2.2.3 Plant Vitality Assessments

The percentage seedling emergence was determined 6 weeks after sowing. The health of the emerged seedlings was assessed after 3 and 7 months based on the standard health score used by the PF Olsen nursery:

0 … dead plant
1 … dying plant: from strongly abnormal needle colour to severe damage especially of branch tips
2 … mainly healthy plant: slight variations from intensely green needles
3 … healthy plant with intense green needles

After 3 and 7 months, 10 seedlings per tray were randomly chosen for non-destructive shoot height measurements and after 7 months, 10 randomly selected seedlings per tray were destructively harvested for shoot diameter, and shoot and root dry weight assessments. The shoot diameter was measured 1 cm above the

potting mix surface using calipers. For the shoot and root dry weights, plants were harvested and adhering potting mix was washed off completely from the roots. The seedlings were then cut at potting mix level and roots and shoots separately oven dried in paper bags to a constant weight for 3 days at 60ºC.

2.2.2.4 *Trichoderma* Population Dynamics

To assess the population dynamics of the three *Trichoderma* isolates, four subsystems of the *P. radiata* root ecosystem were analysed: bulk potting mix, rhizosphere potting mix, rhizoplane potting mix and endorhizosphere. *Trichoderma* populations, expressed as colony forming units (cfu) per gram of oven-dry potting mix, were assessed using the soil dilution plating onto semi-selective TSM-LU medium method outlined in **Section 2.2.2.5**.

Three seedlings with an intact root system and associated potting medium were randomly harvested for each treatment. Bulk potting mix, rhizosphere potting mix and lateral roots with adhering rhizoplane potting mix for each seedling were separated, with the potting mix from each subsystem for the three seedlings combined and considered as one subsample (**Figure 2.2**).

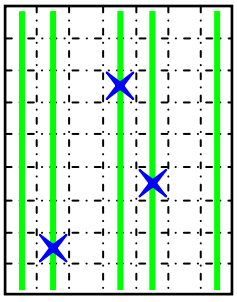


Figure 2.2 Schematic drawing illustrating the sampling procedure. At each sampling time, 3 cells (X) were randomly selected from each tray (7 x 9 cells) and considered as one sub-sample. Lines show rows of seedlings.

Bulk potting mix was defined as the potting mix more than 1 cm away from the roots and was taken from three different depths within the cell: 1.5 cm, 4 cm and 6.5 cm. These different potting mix samples provide information on the ability of the *Trichoderma* isolates to proliferate in the bulk potting mix. Bulk potting mix assessment was only possible until the seedling roots had completely colonised the potting mix which occurred after 8 weeks. Potting mix within a distance of 0.5 cm around the roots, but still adhering loosely was defined as rhizosphere potting

mix (Killham, 1994). After sampling the rhizosphere potting mix, lateral roots were shaken vigorously and any potting mix which was still firmly adhered to the root surface was defined as rhizoplane potting mix. After the rhizoplane potting mix was washed off, roots were used to determine the endorhizosphere *Trichoderma* populations. The roots were first surface sterilised (1 min soak in 70% ethanol, 1 min soak in 2.5% sodium hypochlorite, three SDW rinses; Shishido *et al*., 1998), then cut into slices (approx. 2 mm long) and plated onto TSM-LU. The plates were incubated in the dark at 20°C and the presence of *Trichoderma* recorded after 7 and 14 days.

There were six potting mix subsamples per tray, three bulk potting mix subsamples, and rhizosphere, rhizoplane and endorhizosphere samples, resulting in 168 subsamples to be processed at each sampling. The potting mix and root samples were stored at 8°C and processed within 2 weeks.

Within the 7 months experimental timeframe, seedlings were destructively sampled and *Trichoderma* populations in the different sub-systems determined 24 h after *Trichoderma* application (bulk potting mix only), directly after seedling emergence (1 month after *Trichoderma* application), and 4, 8, 16 and 24 weeks after emergence.

2.2.2.5 Soil Dilution Plating Protocol

The *Trichoderma* population in the potting mix samples was assessed using soil dilution plating (McLean *et al*., 2005). For both the bulk and rhizosphere potting mix, a 10 g potting mix sample was placed into a 100 mL Schott bottle, filled with 90 mL of sterile 0.01% distilled water agar (DWA) and shaken for 10 min on a Griffin® flask shaker. After the flasks were left to stand for a further 10 min, serial dilutions were made and then 1 mL of the soil dilution was pipetted into a 25 mL test tube filled with 9 mL of SDW and shaken. This process was repeated until a 10^{-4} dilution was obtained. Then, aliquots (100 µL) of each dilution from 10^{-2} to 10^{-4} were spread onto three replicate plates of TSM-LU. The plates were then incubated in the dark at 20°C. Characteristic *Trichoderma* colonies were then recorded after 7 and 14 days and cfu per gram of oven-dry potting mix determined. To determine the potting mix water content, 1 g of each bulk potting mix and rhizosphere subsample was weighed out to +/- 0.01 g (DeltaRange® PB 3002-S) and dried at 105°C for 24 h.

For rhizoplane potting mix, roots from three seedlings and adhering potting mix sample was shaken in 90 mL of sterile 0.01% DWA and processed as described for the bulk and rhizosphere potting mix. After processing, the roots were removed from the Universal bottles and blotted on filter paper to remove excess water. The roots were reweighed to calculate the weight of rhizoplane potting mix. *Trichoderma* colonies on TSM-LU were frequently subcultured onto PDA to verify *Trichoderma* identification.

2.2.2.6 Morphological Characterisation

The proportion of introduced *Trichoderma* to indigenous *Trichoderma* populations was determined once after 20 weeks by morphological identification of the *Trichoderma* colonies based on the modified method of Bourguignon (2008) as follows. Twenty four randomly selected *Trichoderma* colonies per treatment and subsample (except endorhizosphere) from twelve 7-day-old TSM-LU plates were subcultured onto half-strength PDA amended with 2.5 µg/mL chloramphenicol (Sigma-Aldrich® Inc., USA) (½ PDA+c) and incubated in the dark at 20ºC for 7 days. For the endorhizosphere, all *Trichoderma* colonies isolated from the roots were taken (between 7 and 12 colonies per treatment). Pure cultures were transferred a second time to ½ PDA+c and incubated for 7 days at 20ºC in the dark to allow optimal colony growth and stimulate starvation-induced sporulation and then another 7 days at 25ºC in constant blue light to initiate light-induced sporulation. The *Trichoderma* isolates were grouped according to their morphology on ½ PDA+c, using the following macroscopic criteria: mycelium density and appearance, conidiation colour, pattern, abundance or absence, and coloration of the medium. Then, between three (for groups with uniform and distinct morphological characteristics) and up to 21 (for groups with inconsistent and indistinct morphological characteristics) representatives of each morphology underwent molecular identification by species-specific sequencing as described in **Section 2.2.2.7**.

2.2.2.7 Molecular Identification of *Trichoderma* Species

Initially, a 50 mL Falcon tube containing 25 mL PDB was inoculated with mycelium from an actively growing *Trichoderma* colony and incubated in the dark for 3 days at 20ºC to allow the growth of a mycelial mat and to prevent conidiation. The

solution was then filtered through sterile mira-cloth, and the remaining PDB squeezed out and the mycelium was wrapped in aluminium foil and transferred to liquid nitrogen. The samples were then stored at –80ºC until further processing.

DNA extraction

Genomic DNA was extracted from all samples using the Puregene® Genomic DNA Purification Kit (Gentra Systems Inc., USA) as per manufacturer's instructions. Approximately 200 mg of frozen mycelium was transferred to a chilled 1.7 mL centrifuge tube containing 600 µL of cell lysis buffer, 30 µg RNaseA and 60 µg Proteinase K (Roche Diagnostics Ltd, Mannheim, Germany). The mixture was immediately homogenised using a sterile micropestle and placed on ice until all samples were processed. Then, samples were incubated at 55ºC for 60 min and inverted periodically to mix. Tubes were cooled to room temperature and 200 µL precipitation buffer added. Tubes were inverted 10 times and placed on ice for 15 min. The solution was centrifuged at 20,000 *g* for 5 min and the supernatant containing DNA transferred to a new tube. The DNA was precipitated by addition of one volume of 100% isopropanol, inverted 10 times and incubated on ice for 5 min, then pelleted by centrifugation at 20,000 *g* for 10 min at 4ºC. The pellet was washed twice with 600 µL 70% ethanol, air-dry and resuspended in 100 µL SDW. The DNA was fully redissolved by incubation at room temperature overnight and then stored at 4ºC. DNA concentrations were estimated by visual comparison of sample DNA intensity with that of the High DNA Mass ladder (Invitrogen, USA) on a 1% agarose gel (**Appendix 1**).

Amplification of the ITS1 region

Amplification of *Trichoderma* nuclear small-subunit rDNA sequence containing the Internal Transcribed Spacer (ITS) 1 and 2 regions and the 5.8S rRNA gene was performed using a combination of two specific primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Invitrogen™ Life Technologies, USA) (White *et al*., 1990). Each 25 µL reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP (Advanced Biotechnologies Ltd), 5 pmol of each primer, 10 ng of *Trichoderma* DNA and 1.25

U Taq DNA polymerase (Roche Diagnostics Ltd, Mannheim, Germany). Controls contained all of the above ingredients except genomic DNA. The rDNA sequence was amplified using an Eppendorf Mastercycler® Gradient PCR machine (Eppendorf-Netherler-Hinz Ltd, Hamburg, Germany) and consisted of an initial denaturation of 6 min at 95ºC, followed by 30 cycles of 30 s denaturation at 95ºC, 30 s primer annealing at 50ºC and 30 s extention at 72ºC, and a final extension of 7 min at 72ºC. The resulting PCR products were then visualised by gel electrophoresis (**Appendix 1**).

DNA sequencing

Each PCR product was added to a sequencing reaction using Big Dye® Terminator v. 3.1 (BDT; Applied Biosystems, USA) as a fluorescent marker. Each 10 µL reaction contained 0.5 µL BDT (Applied Biosystems, USA), 2.0 µL sequencing buffer (Applied Biosystems, USA), 5 pmol primer ITS4 and 0.4 µl DNA template according to the manufacturer's instructions. The thermal cycling conditions were an initial denaturation of 1 min at 96ºC, followed by 25 cycles of 10 s at 96ºC, 5 s at 50ºC and 4 min at 60ºC. The sequencing PCR was separated using the automated sequencer ABI PRISM® 3100-*Avant* Genetic Analyzer (Applied Biosystems, USA). Forward and reverse sequences were obtained and aligned for each DNA template using the software DNAMAN version 4.0a (Lynnon Biosoft®) based on a ClustalW algorithm (Feng & Doolittle, 1990; Thompson *et al*. 1994) to obtain a complete sequence (~ 600-620 bp) of the ITS 1 and 2 regions. The sequences were then submitted to a BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/) in the GenBank EST database and only matched to *Trichoderma* sequences lodged by authors prominent in the field such as J. Bissett, S.L. Dodd, I. Druzhinina, C.P. Kubiceck, K. Kuhls, E. Lieckfield or G.J. Samuels. The sequences were also submitted to a *Tricho*BLAST v. 1.0 search (http://www.isth.info/tools/blast/index.php) in the ISTH database for reliability.

2.2.3 Experiment 2: Verification of *P. radiata* Growth Promotion by *T. hamatum* LU592

Subsequent to experiment 1, a large-scale experiment at the PF Olsen commercial nursery was carried out to reconfirm the beneficial impacts of *T. hamatum* LU592 on *P. radiata*. The experimental conditions were similar to experiment 1 with the following modifications. Three treatments were applied to *P. radiata* seeds: (i) *T. hamatum* LU592 seed coat, (ii) *T. hamatum* LU592 spore suspension (including uninoculated seed coat), (iii) water control (including uninoculated seed coat). Each treatment was applied to 60 root-pruning trays (BCC Sweden) within 15 completely randomised blocks (four replicates per block). Seeds were sown and trays placed outside on a wire system (no glasshouse period; **Figure 2.3**). The experiment started on October 2008 and seedlings were grown for a duration of 9 months. During emergence, pots were irrigated every 2 hours for 2 min during daytime and covered with a mesh to protect seeds from birds; after emergence, seedlings were irrigated once per day for 30 min using spray nozzles. A biological fertiliser (Peters Plant Starter; Scotts, The Netherlands) was added to the water at an adjusted electrical conductivity of 1.1 to 1.2 siemens per metre. The herbicide Valzine500 (AgPro NZ Ltd, New Zealand) was added once per month to the water at a concentration of 1 L/ha to reduce weed competition. After 6 months, shoot tips were cut to maintain a favourable root/shoot ratio following commercial practice.

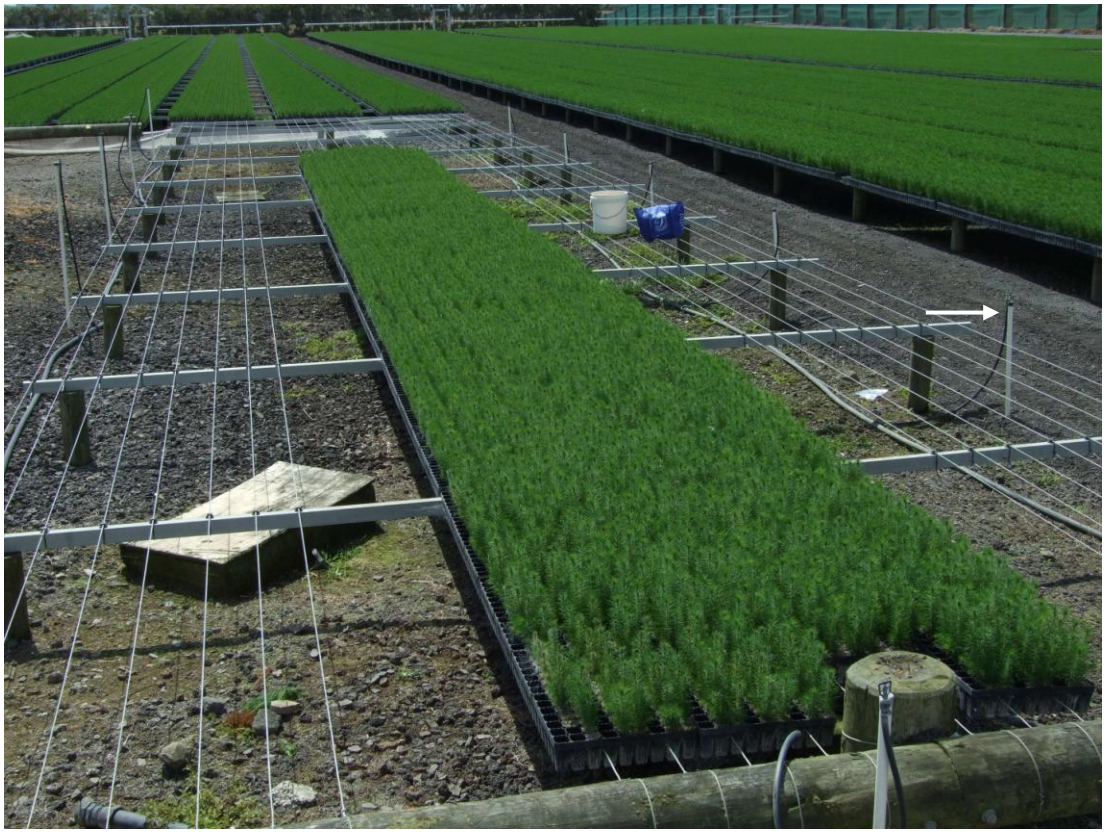


Figure 2.3 Experimental set up (front) at the PF Olsen nursery illustrating the irrigation and wire system. Trays were placed next to each other on wires, irrigation nozzles (white poles, arrow) surrounding the set up.

2.2.3.1 Plant Vitality Assessments

Seedlings were evaluated after 2, 4, 6 and 9 months. At each sampling time, 10 seedlings were randomly chosen for all non-destructive measurements. The same 10 seedlings were observed in successive measurements. For destructive sampling, three randomly chosen seedlings were harvested. The edge plants of each tray were considered as a buffer zone and not included in the measurements. Potting mix adhering to the harvested seedlings was completely washed off from the roots. The seedlings were cut at potting mix level and roots and shoots were transferred into zip-lock plastic bags and stored in chiller bags for transportation. Seedlings were then stored at 8ºC and processed within 1 week.

The following data was collected throughout the experiment: percentage seedling emergence, shoot height and stem diameter, root tip count, root and shoot dry weights, qualitative assessment of ectomycorrhizal formation, assessment of affected shoot tips (potential infestation of *Thrips tabaci* occured after 4 months), percentage commercially marketable seedlings and seedling mortality rate. **Table 2.1** shows the chronology of these measurements. The criteria to identify marketable seedlings were provided by PF Olsen Nursery Ltd and include healthy growing shoots (shoot tips in particular) free from pathogen infections and water and nutrient deficiencies. Seedlings also have to grow reasonably uniformly and meet the requirements of a shoot height of >25 cm and a stem diameter of >3 mm. The root system must be distributed densely throughout the entire cell without any indication of root infections.

Table 2.1 Chronological sequence of seedling health and growth assesments.

	Time [month]			
Assessment	2	4	6	9
% seedling emergence	X	X		
shoot height	X	X	X	
stem diameter	X	X		
root tips count	X			
root & shoot dry weights	X	X		
ectomycorrhizal formation		X		
affected shoot tips count		X		
% marketable seedlings				X
mortality rate				X

For the root tip count assessment, harvested roots were scanned using an image scanner to determine the number of root tips. For the assessment of ectomycorrhizae (ECM), roots were sampled to estimate the proportion of ectomycorrhizal colonisation giving a factor of 0 (no ECM) to 3 (ECM along the whole root system) based on observations by eye. For dry weight assessments, roots and shoots were separately oven dried in paper bags for 3 days at 60ºC. For

the affected shoot tip evaluation, seedling shoot tips were categorised into four groups:

0 … dead or dying shoot tip
1 … obvious abnormality in shoot tip growth including distorted needles and bent shoot tip
2 … mainly healthy shoot tip with slight indications of abnormal shoot tip and needle growth
3 … healthy shoot tip with intense green needles

2.2.4 Statistical Analyses

All data acquired in this experiment was analysed using standard (general unblocked) analysis of variance (ANOVA) with factorial treatment structure and interactions, unless stated otherwise. *Trichoderma* population assessment data were $\log_{10}$-transformed and seedling mortality assessments were arcsin-transformed to satisfy the assumption of normality for ANOVA (Olsen, 2003). Mean separation between each *Trichoderma* treatment and the control was analysed using the unrestricted least significant difference (LSD) test according to Saville (2003). Data was analysed using the statistical software GenStat v. 9.0 (VSN Internation Ltd) and unless stated otherwise all presented data is shown as the back transformed mean.

2.3 Results

2.3.1 Experiment 1: *P. radiata* Growth Promotion and Rhizosphere Competence by *Trichoderma* spp.

2.3.1.1 *Trichoderma* Inoculum Application

The percentage of germinated spores was determined for each *Trichoderma* spray application. Spore viability was high for the spore suspensions of all three *Trichoderma* isolates with 89%, 97.3% and 100% for *T. harzianum* LU686, *T. hamatum* LU592 and *T. atroviride* LU132, respectively. This equates to 4.5 x 10^6, 4.9 x 10^6 and 5.0 x 10^6 spores/cell for LU686, LU592 and LU132, respectively (**Table 2.2**). The number of viable *Trichoderma* spores per seed was determined as being 4.9 x 10^4, 5.7 x 10^4 and 9.1 x 10^4 spores/seed for LU592, LU686 and LU132, respectively.

Table 2.2 Inoculum concentration for three *Trichoderma* isolates applied as either spray application (SA) or seed coat (SC).

***Trichoderma* isolate**	Spore viability (SA) [spores/cell]	Spore concentration (SC) [spores/seed]
T. atroviride LU132	5.0 x 10^6	9.1 x 10^4
T. hamatum LU592	4.9 x 10^6	4.9 x 10^4
T. harzianum LU686	4.5 x 10^6	5.7 x 10^4

Values are means of 3 replicates.

2.3.1.2 Plant Vitality Assessments

Plant Health Assessment

The percentage of emerged seedlings was evaluated 6 weeks post-inoculation (wpi; **Table A2.1, Appendix 2**). No significant effects could be detected, with the means ranging between 91.1% for the seed coat control (SCcontrol) and 94.6%

for both seed coat (SC) and spray (SA) applications of *T. harzianum* LU686. The mean emergence for the SAcontrol was 93.5%.

Representatives of each of the different health scores are shown in **Figure 2.4**. **Figure 2.4**a shows a dead plant (health score 0). A health score of 1 was given either when the seedling developed an atypical star pattern with abnormal shoot growth (**Figure 2.4**b) or when most of the needles turned brownish without a healthy growing shoot tip present (**Figure 2.4**c). A health score of 2 was given either when brown coloured needles were seen on the upper part of the shoot but a healthy growing shoot tip was present (**Figure 2.4**d) or when the majority of the seedling needles were a palish green colour (**Figure 2.4**e, right seedling). **Figure 2.4**e (left seedling) and f show completely healthy seedlings with deep green coloured needles and a healthy shoot tip. These were given a health score of 3. In all treatments, ectomycorrhizal formation was observed on the roots of 7-month-old seedlings (**Figure 2.4**g).

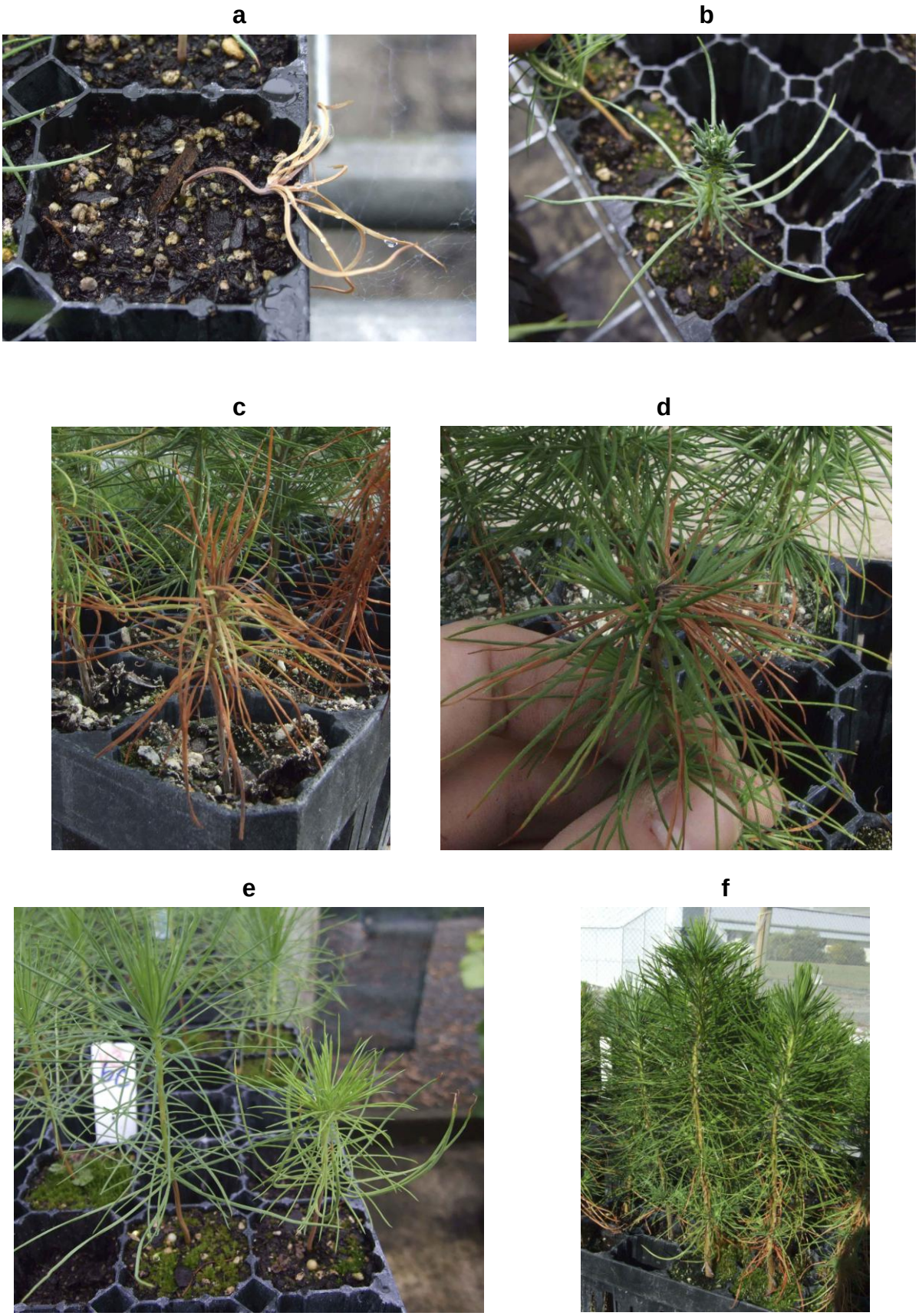


Figure 2.4 Photographs of representative seedlings illustrating the health score: 0 (a), 1 (b, c), 2 (d, e right seedling), 3 (e left seedling, f). Ectomycorrhizal colonisation of roots of a 7-month-old seedling (g).

g

Figure 2.4 Continued.

When the data was analysed using ANOVA, there was no significant difference in the plant health scores between the different treatments either at 3 or 7 months (**Table A2.1, Appendix 2**). The health score for the seedlings at the 3 months assessment was between 2.68 for *T. harzianum* LU686 seed coat (SC686) and 2.90 for the spray application of *T. hamatum* LU592 (SA592). The mean health score for SAcontrol was 2.79. An insufficient irrigation over the Christmas break (after 4 months) combined with warm and dry north-west winds caused stress and death to some plants, particularly for the edge plants facing north and west. The health score after 7 months was unaffected with no significant differences between treatments and ranged between 2.65 and 2.90 for SC132 and SA592, respectively. The mean for the SAcontrol was 2.68.

The proportions of dead seedlings were analysed after 3 and 7 months. After 3 months, the lowest mortality rate was found for SC592 with 0.19% and was signifcantly different to the highest mortality rate of 5.2% for SCcontrol (**Figure**

2.5). In addition, there was a significant reduction in seedling mortality of 3.8% when applied with *T. hamatum* LU592 (SC and SA combined) compared to the control (SC and SA combined) after 3 months. The mean of SAcontrol was 2.6%. The mean proportion of dead plants after 7 months were not significantly different to the control and ranged between 0.97% for SA592 and 5.8% for SCcontrol (**Table A2.1, Appendix 2**). The percentage of dead seedlings for SAcontrol was 3.8%.

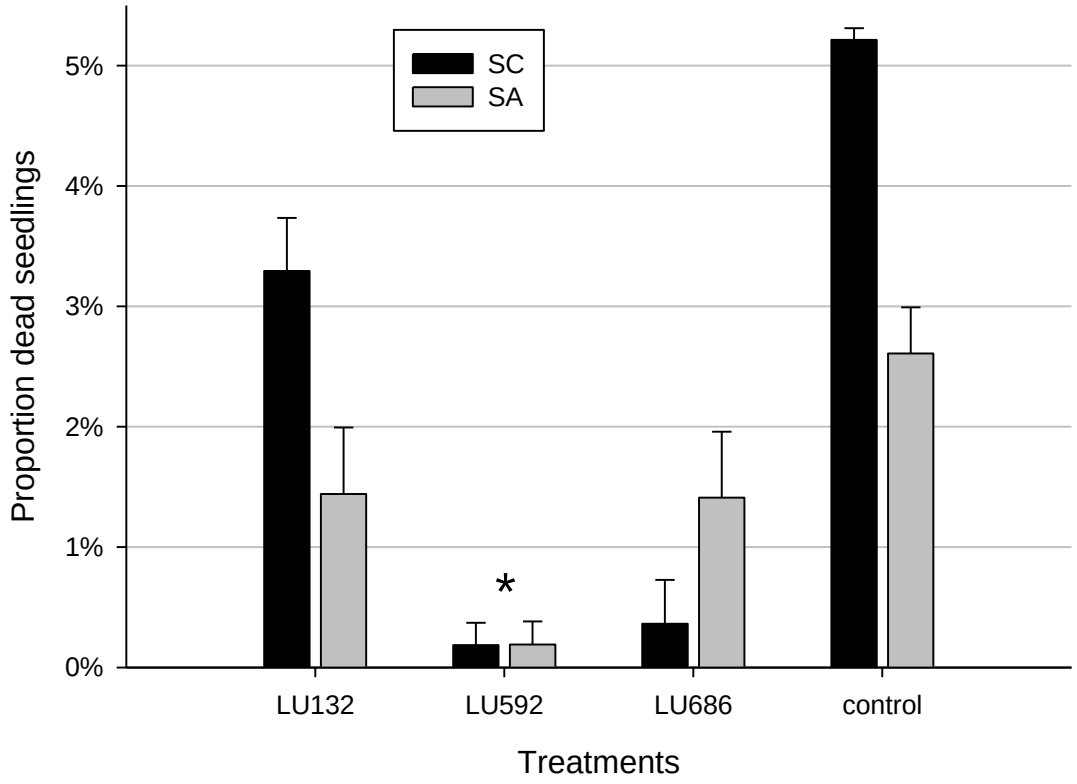


Figure 2.5 Proportion of dead seedlings assessed after 3 months for each *Trichoderma* isolate as either a seed coat (SC) or spray application (SA) treatment. The values are back transformed means (n = 4). Asterisk indicates significant difference to the control (SC and SA combined). Error bars represent the standard error of the means.

Plant Growth Assessments

Seedling shoot heights were measured after 3 and 7 months for each treatment (**Table A2.2, Appendix 2**). Analyses of variances did not detect any significant effects. The seedling shoot height means at the 3 months assessment varied by

20% between SC592 (8.3 cm) and SCcontrol (6.9 cm). The seedling shoot height for the SAcontrol was 7.1 cm. The seedling shoot height at the 7 months assessment was between 12.4 cm for SCcontrol and 14.9 cm for SA132 with 13.6 cm for the SAcontrol.

No treatment had any significant effect on the diameter of seedling trunks, with diameter measurements ranging from 2.8 mm for SC132 to 3.1 mm for SA592 and SAcontrol (**Table A2.2, Appendix 2**).

Seedling root and shoot dry weights were assessed after 7 months for each treatment (**Table A2.2, Appendix 2**). The roots entirely colonised the small 100 mL cells at that time. Potting mix particles were strongly enclosed by the root system and the separation of the roots from the potting mix led to a loss of fragile lateral roots. No significant treatment effect was detected. Root dry weights ranged between 0.50 g/root and 0.65 g/root for SC132 and SA132, respectively. The root dry weight for the SAcontrol treatment was 0.55 g/root. Shoot dry weights ranged between 1.1 g/shoot for SC132 and 1.5 g/shoot for SA132 with the SAcontrol being 1.4 g/shoot. The analyses of the ratio of root/shoot dry weights did not show significant differences between any of the *Trichoderma* treatments and their controls. However, an isolate effect indicated a significantly higher root/shoot ratio for LU132 compared to LU592.

2.3.1.3 *Trichoderma* Population Dynamics

Pinus radiata seedlings were harvested 4, 8, 12, 20 and 28 weeks after *Trichoderma* inoculation. **Figure 2.6** shows representative seedling roots at the different assessments. **Figure 2.6a** shows a 4-week-old root with firmly attached potting mix defined as rhizoplane. The root system of an 8-week-old seedling is shown in **Figures 2.6**b and c. Lateral roots are seen through the middle and lower part of the potting mix (**Figure 2.6b**) with rhizoplane potting mix seen firmly attached along the roots (**Figure 2.6c**). A 12-week-old seedling with the separated rhizosphere potting mix is shown in **Figure 2.6d**. After 20 weeks, seedlings became root bound with the root system equally distributed throughout the cell **(Figure 2.6e**) and after 28 weeks the root system was seen to have well developed ectomycorrhizal root tips (**Figure 2.6f**).

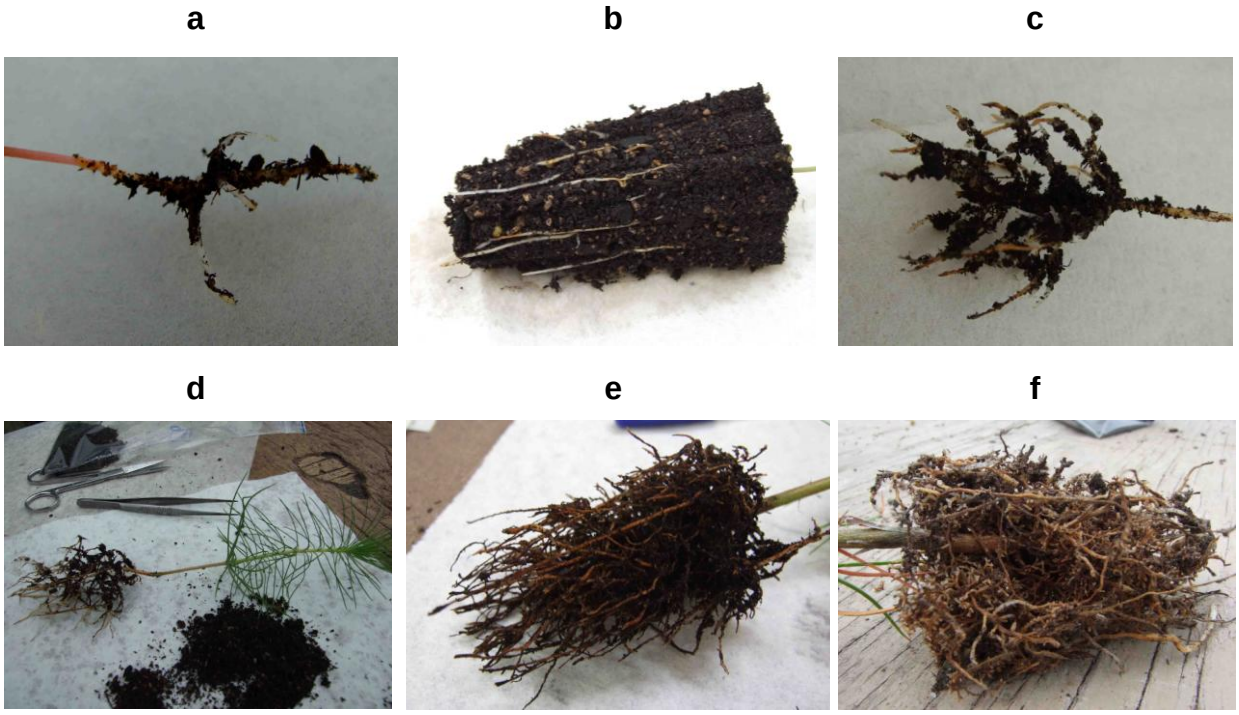


Figure 2.6 Representative seedlings illustrating the different stages of root development: 4 weeks post-inoculation (wpi) (a), 8 wpi (b, c), 12 wpi (d), 20 wpi (e) and 28 wpi (f).

Potting mix and root samples were sampled at each assessment and *Trichoderma* colony-forming units (cfu) recovered on TSM-LU. *Trichoderma* colonies (**Figure 2.7**) were subcultured onto PDA to verify the correct identification of *Trichoderma* using typical morphological characteristics.

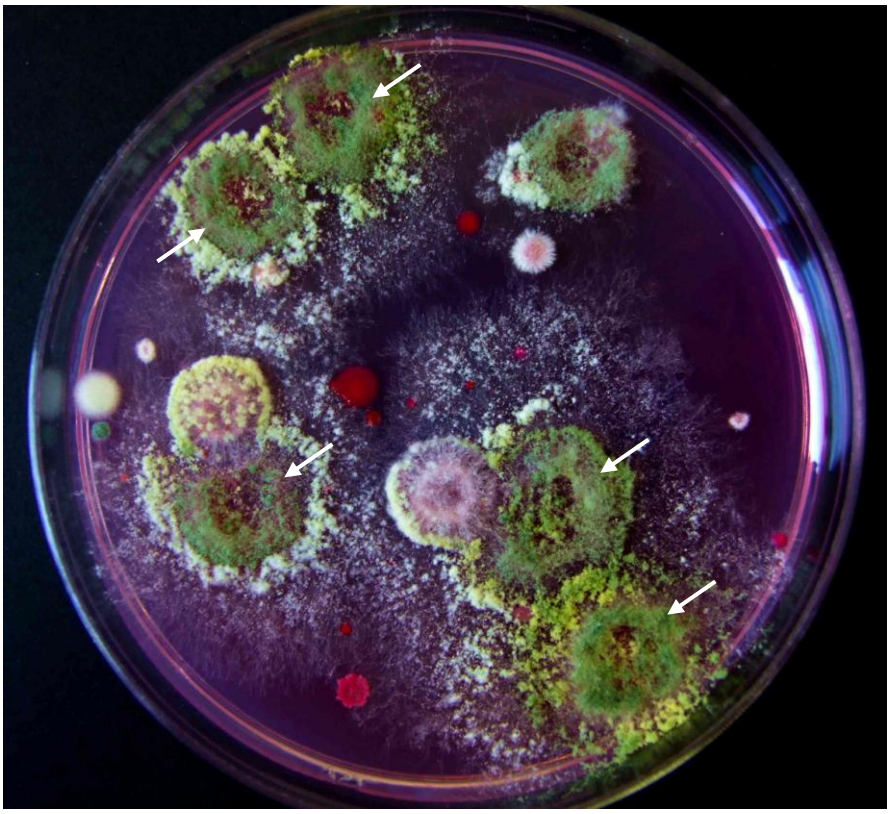


Figure 2.7 *Trichoderma* colonies (mostly characterstic of *T. harzianum*, arrows) growing on TSM-LU.

Trichoderma populations across all treatments in the top bulk potting mix and rhizosphere established at similar levels around 2.5 x 10^4 cfu/g dry potting mix within the first 12 weeks post-inoculation (wpi) (**Figure 2.8**). However, *Trichoderma* concentrations in the top bulk potting mix declined significantly after 12 wpi, whereas *Trichoderma* concentrations in the rhizosphere and rhizoplane significantly increased during the same period, with the highest *Trichoderma* concentrations in the rhizoplane at 20 wpi (4.7 x 10^5 cfu/g dry potting mix). Overall, *Trichoderma* populations across time and treatments in the rhizoplane were significantly higher compared with the rhizosphere and *Trichoderma* levels in the rhizosphere were significantly higher than the top bulk potting mix. *Trichoderma* populations across subsamples and treatments significantly decreased between 8

and 12 weeks corresponding to the time of seedling transfer from the glasshouse to the outside.

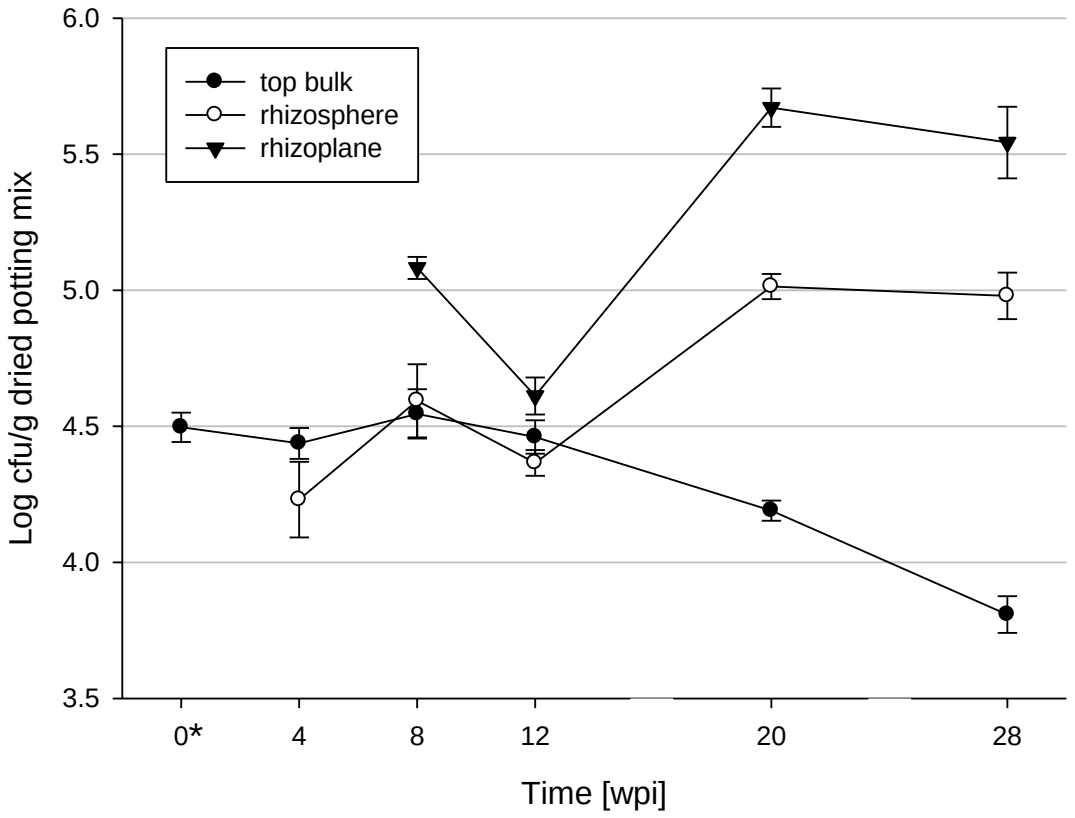


Figure 2.8 *Trichoderma* population (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from the top 1.5 cm of bulk potting mix, rhizosphere and rhizoplane of *Pinus radiata* seedlings treated with three different *Trichoderma* isolates (LU132, LU592 and LU686) as either seed coat (SC) or spray application (SA) treatments over time. Data for all treatments have been combined (across all treatments). Error bars represent the standard error of the means (n = 4). Wpi = weeks post-inoculation (*: first sampling was 24 h post-inoculation).

For each *Trichoderma* isolate, *Trichoderma* populations were significantly higher in the spray application treatment compared with corresponding seed coat treatment in both the top bulk potting mix (**Figure 2.9)** and rhizosphere (**Figure 2.10)**. Overall *Trichoderma* population means (averaged over time) in the top bulk potting mix were significantly different between treatments. Significantly higher *Trichoderma* populations were recovered from the top (1.5 cm) bulk potting mix for all *Trichoderma* spray application treatments (SA132, SA592 and SA686)

compared with the spray control (5.6 x 10^3 cfu/g dry potting mix) equating to 7.8 times for SA686 (4.4 x 10^4 cfu/g dry potting mix), 11 times for SC132 (6.5 x 10^4 cfu/g) and 39 times for SA592 (2.2 x 10^5 cfu/g). None of the *Trichoderma* seed coat treatments (SC592, SC686 and SC132) significantly increased *Trichoderma* populations in the bulk potting mix compared with the SCcontrol. *Trichoderma* populations for all *Trichoderma* spray application treatments were significantly higher compared with the SAcontrol treatment at each assessment except the last sampling time (28 wpi) when the *Trichoderma* population for SA686 rapidly decreased and was not different to the SAcontrol (**Figure A2.2, Appendix 2**). Further, SC686 was the only SC treatment (including control) causing a significant decrease in *Trichoderma* cfu levels over the period of this experiment.

The centre and bottom bulk potting mix were only sampled in the first 4 weeks as after this time the potting mix was completely colonised by the roots (**Figure 2.6**a). After 4 weeks, an isolate effect was detected in the centre bulk potting mix. Cfu levels for LU686 (SC and SA combined) were significantly higher compared with the control (SC and SA combined) by 3.6 times (**Table A2.3, Appendix 2**).

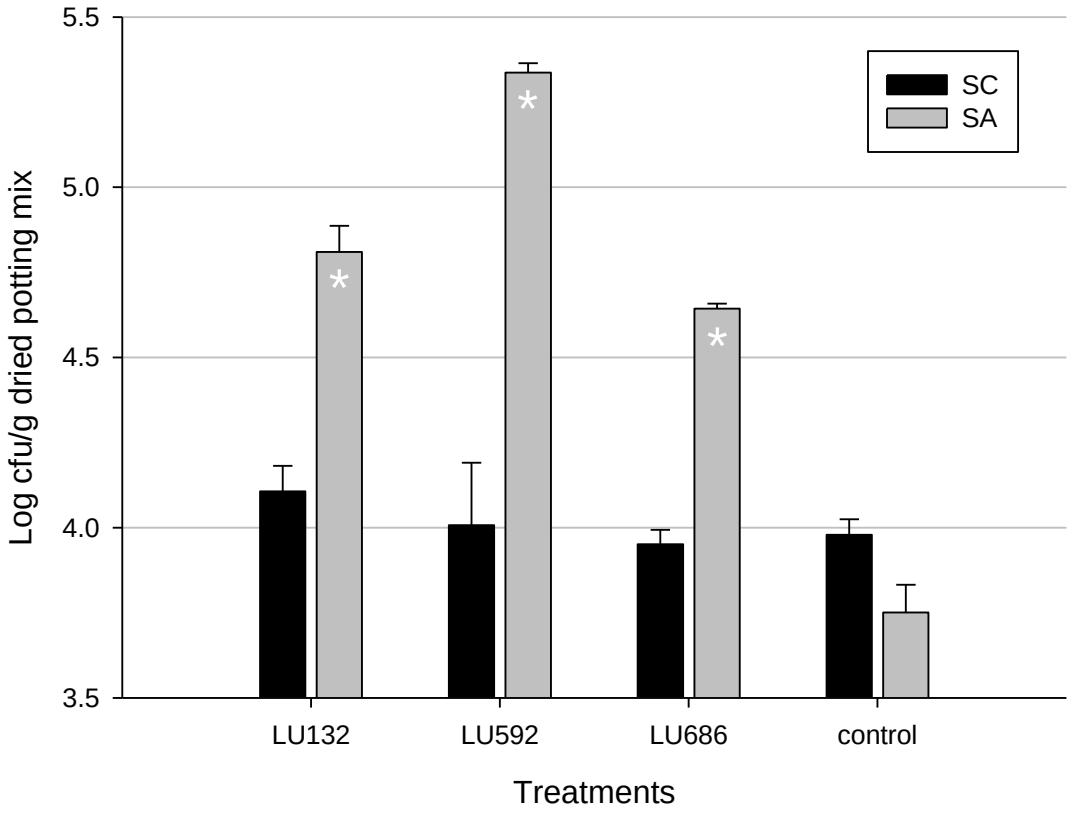


Figure 2.9 Overall *Trichoderma* populations (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from **top bulk** potting mix of *Pinus radiata* seedlings treated with three different *Trichoderma* isolates as either seed coat (SC) or spray application (SA) treatments (means of all sampling times 1 – 6). Asterisks indicate significant differences to the respective control. Error bars represent the standard error of the means (n = 24).

Main treatment effects were detected for the *Trichoderma* population means (over time) within the rhizosphere potting mix. *Trichoderma* populations of all spray application treatments were significantly higher compared with the SAcontrol treatment (2.7×10^4 cfu/g dry potting mix) by 2.1 times for SA132 (5.5×10^4 cfu/g dry potting mix), 2.9 times for SA686 (7.6×10^4 cfu/g) and 3.3 times for SA592 (8.7×10^4 cfu/g) (**Figure 2.10**). There were no significant effects between the SC treatments.

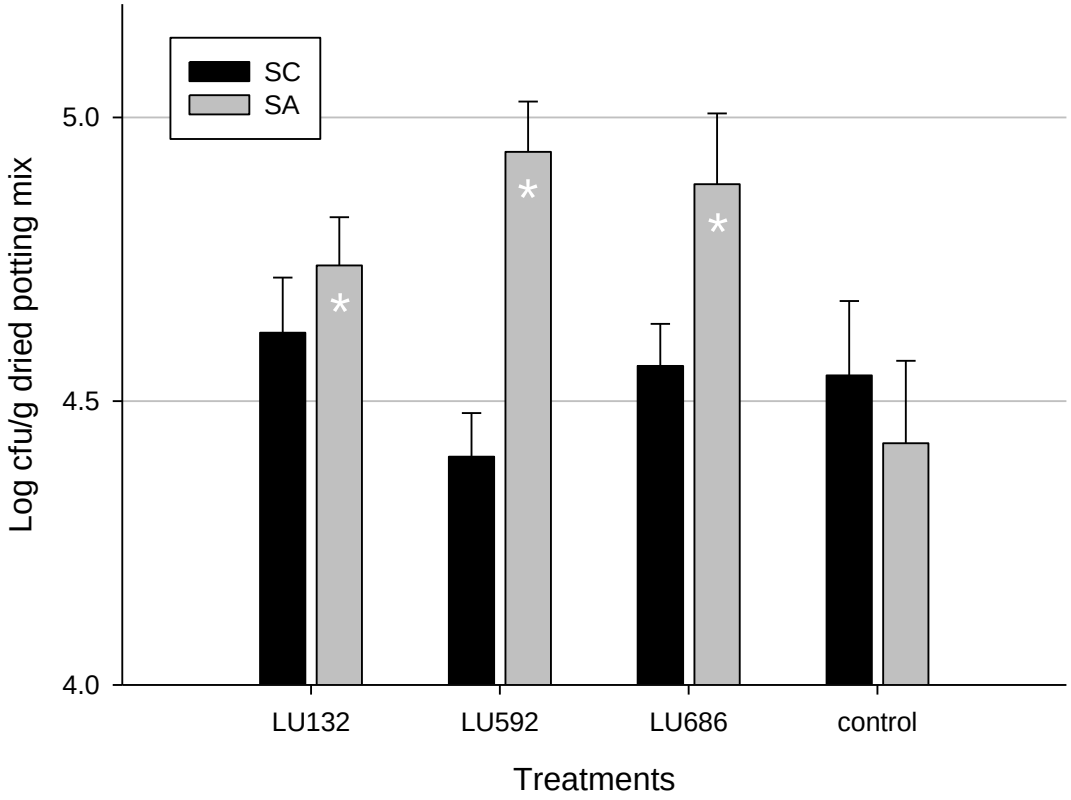


Figure 2.10 Overall *Trichoderma* population (log_{10} colony forming unit (cfu)/g dry potting mix) in the **rhizosphere** of *Pinus radiata* seedlings treated with three different *Trichoderma* isolates as either seed coat (SC) or spray application (SA) treatments (means of sampling times 2 – 6). Asterisks indicate significant differences to the respective control. Error bars represent the standard error of the means (n = 20).

Overall, *Trichoderma* populations increased in the rhizosphere between 4 and 8 weeks, decreased between 8 and 12 weeks (during seedling transfer from the glasshouse to the shade house) followed by a second increase between 12 and 20 weeks (**Figure 2.11**). At 4 wpi, the *Trichoderma* populations recovered from SA592 and SA686 treatments were significantly higher compared with the SAcontrol by 5.5 and 6.4 times, respectively. Of these treatments, only *Trichoderma* populations in the rhizosphere of SA686 treated seedlings remained at relatively high levels until 12 wpi. *Trichoderma* cfu levels in the rhizosphere of LU686 treated seedlings (SC and SA combined) were significantly higher than the control (SC and SA combined) at 4 and 8 wpi. *Trichoderma* populations in the rhizosphere of SA592 treated seedlings increased after 12 weeks being

significantly different to the control at 28 wpi (3.8 x 10^5 cfu/g dry potting mix), being 10.3 times higher than SAcontrol. From 8 wpi onwards, no significant differences were observed between the SC and SA application.

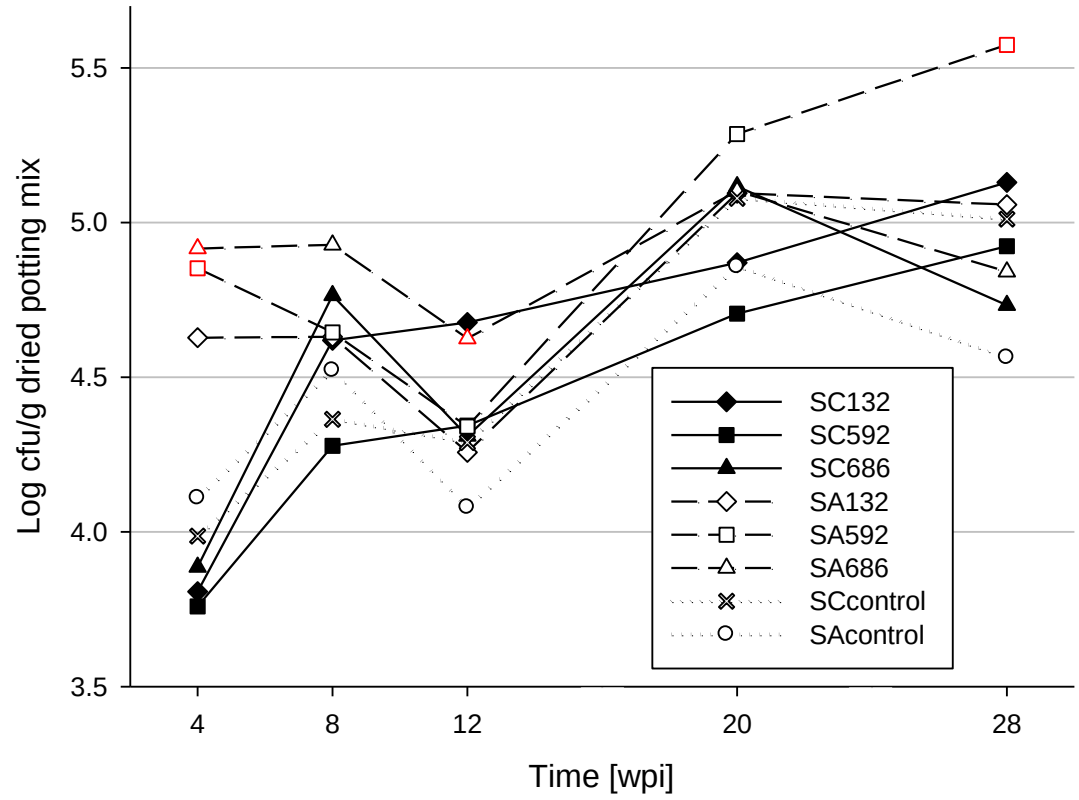


Figure 2.11 Mean *Trichoderma* population (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from the **rhizosphere** of *Pinus radiata* seedlings treated with three different *Trichoderma* isolates (LU132, LU592 and LU686) as either seed coat (SC) or spray application (SA) treatments over time. Red values indicate significant differences to the respective control within each sampling time (n = 4). Wpi = weeks post-inoculation.

Overall *Trichoderma* population means (over time) were not different between treatments in the rhizoplane and ranged from 1.5 x 10^5 cfu/g dry potting mix for SA686 to 4.9 x 10^5 cfu/g dry potting mix for SAcontrol. At 8 wpi, *Trichoderma* cfu levels for SA132 were significantly lower than for the SAcontrol by 74% (**Figure A2.2, Appendix 2**). At 12 wpi, *Trichoderma* populations for SA592 did not significantly decrease and were significantly higher compared to the SAcontrol by

6.0 times. At 28 wpi, the only treatment with significantly lower *Trichoderma* populations compared to the control was SA686 which was 86% lower. *Trichoderma* could be recovered from both surface-sterilised lateral and tap roots throughout all treatments from 8 wpi onwards. No *Trichoderma* grew out of the roots at the 4 wpi assessment.

2.3.1.4 Morphological and Molecular Characterisation

Trichoderma colonies appearing on TSM-LU plates at 20 wpi were morphologically characterised in an attempt to differentiate the introduced isolates from the indigenous *Trichoderma* population. Treatment replicates were not kept separate due to the large number of subculture plates to be processed. Therefore, statistical analyses were not possible and the results can only be used as indicator of the presence or absence of the introduced isolates. *Trichoderma* isolates growing on ½ PDA+c displayed six morphologies with the different morphological groups designated as A, B, C, D, E1 and E2 (**Figure 2.12**). The morphology groups A – D showed uniform and distinct characteristics, whereas groups E1 and E2 showed a wide range of morphological characteristics and were considered to be inconsistent and indistinct. Representative colonies of each morphology group (depending on morphology consistency: three replicates for groups A, B and C, five replicates for D, 15 replicates for E1 and 21 replicates for E2) were sequenced to identify *Trichoderma* at the species-level (**Table 2.3**).

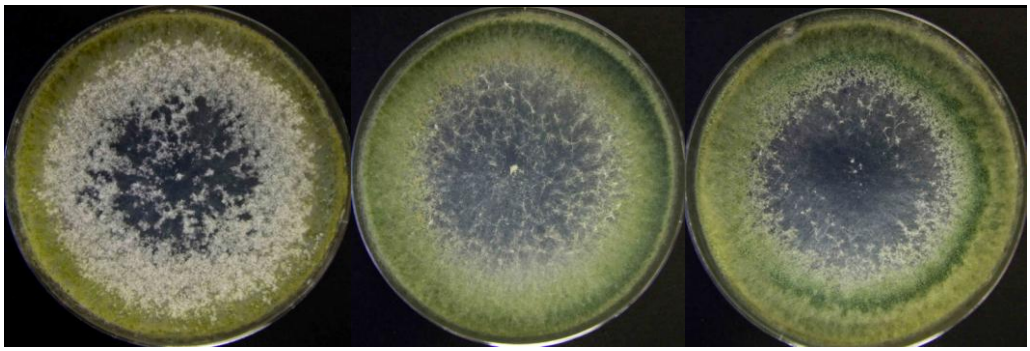
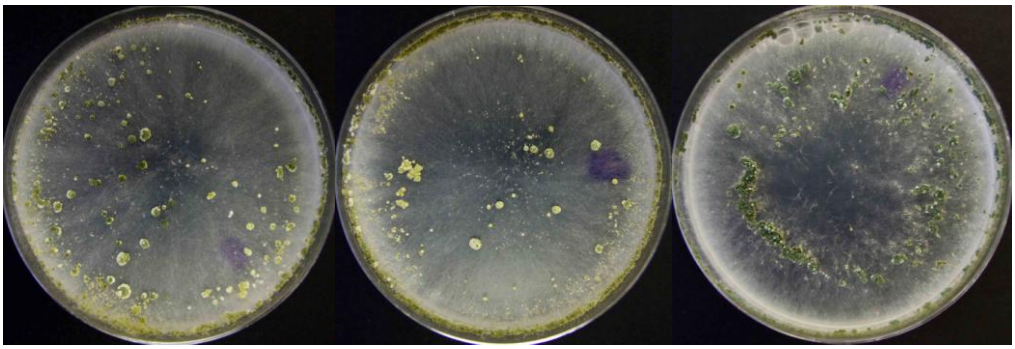
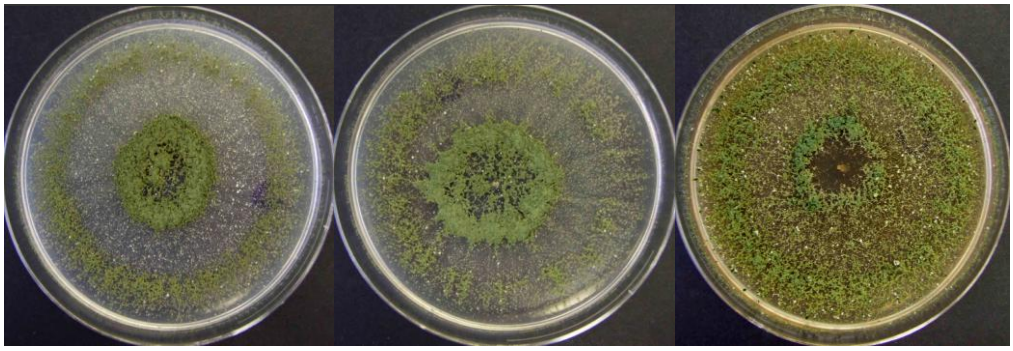
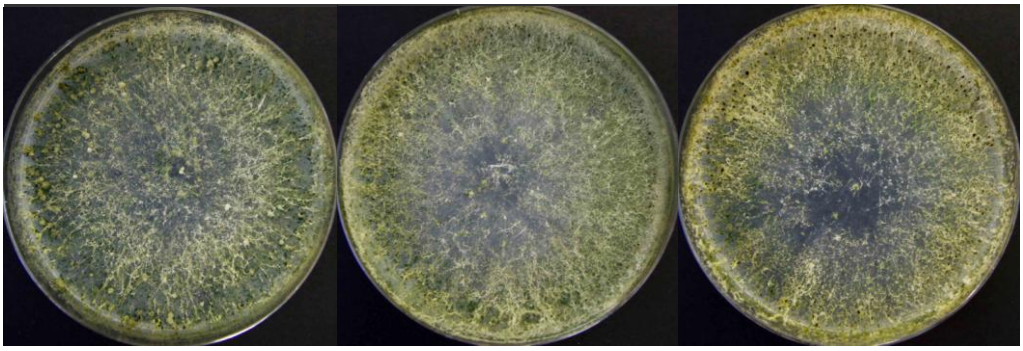
Morphology group	*Trichoderma* morphologies
A	
B	
C	
D	

Figure 2.12 Representative *Trichoderma* isolates illustrating the range of morphologies for each of the morphological groupings (A - E2) and comparisons to pure cultures of *T. atroviride* LU132, *T. hamatum* LU592 and *T. harzianum* LU686 obtained after 14 d incubation on ½ PDA+c.

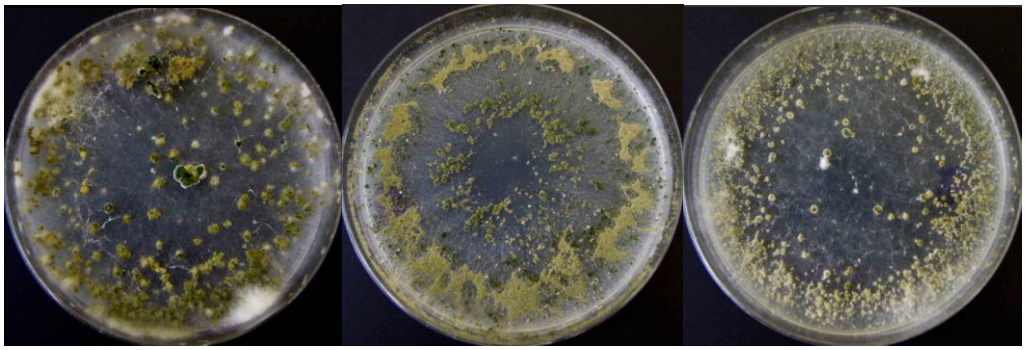
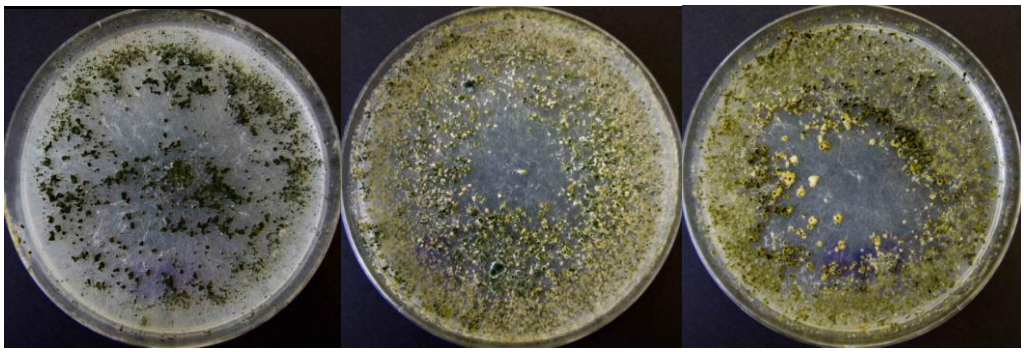
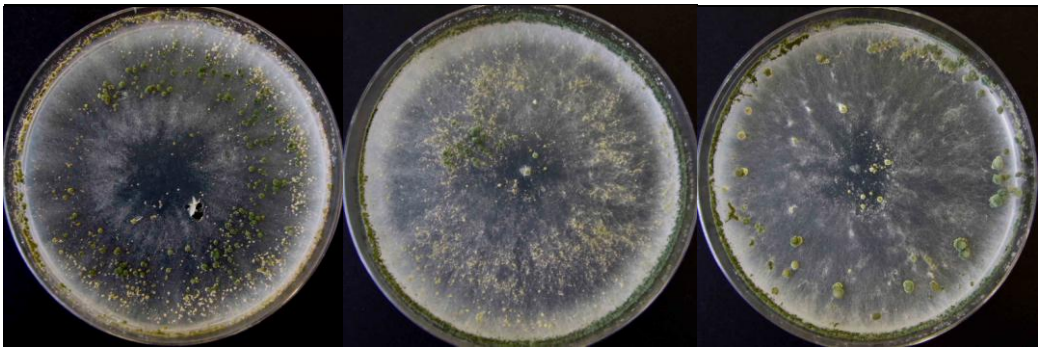
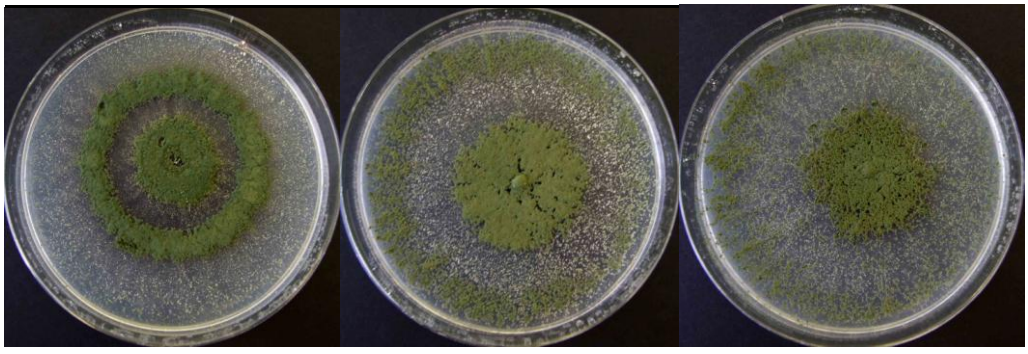
Morphology group	*Trichoderma* morphologies
E1	
E2	
LU132	
LU592	
LU686	

Figure 2.12 Continued.

Table 2.3 Sequencing results for *Trichoderma* strains isolated from experiment 1 after 20 weeks.

Morphology group	No. of replicates	*Trichoderma* species	ITS Gene bank accession no.	Max. identity	Reference
A	3/3	*T. atroviride*	AF456918.1	100%	Dodd *et. al.* 2001
B	3/3	*T. hamatum*	EU280105.1	100%	Hoyos *et. al*. 2007
C	3/3	*T. harzianum*	AF345948.1	100%	Samuels *et. al.* 2002
D	4/5	*T. asperellum*	AJ230668.1	100%	Lieckfeldt *et. al.* 1999
	1/5	*T. asperellum*	AJ230669.1	100%	Lieckfeldt *et. al.* 1999
E1	9/16	*T. hamatum*	EU280105.1	100%	Hoyos *et. al*. 2007
	7/16	*T. viride*	AF486010.1	100%	Kubicek *et. al.* 2003
E2	16/20	*T. viride*	AF486010.1	100%	Kubicek *et. al.* 2003
	2/20	*T. atroviride*	AF456918.1	100%	Dodd *et. al.* 2001
	1/20	*T. viridescens*	EU280104.1	100%	Hoyos *et. al*. 2007
	1/20	*T. saturnisporum*	AF359403.1	99%	Park *et. al.* 2001
LU132	3/3	*T. atroviride*	AF456918.1	100%	Dodd *et. al.* 2001
LU592	3/3	*T. hamatum*	EU280105.1	100%	Hoyos *et. al*. 2007
LU686	3/3	*T. harzianum*	AF345948.1	100%	Samuels *et. al.* 2002

Morphology groups A, B and C showed clear similarities with the morphologies of LU132, LU592 and LU686, respectively. DNA sequencing of the ITS1 region confirmed these morphology groups to be *T. atroviride*, *T. hamatum* and *T. harzianum*. Similarly, morphology group D was identified as *T. asperellum*. The previously described inconsistent morphology groups E1 and E2 showed less reliabilty. For E1, 9 out of 16 colonies were identified as the species *T. hamatum* and the remaining 7 colonies as *T. viride*. Most of the colonies of the morphology groups E2 (16/20) were identified as *T. viride*, two colonies as *T. atroviride*, one as *T. viridescens* and one as *T. saturnisporum* (less reliable with max. identity of 99%).

Based on the information provided from morphological and molecular characterisation, the morphology groups A, B and C are assumed to be the introduced isolates *T. atroviride* LU132, *T. hamatum* LU592 and *T. harzianum* LU686, respectively. Morphology group D was identified as the species *T. asperellum*. E1 is designated as *T. viride/hamatum* and E2 as *T. viride*.

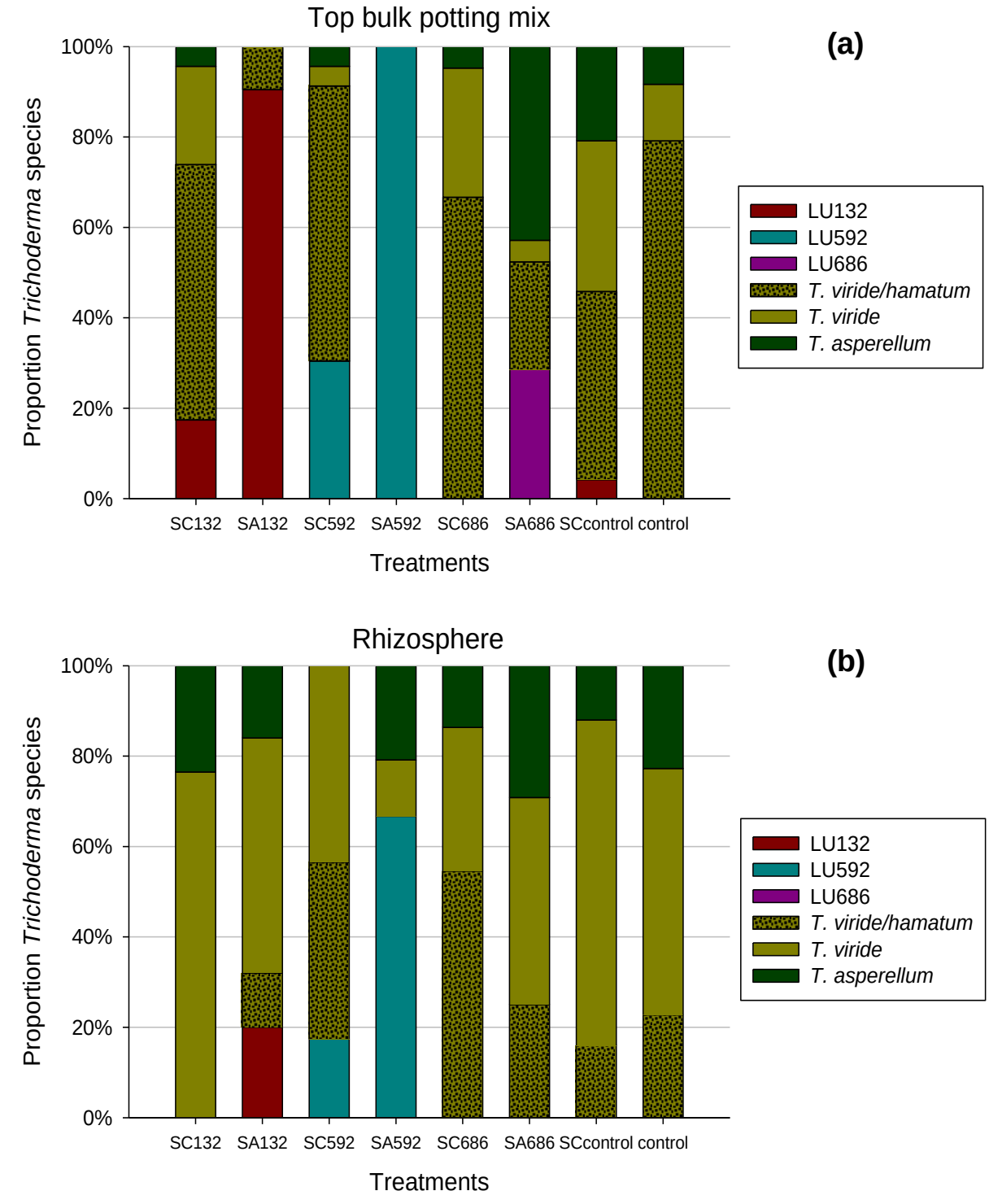


Figure 2.13 Estimated proportion of each morphology group recovered from the **top bulk** potting mix (a) and rhizosphere (b) of *Pinus radiata* seedlings treated with three different *Trichoderma* isolates (*T. atroviride* LU132, *T. hamatum* LU592 and *T. harzianum* LU686) as either a seed coat (SC) or spray application (SA) treatments 20 weeks post inoculation.

Isolates with morphologies characteristic of the introduced *Trichoderma* species were found in all SA treatments in the top bulk potting mix with the proportion of the total *Trichoderma* levels being 90% and 100% for SA132 and SA592, respectively (**Figure 2.13**a). *T. harzianum* established with a proportion of 29% for SA686. For the seed coat treatments, the introduced isolates established with levels of 17% and 30% for SC132 and SC592, respectively. Colonies characteristic of *T. harzianum* were not recovered from SC686.

In the rhizosphere, the introduced *Trichoderma* species established with 20% and 67% of the total cfu for SA132 and SA592, respectively (**Figure 2.13**b). Colonies characteristic of *T. harzianum* were not recovered from either the seed coat or the spray application treatments of LU686. When applied as a seed coat formulation, only colonies characteristic of *T. hamatum* LU592 were found to be present in the rhizosphere with a proportion of 17%.

Similar results to the rhizosphere were detected for the rhizoplane with the proportions for the respective introduced species in the spray application treatments being 25% for SA132, 58% for SA592 and 4% for SA686 (**Figure 2.14**a). For the seed coat treatments, proportions of 4% and 23% were detected for SC132 and SC592, respectively. *T. harzianum* was not recovered from SC686 treated seedlings.

Trichoderma hamatum when applied as a spray application clearly dominated all four subsystems and was the only species out of the introduced ones which was found within the roots (92%; **Figure 2.14**b). The majority of the indigenous *Trichoderma* species were identified as *T. viride*, *T. asperellum* and *T. hamatum* for all subsystems.

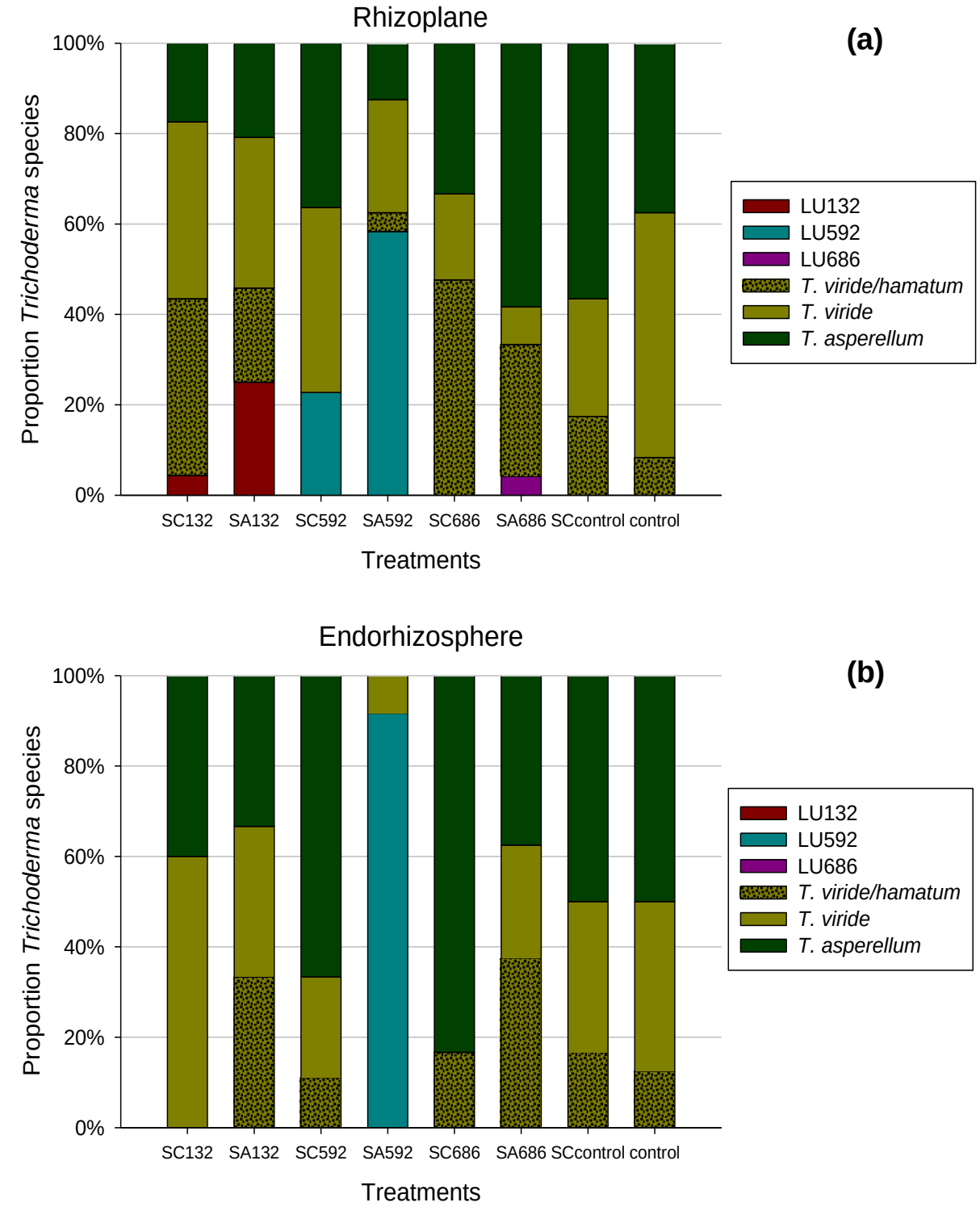


Figure 2.14 Estimated proportion of each species recovered from the **rhizoplane** (a) and **endorhizosphere** (b) of *Pinus radiata* seedlings treated with three different *Trichoderma* isolates (*T atroviride* LU132, *T. hamatum* LU592 and *T. harzianum* LU686) as either a seed coat (SC) or spray application (SA) treatments 20 weeks post inoculation.

2.3.2 Experiment 2: Verfication of *P. radiata* Growth Promotion by *T. hamatum* LU592

2.3.2.1 *Trichoderma* Application

As for the previous experiment 1, the proportion of germinated spores was determined for the *T. hamatum* LU592 spray application. Spore viability was similar to the previous experiment 1 with a germination rate of 98.5% which equates to 4.9×10^6 spores/pot. The number of viable *Trichoderma* spores per seed was determined as being 2×10^4 spores/seed.

2.3.2.2 Plant Vitality Assessments

Pinus radiata seedling emergence did not differ between treatments. After 2 months, seedling emergence was 83.1%, 84.3% and 84.4% for SA592, SC592 and the control, respectively (**Table A2.5, Appendix 2**). The assessment was repeated after 4 months. The proportion of emerged seedlings significantly increased for all treatments compared to the 2 months assessment, but again emergence was not influenced by *T. hamatum* LU592 and was 93.2% for SA592, 94.1% for SC592 and 93.8% for the control.

Seedling shoot height was assessed three times at 2-month intervals. Both *Trichoderma* treatments significantly increased growth rates compared with the untreated control at all assessment times. Differences in shoot height compared with the control became more obvious over time, with 3.2% and 5.5% increases at 2 months post-inoculation (mpi) and up to 7.4% and 9.5% higher seedlings at 6 mpi for SC592 and SA592, respectively (**Figure 2.15**i). Similarly, *P. radiata* stem diameter significantly increased by 8.9% and 9.4% after 4 months for SC592 and SA592, respectively (**Figure 2.15**iii). Shoot and root dry weights of the *Trichoderma* treated seedlings were also significantly higher with 22.5% and 24.4% increase in shoot dry weights and 16.8% and 20.7% increase in root dry weights for SC592 and SA592, respectively (**Figure 2.15**iv). A higher root/shoot ratio was determined for SC592 with a 6.4% increase after 2 months and a 5.4% increase after 4 months compared to the untreated control (**Table A2.5, Appendix 2**). SA592 also significantly increased the root/shoot ratio after 2 months by 4.2%.

The number of root tips assessed after 2 months was significantly higher than the control by 11.1% when treated with SC592.

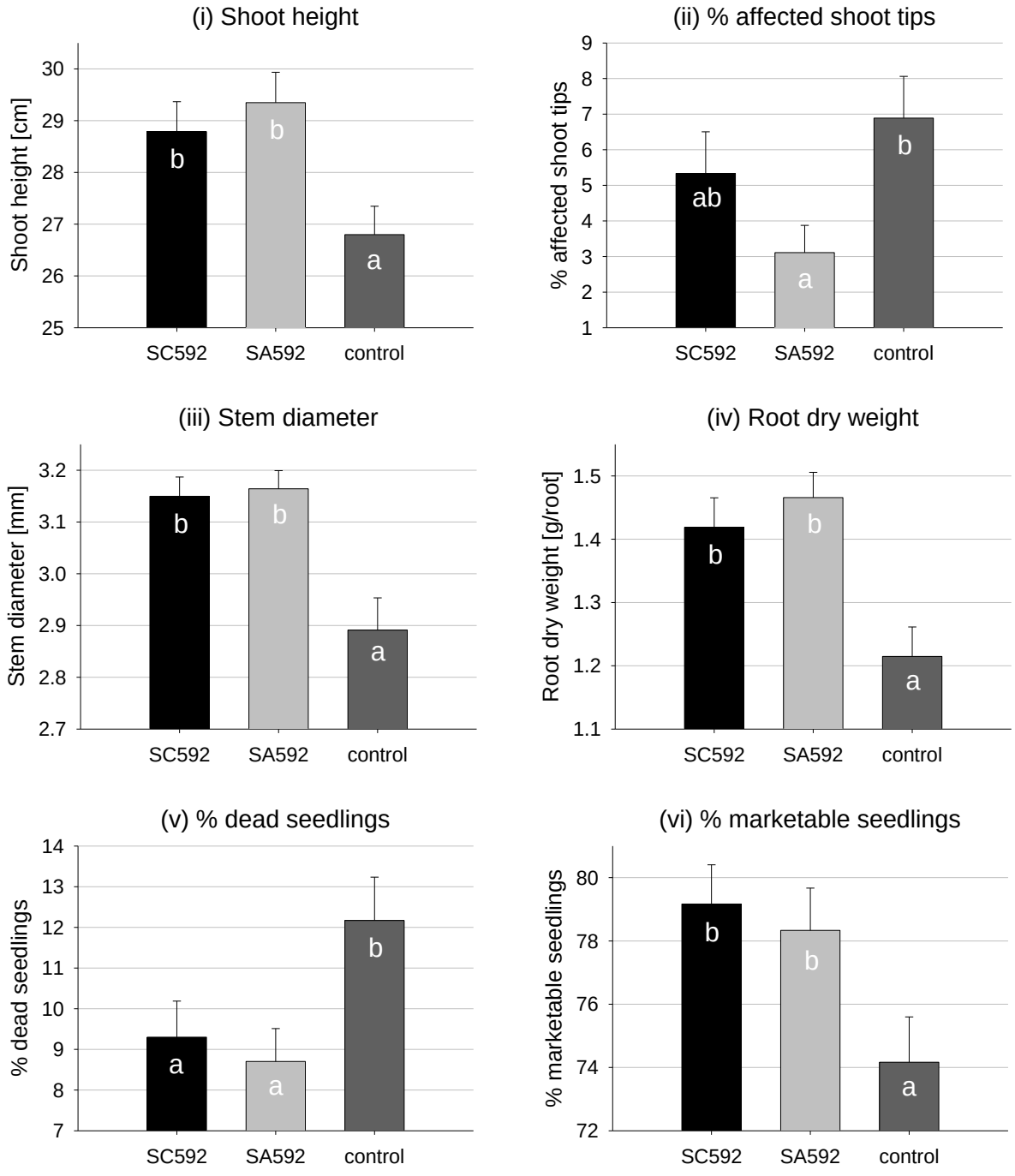


Figure 2.15 Plant performance assessments after treatment with *T. hamatum* LU592 either as seed coat (SC) or spray application (SA) and the control at different times of *P. radiata* seedling growth: 4 months post-inoculation (mpi; i, ii), 6 mpi (iii, iv) and 9 mpi (v, vi). Bars with different letters are significantly different. Error bars represent the standard error of the means (n = 60 for i, v and vi; n = 30 for ii, iii, iv).

After 4 months, seedling shoot tips were observed to be deformed. Some shoot tips were bent and shoot tip needles crinkled (**Figure 2.16**). Samples were sent to Margaret Dick (Scion, Rotorua) who associated these symptoms with a possible onion thrips infestation caused by *Thrips tabaci*. Therefore, shoot tip health was evaluated to identify potential differences between treatments. There were no significant differences between treatments in the shoot health scores which ranged between 2.26 for the control and SC592 and 2.40 for SA592 (**Table A2.3, Appendix 2**). Analysis of the proportion of seedlings with severely affected tips (score <2) also showed no significant effects between treatments with 4.4% severely affected shoot tips for SA592, 8.9% for SC592 and 12.2% for the untreated control. Two months later, the assessment was repeated with the seedlings having significantly recovered across all treatments. Significantly fewer seedlings had severely affected shoot tips in treatment SA592 with 3.1% compared to the untreated control with 6.9%. (**Figure 2.15**ii).

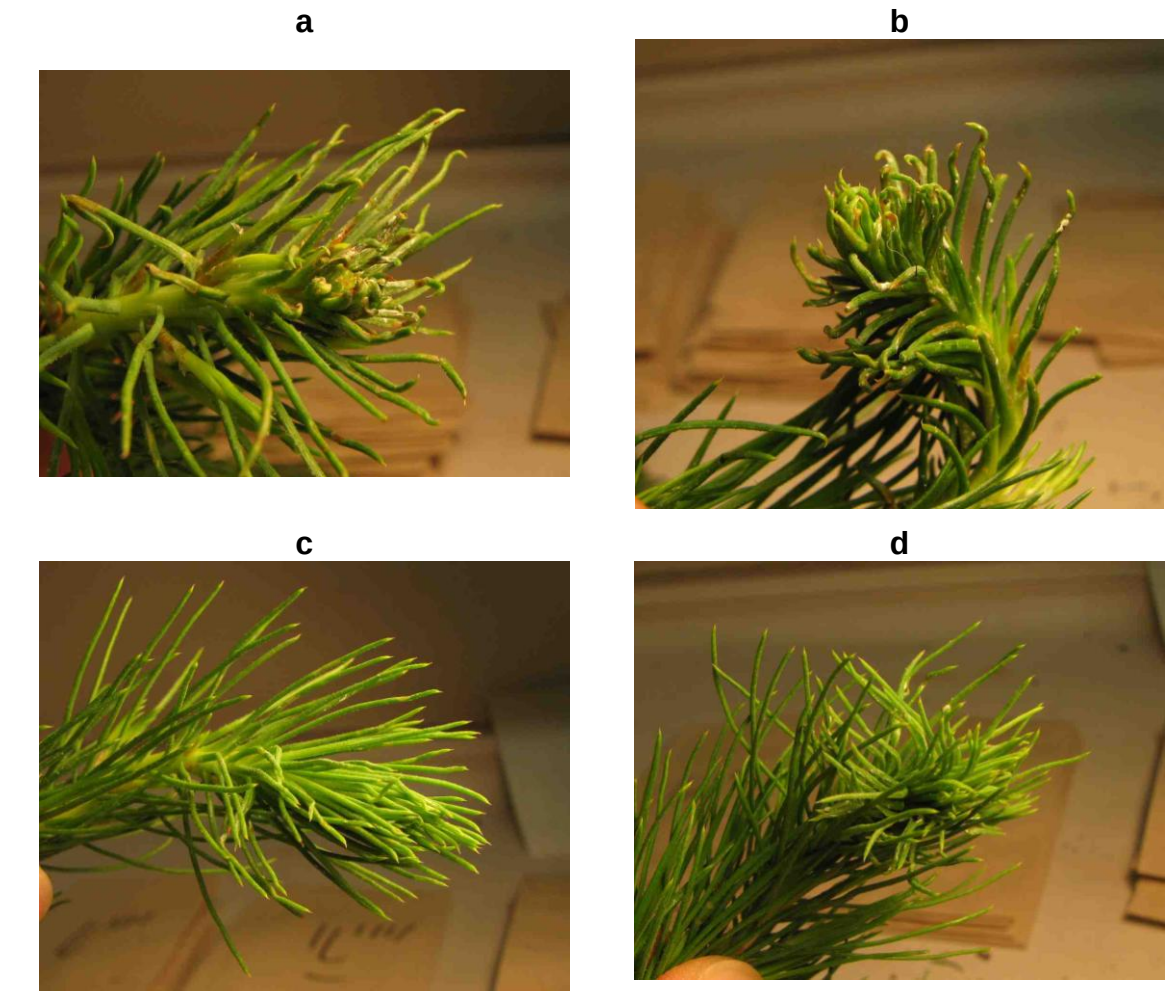


Figure 2.16 Photographs of representative seedlings (4 months old) illustrating deformation of shoot tip and needles (presumably caused by *Thrips tabaci*). Score 1 for severe damage (a, b) and score 2 for less severe symptoms (c, d).

The qualitative assessment of ECM root coverage revealed significantly higher scores when treated with *T. hamatum* LU592. The root coverage scores were 1.78 and 1.88 for SC592 and SA592, respectively, compared with the control score of 1.20. **Figure 2.17** shows representative root systems ranging from extensive ECM coverage (**Figure 2.17**a; Score 3) and no visible ectomycorrhizal structures (**Figure 2.17**a; Score 0).

Seedling mortality and proportion of marketable seedlings (meeting PF Olsen nursery specifications) were determined after 9 months. Seedling mortality rate was significantly reduced from 12.2 % for the control to 9.3% and 8.7% for SC592

and SA592, respectively (**Figure 2.15**v). The number of marketable seedlings significantly increased with 6.7% and 5.5% more marketable seedlings after 9 months when treated with SC592 and SA592, respectively (**Figure 2.15**vi). The proportion of marketable seedlings for the control was 74.2%.

Figure 2.17 Representative root systems (4 months old) illustrating the different stages of ectomycorrhizae (ECM) coverage. Score 3: Ectomycorrhizal formations throughout the entire root system (a). Score 2: Coverage of the upper half of the root (b). Score 1: Sparse ECM colonisation on the upper part of the root (c). Score 0: Virtually no ECM found (d). White arrowheads show typical ECM colonised root tip.

c

d

Figure 2.17 Continued.

2.4 Discussion

This study successfully identified a growth promoting and rhizosphere competent *Trichoderma* isolate in the root ecosystem of *P. radiata* seedlings. *Trichoderma hamatum* LU592 was the most promising bio-inoculant based on its benefical biological impacts on *P. radiata* seedling's growth and health and its ability to proliferate and establish in all assessed niches of the plant root system. LU592 was the predominant strain within a rich *Trichoderma* community in the *P. radiata* rhizosphere, rhizoplane and endorhizosphere and improvements of various health and growth factors were confirmed. However, a high indigenous *Trichoderma* population with similar population dynamics to the introduced isolates revealed the limitations of the dilution plating technique. It was also shown that the assessment of growth promotion effects is subject to high variability.

***Trichoderma* Application**

The inoculum concentrations were chosen according to the standard procedures at the PF Olsen nursery. As a result, the inoculum rates between SC and SA differed by a factor of 10 and direct comparisons between the two application methods in terms of *Trichoderma* population dynamics might be misleading. However, these concentrations resulted in best growth promotion effects of *Trichoderma* spp. in previous studies (GroChem NZ Ltd, commercial trial). Considering that the SC method introduces the isolate in a more concentrated form, it is believed that inoculum densities, rather than absolute numbers are the driving factor for the isolate's establishment. This assumption is supported by previous reports demonstrating that initial population distribution and density determine the success or failure of a microorganism to establish in a new environment (Bonde *et al.*, 2004; Aspray *et al.*, 2006; Kadam & Velicer, 2006).

A spore viability assay is a standard way to identify potential negative impacts of an application method on spore germination efficiency. For the spray applications, the germination rates of all three isolates remained at high levels of >89%. Initially, a spore viability assay for the seed coat preparation was planned. However, high background levels of other fungal communities impeded the accurate selection

and counting of *Trichoderma* spores and germ-tube length measurements (**Figure A2.1, Appendix 2**). In future, a small aliquot of seeds should be surface-sterilised before the coating procedures to overcome this problem. The spore concentrations on the seed surface were up to 10 times lower than expected (4 x 10^5 spores/seed were applied). However, it was shown that *Trichoderma* grew from the seed surface onto PDA after being washed. This indicates an incomplete removal of spores from the seed surface. In addition, loss of spore viabilty during the seed coating process could have occured.

Plant Performance

Some experiment-specific issues of experiment 1 need to be discussed in order to be able to comprehensively interpret the results of the health and growth assessments:

In experiment 1, an attempt was made to simulate the growing conditions of commercial New Zealand *P. radiata* nursery sites. In doing this, there were restrictions on the experimental design. Seedlings were grown in root-pruning containers with 45 seedlings/container (pseudo-replicates). Considering the detailed assessments and the dimension of this trial to be processed by one person, the scale of this experiment was limited to 1200 seedlings, having only four 'true' replicates per treatment. This was not enough to obtain the same analytical power as in the previous large-scale experiment in 2004 with 15,000 seedlings (Paderes *et al.*, 2006a). Further, potential heterogeneity of various factors such as irrigation led to large differences in variability between treatments. Even though all appropriate data-transformations were attempted, equal variances as required for ANOVA could not always be achieved.

The 7-month data of experiment 1 must be interpreted cautiously due to the following reasons: (1) Seedlings became root-bound in these small (100 mL) containers (**Figure 2.6**e**, Section 2.3.1.3**). Considering the small scale (4 replicates) of this experiment, 7 months were probably too late to identify subtle effects. (2) The temporary problems with the irrigation system for two weeks at 4 mpi negatively affected the 7-month assessments. A potential occurrence of edge effects and other causes of variation were taken into consideration for the PF Olsen experiment by excluding edge seedlings from the assessment and

increasing the number of replicates from 4 to 60 per treatment. In addition, root dry weight measurements proved to be difficult after 7 months due to the difficulties in separating the potting mix from the strongly enclosed root system. Therefore, root dry weight assessments of experiment 2 were carried out at an earlier stage, after 2 and 4 months, and the number of root tips were evaluated as an indicator of root development and branching. In future work, to enable root dry weight evaluations at later growth stages, root systems could be cut into smaller sections to enable the potting mix to be removed more easily. In conclusion, pot experiments under nursery conditions are subject to highly variable environmental conditions. Experiment 2 and the preceding experiment (Paderes *et al.*, 2006a) demonstrated that increasing the replicate number of an experiment is an effective means to deal with variable measurements. Therefore, it is suggested that separate experiments should be set up to study plant vitality assessments and fungal population dynamics. However, the size of the experiment would depend on the number of personnel available to harvest and process the samples.

Health scores have been used in the past to identify impacts of treatments on resilience to pathogen attacks and on the uptake of nutrients and water (R.A. Hill, pers. comm.). The experiments were set up in an environment with little infectious pathogen pressure and optimised water and nutrient supply. In experiment 1, a health factor of 3 (highest score) was given for most of the seedlings (between 90% and 97%). Consequently, all treatment means were close to 3 and statistical analyses could not identify major differences to the control. However, the treatment of *T. hamatum* LU592 achieved the best health score for 3-month old as well as for 7-month old seedlings. Papavizas (1981) also raised concerns about the use of health scores and concluded it could be a subjective method when an “arbitrary” scale is used to estimate early disease progression.

A more meaningful plant vitality measure proved to be the assessment of seedling mortality. Several factors may cause the death of a seedling including water and nutrient deficiencies or pathogen infection. The proportion of dead seedlings was reduced from 5.2% to 0.2% with application of the SC treatment of LU592. The large-scale experiment at the PF Olsen nursery confirmed these findings. Another robust measure was used to identify effects on plant vigour. Seedlings were assessed after 9 months by the use of the binomial indicator of marketable versus

non-marketable seedlings. In commercial nurseries, seedlings have to meet certain specifications before they can be sold and transplanted into forest plantations. Around 25% of seedlings in New Zealand nurseries do not meet these specifications (K. Haine, pers. comm.). The number of marketable seedlings was increased by up to 6.7% when *P. radiata* seeds were treated with *T. hamatum* LU592. This factor on its own increases the turnover of a tree nursery on the scale such as the PF Olsen nursery by ~NZ$56k per year. If this effect can be confirmed for bare-root seedlings, the increase in turnover could reach ~NZ$2.7M per year for New Zealand nurseries alone, based on the peak tree stock sales in 1996 (NZFAO, 2009).

Experiment 2 confirmed results from the previous large-scale experiment in 2004 (Paderes *et al.*, 2006a) showing a substantial increase in growth rate for up to 6-month old seedlings when treated with *T. hamatum* LU592. In contrast to experiment 1, the percentage differences at PF Olsen steadily increased from 2 to 6 months. In commercial nurseries, shoot height measurements are not relevant after 6 months because shoot tips are cut to maintain a favourable root/shoot ratio and stimulate the plant to develop a thick stem, resulting in more robust and vigorous seedlings (K. Haine, pers. comm.). For experiment 1, no significant growth promotion effects were detected, although shoot growth increased compared to the control by 20% and 14% for SC592 and SC686, respectively. This might be due to the above mentioned limitations of the experimental design. However, the performance of bio-inoculants is sensitive to environmental factors. Variability and inconsistency in the growth promotion effects on plants by *Trichoderma* spp. and other growth promoting microbes have been reported in the past (Rabeendran *et al.*, 2000; Dey *et al.*, 2004; Vestberg *et al.*, 2004; Algam *et al.*, 2005). Despite the fact that shoot tips are trimmed after 6 months, shoot growth promotion is a valuable indicator of a beneficial plant impact and must be acknowledged as evidence of an influential biological activity.

Similar to the shoot height results, effects of LU592 on the ratio of root and shoot dry weights and stem diameter were only detected in the large-scale experiment at the PF Olsen nursery. The stem width increased by ~9% when the seedlings were treated with either LU592 treatment. This is a desired outcome as thicker stems usually indicate more robust seedlings and result in a better establishment after

outplanting. Shoot and root dry weights increased by 24 and 21%, respectively, and the number of root tips increased by 11%. These are strong indicators of improved root development leading to more vigorous and consequently more valuable seedlings.

Ectomycorrhizal formations were found on roots in all treatments after 7 months in experiment 1. Qualitative assessments of ectomycorrhizal colonisation were conducted after 4 months in the PF Olsen experiment to identify potential impacts of *T. hamatum* LU592. The observations of both the reduction in the number of affected shoot tips and the higher coverage of ectomycorrhizal formations were not investigated further and are probably influenced by a general improvement in root development. This is supported by a previous study which showed that *Trichoderma* spp. did not directly affect the formation of ECM on *P. radiata* seedlings (R. Minchin, 2010). However, there was no correlation between the ECM score and root dry weights. What was then affecting the higher ECM root coverage for the *Trichoderma* treatments? One plausible explanation is based on a report by Zadworny *et al.* (2008) who reported that *Trichoderma* spores provide a P-source for the mycorrhizal fungi *Laccaria laccata* and *Suillus bovinus* on *P. sylvestris* roots. Both genera are known to colonise *P. radiata* in New Zealand (Orlovich & Cairney, 2004, Walbert *et al.*, 2010).

Trichoderma Population Dynamics

The *Trichoderma* population results of this study support recent reports that show *Trichoderma*'s general affinity to establish in the rhizosphere of plants (e.g. Harman *et al.*, 2004a; Miranda *et al.*, 2006; Longa *et al.*, 2009). *Trichoderma* spp. established at significantly higher levels in the rhizophere and rhizoplane compared to the bulk potting mix where the *Trichoderma* population declined. Further, *Trichoderma*'s capability to penetrate *P. radiata* roots was demonstrated. These are valuable attributes for a bio-inoculant (Yedida *et al.*, 2000; Harman, 2006).

In the rhizosphere, the *Trichoderma* population means in the SC132 and SC686 treatments increased substantially between 4 and 8 weeks compared with the control. Spray applications of LU132 and in particular LU686 resulted in high *Trichoderma* levels at that time indicating that these two isolates established in the

rhizosphere at an early stage. *T. hamatum* LU592 increased considerably after 12 weeks in the rhizosphere at the time when planting tray cells became root-bound with an abundancy of lateral roots in the centre and bottom section. These results suggested that *T. harzianum* LU686 was an early rhizosphere coloniser and *T. hamatum* LU592 a rhizosphere coloniser at a later stage of the nursery growing period. However, the limitations of the assessment method (difficulty in distinguishing introduced strains from background population, and potential discrepancy between population counts and actual fungal activity) do not allow definite conclusions about the isolates. Further, these indications of early rhizosphere competence could be an isolate-specific trait and, therefore, do not disprove previous reports of *T. harzianum* not being rhizosphere competent (Papavizas, 1981; Ahmad & Baker, 1987).

In the rhizoplane, overall *Trichoderma* populations of all treatments established at high levels (~5 x 10^5 cfu/g dry potting mix) towards the second half of the experiment. The highest variations within replicates across all treatments were found in the rhizoplane. Accurate measurements seem to be restricted to a short growing period, between the time when the roots were big enough to supply at least 1 g of rhizoplane up to the point when the roots become container-bound. In this experiment, it was decided to keep the root system intact to be able to process and transfer the roots more easily and minimise root loss. After 3 months, it was challenging to separate the rhizosphere from the roots and also, the rhizoplane may not have been fully shaken off the roots when the entire root system was shaken in only 90 mL of water. In future, sampled root systems should be cut into three or four parts. This will help to remove the rhizosphere potting mix and to let the roots float more freely while shaking. Further, it is recommended to shake roots in bigger volumes of water (e.g. 250 mL or 500 mL) for seedling root systems older than 3 months.

Trichoderma spp. were recovered from roots of all treatments after surface sterilisation between 8 and 28 wpi. *Trichoderma* spp. were not recovered from 4-week old seedlings (just emerged). The reasons for this might either be that (i) *Trichoderma* spp. start to penetrate roots at a later stage (8 wpi), or (ii) the root surface sterilisation method was too harsh for very young seedlings. It certainly demonstrates the effectiveness of the sterilisation method, which was strong

enough to eliminate all root surface-colonising *Trichoderma* spp. (4 wpi), and also sensitive enough not to affect *Trichoderma* spp. in the endorhizosphere once the root system matured.

Several factors prompted the attempt to identify the introduced isolates by morphological characterisation. As mentioned previously, high levels of indigenous *Trichoderma* with similar population dynamics to the introduced isolates were found. Further, high variability within replicates, particularly in the rhizoplane, often did not allow detection of significant differences to the *Trichoderma* populations in the control. Difficulties in interpreting colonisation dynamics of introduced *Trichoderma* isolates due to high indigenous *Trichoderma* populations have been reported earlier (Mousseaux *et al.*, 1998; Lo *et al.*, 1998).

Bourguignon (2008) successfully distinguished different *Trichoderma* at the species level on the basis of morphological characteristics. This approach was primarily used to verify the rhizosphere competence of the introduced isolates. It was also important to verify whether the *Trichoderma* colonies recovered from the endorhizosphere were introduced or indigenous strains. This method was particularly valuable for the identification of the introduced isolates. All three isolates showed uniform and distinct morphologies which were consistent with the morphologies of each isolate in pure culture. The morphologies of the indigenous isolates were less uniform and often variable, with the exception of the morphology of *T. asperellum* which also appeared to be uniform and distinct. More than 50% of morphology group E1 was identified as *T. hamatum* which appears to be an indigenous *T. hamatum* strain. Not only was this morphology quite distinctive from the introduced *T. hamatum* strain (see **Figure 2.12**), but was also recovered across all treatments. About 10% of the morphology group E2 could be identified as *T. atroviride*. Similarly, this morphology could be found throughout all treatments and subsystems and is unlikely to be the introduced isolate.

The morphological characterisation was carried out after 20 weeks. The results clearly verify the presence of the introduced isolates. Generally, the spray applications promoted the isolates' establishment. *T. harzianum* LU686 declined at this time which was already indicated by the overall *Trichoderma* population assessments. Both, *T. atroviride* LU132 and *T. hamatum* LU592 were predominant

in the top bulk potting mix when spray applied, but only LU592 was also clearly dominant in the rhizosphere (67%), rhizoplane (58%) and endorhizosphere (92%).

Conclusions

Trichoderma hamatum LU592 was the only introduced isolate predominant in all subsystems for 20-week old seedlings and was shown to increase various health and growth factors in two large-scale nursery experiments under commercial conditions. A significant growth promotion effect of LU592 was not detected in experiment 1. However, the vitality analyses were compromised by the previously mentioned limitations. Rhizosphere population densities of ~10^5 cfu/g dry potting mix for LU592 were similar to those reported for rhizosphere competent strains (Ahmad & Baker, 1987; Tsahouridou and Thanassoulopoulos, 2002). No biological impact of *T. atroviride* LU132 was observed on seedling's growth and health in two independent experiments (including the previous large-scale experiment in 2004; Paderes *et al.*, 2006a). LU132 appeared to be a weak rhizosphere coloniser of 20-week old *P. radiata* seedlings. However, LU132 has been shown to successfully colonise the rhizosphere of onion when applied with a food base (McLean *et al.*, 2005). It was reported that some *Trichoderma* isolates are subject to soil fungistasis when no additional nutrients were added (Bae *et al.*, 2000; Green *et al.*, 2001; McLean *et al.*, 2005) and this may be the case here. Growth promotion effects by *T. harzianum* LU686 were not shown in this study. The previous large-scale experiment detected growth promotion only as part of a mixture of six *Trichoderma* isolates. LU686 was barely detected in the rhizosphere after 20 weeks, although there are indications of LU686 being an early rhizosphere coloniser (highest overall levels within the first 12 weeks).

A relationship between high *Trichoderma* populations and plant growth promotion was reported in soil for a range of horti- and floricultural plants (Chang *et al.*, 1986) and in potting mix for *Ficus benjamina* (Srinath *et al.*, 2003) when treated with *T. harzianum*. However, these studies did not report any temporal dynamics of *T. harzianum* in these systems. Chacón *et al.* (2007) related qualitative observations of root colonisation and penetration to plant growth promotion by *T. harzianum* in *in vitro* grown *Nicotiana benthamiana* and hydroponically grown tomato plants (*Lycopersicum esculentum*). A positive correlation between root penetration of a *T.*

harzianum strain and biocontrol activity against *Sclerotium cepivorum* in field grown onion was observed for a period of 6 months (Miranda *et al.*, 2006).Similarly, a significant correlation between *Trichoderma* population levels in the rhizosphere and its ability to antagonise *S. rolfsii* and improve plant performance parameters was determined for axenically grown tomato plants when seed coated with a strain of *T. koningii* (Tsahouridou and Thanassoulopoulos, 2002).

In the present study, the spray application treatments clearly promoted the establishment of the introduced isolates. However, as previously discussed, there is no information as to whether this promotion was due to differences between the application methods or an effect of different inoculum concentrations (SC: 4×10^5 spores/pot vs SA: 5×10^6 spores/pot). Successful biocontrol activities have been reported for both seed coat and spray application (Mousseaux *et al.*, 1998; Bell *et al.*, 2000; Tsahouridou and Thanassoulopoulos, 2002; Vanneste *et al.*, 2002; Lu *et al.*, 2004). The seed coat application method has been shown to promote the establishment of *Trichoderma* spp. in critical niches such as the spermosphere and the developing rhizosphere (Green *et al.*, 2001). However, this method does not always result in sufficient *Trichoderma* levels in the rhizosphere (McLean *et al.,* 2005).

Both the seed coat and the spray application improved the plant performance equally in experiment 2. Why were there no differences in growth promotion even though clear differences in rhizosphere and root colonisation were identified? Is it really as simple as concluded by Baker & Cook (1974) that an effective bio-inoculant must produce abundant propagules to survive, grow and interact in the soil and rhizosphere? Lewis & Papvizas (1984) achieved *Trichoderma* population densities of up to 10^8 cfu/g in natural soils and high application rates of $>10^6$ spores/g of soil are not uncommon (Bell *et al.*, 2000; Rabeendran *et al.*, 2006) and even *Trichoderma* inoculum concentrations of 10^8 cfu/g dry matter are reported (Green *et al.*, 2001; Longa *et al.*, 2008). The decline of introduced *Trichoderma* populations in the rhizosphere after such high inoculum concentrations was sometimes associated with rhizosphere incompetence which can lead to discounting of potential bio-inoculants (Papavizas *et al.*, 1981; McLean *et al.*, 2005). However, R.A. Hill (pers. comm.) suggested that there is an optimum

inoculum concentration for *Trichoderma* based on a commercial *P. radiata* pot experiment in 2004 where three different spore application rates between 5 x 10^4 and 2 x 10^5 spores/g dry potting mix were tested (GroChem NZ Ltd, commercial trial). The beneficial impact on plant performance was highest when 5 x 10^4 spores/g dry potting mix were applied. Paulitz (2000) suggested that most effective biocontrol activity occurs at ~10^5 cfu/g root and higher BCA concentrations do not result in further improvements. Recently, Arcia & Pérez-Pivat (2008) demonstrated biological impacts in non-sterile systems at inoculum concentrations down to 10^2 cfu/g soil with an optimum impact at 10^4 cfu/g soil. In another study, *T. hamatum* showed similar biocontrol activity for two different application methods (transplant or maize-perlite mix) where the transplant application resulted in differences in the cfu numbers in the rhizosphere of lettuce roots by two orders of magnitude (Rabeendran *et al.*, 2006). However, it was shown that mycoparasitism was the mode of action of this BCA and thus this strain might not be as population dependent as those BCAs that work through competition (A. Stewart, pers. comm.).

It is still unknown whether proliferation of an introduced isolate is directly related to growth promotion effects. One assumption, based on the results of this study, is that both the seed coat and spray application inoculum concentrations of 4 x 10^5 spores/cell and 5 x 10^6 spores/cell, respectively, reflect reasonable application rates around the optimum range. They resulted in different colonisation patterns in experiment 1, but similar enough to show the same impacts on *P. radiata* growth and health in experiment 2. This is supported by the fact that both application methods resulted in reasonable establishment in the rhizosphere (17% for SC, 67% for SA), rather than being either not detectable or fully replacing the indigenous *Trichoderma* spp. Whereas, overdosing plant ecosystems can result in detrimental side-effects to the plant caused by competitive displacement of beneficial microorganisms, phytotoxicity or phytopathogenicity (Ousley *et al.*, 1993; Rousseau *et al.*, 1996; Brimner & Boland, 2003).

It was demonstrated that the different *Trichoderma* isolates can have different colonisation behaviours. This information can help to identify multi-strain formulations with synergistic effects. Whipps (2001) suggested combinations of bio-inoculants could perform superior to any one isolate used on its own,

particularly when agents are chosen with complementary modes of action or different colonisation behaviours in the root ecosystem. Similarly, Miranda *et al.* (2006) proposed multiple applications of different strains. Initially, they suggested introducing an early root coloniser, with an isolate with good soil dispersion capacity and activity against pathogens applied later in the growing cycle. However, the identification of such mixtures to improve plant performance has been poorly investigated (Harman, 2004). A better understanding of the diversity of colonisation patterns of different *Trichoderma* spp. is needed to be able to create successful mixtures.

In this study, only one potential bio-inoculant was identified. However, it is worthwhile to include LU686 in future screening trials. This early rhizosphere coloniser could complement LU592 which was shown to establish at a later stage. Future work would then be required to determine synergistic effects of combined applications of both LU592 and LU686. Another issue is whether LU592 would be better applied at a later stage in the seedling growing period (after 12 or 20 weeks) because its predominance within the *Trichoderma* community appears to peak around this time. However, even LU592 applied as a seed coat with weaker rhizosphere colonisation showed beneficial impacts on *P. radiata* seedlings at an early stage (2 month). It is not known whether “low” cfu concentrations can be related to weak rhizosphere colonisation and whether rhizosphere colonisation is the driving factor for growth promotion in the first place. Therefore, caution needs to be exercised when drawing conclusions based on cfu counts alone. Further, it is also unknown how *Trichoderma* impacts *P. radiata* growth when applied at low levels. Because an isolate cannot be detected with current detection methods, does not necessarily mean it is not present or has no impact. Chapter 4 experiments will address these issues by determining isolate-specific dynamics of a *gfp/hph*-tagged *T. hamatum* LU592 to help understand its population behaviour in more detail.

This study has contributed to a better understanding of the ecology of *Trichoderma* in the *P. radiata* root ecosystem. The *Trichoderma* population assessments provided initial valuable information which was examined more closely in Chapter 4 experiments. The dilution plating method has been proven to provide robust and consistent data for *Trichoderma* populations in this potting mix ecosystem. The

validity of this quantification method was recently verified by Cordier *et al.* (2007). They compared the enumeration of cfu by the dilution plating technique with number of target DNA copies by quantitative real time PCR (qPCR). In two soils, both methods gave similar results. In addition, limitations were encountered in distinguishing between introduced and indigenous *Trichoderma* isolates. This drawback has partly been overcome by estimation of species proportions through morphological characterisation. However, this approach is rather subjective when it is used to distinguish between isolates of the same species and is reasonably time consuming. Another disadvantage of the dilution plating method remains the unknown physiological state of the colony-forming unit and consequently insufficient knowledge of the biological activity of the introduced isolates (Green *et al.*, 2001). it needs to be confirmed whether LU592 really is a “late” rhizosphere coloniser. If so, further improvements could be made by the addition of an external food source to promote an early proliferation in the root system, particularly for the seed coat application. Promising results of improved distribution of *T. harzianum* with the addition of an external food source were reported in root ecosystems of horticultural crops (Chang *et al.*, 1986; Kleifeld & Chet, 1992; Bell *et al.*, 2000). However, other studies with shortleaf pine (Kelley, 1976), bean (Elad, 1980) peas (Campbell, 1989), and cucumbers (Wolffhechel & Jensen, 1992) documented the risk of attracting competing pathogens such as *Phytophthora*, *Pythium*, and *Rhizoctonia* when organic substrates are supplemented to *Trichoderma* inoculum preparations, causing increased disease incidences.

Chapter 3 will report the construction of a genetically transformed strain of LU592 carrying a fluorescent marker gene and an antibiotic resistant gene. This modified strain will be used in Chapter 4 experiments to enable LU592 to be distinguishable from the indigenous populations and provide some conclusions about the physiological state in the root zone at different times.

Chapter Three

Agrobacterium-mediated Transformation of *Trichoderma hamatum* LU592 with Two Marker Genes

3.1 Introduction

Traditionally, quantification of fungal biomass was attempted based on serial dilution and plate counts. However, two major drawbacks limit the use of dilution plating techniques. Firstly, the recovered propagules (mycelia, conidia, chlamydospores) cannot be distinguished and, therefore, are not necessarily a measure of biological activity (Warcup, 1955). High proportions of actively growing mycelium would suggest stronger bioactivity compared to high proportions of resting structures such as conidia and chlamydospores. Secondly, the specificity of the method relies on the medium on which the propagules are recovered. *Trichoderma* semi-selective media have been developed which allow preferential growth of *Trichoderma* spp., but this method still depends on a subjective assessment of the colonies. In most cases, *Trichoderma* colonies can be clearly distinguished from other genera of fungi. However, no established procedure is described to differentiate species within the genus *Trichoderma* on these selective media. This obstacle makes it nearly impossible to distinguish between introduced and indigenous *Trichoderma* isolates (Garrett, 1981; Lumsden *et al.*, 1990; Knudsen & Bin, 1990; Green & Jensen, 1995; Lo *et al.*, 1998). Other methods which partially overcome these drawbacks include the quantification of biochemical components of fungal cells, measurement of soil respiration, and direct microscopy (Parkinson & Coleman, 1991) or by the use of mutant strains resistant to specific toxicants (Ahmad & Baker, 1987). However, all of these methods lack in either specificity or correlation to biological activity.

In the last 10 to 15 years, major advances in ecological studies of filamentous fungi in plant ecosystems have been achieved by the use of marker genes inserted into the target organism's genome (Thrane *et al.*, 1995; Lorang *et al.*, 2001; Lübeck *et al.*, 2002; Sarrocco *et al.*, 2006). Constitutively expressed genetic markers offer new opportunities to monitor and quantify specific isolates in soil and other non-sterile environments. A novel marker system, the green fluorescent protein (GFP), was first successfully expressed in a filamentous fungus (*Ustilago maydis*) in 1996 (Spellig *et al.*, 1996) and three years later in *Trichoderma* (*T. harzianum*; Zeilinger *et al.*, 1999). *Gfp* (green fluorescent protein gene), together with an antibiotic resistance gene such as *hph* (hygromycin B phosphotransferase gene), give the fungus unique characteristics enabling (i) visualisation of fungal colonisation behaviour and (ii) selective recovery via dilution plating from soil/plant sample. For instance, Lu *et al.* (2004) successfully elucidated *Trichoderma*-plant-pathogen interactions on cucumber seeds using constitutive and inducible GFP marker systems. Similarly, a GFP-expressing *T. harzianum* strain was quantified in non-sterile soil by an image analysis approach (Orr & Knudsen, 2004) and *hph* was used to quantify *Trichoderma* in the rhizosphere and phylloplane of creeping bentgrass (Lo *et al.*, 1998).

Transformation of filamentous fungi with marker genes can be achieved by e.g. protoplast electroporation, particle bombardment or *Agrobacterium tumefaciens* T-DNA transfer (Zwiers & De Waard, 2001; Lorang *et al.*, 2001). The *Agrobacterium*-mediated transformation technique is considered superior to the others as the efficiency was shown to be up to 1000-fold higher for a range of different filamentous fungi including *Trichoderma* (De Groot *et al.*, 1998). More recently, *Agrobacterium*-mediated transformation in general resulted in higher transformation efficiencies compared to polyethylene glycol-based protoplast transformation for four different *Trichoderma* spp. representing three of the four sections contained in this genus (Cardoza *et al.*, 2006). Moreover, conventional protoplast or biolistic transformation protocols shown to be reliable for one species (*T. reesei*; Suominen *et al.*, 1993) might not be easily adapted for other species such as *T. harzianum* and *T. atroviride* (Woo *et al.*, 1999; Peterbauer *et al.*, 2002). This issue became even more evident when pleiotropic effects were observed. Whereas, *Agrobacterium*-mediated transformation mainly results in single copy

integrations and, therefore, lowers the risk of pleiotropic effects and also reduces the chance of gene disintegration. The gene transfer is efficient for intact cells, making the step of protoplast production redundant (Zeilinger *et al.*, 1999; Zwiers & De Waard, 2001).

In this study, Zeilinger's (2004) protocol for *Agrobacterium*-mediated transformation of a *T. atroviride* strain was used to transform *T. hamatum* LU592. This study reports on the successful integration of the two marker genes *gfp* and *hph* into the genome of *T. hamatum* LU592. The transformation was verified using four indicators: (i) growth on hygB-amended medium, (ii) visualisation of the green fluorescence under excitation, (iii) *gfp*-targeted polymerase chain reaction (PCR) and *hph*-targeted Southern hybridisation as molecular-based confirmations and (iv) 10x subculturing on non-selective medium to verify the mitotic stability of the insertion. In addition, the ecological fitness was compared to the wild type to confirm phenotypic similarity. Ultimately, the transformed isolate was visualised in and recovered from non-sterile potting mix.

3.2 Materials and Methods

3.2.1 GFP Vector Construction

The construction of the binary vector was carried out by M. Carpenter and is described in Carpenter *et al.* (2008). The general method is outlined below.

A binary vector for constitutive expression of GFP (p0300GFP) was constructed by digesting pCT74 as described in Lorang *et al.* (2001) with *Eco*RI and *Kpn*I (Fermentas, USA) to remove the *hph* and *gfp* cassettes onto a single fragment which was then cloned into the multiple cloning site (MCS) of p0300. p0300 was made from pCAMBIA 2300 by digestion with *Bst*XI and XhoI (New England Biolabs, USA) to remove the NPTII coding region and CaMV 35S promoter. The fragments were blunt-ended with T4 DNA polymerase and religated to produce the binary vector p0300 in which the only sequences between the left and right borders was the MCS in the LacZ alpha gene and the redundant CaMV 35S terminator. The p0300GFP vector was then transformed into *Agrobacterium tumefaciens* EHA105 by electroporation and transformants were selected on Luria Bertani broth (LB broth; Bio101, USA) agar amended with 50 µg/ml kanamycin and 10 µg/ml rifampicin (LB+kan+rif; Sigma-Aldrich® Inc., USA).

3.2.2 Hygromycin B sensitivity of *T. hamatum* LU592

Prior to transformation, a hygromycin B (hygB) sensitivity assay was carried out to determine the concentration of hygB at which mycelial growth of wild type *T. hamatum* LU592 was fully inhibited. Discs (3 mm) cut from the edge of actively growing colonies of LU592 on potato dextrose agar (PDA; Difco™ Laboratories, USA) were placed in the centre of hygB-amended PDA (three replicates) and mycelial growth was recorded daily. The concentrations tested were: 50, 100, 200, 300 and 500 µg/mL PDA.

3.2.3 *Agrobacterium*-mediated Transformation Protocol

The method was based on the transformation procedure described by Zeilinger (2004) with the following modifications. Five millilitre of LB+kan+rif was inoculated with the *Agrobacterium* strain carrying the p0300GFP plasmid and cultured

overnight at 28ºC shaking at 250 revolutions per minute (rpm). Two times 10 mL of minimal medium+kan+rif were inoculated with 0.1 mL of the overnight culture and incubated overnight at 28ºC and 250 rpm Then, the culture was centrifuged at 3000 *g* for 10 min at room temperature (RT) and the pellet resuspended in induction medium acetosyringone (IMAS; Sigma-Aldrich® Inc., USA) at OD_{660} = 0.15. The resuspension was then transferred into a wide-bottom conical flask and incubated at 28ºC and 70 rpm (Orbital Mixer Incubator, Ratek Instruments Pty. Ltd, Australia) for at least 6 h until an OD_{660} of 0.25 – 0.3 was obtained. A pure spore-suspension of 5 x 10^8 spores/mL was obtained from a 1-week old sporulating *Trichoderma* colony on PDA (**Section 2.2.2.2**) and adjusted to the required concentration. The *Agrobacterium* culture was mixed with a spore suspension of 10^8 spores/mL in a ratio of 2:1 V:V. Three hundred microlitre aliquots were then plated on sterile cellophane on IMAS plates (60) and incubated at 22ºC for 2 d. PDA plates and ½ strength PDA for overlay were prepared, both amended with 300 µg/mL hygB and 200 µg/mL timentin (tim; GlaxoSmithKline Plc., UK). The cellophane was then transferred to the prepared PDA plates, overlaid with 4 ml ½ strength PDA+hygB+tim (>40ºC) and incubated at 22ºC for 3 to 10 days. Emerging *Trichoderma* colonies were transferred onto fresh PDA+hygB+tim plates and incubated at 22ºC.

3.2.4 Verification of Stable Transformants

Trichoderma colonies that grew after the first transfer to PDA+hygB+tin, were subcultured twice onto the same type of agar. Actively growing mycelium of the third subculture was scraped off for DNA extraction using the Puregene® Genomic DNA Purification Kit (Gentra Systems Inc., USA) (**Section 2.2.2.7**) and the presence of *gfp* was verified by PCR (**Appendix 1**). The primers GFPIDfwd (5`-ACGTAAACGGCCACAAGTTC-3`) and GFPIDrev (5`-TGCTCAGGTAGTGGTTGTCG-3`) were used. The resulting PCR products (546 bp expected) of extracted DNA from LU592 wild type (negative control), LU592 transformants and p0300GFP plasmid DNA (positive control) were then visualised by gel electrophoresis (**Appendix 1**). In addition, GFP fluorescence was detected

using epifluoroscence microscopy (Olympus UIS2 fluorescent microscope[1], Olympus New Zealand Ltd, Auckland, New Zealand). Transformants which were positive for *gfp* were purified by three rounds of single-spore isolation (**Appendix 1**) on PDA-hyg and spores were stored long-term in 25% glycerol at -80ºC after each purification. Subsequently, Southern hybridisations were carried out as described by Sambrook *et al.* (1989) (**3.3.5 Southern Hybridisation Analysis**). In addition, mitotic stabilisation was tested by subculturing actively growing hyphae 10 times onto non-selective PDA. Growth rates of the subcultured transformants were then compared with the -80ºC pure cultures on hygB-amended PDA.

3.2.5 Southern Hybridisation Analysis

3.2.5.1 DNA preparation

Two millilitre of a conidial suspension (previously stored in 25% glycerol at -80ºC) of LU592 wild type and all LU592 transformants were used to inoculate 5 x 25 mL potato dextrose broth (PDB; Difco™ Laboratories, USA) in 50 mL Falcon tubes and incubated at 20ºC in the dark while gently shaken. After 3 days, the solution including a dense mycelium mat was filtered through sterile miracloth, PDB was squeezed out and mycelium immediately wrapped in aluminium foil to store at -80ºC. gDNA was extracted from approximately 200 mg of frozen mycelium using the Puregene® Genomic DNA Purification Kit (Gentra Systems Inc., USA) (**Section 2.2.2.7**). For each isolate, 3 µg of extracted gDNA was digested to completion with either *Eco*RI or *Acc*65I (neoschizomer of *Kpn*I; New England Biolabs, USA) according to manufacturer's instruction. Digestion was verified by gel electrophoresis. Digested gDNA was then reprecipitated, redissolved in 15 µL SDW and size fractionated by 0.8% gel electrophoresis. The gel was rinsed in SDW then submerged in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and agitated for 2 x 15 min, followed by another rinse in SDW. The gel was then submerged in neutralisation solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 2 x 15 min with gentle agitation, then transferred to 20x sodium chloride sodium citrate (SSC; 3M NaCl, 0.3M NaCitrate, pH 7) and allowed to equilibrate for 10 min.

[1] fluorescence mirror unit U-MWIBA3: excitation wavelengths 460-495 nm, emission wavelengths 510-550 nm and a dichroic mirror peak wavelength of 505 nm

3.2.5.2 Capillary Transfer

A gel casting tray was placed upside down in a large pyrex dish and transfer buffer, 20x SSC, added until just beneath the tray. A wick made from three pieces of 3 mm blotting paper (Whatman; W and R Balston Ltd, New Zealand) soaked in transfer buffer was placed on top of the tray. The agarose gel was placed directly onto the wick and strips of parafilm arranged 2-3 mm under the edges of the gel. A section of Hybond-N+ nylon membrane (Amersham Pharmacia Biotech Ltd, UK) was cut to size and carefully placed on the gel avoiding creating air bubbles and this was covered with three layers of blotting paper cut to size and soaked in transfer buffer. A 5 cm stack of paper towels was placed on top of the blotting paper, followed by a glass plate and a 750 g weight. Capillary transfer was allowed to proceed overnight and in the morning the transfer apparatus was disassembled, the position of the wells marked on the blot with a chinograph pencil and the membrane baked for 2 h at 80°C to fix the nucleic acid.

DIG Chemiluminescent Detection of *hph* DNA:

Plasmid DNA containing a portion of the *hph* gene was used to generate a probe to detect construct DNA on the Southern blot. Using primers *Hph*Cod-F (5'-GATGTAGGAGGGCGTGGATA-3') and *Hph*Cod-R (5'-GATGTTGGCGACCTCGTATT-3') (designed previously, M. Carpenter, pers. comm.) and pYT6, a 600 bp region of the *hph* gene was labelled using the DIG PCR Probe Synthesis Kit (Roche Diagnostics Ltd) with an annealing temperature of 60°C, according to manufacturer's instructions.

Chemiluminescent detection of DNA was performed using the DIG Luminescent Detection Kit (Roche Diagnostics Ltd) according to manufacturer's instructions. For each reaction, 250 ng of DIG-labelled DNA was hybridised in tubes (Thermo Hybaid) overnight to the immobilised DNA in 20 mL DIG Easy Hyb. Blots encased in mylar sheets were placed in an X-ray film cassette and a sheet of Kodak BioMax XAR Film (Kodak [Australia] Pty., Ltd) placed on top. The cassette was closed and the film exposed for 3 min. Films were developed manually using the Kodak GBX system (Kodak [Australia] Pty., Ltd).

3.2.6 Comparison of Physiological Traits

After mitotic stabilisation of the transformants by single-spore isolation, the three transformants were compared to the wild type by measurement of the following physiological characteristics: mycelial growth rates on different media, sporulation behaviour and spore germination efficiency.

3.2.6.1 Mycelial Growth Rate and Sporulation Behaviour

PDA discs cut from the edge of actively growing colonies of the transformed isolates of *T. hamatum* LU592 and its wild type were transferred to the centre of standard Petri dishes containing either a rich nutrient medium PDA or a poor nutrient medium soil extract agar (SEA, **Appendix 1**). Three replicate plates for each treatment were incubated at 20 °C in 12 h light/12 h dark conditions and the growth rate of the mycelium was measured each day (mm/day). The morphology and sporulation pattern of the transformants were compared with the wild type by taking photographs of the colonies after 14 days. Spores were then collected in 5 mL SDW, filtered through sterile miracloth and counted using a haemocytometer (**Section 2.2.2.2**).

3.2.6.2 Spore germination assay

Each spore suspensions from **Section 3.2.6.1** were diluted with PDB to 10^6 spores/mL. Aliquots (1 mL) of this spore suspension were then placed in sterile Eppendorf tubes and incubated at 20°C for 24 h while slowly being rotated. Three samples from each tube (three replicates for each isolate) were taken, 2 x 50 spores/germ tubes per sample counted and the percentage germination of spores determined. A spore was assessed as being germinated when the germ tube length was equal or greater than the diameter of the spore.

3.2.6.3 Soil-Sandwich Bioassay

The soil-sandwich technique, as described by Wakelin *et al*. (1999), was used to compare the mycelial growth of transformants and their wild types across a filter-membrane in a non-sterile environment.

Five replicate deep Petri dishes (9 cm) were half-filled with 27 g of non-sterile potting mix (**2.3.2.3 Experimental Setup**) adjusted to 80% water holding capacity (WHC; determined gravimetrically) and slightly compressed to form a flat surface.

Discs (3 mm) cut from the edge of actively growing colonies on SEA of the transformed isolates of *T. hamatum* LU592 and its wild type were placed in the centre of sterile nitrocellulose filter-membranes (9 cm diameter, 0.45 µm pore size; Sartorius plc, Germany) and then covered by another layer of filter-membrane. A second 27 g portion of potting mix (80% WHC) was added on top of the filter-membranes. Petri dishes were sealed tightly with Glad Wrap to minimise moisture loss over the incubation period. Plates were incubated at 20 °C. After 6 days, each soil-sandwich was opened and the filter-membranes were removed from the potting mix. The two filter-membranes were then separated from each other and fungal hyphae stained for 2 min in lactophenol cotton blue with excess stain removed by washing in SDW for 5 min. Filter-membranes were then air-dried and colony diameter measured 8 times per filter-membrane using a stereo microscope. The highest and lowest value from each filter-membrane were not considered for statistical analyses to normalise variations.

3.2.6.4 Statistical Analyses

All data was analysed using standard analysis of variance (ANOVA) with factorial treatment structure and interactions. Unless stated otherwise this ANOVA was used in all analyses. Mean separation between treatments was analysed using the unrestricted least significant difference (LSD) test according to Saville (2003). Data was analysed using the statistical software GenStat v. 9.0 (VSN International Ltd).

3.2.7 Visualisation and Recovery of Transformed *Trichoderma hamatum* LU592/C in Non-Sterile Potting Mix

Standard Petri dishes were filled with 30 g non-sterile potting mix (**Section 2.3.2.3**) and 5 mL of a 10^5 spores/mL suspension (**Section 2.3.2.2**) of the GFP-marked *Trichoderma* transformant LU592/C applied to the potting mix. Plates were then incubated at 20ºC. After 2 weeks, samples were taken and fluorescing hyphae and conidiophore structures visualised with an Olympus UIS2 fluorescent microscope, set up as previously described.

The isolate-specific recovery of *T. hamatum* LU592/C from *P. radiata* growing in non-sterile potting mix was determined. Stratified *P. radiata* seeds were sown into 50 mL pots containing non-sterile potting mix (**Section 2.3.2.3**). Five pots were

inoculated with 5 x 10^6 spores/pot of LU592/C (**Section 2.3.2.2**) and incubated at 20ºC and 12h/12h light/dark conditions. For the untreated control SDW was applied to each pot. Seedlings were irrigated by application of 6 mL of SDW every two days. After 3 weeks, rhizosphere and top bulk potting mix were sampled and *Trichoderma* cfu levels determined by dilution plating (**Section 2.3.2.6**) onto TSM-LU and TSM-hyg (TSM without Terrachlor amended with 300 µg/mL hygromycin B). On TSM-LU, all *Trichoderma* colonies (indigenous + introduced *Trichoderma*) and fluorescent colonies (introduced *Trichoderma* only) were recorded after 7, 14 and 21 days using an Olympus UIS1 fluorescent microscope, set up as described previously. Simultaneously, *Trichoderma* cfu were recorded on TSM-hyg after 7, 14 and 21 days.

3.3 Results

3.3.1 *Agrobacterium*-mediated Transformation

Initially, a spore suspension of *T. hamatum* LU592 was used as inoculum for the hygB sensitivity assay. Spore germination was entirely inhibited at a hygB concentration of 100 µg/mL and, consequently, 200 µg/mL was chosen for selection of transformants (as used by Zeilinger for *T. atroviride*). After two rounds of transformation, nine colonies were isolated on the selection plates. However, the PCR verification of *gfp* detected the cassette in only one of the putative transformants which showed slow and untypical mycelial growth on PDA and weak GFP-fluorescence.

The hygB resistance assay was repeated with freshly growing mycelium instead of spores as the starter inoculum and little growth was observed (1-2 mm after 3 days) at 100 µg/mL. No mycelial growth was observed at 200 µg/mL and a concentration of 300 µg/mL was chosen for selection in subsequent transformation rounds. The following two transformation rounds did not result in any transformants. Therefore, the original protocol was modified as followed: The initial *Agrobacterium* concentration was reduced from OD = 0.2 down to 0.15 to allow growth for at least 6 h in the induction medium until an OD of 0.25 - 0.3 was reached. Further, the *Agrobacterium* : *Trichoderma* spore ratio was increased from originally 1:1 V:V to 2:1 V:V. The following two transformation cycles resulted in three putative transformants, these were labelled LU592/A, LU592/B and LU592/C.

Initial observations of the transformants showed uniform colony growth of 2-3 mm/day on hygB-amended PDA and mycelium showed fluorescence under excitation.

3.3.2 Verification of Stable Transformation with *gfp/hph*

For all three transformants, fluorescence of all parts of the young and actively-growing mycelium was observed (**Figure 3.1**), whereas, fluorescence of mycelium older than 7 days (colony centre) began to weaken (data not shown). Mycelium of

LU592/C showed the strongest fluorescence indicated by brighter background glare in the photographs of LU592/A and LU592/B due to longer exposure times. Conidia of all three transformants showed fluorescence with variations in intensity (**Figure 3.2**).

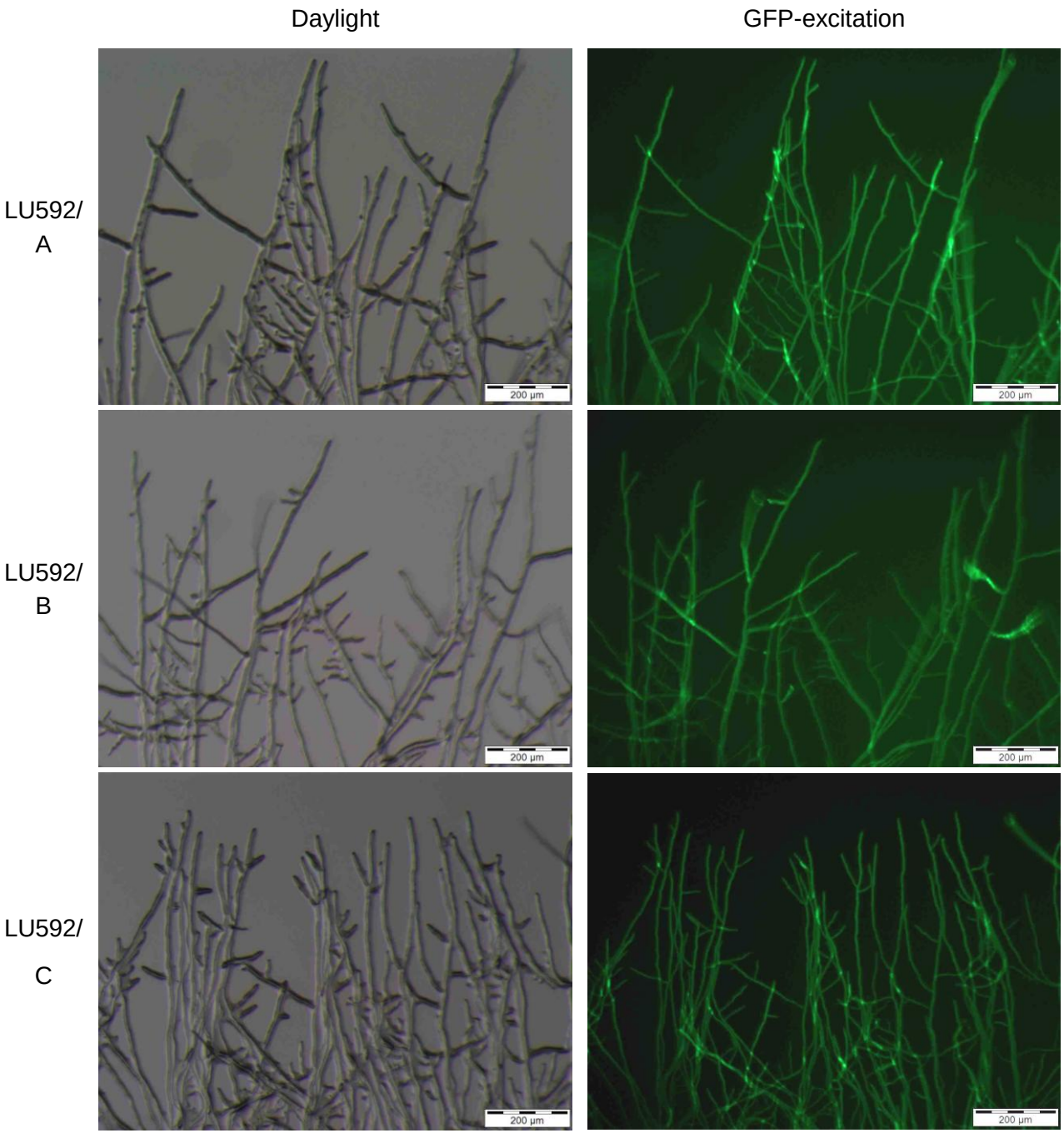


Figure 3.1 Microscopic observations of mycelium of three transformed LU592 isolates (LU592/A, LU592/B and LU592/C) on PDA under daylight (left) or GFP-excitation (right) using an Olympus UIS2 fluorescent microscope.

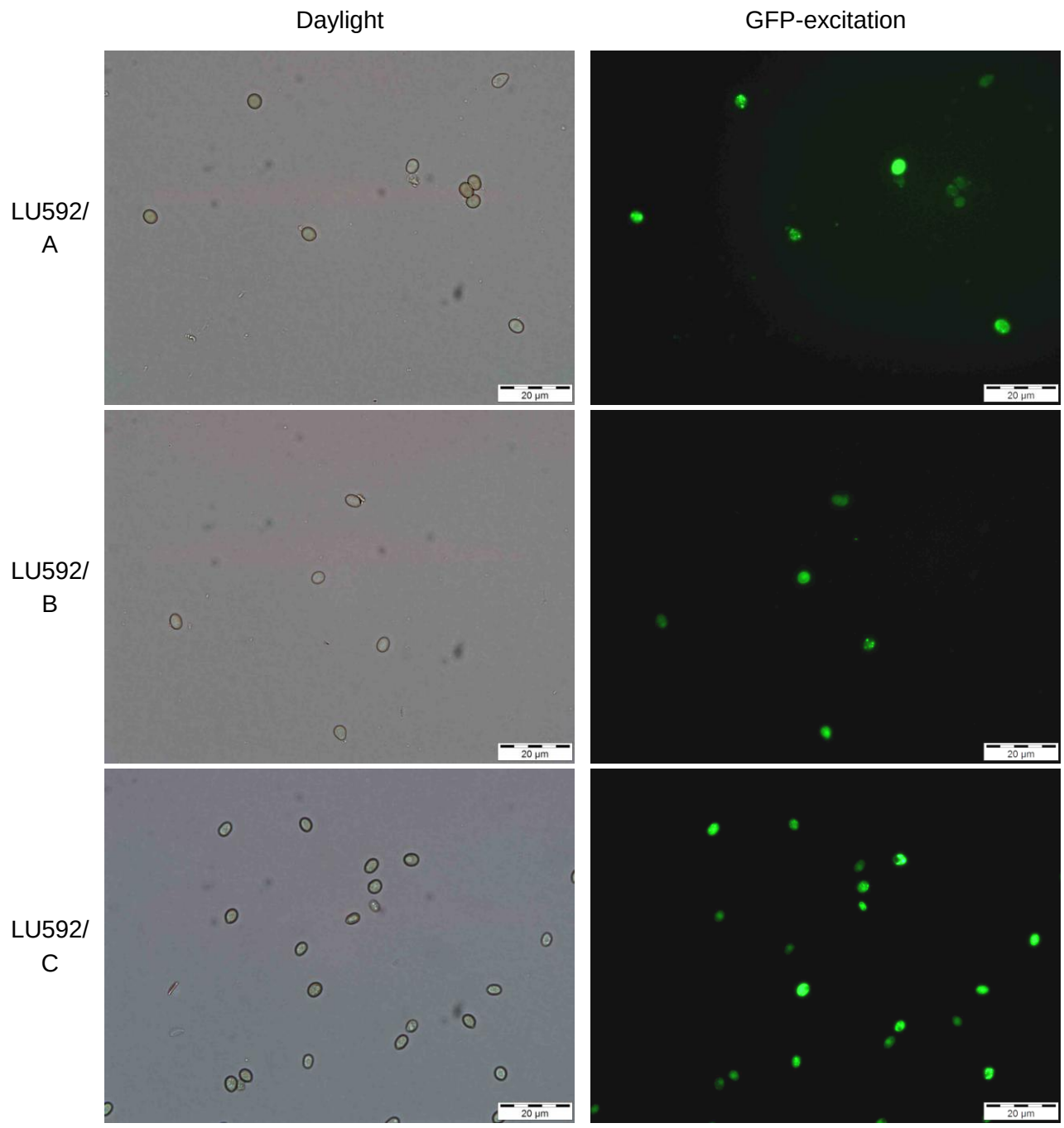


Figure 3.2 Microscopic observations of spores of three transformed LU592 isolates (LU592/A, LU592/B and LU592/C) on PDA under daylight (left) or GFP-excitation (right) using an Olympus UIS2 fluorescent microscope.

DNA was extracted from mycelium of each transformant and the presence of the *gfp*/*hph* cassette was verified by PCR for all three transformants with a fragment size of 546bp (**Figure A3.1, Appendix 3**).

After ten subcultures on non-selective PDA, all three transformants showed fluorescence of conidia and mycelium and were able to grow on hygB-amended PDA. No differences were revealed between the mean growth rates of colonies originating from the original -80ºC cultures and after ten subcultures on non-selective PDA confirming the mitotic stability of the *gfp*/*hph* cassette insertion (**Table 3.1**). However, after ten subcultures LU592/A showed irregular growth rate patterns being significantly different from the original culture at each time interval assessed (**Figure A3.2, Appendix 3**).

Table 3.1 Mycelial growth of the three transformants on hygB-amended PDA originating from either the original culture or after ten subcultures on non-selective PDA.

	Growth rate on hygB-amended PDA [mm/day]			
Transformants	Original culture	After 10 subcultures on non-selective PDA	F pr.	LSD (*P* = 0.05)
LU592/A	3.55	3.92	0.334	1.265
LU592/B	2.40	2.02	0.283	1.135
LU592/C	2.90	2.44	0.261	1.253

Values are means from three consecutive measurements (n = 3). HygB = hygromycin B, PDA = potato dextrose agar, F pr. = ANOVA probability value, LSD = least significant difference.

The Southern hybridisation confirmed the successful insertion of the construct into the genome of all three transformants (**Figure 3.3**). Both digestion enzymes demonstrated a single copy integration with similar fragment sizes for LU592/B and LU592/C of ~8 kb and ~11 kb for *Eco*RI and *Acc*65I, respectively. The fragment sizes for LU592/A were ~4.5 kb and ~6.5 kb for *Eco*RI and *Acc*65I, respectively. All fragment sizes were of higher molecular weight than the 3 kb *gfp*/*hph* cassette indicating a successful integrative transformation for all transformants. There were no background signals for the wild type genomic DNA.

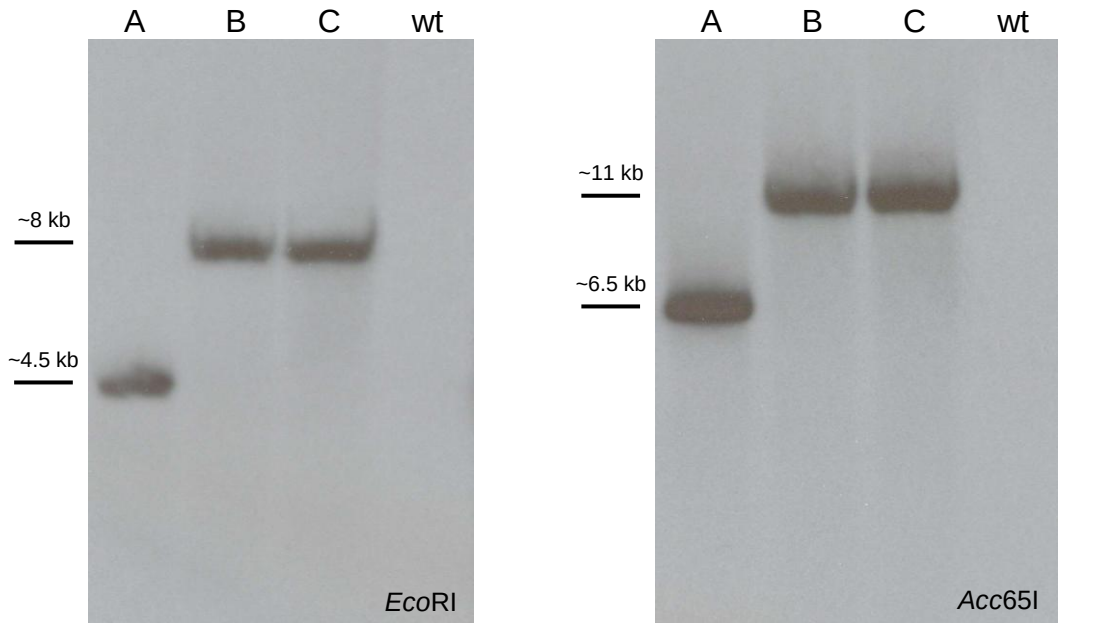


Figure 3.3 Southern hybridisation analyses showing the successful single copy integration of the *gfp*/*hph* cassette into the genome of the three transformants LU592/A (A), LU592/B (B) and LU592/C (C) digested with either *Eco*RI (left) or *Acc*65I (right). Wt = LU592 wild type.

3.3.3 Comparison of Physiological Traits

Prior to carrying out ecological studies, phenotypic characteristics of the transformants were compared with the wild type to ensure that ecological fitness has not been compromised. **Table 3.2** summarises the comparisons of spore production, germination, and growth rates on a nutrient-rich medium PDA, a nutrient-poor medium SEA and in a non-sterile potting mix system using the soil sandwich assay. LU592/A and LU592/C did not differ to the wild type in any of the physiological traits. The only significant difference to the wild type was detected for LU592/B on SEA where mycelial growth was reduced by 2.7%. In addition, sporulating cultures were photographed and sporulation patterns were similar for all three transformants compared with the wild type as demonstrated for LU592/C (**Figure A3.3, Appendix 3**). The soil sandwich assay showed similar mycelium densities on the membrane for LU592 wild type compared with LU592/C at the centre and edge of the colonies (**Figure A3.4, Appendix 3**).

Overall, all three transformants were shown to be mitotically stable, constitutively expressing GFP, and were phenotypically similar to the wild type. Therefore, the efficiency of the modified transformation protocol can be determined as being three transformants per two transformation rounds. Of the three transformants, LU592/C was the most characteristic of the wild type in terms of colony morphology, growth, spore production and germination and was chosen for further studies.

Table 3.2 Comparisons of the three transformants with LU592 wild type for spore production, spore germination and growth rate.

	Spore production [spores/mL]		Spore germination [%]	Growth rate [mm/day]		
Treatment	PDA	SEA		PDA	SEA	SSA
LU592/A	4.5×10^8	1.6×10^6	99.2	30.7	18.7	9.8
LU592/B	6.0×10^8	1.6×10^6	98.8	30.2	**17.9**	10.6
LU592/C	4.5×10^8	2.4×10^6	98.5	30.0	18.2	10.7
Wild type	7.4×10^8	2.7×10^6	98.5	29.9	18.4	10.6
F pr.	0.097	0.203	0.330	0.448	0.030*	0.261
LSD (P = 0.05)	3.53×10^8	1.3×10^6	0.90	1.178	0.484	1.04

Values are means of three replicates. * indicates F probability <0.05 and **bold** values show significant difference to the wild type. PDA = potato dextrose agar, SEA = soil extract agar, SSA = soil sandwich assay, F pr. = ANOVA probability value, LSD = least significant difference.

3.3.4 Visualisation and Recovery of *Trichoderma hamatum* LU592/C in Non-Sterile Potting Mix

Initially, recovery of LU592/C from non-sterile potting mix was attempted on hygB-amended TSM-LU. However, the isolate could not be recovered on this medium. The TSM-LU contains the antimicrobial substances rose bengal, Terrachlor and chloramphenicol. It was concluded that the combination of these chemicals with the selective antibiotic hygB had synergistic inhibitory effects. Therefore, a test was carried out to identify a suitable selection medium. Three different

modifications of the TSM-LU were tested all of which included 300 µg/mL hygB in the absence of either of the antimicrobial substances (TSM[+hygB-rose bengal], TSM[+hygB-Terrachlor] and TSM[+hygB-chloramphenicol]). LU592/C was only recovered on media absent of Terrachlor. The modified selection medium (TSM[+hygB-Terrachlor]) was subsequently designated as TSM-hygB.

Hyphal and conidiophore structures were successfully identified in non-sterile potting mix 2 weeks after inoculation (**Figure 3.4**). The conidiophores showed strong and uniform fluorescence, whereas hyphal structures had weaker and more variable fluorescence intensity.

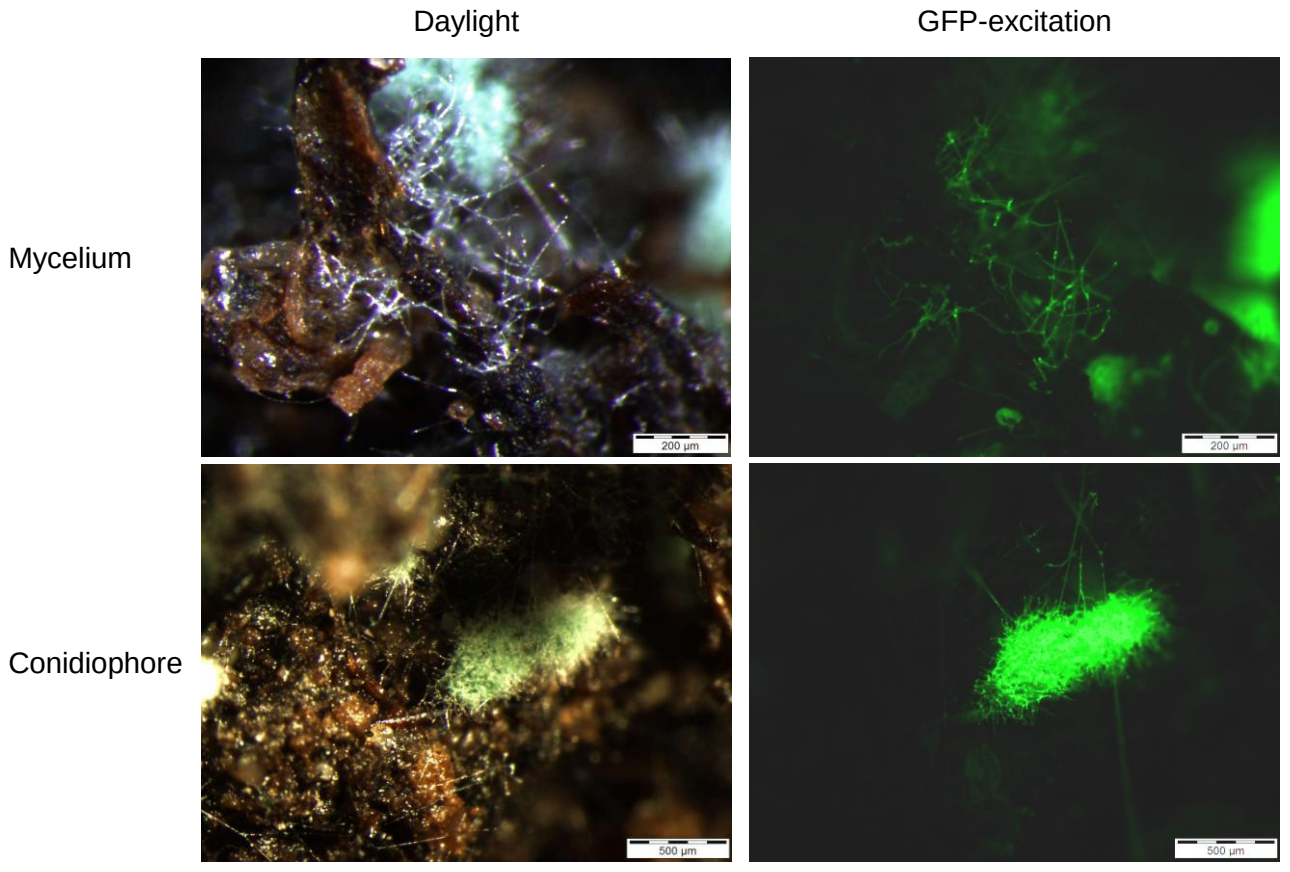


Figure 3.4 Visualisation of mycelium and conidiophores of LU592/C in non-sterile potting mix under either daylight or GFP-excitation using an Olympus UIS2 fluorescent microscope.

LU592/C was successfully recovered from the *P. radiata* rhizosphere and bulk potting mix by dilution plating onto TSM-hygB (**Figure 3.5**). No differences were

observed between LU592/C cfu levels determined from the number of fluorescent colonies on TSM-LU (fluorescent colonies; **Figure 3.6**) and the number of colonies on TSM-hyg in both the top bulk and rhizosphere potting mix indicating that selection pressure did not influence the recovery rate. Inoculation with LU592/C did not result in significantly higher *Trichoderma* cfu levels compared with the un-inoculated control. Overall *Trichoderma* populations, determined on TSM-LU, were significantly higher than the population of hygB-resistant *Trichoderma* colonies of LU592/C determined on TSM-hyg from both the top bulk and rhizosphere potting mix.

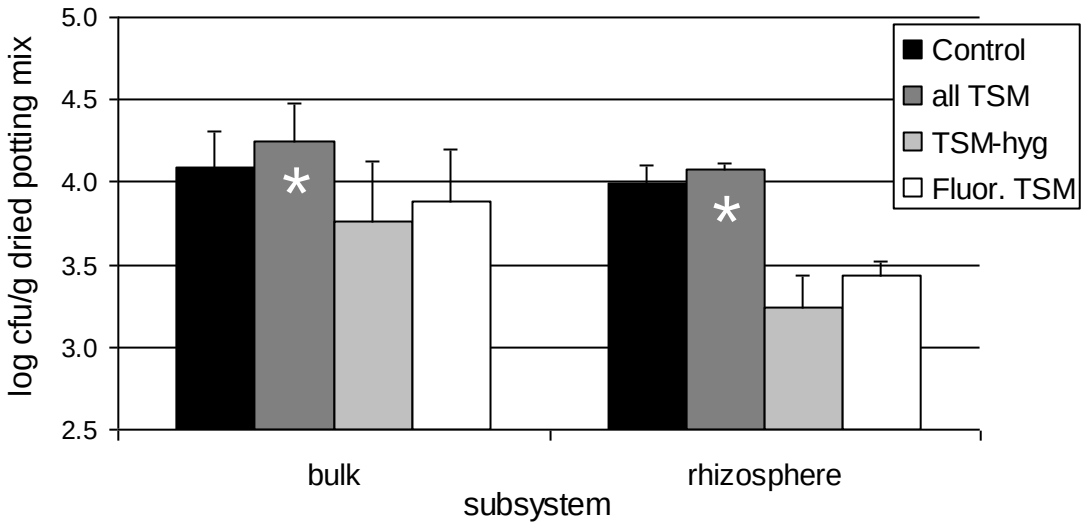


Figure 3.5 *Trichoderma* population means (log_{10} colony forming unit (cfu)/ g dry potting mix) recovered from top bulk potting mix and rhizosphere of 3-week old *P. radiata* seedlings inoculated with LU592/C. Asterix indicate significant difference to 'TSM-hyg'. Treatments: All TSM = all *Trichoderma* colonies on TSM-LU, TSM-hyg = *Trichoderma* colonies on hygromycin B-amended TSM, Fluor. TSM = fluroescent colonies on TSM-LU. Error bars represent the standard error of the means (n = 3).

Daylight GFP-excitiation

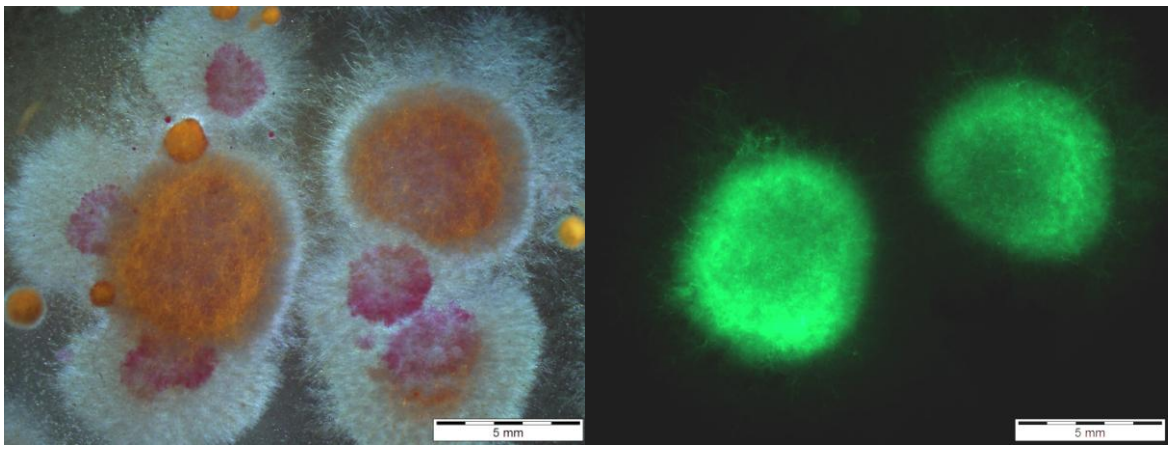


Figure 3.6 Microscopic photographs demonstrating the identification of GFP-marked LU592/C on TSM-LU. Daylight: Colonies of LU592/C surrounded by colonies of indigenous *Trichoderma*. GFP-excitation: Colonies of fluorescing LU592/C under GFP-excitation using an Olympus UIS2 fluorescent microscope.

3.4 Discussion

Agrobacterium-mediated Transformation

The *gfp/hph* cassette was successfully integrated into the genome of *T. hamatum* LU592. The transformant LU592/C was mitotically stable, showed phenotypical similarity to its wild type and could be visualised in and recovered from non-sterile potting mix.

The occurrence of the nine putative transformants (false positives) obtained before the modification of the protocol was possibly a result of natural resistance of the wild type to low concentrations of hygB determined by using conidia for the hygB sensitivity assay as suggested by Zhong *et al.* (2007). Their pre-determined hygB level of 150 µg/mL was successfully used to transform *T. reesei*. However, up to 50% of the putatively transformed *Trichoderma* spp. were found to be mitotically unstable when conidia were used to determine the background hygB sensitivity of four species of *Trichoderma* (Cardoza *et al.*, 2006). It was assumed that actively-growing mycelium would be able to tolerate hygB at higher concentrations compared to conidia since germination may be more sensitive under such stress. This assumption was confirmed in this study as growth was observed at 100 µg/mL from mycelium but not conidia. No transformants were obtained in the following two transformation rounds after the increase in hygB concentration and 1.5 transformants per transformation round was achieved after additional modifications of the protocol. This indicates that the genetic diversity of the genus *Trichoderma* may influence the success of the transformation procedure.

Zeilinger's protocol was used to successfully transfer T-DNA into the genome of *T. atroviride* resulting in 30-100 stable transformants per transformation round (Zeilinger *et al.*, 2004; Cardoza *et al.*, 2006), whereas strains of the species *T. harzianum* were reported to be more difficult to transform (Bae *et al.*, 2000; Cardoza *et al.*, 2006; McLean *et al.*, 2009). In fact, polyethylene glycol (PEG)-mediated transformation of *T. harzianum* protoplasts resulted in up to 100 false positives (McLean *et al.*, 2009). When transformed via *Agrobacterium* T-DNA transfer, genomic integration of the selection cassette could not be confirmed for *T. harzianum*, although transformants showed initial resistance to hygB (Cardoza

et al., 2006). Maor *et al.* (1998) hypothesised that potential reasons for the absence of GFP-fluorescence in their hygB-resistant mutants could be due to instability of mRNA or protein, compromised promoter functionality or mitotic instability of the DNA. All these causes could apply to the nine false positives initially produced in this study. Mitotic instability is the most likely explanation besides the above mentioned possibility of natural resistance of the wild type to hygB. RNA silencing-related or protein phenomena can possibly be ruled out since the putative transformants showed initial fluorescence, and the toxA promoter, used in this study, has been proven to give constitutive and high-level gene expression in a range of ascomycota including *Trichoderma* spp. (Lorang *et al.*, 2001; McLean *et al.*, 2009).

After the increase in selection pressure, transformants were produced after modifying the protocol as follows: (i) the growth period of *A. tumefaciens* in the induction medium was extended to >6 h, since acetosyringone has been shown to be an important inductor of its virulence genes (De Groot *et al.*, 1998; Zeilinger, 2004; Zhong *et al.*, 2007); and (ii) the volume ratio of *Agrobacterium* suspension to *Trichoderma* conidia was doubled, to enhance the likelihood of virulence occurence. The transformation efficiency of 1.5 transformants per transformation round is comparable to levels reported by Zwiers & De Waard (2001) with five stable (mitotically and phenotypically stable) transformants per round for *Mycosphaerella graminicola*, Bae *et al.* (2000) with one stable transformant per round and McLean *et al.* (2009) with one stable transformant per two transformation rounds, both for *T. harzianum*. However, *Agrobacterium*-mediated transformation of *T. reesei* and *T. atroviride* resulted in up to 100-fold higher efficiencies (De Groot *et al.*, 1998; Zeilinger, 2004; Cardoza *et al.*, 2006).

Further modifications of the procedure to transform *T. hamatum* seem necessary to improve the efficiency of gene integration. Recently, our research group has substantially increased the number of transformants by excluding the overlay procedure (J. Steyaert, pers. comm.), which was introduced by Lübeck *et al.* (2002) to identify stable transformants more easily at high efficiency rates. It was postulated that the heat of the overlay (~40ºC) may have irreversibly harmed the already fragile transformants after the *Agrobacterium* ‘attack’. Excluding weaker and potentially unstable transformants is a sensible strategy at high efficiency

rates, but reduces the chance of recovering initially weaker but potentially stable transformants at low efficiency rates (J. Steyaert, pers. comm.). Other modifications of the procedure included optimisation of the concentration of *Trichoderma* conidia and the ratio of *Agrobacterium* to *Trichoderma*, both were essential factors which have been investigated for various target organisms in earlier reports (De Groot *et al.*, 1998; Zeilinger, 2004; Zhong *et al.*, 2007). In addition, the transformation efficiencies for different *Trichoderma* spp. seem to be dependent on the transformation technique, and also the choice of the antibiotic marker gene. Although *Agrobacterium*-mediated transformation was proven to be generally superior compared to other techniques, PEG-mediated protoplast transformation showed similar or even better efficiencies for *T. harzianum* (Cardoza *et al.*, 2006). The results of this study revealed initial difficulties in using a protocol which was well established for *T. atroviride* for *T. hamatum* (Zeilinger, 2004; Cardoza *et al.*, 2006) and adds support to the conclusion that a transformation procedure efficient for one *Trichoderma* species may not be easily transferred to all species of *Trichoderma*, even when phylogenetically similar.
More research is needed to better understand the genetic diversity of *Trichoderma* and the mechanisms of action of *Agrobacterium*-mediated transformation, which will ultimately help to establish highly efficient transformation protocols.

Comparison of Physiological Traits

Three transformants were selected and examined to determine whether the insertion compromised essential genes of *T. hamatum* LU592 by comparing their physiological characteristics with the wild type. Measurements of growth rate on rich and poor substrates as well as sporulation and germination abilities were considered useful indicators of potential ecological behaviour (Thrane *et al.*, 1995; Lo *et al.*, 1998; Lübeck *et al.*, 2002). Previous studies of transformed fungi have shown that the insertion of marker genes into the fungal genome did not compromise pathogenicity and virulence of transformants (Spellig *et al.,* 1996; Maor *et al.*, 1998; Jones *et al.*, 1999; Nahalkova and Fatehi, 2003; Visser *et al.*, 2004; Wu *et al.*, 2008). However, insertional interferences of one or more essential genes are not uncommon. For instance, three out of four tested *T. harzianum hph*-transformants showed significantly different growth rates on two different media

(Lo *et al.*, 1998) and the growth of all tested *T. harzianum* transformants produced by Thrane *et al.* (1995) and Bae *et al.* (2000) was compromised on at least one growth medium.

Apart from genetic stability, the transformed strain must maintain its ecological fitness after inoculation into soil or other plant growth media such as potting mix. It was, therefore, important to compare these physiological traits with the wild type before carrying out these ecological studies. All three transformants from this study were similar to the wild type in all evaluated criteria. Growth rate of LU592/B on SEA was significantly lower than for the wild type, but a reduction of 2.7% is unlikely to have a significant biological effect. In addition, this reduced growth was not observed with the soil sandwich assay using non-sterile potting mix, the medium to be used for future ecological studies. LU592/A exhibited an irregular growth rate pattern on hygB selective medium after 10x subcultures onto non-selective medium. Mitotic instability or compromised expression of the *hph* gene are possible explanations and suggest the strain should be used with caution if chosen for ecological studies.

The growth rate of the three transformants on hygB-amended PDA was about ten times slower than on PDA without selection pressure. Thrane *et al.* (1995) produced *gfp/hph*-transformants whose growth rate was not compromised when grown on selective medium. The reduced growth rates of the transformants in this study might be explained by lower *hph* gene expression. However, all of the transformants used by Thrane *et al.* (1995) showed significant differences in growth rate compared to the wild type suggesting that higher expression levels of foreign genes increase the risk of a changed phenotype. LU592/C was chosen for visualisation and recovery from non-sterile potting mix since it showed the most similar phenotype and behaviour compared to the wild type, and was also found to fluoresce strongest out of the three tested transformants.

Visualisation and Recovery of *T. hamatum* LU592/C in Non-sterile Potting Mix

Initially, synergistic inhibitory effects were observed when both Terrachlor and hygB were present in the medium which did not allow the hygB-resistant transformants to grow. Lübeck *et al.* (2002) tested their *Clonostachys rosea* wild type strain onto hygB-amended PDA and hygB-amended TSM (also containing Terrachlor) and observed that a higher hygB concentration was needed in PDA as opposed to TSM to inhibit *C. rosea* growth. However, their conclusion was that the hygB activity must be compromised in PDA.

LU592/C was able to be recovered on hygB-amended TSM without Terrachlor. It was demonstrated that the efficiency to recover propagules from two different systems, bulk potting mix and *P. radiata* rhizosphere, was not affected by the addition of hygB. The advantage of the *hph* gene was shown since inoculation with LU592/C did not increase the overall *Trichoderma* cfu and the presence of the introduced isolate was masked by high levels of indigenous *Trichoderma* on standard TSM-LU. However, on hygB-amended agar, the introduced LU592/C could be selectively isolated.

The GFP-fluorescence of LU592/C was strong enough to identify the transformed strain in non-sterile potting mix, despite the presence of higher background fluorescence of the potting mix particle compared to the previously tested PDA. Strong GFP intensity was observed for conidiophores. This might be explained by their metabolically active nature and a relatively high density of cells in these structures. Mycelia of LU592/C were also detected, however, intensity of fluorescence was more variable. The metabolic activity of mycelium differs depending on age and location/environment, and this is likely to have led to different expression levels of the constitutively expressed *gfp* gene.

GFP is expressed in the cytoplasm of fungal cells. The fact that older mycelia cells contain less cytoplasm (due to cell shrinking and the expansion of vacuoles) could have accounted for a decrease in GFP-fluorescence intensity. In pure culture, a reduction in GFP fluorescence of 7-day old mycelium on hygB-amended PDA was observed. Therefore, this indicates that the variability in fluorescence observed in the potting mix system after 2 weeks could also be due to the fading of GFP-fluorescence caused by a decrease in metabolic activity of older mycelia. Similar

conclusions were drawn when microconidia of a DsRed-transformed *Fusarium oxysporum* varied in fluorescence intensity (Nahalkova & Fatehi, 2003). Inglis *et al.* (1999) observed granular GFP accumulation in older cultures of a transformed *T. harzianum* compared to uniformly distributed fluorescence of young and fast-growing hyphae. Low fluorescence intensities were also found in older hyphae and chlamydospores of a strain of *T. harzianum* after inoculation in natural soils (Bae *et al.*, 2000). Previous ecological studies of fungi transformed with fluorescent marker genes have mainly assessed fluorescence within 72 hours of inoculation but in some cases extending the period to 14 or 21 days (Bae *et al.*, 2000; Lagopodi *et al.*, 2002; Orr & Knudsen, 2004; Lu *et al.*, 2004; Chacon *et al.*, 2007). LU592/C is used to look at colonisation of the *P. radiata* root system in the next Chapter. Both selective isolation using hygB resistance and visualisation using GFP will elucidate the ecology of *T. hamatum* LU592 in more detail. Experiments will run for several months and it still remains unclear how feasible microscopic observations will be over these longer time frames. However, the recovery of older, yet viable propagules via the *hph* gene should not be compromised.

Chapter Four

Rhizosphere Competence and Root Colonisation by *Trichoderma hamatum* LU592/C in the Root Ecosystem of *Pinus radiata*

4.1 Introduction

One of the biggest challenges for the integration of beneficial microorganisms in commercial agricultural systems remains their inconsistent performance (Harman, 2000; Stewart, 2010). Biocontrol efficacy as well as growth promotion effects are affected by biotic and abiotic factors. Extensive work has been carried out to investigate the interactions between biological control agents (BCA) and various plant pathogens (Yedidia *et al.*, 2000). However, there is still a great need to better understand the agent's population dynamics in the plant ecosystem to successfully and more predictably use those beneficial microbes. Further, knowledge about the population size, survival period and distribution need to be related to the vitality of the introduced isolate within the root ecosystem (Green & Jensen, 1995; Lo *et al.*, 1998; Paulitz, 2000; Orr & Knudsen, 2004). This information can further form the basis to study naturally occuring three-way BCA-plant-pathogen interactions (Vinale *et al.*, 2007).

More recently, microbial ecologists have incorporated a range of new molecular technologies in their studies to facilitate the investigation of microorganisms in complex soil environments. Marker genes such as the green fluorescent protein (GFP) and ß-glucuronidase (GUS) genes, often in combination with various antibiotic resistance genes, revolutionised the way ecological studies were conducted. Besides the importance of isolate-specific detection, it was realised that not only was actual numbers, but also the activity of the strain a key criterion to identify an effective establishment in the root ecosystem. For instance, several

strains of *Fusarium oxysporum* were monitored and quantified in the tomato root system by microscopic observations and activity measurements using constitutively expressed GUS or GFP as markers (Bao *et al.*, 2000; Lagopodi *et al.*, 2002; Papadopoulou *et al.*, 2005). The colonisation behaviour of *Trichoderma* spp. on tomato and cucumber roots was elucidated using GFP reporter systems (Lu *et al.*, 2004; Chacón *et al.*, 2007). However, all of the above studies (and others such as Rincón *et al.*, 2005; Sarrocco *et al.*, 2006) worked with sterile, artificial systems or in the absence of a plant (Bae *et al.* 2000; Orr & Knudsen, 2004). Only a few studies have attempted to quantify *Trichoderma* populations in non-sterile plant ecosystems using GUS activity or hygromycin B (hygB) resistance (Green & Jensen, 1995; Lo *et al.*, 1998) or, prior to this, using benomyl-resistant mutants (Papavizas, 1982; Ahmad & Baker, 1987). Green *et al.* (2001) and De Souza *et al.* (2008) concluded a reduced ability of *Trichoderma* spp. to colonise and penetrate roots in non-sterile conditions compared to sterile conditions. Further, previous work on bacterial bio-inoculants demonstrated that *in vitro* performance does not necessarily correlate with *in vivo* behaviour (Milus & Rothrock, 1997; Schottel *et al.*, 2001; Faltin *et al.*, 2004; Gravel *et al.*, 2005; Adesina *et al.*, 2009).

There are still major challenges when it comes to studying the population dynamics of bio-inoculants in non-sterile plant ecosystems. Qualitative microscopic observations, although valuable in themselves, need to be supplemented with quantitative assessments to allow conclusions about the root colonisation behaviour. Quantification attempts were mostly based on visual assessments of marker gene expression levels whose accuracy is always compromised by factors such as background noise and fungal fragments that are hidden by soil particles. Even when isolate-specific recovery was achieved using strains resistant to inhibitors, there was little correlation to actual biological activity of the fungus. For instance, Bae *et al.* (2000) demonstrated that a shift from hyphal growth to abundant spore production (high propagule levels) decreased the biocontrol efficacy of a *gfp*-tagged strain of *T. harzianum*.

T. hamatum LU592 was demonstrated to improve growth and vitality of commercially grown *P. radiata* seedlings (Chapter 2). Preliminary information was obtained about its ability to colonise and penetrate the *P. radiata* root zone. This

isolate was successfully transformed with the *gfp* and hygB resistance (*hph*) genes to enable isolate-specific monitoring and recovery of LU592/C in a non-sterile environment (Chapter 3) and will be used to investigate the ecology of *T. hamatum* LU592 in relation to *P. radiata* root development in this Chapter. A modified dilution plating technique was developed to quantitatively differentiate mycelia and spores. Both root penetration ability and mycelia enumeration were incorporated into all assessments of overall propagule levels. In this study, these measures were used as indicators of fungal activity to overcome the obstacles mentioned previously.

A meaningful way to study the population dynamics of *Trichoderma* was shown by Chao *et al.* (1986) and Ahmad & Baker (1987) who evaluated population dynamics as a function of root depth. The identification of preferred root penetration sites will also help to understand the agent's mode of action. This information can then be used to match to colonisation patterns of potential root pathogens. It is also of interest to assess the influence of different inoculum concentration on the establishment of LU592/C in the *P. radiata* root ecosystem. Bull *et al.* (1991) concluded a positive relationship between inoculum concentration and root colonisation by a strain of *Pseudomonas fluorescens*. However, as mentioned in Chapter 2, growth promotion effects of *Trichoderma* spp. were not always best with highest inoculum applications (Paulitz, 2000; Arcia & Pérez-Pivat, 2008; GroChem NZ Ltd, commercial trial; P. Chohen, unpublished data). Further, two different inoculum concentrations of *T. hamatum* LU592 applied as either seed coat or spray application resulted in similar growth benefits for *P. radiata* seedlings. Determining which inoculum concentration results in the most efficient root and rhizosphere colonisation is therefore required.

4.2 Materials and Methods

4.2.1 Differentiation of *Trichoderma* Spores and Mycelia Using a Modified Dilution Plating Technique

This experiment aims to indirectly determine the proportions of the overall colony-forming units (cfu) as mycelia by subtracting the cfu levels arising from spores from the overall cfu levels.

A pure mycelial suspension was obtained as follows: 50 mL conical centrifuge tubes were filled with 25 mL potato dextrose broth (PDB; Difco Laboratories, USA) and inoculated with 10^4 spores of *T. hamatum* LU592/C and then incubated at 20ºC in the dark. After 3 days, the solution including a dense mycelia mat was filtered through sterile miracloth, and the excess PDB squeezed out. The fresh mycelial was resuspended in sterile distilled water (SDW). A 10-fold dilution was vortexed for 5 min to homogenise the suspension and cfu/g fresh mycelia was determined by dilution plating onto *Trichoderma* Selective Medium - Lincoln University (TSM-LU; **Appendix 1**). A pure spore-suspension of 3 x 10^8 spores/mL was obtained from a sporulating *T. hamatum* LU592/C colony on PDA (**Section 2.2.2.2**). Both the mycelia- and spore-suspensions were mixed with potting mix to achieve two different concentrations of 10^6 and 10^4 cfu/g fresh potting mix (**Section 2.2.2.3**). Dilution plating onto hygromycin B-amended TSM (300 µg/mL; TSM-hyg; **Section 3.2.7**) was carried out to confirm the concentrations. The first dilution of each sample was then taken and filtered through either two, three or four layers of sterile miracloth. Different numbers of layers were chosen to identify optimum filtration efficacy. The spores-only cfu levels of the filtered samples were then determined by dilution plating and compared with the overall cfu levels before filtration.

4.2.2 Experiment 1: Verification of Rhizosphere Competence of *Trichoderma hamatum* LU592/C Using Two-Compartment Rhizosphere Study Container

This experiment aims to determine the rhizosphere competence of *T. hamatum* LU592/C when applied at three different inoculum concentrations. The colonisation at different distances from the root surface was also determined.

4.2.1.1 Experimental Setup

Rhizosphere Study Containers (RSC) described by Chen *et al.* (2002) were used to determine the rhizosphere competence of LU592/C. RSC consisted of a two-compartment PVC cylinder (50 mL each; height: 40 mm, internal diameter: 40 mm) separated by a 25 µm nylon membrane (**Figure 4.1**). The membrane prevented the roots from growing into the lower compartment, but allowed root hairs and fungal hyphae to penetrate. Further, the lower compartment could be divided in two rings (13 and 27 mm depth) to allow the separation of rhizosphere and bulk potting mix. A 100 µm mesh was attached to the bottom of the lower compartment.

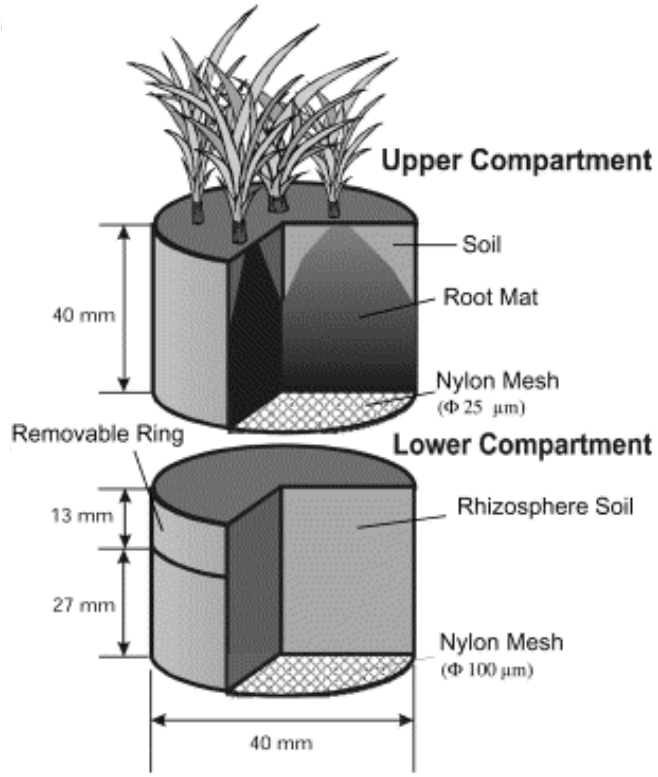


Figure 4.1 Schematic illustration of the Rhizosphere Study Container (Chen *et al.*, 2002).

Both compartments were filled with potting mix (**Section 2.2.2.3**), combined and sealed with duct tape. Stratified *P. radiata* seeds (**Section 2.2.2.1**) were sown in the upper compartment at 0.5 - 1 cm depth (1 seed per RSC). Three spore-suspensions of *T. hamatum* LU592/C of 10^3, 10^5 and 10^7 spores/mL or a water control (SDW) were prepared (**Section 2.2.2.2**) and 1 mL of each suspension applied to the top of the RSC. The spore viability of each suspension was determined as described in **Section 2.2.2.2**. Each treatment was applied to 10 RSC with 5 RSC/treatment randomised on each of two trays (2 blocks). The trays contained drainage holes to prevent cross-contamination and placed within bigger trays to collect excess water. Seedling growing conditions are described in **Table 4.1**. RSC were irrigated every day by applying water (3 mL within the first 2 month and 6 mL after 2 month) to the individual pots taking care not to cross contaminate. Once a month, 0.6 g of a solid fertiliser (Osmoform®, Scotts, The

Netherlands) was added to each RSC. The seedlings were then grown for 6 months (3 months to allow the establishment of a dense root mesh above the nylon membrane and another 3 months to allow the establishment of the rhizosphere underneath the nylon membrane).

Table 4.1 Growing conditions of *P. radiata* seedlings grown in Rhizosphere Study Container for a period of 6 months.

Time/Period	Temperature [ºC]	Light [µmol s^{-1} m^{-2}]	Relative humidity [%]
6 am - 9 am	20	~200	76
9 am - 6 pm	22	~500	69
6 pm - 9 pm	20	~200	76
9 pm - 6 am	18	0	86

* Solid fertiliser Osmoform®

4.2.1.2 Assessments

After 6 months, the two compartments were separated and the upper ring of the lower compartment was taken to sample rhizosphere discs of 0-2, 3-5 and 8-10 mm below the nylon membrane. Bulk potting mix was sampled in the lower ring of the lower compartment (25-30 mm below the nylon membrane as described by Chen *et al.*, 2002). The filtration dilution plating technique (**Section 4.2.1**) was used to determine overall and spore only LU502/C cfu levels in each sample. The root system in the upper compartment was separated from the potting mix, divided into three sections (upper 1/3, middle 1/3 and lower 1/3 of the root) and surface sterilised (**Section 2.2.2.4**). The roots were then cut in ~1 cm parts and six randomly selected portions from each section were plated onto TSM-hyg and incubated for 7 days at 20ºC in the dark. The number of root segments where LU592/C was observed growing out were counted and the proportion of root segments colonised by LU592/C determined for every treatment.

4.2.2 Experiment 2: *Pinus radiata* Root Colonisation by *Trichoderma hamatum* LU592/C

This experiment aims to examine the root colonisation behaviour of *T. hamatum* LU592/C using epifluorescence microscopy and to determine if LU592/C colonises the *P. radiata* root system at preferred sites.

4.2.2.1 Experimental Setup

Square Petri dishes (10 x 10 x 2 cm) were used as the growing container to allow observations of the root system without destructive sampling. The Petri dishes had one side cut open to form the top of the microcosm and holes on the other side to allow drainage of excess water (**Figure 4.9**). The lid was taped to the plate and wrapped in aluminium foil to protect the roots from exposure to light. The microcosms were then filled with potting mix (**Section 2.2.2.3**) and stratified *P. radiata* seeds (**Section 2.2.2.1**) sown in the centre of each microcosm at 0.5 - 1 cm depth. At the time of sowing, 1 mL of spore-suspensions of LU592/C were applied to the top of each microcosm at one of three different inoculum concentrations of 10^3, 10^5 and 10^7 spores/microcosm. Microcosm treated with sterile water served as the untreated control. Spore suspension preparation, application and spore viability verification are described in **Section 4.2.2.1**. Each treatment was randomly applied to 5 microcosms placed in a tray. The tray contained drainage holes to prevent cross-contamiation and placed within a bigger tray to collect excess water. The microcosms were grown at a 70º angle to allow root elongation against the transparent lid and incubated and irrigated as described in **Section 4.2.2.1** for 16 weeks.

4.2.2.2 Assessments

After 3, 7 and 16 weeks post-inoculation (wpi), the root systems were revealed by removal of the microcosms' lid and fluorescent fungal structures were then observed using epifluorescence microscopy (**Section 3.2.4**). Whenever fluorescent structures were observed in proximity to roots, the respective root sections (2-3 mm) were sampled, surface sterilised and plated on TSM-hyg (**Section 3.3.4**). Plates were then incubated at 20ºC in the dark for 7 days to determine the potential root penetration by LU592/C. In addition, the location of

fluorescent structures was recorded to enable root colonisation patterns to be qualitatively determined:

Colonisation of:

- Top vs bottom part of the roots
- Lateral vs taproot
- Healthy vs damaged roots
- Root tips vs straight parts of the roots vs root intersections

In addition, three bulk potting mix samples (3 g each) were randomly taken and LU592/C recovered by dilution plating (**Section 2.2.2.5**) onto TSM-hyg and cfu/g dry potting mix were determined.

At 16 wpi, a quantitative approach was taken to examine internal root colonisation behaviour. Six portions of each root section, root tips (<1 mm in length), straight root parts and root intersections (<2 mm) were randomly sampled for each microcosm, surface-sterilised (**Section 2.2.2.4**) and plated on TSM-hyg. Plates were incubated at 20ºC in the dark for 7 days with the number from which LU592/C grew out of the roots recorded. The proportion of colonised root segments were then determined.

4.2.3 Experiment 3: Spatio-Temporal Distribution of *Trichoderma hamatum* LU592/C down the Root System

This experiment aims to determine the spatio-temporal distribution of *T. hamatum* LU592/C in the presence and absence of *P. radiata* seedlings.

4.2.3.1 Experimental Setup

A root containers method modified from that described by Bourguignon (2008) was used. Each root container was made using 50 mL conical centrifuge tubes. The tubes were cut vertically into two equal halves and the bottom tip removed to allow drainage of excess water. The two halves of each tube were combined and sealed with duct tape. The root containers were filled with potting mix (**Section 2.2.2.3**)

and stratified *P. radiata* seeds (**Section 2.2.2.1**) sown at a depth of 0.5 - 1 cm. A *Trichoderma* spore-suspension of LU592/C was prepared (**Section 2.2.2.2**), adjusted to 5 x 10^6 spores/mL and 1 mL applied to the top of each root container containing either potting mix and seeds or potting mix only. One mL of sterile water was applied to seed-containing root containers as the untreated control. Holes (diameter 17 mm) were drilled into 2 styrofoam plates (thickness: 5 cm) and 20 root container of each treatment randomly placed in the holes of each plate (2 blocks giving 40 replicates per treatment). The root containers were incubated as described in **Section 4.2.2.1** for 16 weeks.

4.2.3.2 Assessments

After 3 dpi (days post-inoculation), 4, 8, 12 and 16 wpi, 8 root containers per treatment were randomly collected at each time (**Figure 4.2**). The centrifuge tubes were split open and ~2 cm^3 discs of potting mix sampled at three different depths using a utility knife (**Figure 4.3**). Overall and spores-only cfu levels of LU592/C were determined for each sample using the filtration dilution plating technique. Roots within each disc were separated from the potting mix, surface-sterilised (**Section 2.2.2.4**), cut into ~1 cm pieces and plated onto TSM-hyg. Plates were then incubated at 20ºC in the dark for 7 days with the number of roots from which LU592/C grew out of recorded. Root and potting mix subsamples (~50 mg) were immersed in 200 µL SDW and transferred to microscope slides (with coverslip) at every sampling time to observe fluorescent fungal structures using epifluorescence microscopy as described previously.

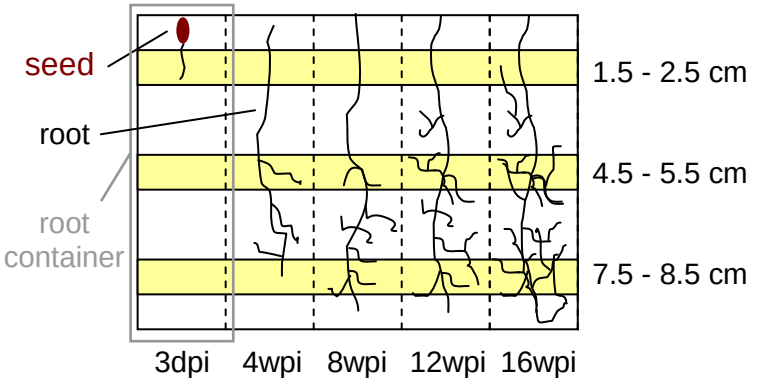


Figure 4.2 Schematic illustration of the sampling procedure for the root container across 5 sampling times. The three depth sampled are shown as shaded. Dpi/wpi = days/weeks post-inoculation.

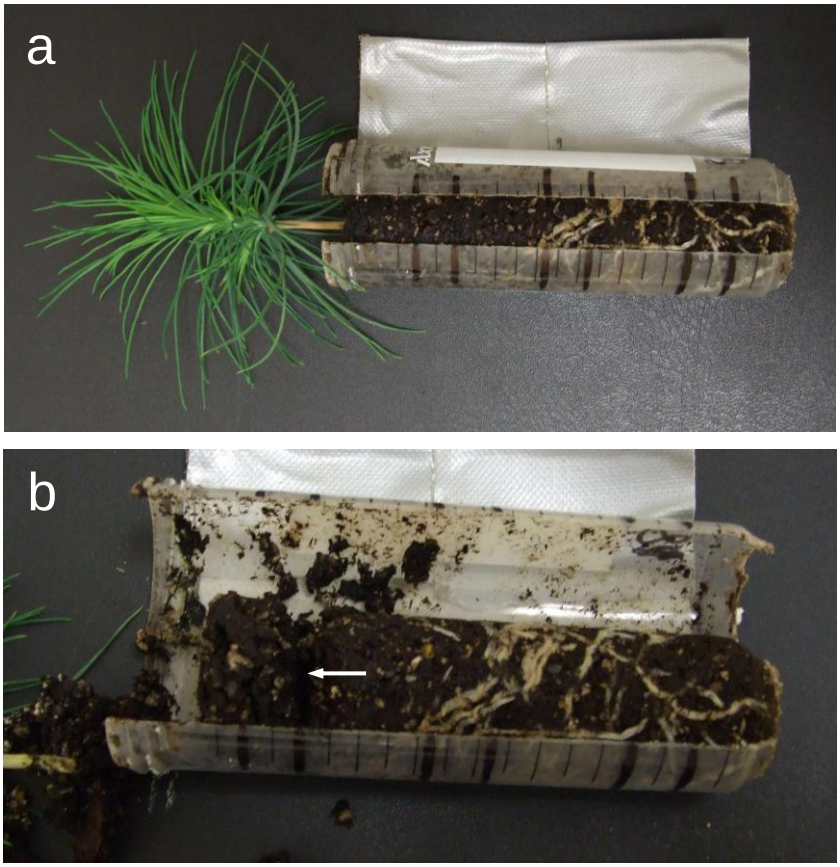


Figure 4.3 Photographs of a representative 4-week old *P. radiata* seedling grown in a conical centrifuge tube, vertically split to enable destructive sampling down the root system. (a): black markings indicate the three sampling depths, (b): top potting mix layer removed and 1-cm-depth-disc cut at the top sampling depth (arrow). Lateral roots seen penetrating the centre and bottom sampling depths.

4.2.4 Statistical Analyses

All data acquired in this experiment was analysed using standard analysis of variance (ANOVA) with factorial treatment structure and interactions. Unless stated otherwise this ANOVA was used in all analyses. *Trichoderma* population assessment data were $\log_{10}$-transformed to satisfy the assumption of normality for ANOVA (Olsen, 2003). Mean separation between treatments was analysed using the unrestricted least significant difference (LSD) test according to Saville (2003). The least significant effect (LSE) test was used to determine whether mycelia

proportion means were significantly different from zero. Data was analysed using the statistical software GenStat v. 9.0 (VSN Internation Ltd) and unless stated otherwise all presented data is shown as the back transformed mean.

4.3 Results

The spore viability was determined for all applications of *T. hamatum* LU592. The proportions of germinated spores ranged between 98.2% and 98.9% for the three experiments.

LU592/C was not recovered from roots or potting mix samples from the untreated controls in any of the three experiments.

4.3.1 Differentiation of *Trichoderma* Spores and Mycelia Using a Modified Dilution Plating Technique

Mycelia of LU592/C was recovered from non-sterile potting mix onto TSM-hyg after being filtered through no, two, three or four layers of miracloth (**Figure 4.4**). Two layers of miracloth significantly reduced mycelia levels by 89.4% and 98.6% when inoculated at 10^4 and 10^6 cfu/g potting mix, respectively. Three and four layers of miracloth significantly reduced mycelia numbers by 98.1-98.8% and 99.8-99.9 % when inoculated at 10^4 and 10^6 cfu/g potting mix, respectively. The filtration efficiency increased with higher mycelia concentrations (by >10 times between the 10^4 and 10^6 application rates). For conidial suspensions applied at 10^4 and 10^6 spores/g potting mix, there was no significant difference in cfu counts when filtered through no, two, three and four layers of miracloth. Based on these results, the filtration dilution plating technique with three or four layers of miracloth was used in the experiments in this Chapter to determine mycelia proportions, since an overestimation of mycelia levels by <2% is considered acceptable.

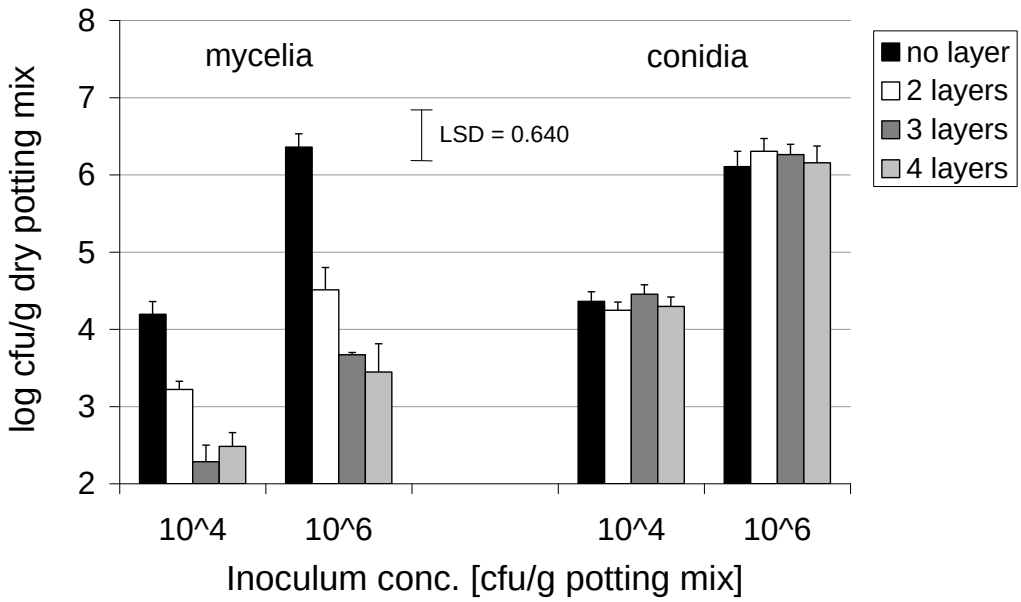


Figure 4.4 *Trichoderma hamatum* LU592/C population (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from potting mix inoculated with two different concentrations (conc.) of either mycelium or spores of LU592/C after filtration through two, three or four layers of miracloth and their respective controls (no layers of miracloth). Error bars represent the standard error of the means (n = 3). LSD = least significant difference (*P* = 0.05).

4.3.2 Experiment 1: Verification of Rhizosphere Competence of *Trichoderma hamatum* LU592/C Using Two-Compartment Rhizosphere Study Container

After the 6-month growing period, *P. radiata* seedlings appeared healthy and vigorous (**Figure 4.5**a). For the destructive sampling, the root, rhizosphere and the bulk potting mix compartments were separated (**Figure 4.5**b). A dense root mesh was seen to have formed above the nylon membrane.

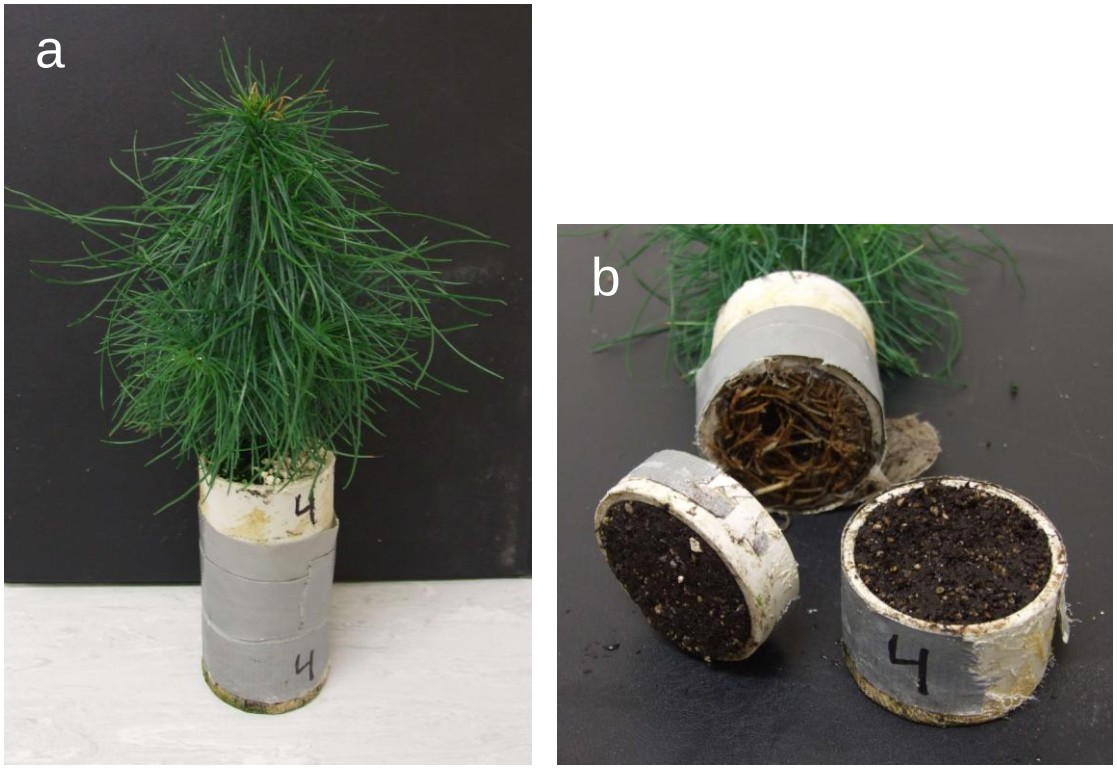


Figure 4.5 Representative 6-month old *P. radiata* seedling grown in a Rhizosphere Study Container, intact (a) and opened system (b). (b) separation of roots (upper compartment, top), rhizosphere (upper ring of lower compartment, left) and bulk potting mix (lower ring of lower compartment, right).

After 6 months, LU592/C established in all the subsystems sampled, the rhizosphere, endorhizosphere and bulk potting mix, at all three inoculum concentrations. Overall LU592/C cfu levels when inoculated with 10^5 spores/RSC (1.5×10^4 cfu/g dry potting mix) were significantly higher by 4.8 and 3.6 times compared with cfu levels when inoculated with 10^3 and 10^7 spores/RSC, respectively (**Figure 4.6**). The proportion of the overall cfu as mycelia was 24.6% and 23.6% when inoculated with 10^5 and 10^7 spores/RSC, respectively. Mycelia proportions were below the detection level when inoculated with 10^3 spores/RSC.

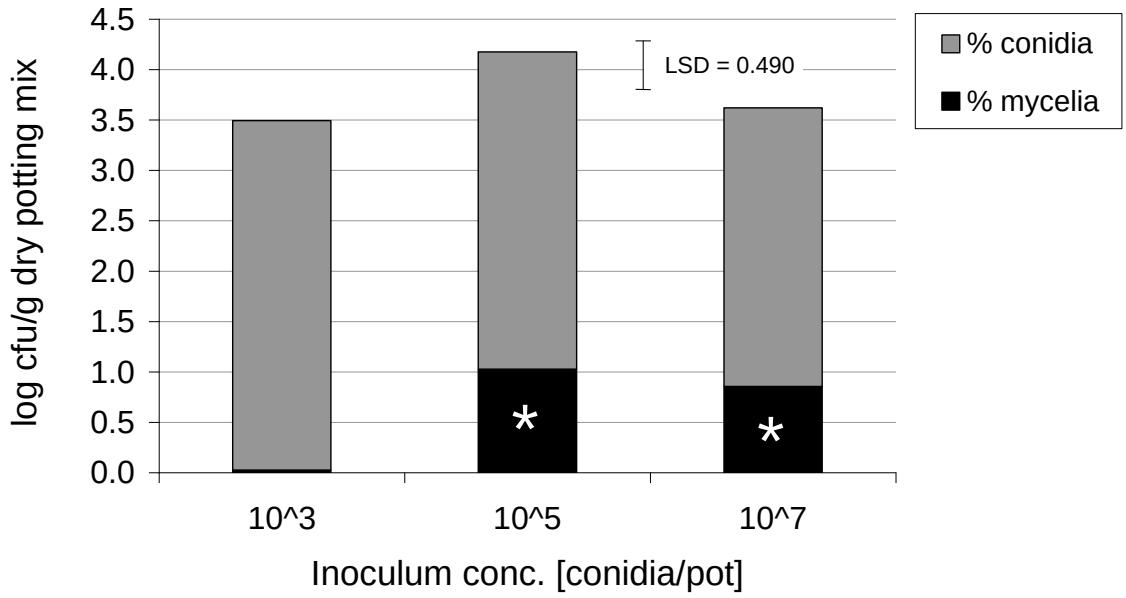


Figure 4.6 *Trichoderma hamatum* LU592/C population (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from potting mix six months after inoculation with three different concentrations (conc.) of LU592/C. Overall bar values are means (n = 10) of all subsamples. Bar areas show the proportion of the overall cfu levels as conidia and mycelia with asterisk indicating mycelia proportion means above the detection level. LSD = least significant difference for overall cfu treatment effects.

The rhizosphere potting mix closest to *P. radiata* roots contained the highest LU592/C cfu levels (mean over all treatments) at 1.3 x 10^4 cfu/g dry potting mix and this was significantly higher than cfu levels at 4-6 mm, 8-10 mm and 25-30 mm (bulk) distances by 2.4, 2.8 and 3.5 times, respectively (**Figure 4.7**). This colonisation pattern was observed for all three inoculum concentrations. The proportion of the total cfu as mycelia for all three rhizosphere subsamples were 25.1%, 20.9% and 19.4%, respectively for the 0-2, 4-6 and 8-10 mm samples. Mycelia proportions of the bulk potting mix were below the detection level.

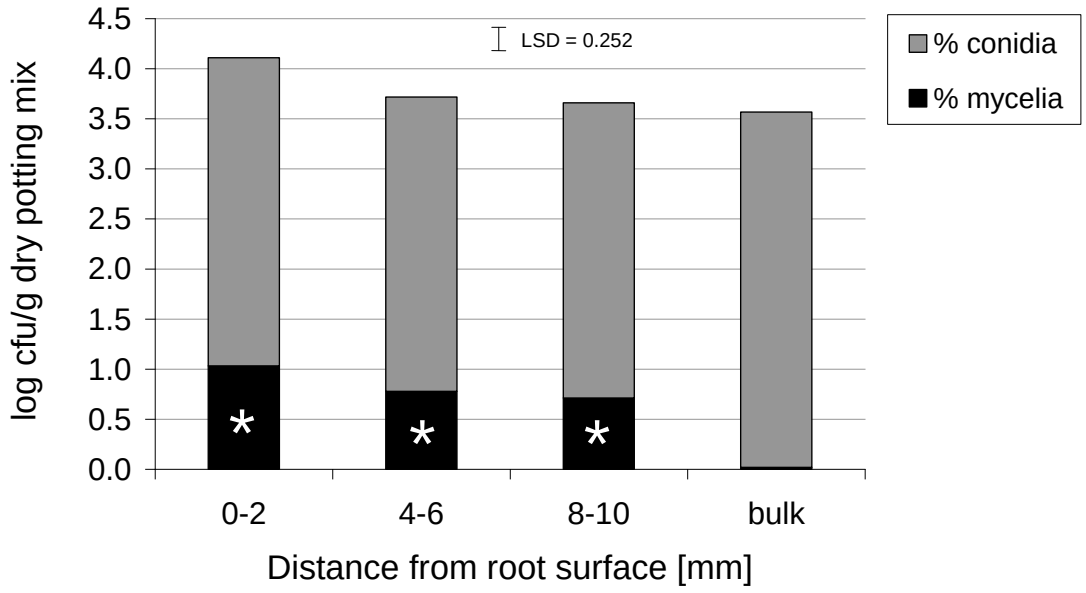


Figure 4.7 *Trichoderma hamatum* LU592/C population (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from potting mix at four different distances from the root surface six months after inoculation with LU592/C. Overall bar values are means (n = 10) of all inoculum concentrations. Bar areas show the proportions of conidia and mycelia of the overall cfu levels with red markings when mycelia proportion means are above the detection level. LSD = least significant difference for overall cfu treatment effects.

LU592/C was isolated from the upper, middle and lower parts of 6-month old *P. radiata* roots. A trend similar to the overall cfu levels in the rhizosphere was observed where the highest proportion of internally colonised root segments (mean over all root parts) occurred when inoculated with 10^5 spores/RSC (80.6%) and this was significantly higher compared to levels when inoculated with 10^3 spores/RSC (51.1%) and 10^7 spores/RSC (66.7%) (**Figure 4.8**). Internal colonisation of the upper (older) part of the root system (mean over all treatments) was significantly higher with 80.0% colonised root segments compared to the recovery of LU592/C from the middle (62.8%) and lower (55.6%) parts of the root. However, this trend was only observed for the 10^5 and 10^7 applications. No significant differences between the three root sections were observed for the 10^3 application.

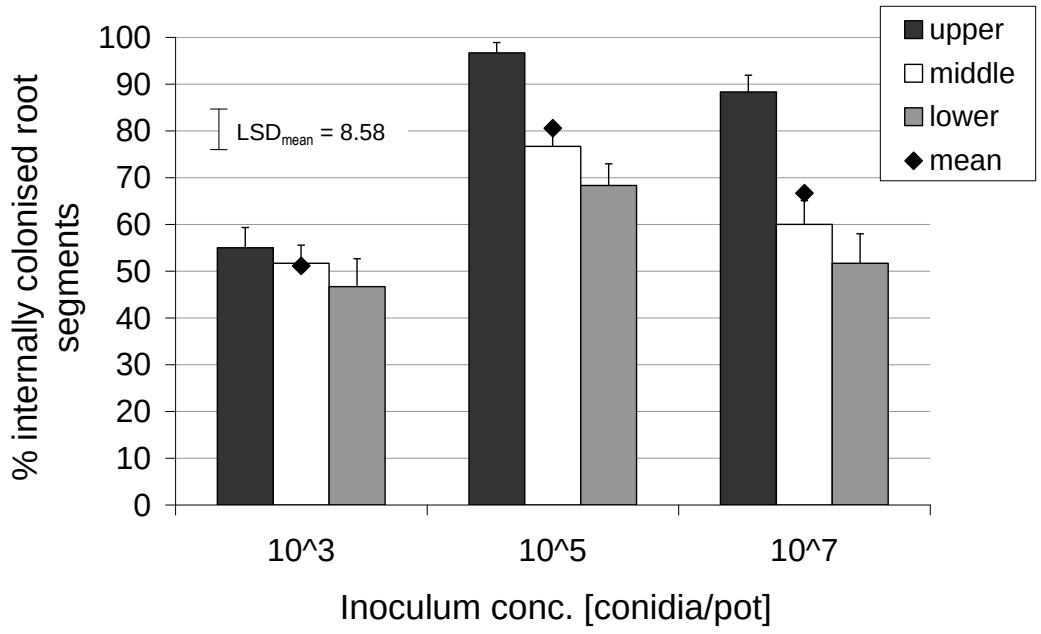


Figure 4.8 Percentage of surface sterilised *P. radiata* root segments where *T. hamatum* LU592/C was isolated from, 6 months post-inoculation with three different *T. hamatum* LU592/C inoculum concentrations (conc.). Bars show means (n = 10) of percentages of internally colonised root segments of the upper, middle and lower section of the root system. Diamond symbols represent means (n = 10) across all subsamples for each inoculum concentration. LSD_{mean} = least significant difference for treatment effects of the mean values.

4.3.3 Experiment 2: *Pinus radiata* Root Colonisation by *Trichoderma hamatum* LU592/C

P. radiata seedlings grown in the microcosms appeared healthy and vigorous during the 4-month growing period (**Figure 4.9**). The taproot and lateral roots were seen to grow against the lid. When the lid was removed, the roots were exposed and root observations and samplings could be made without major disruption to the root ecosystem.

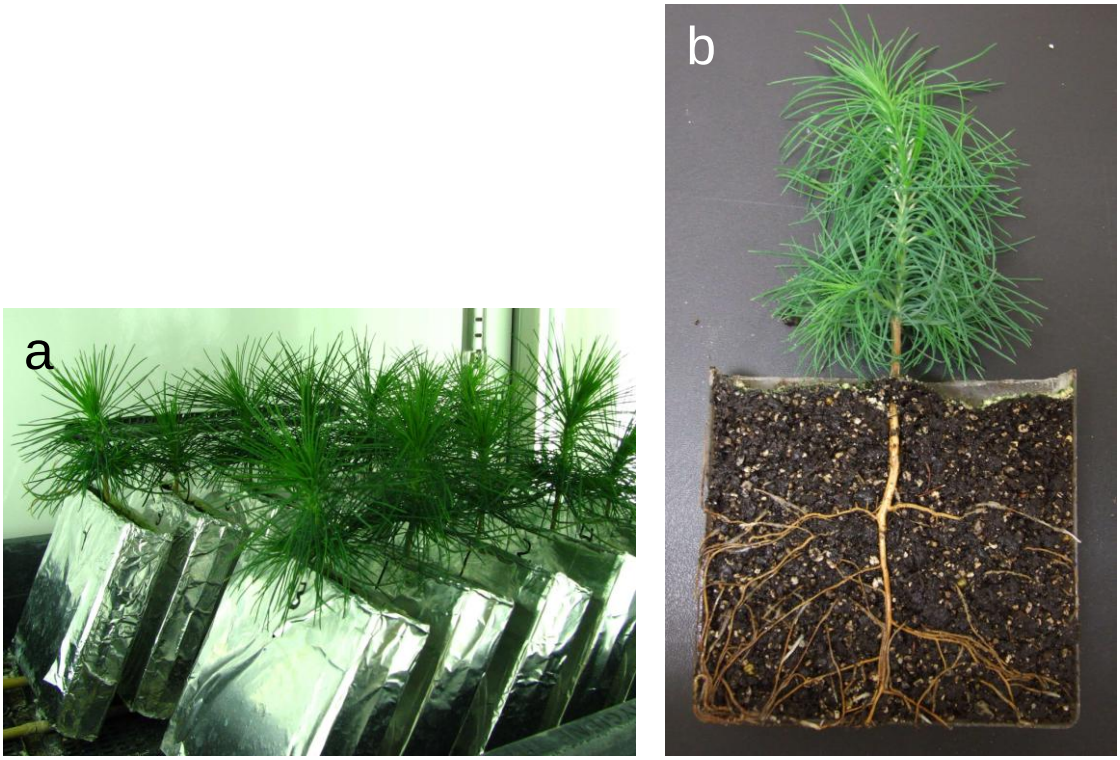


Figure 4.9 Representative *P. radiata* seedlings growing in microcosms. (a) 10-week old seedlings grown at a 70º angle, pots are covered in aluminium foil to protect the roots from exposure to light. (b) 16-week old seedling after opening the microcosm revealing the root system which had grown against the microcosm's lid.

After 3 weeks, no fluorescent fungal structures were observed around the roots or in the potting mix. The isolate was recovered at 3.8 x 10^2 and 1.2 x 10^4 cfu/g dry potting mix when inoculated with 10^5 and 10^7 spores/microcosm, respectively. LU592/C was not recovered from microcosms inoculated with 10^3 spores/microcosm at this assessment time.

After 7 weeks, fluorescent mycelia was seen colonising the root surface, including root hairs and bark particles in proximity to roots (**Figure 4.10**). Wherever GFP-fluorescent fungal structures were found on the root surface, LU592/C was recovered from within the roots indicating internal root colonisation ability at that time. Microscopic examination of samples for LU592/C was laborious and time-consuming. LU592/C was only detected in a few observations and, therefore, no conclusions on colonisation patterns could be drawn.

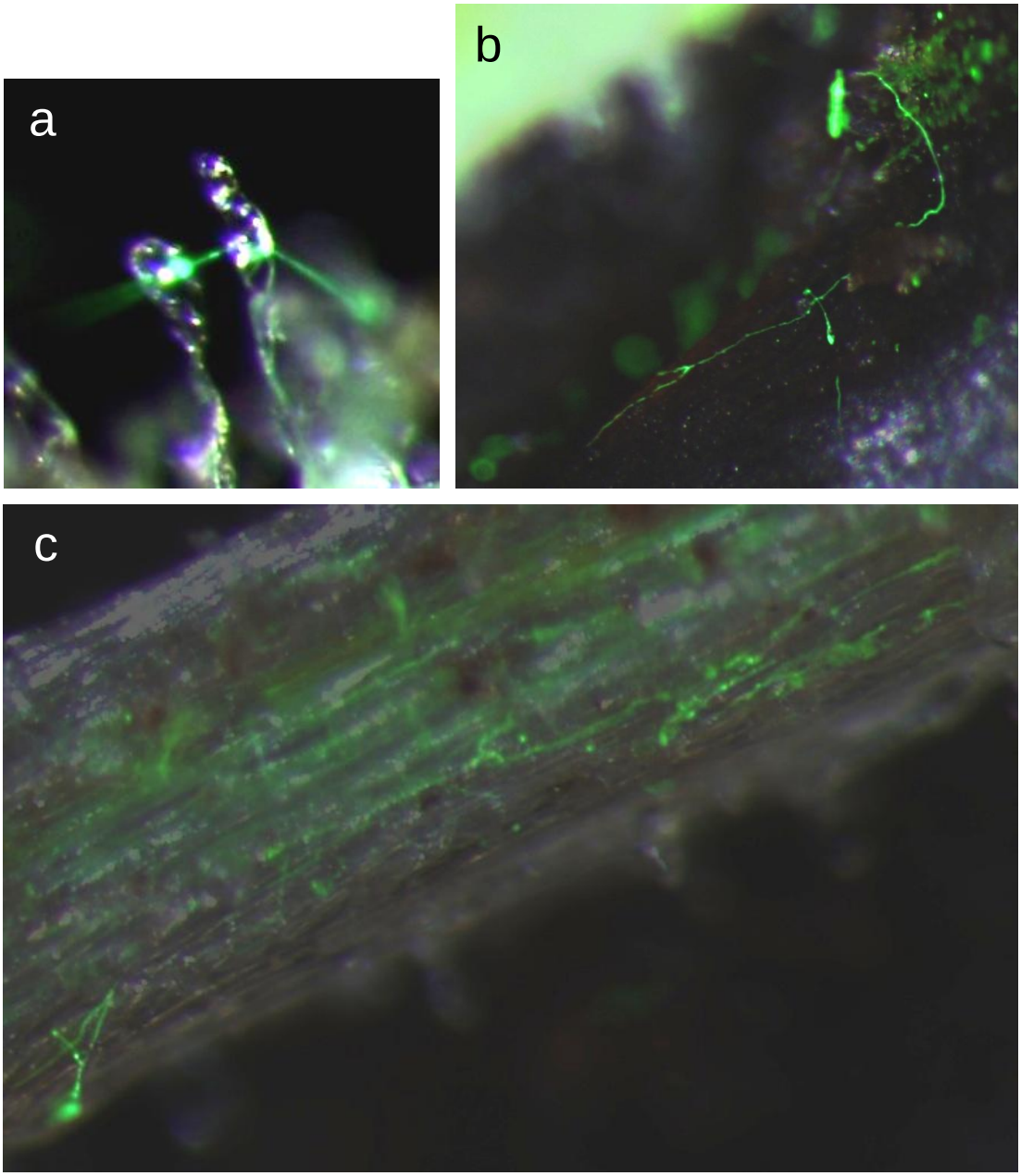


Figure 4.10 Representative photographs of *P. radiata* root colonisation by fluorescent *T. hamatum* LU592/C 7 weeks post-inoculation. (a) fluorescent mycelia colonising root hairs, (b) fluorescent mycelia colonising bark particle next to a lateral root (upper left corner), (c) fluorescent mycelia colonising the root surface.

After 16 weeks, although observations of root colonisation by LU592/C was still time-consuming, LU592/C root colonisation was more frequently detected than at 7 weeks post-inoculation (wpi; **Figure 4.11**). LU592/C was seen to colonise all

parts of the root system. However, the occurrence of fluorescent fungal structures was not frequently detected and, therefore, no conclusions could be drawn about preferred root colonisation sites. Fluorescent mycelia were observed on healthy root tips (**Figure 4.11**a1) and along lateral roots (**Figure 4.11**b1). Again, LU592/C was isolated from surface-sterilised root sections wherever fluorescent mycelia were found. Fluorescent conidiophore structures were observed along lateral roots (**Figure 4.11**c1, c2, d1). Conidiophores were easily detectable due to their bright fluorescence and concentrated occurrence. Non-fluorescent fungal structures were often found in proximity to fluorescent fungal structures (**Figure 4.11**c, c1 (arrows), d, d1). In addition, variation in fluorescence intensity was observed for hyphae of LU592/C at some locations. No fluorescent fungal structures were observed for the untreated control.

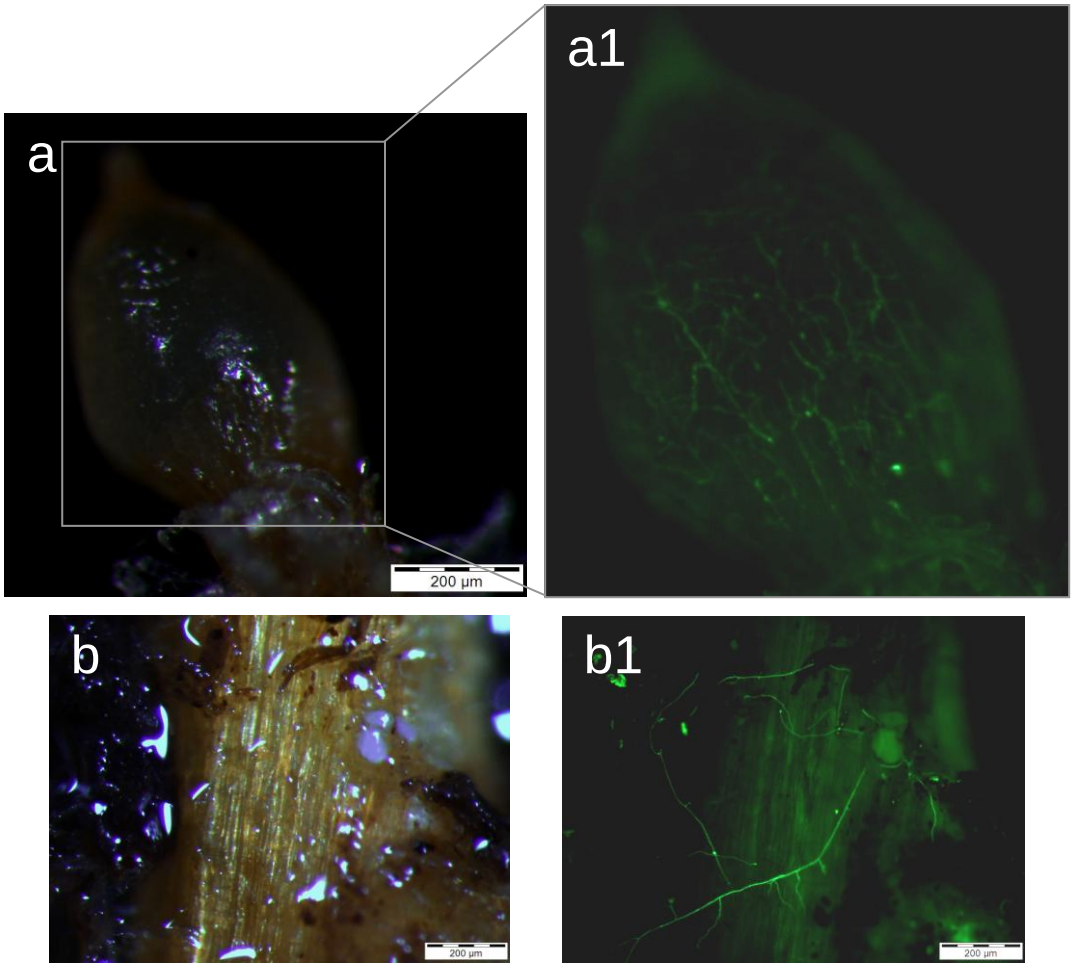


Figure 4.11 Representative photographs of *P. radiata* root colonisation by fluorescent *T. hamatum* LU592/C 16 weeks post-inoculation. (a, a1) fluorescent mycelia colonising elongating root tip, (b, b1) fluorescent mycelia colonising a lateral root, (c, c1, c2) fluorescing conidiophore structures attached to the root surface next to a non-fluorescent conidiophore structure (c & c1, arrow), (d, d1) fluorescent conidiophore structure next to a lateral root and surrounded by non-fluorescent mycelia. Photographs a, b, c, and d were taken under daylight, photographs a1, b1, c1, c2, and d1 were taken under GFP-excitation.

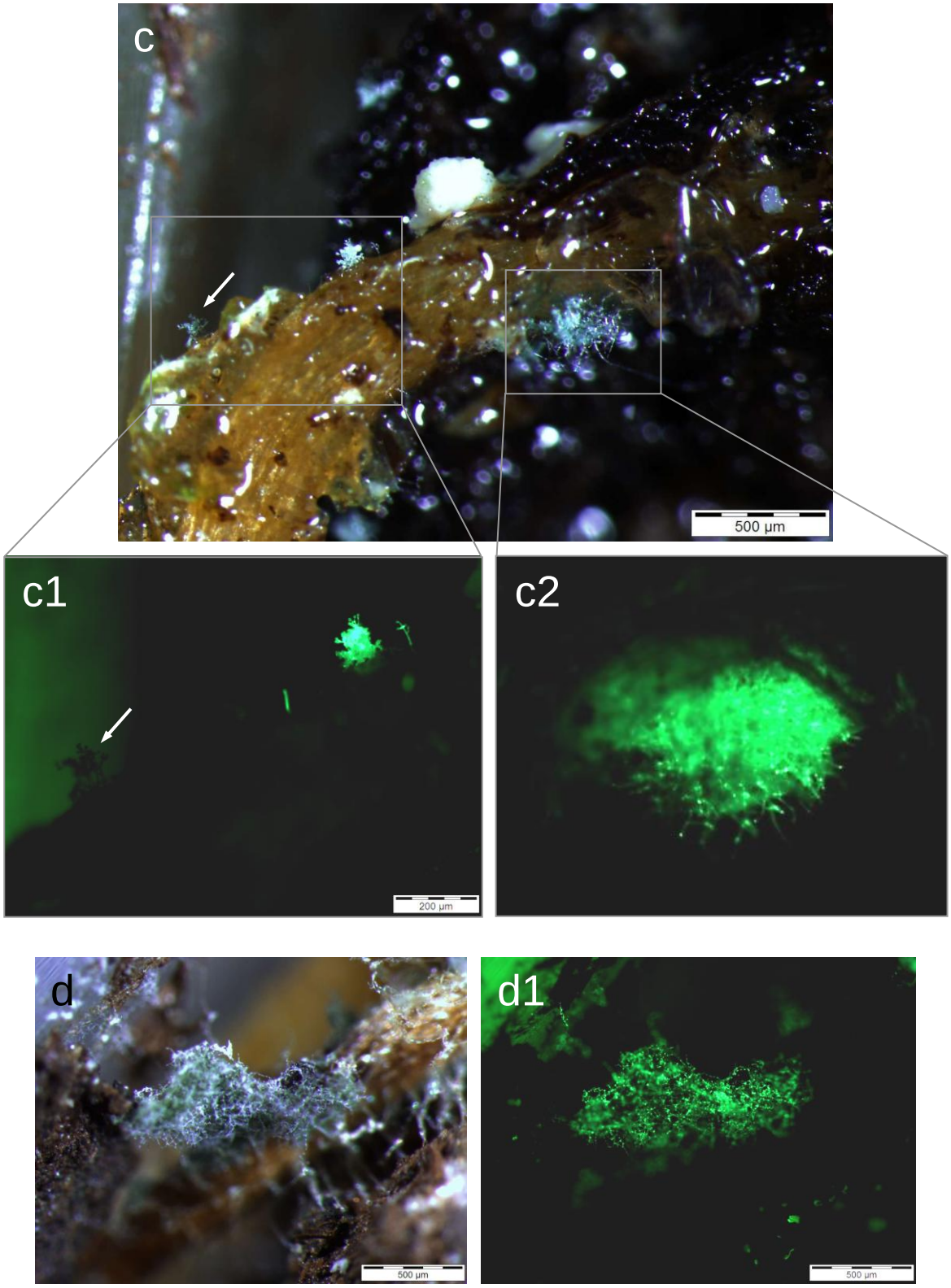


Figure 4.11 Continued.

At the 16-week assessment, a quantitative approach aimed to determine the proportions of penetrated root segments comparing three different parts of the root system. However, LU592/C was only recovered from within six of the 162 root segments sampled which resulted in poor analytical power. No significant effects were detected. The results from this assessment are summarised in **Table A4.5, Appendix 4** and show the proportion of internally colonised root segments by LU592/C across treatments and root samples. LU592/C was not recovered from any root section applied with 10^3 spores/microcosm. LU592/C was recovered from the root tip and intersection sections (each 1 out of the 18 (1/18) root segments) applied with 10^5 spores/microcosm. The 10^7 application rate resulted in LU592/C being recovered from three different root types, however, at a low number (2/18 for the root tip section and 1/18 for both the straight and intersection sections).

4.3.4 Experiment 3: Spatio-Temporal Distribution of *Trichoderma hamatum* LU592/C in the *P. radiata* Root Ecosystem

P. radiata seedlings appeared healthy and vigorous during the 4-month growing period. The moist potting mix discs were easily sampled. Within the first 4 weeks, the taproot penetrated to the bottom of the pot with lateral roots spreading vertically in the centre and bottom sections of the pot system (**Figure 4.3, Section 4.2.3.2**).

At 3 dpi, most of the *P. radiata* seeds had germinated (>90%) and the emerging radicle had penetrated into the top sampling depth. Root surface and internal colonisation by LU592/C was not detected for these radicles. LU592/C cfu levels for both treatments (plant present or absent) were below 10^3 and 10^2 cfu/g dry potting mix in the top and centre sections, respectively (**Table A4.6, Appendix 4**). LU592/C was not recovered from the bottom section.

LU592/C established throughout the system in the presence of *P. radiata* seedlings with cfu levels increasing over the 4-month growing period (**Figure 4.12**). This increase was significantly greater compared to the bulk potting mix (absence of the plant). Populations of LU592/C were significantly higher in the root zone compared with the bulk potting mix at all assessments between 4 and 16 wpi. Overall cfu levels (mean across all sampling times) in the presence of the

plant were recovered at 1.2 x 10^3 cfu/g dry potting mix and this was significantly higher by 8.1 times compared to the cfu levels in the bulk potting mix (1.5 x 10^2 cfu/g dry potting mix).

At the end of the experiment, the difference in the LU592/C cfu levels between the two treatments increased to 79.6 times. Between 3 dpi and 4 wpi, LU592/C cfu levels in the root zone increased significantly by 63 times compared to 4 times in the bulk potting mix (**Figure 4.12**). The decrease in LU592/C population between 4 and 8 wpi relative to the overall increase (measured using the regression slope) was significantly greater in the root zone compared with the bulk potting mix.

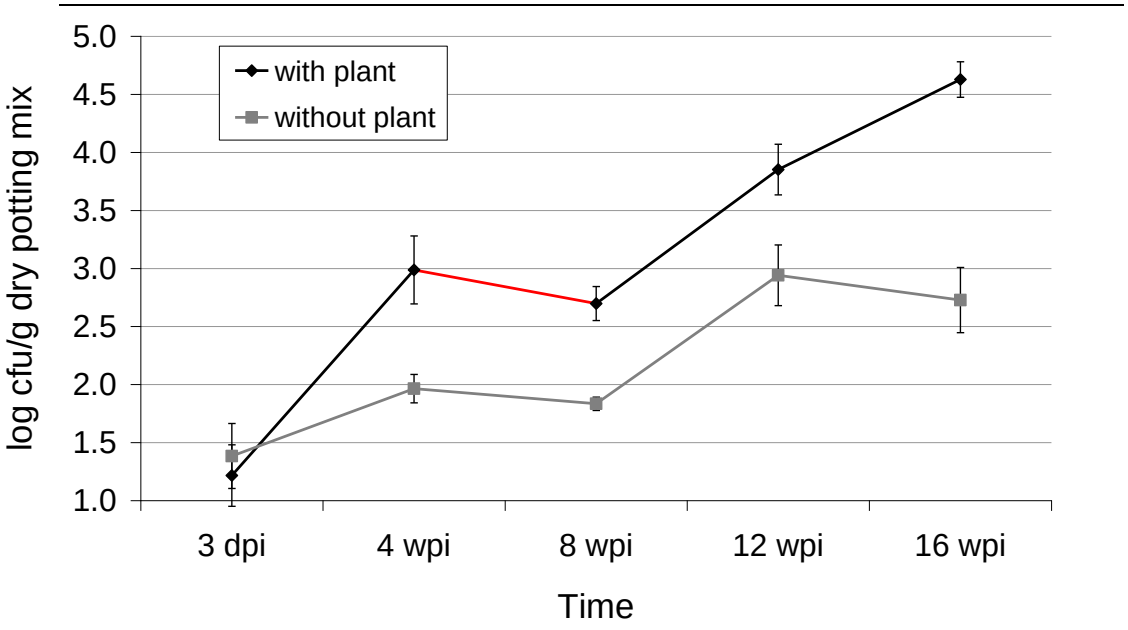


Figure 4.12 *Trichoderma hamatum* LU592/C population (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from potting mix over time in either presence or absence of *P. radiata* seedlings. Values are means (n = 8) of all depths. The red line indicates a significant difference between the two treatments in the decrease of cfu between 4 and 8 wpi relative to the overall increase. Dpi/wpi = days/weeks post-inoculation. Error bars represent the standard error of the means.

LU592/C colonised the centre and bottom sections of the pot at a faster rate when the plant was present (**Table A4.6, Appendix 4**). At the end of the experiment, the population of LU592/C was 1.4 x 10^4 cfu/g dry potting mix in the bottom section compared to 35 cfu/g dry potting mix without the plant (**Figure 4.13**). The cfu levels in the top section of the root were 1.7 x 10^5 cfu/g dry potting mix which was 11.9 times higher than the levels in the bottom section, compared to a difference of 190 times between the top and bottom sections without the plant.

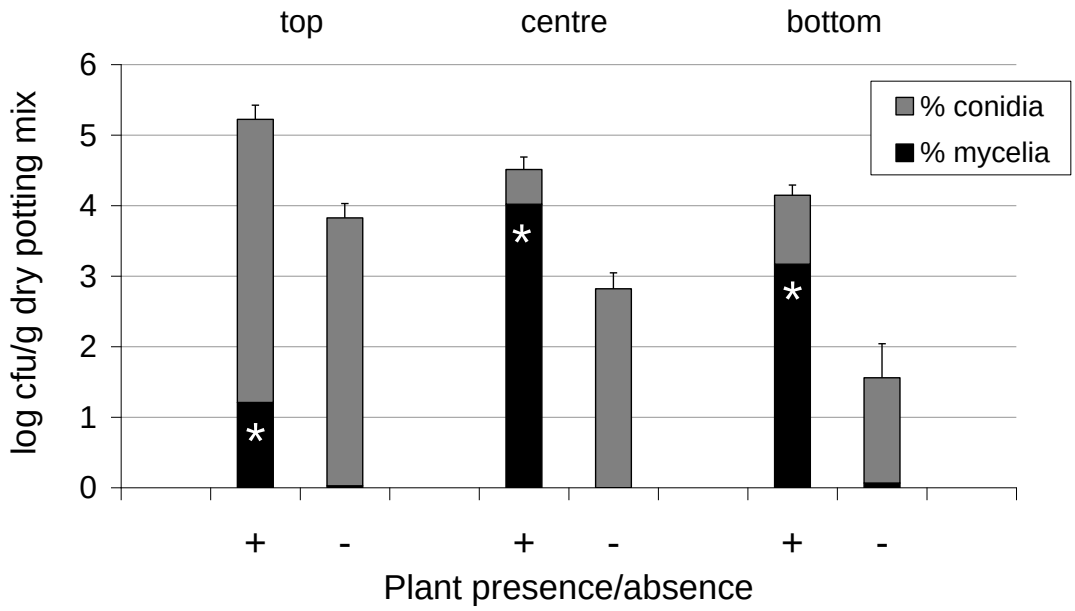


Figure 4.13 *Trichoderma hamatum* LU592/C population (log_{10} colony forming unit (cfu)/g dry potting mix) recovered from potting mix 16 weeks post-inoculation at three different depths (top: 1.5 - 2.5 cm, centre: 4.5 - 5.5 cm and bottom: 7.5 - 8.5 cm) in either presence (+) or absence (-) of *P. radiata* seedlings. Overall bars are means (n = 8) across five sampling times. Bar areas show the proportions of conidia and mycelia of the overall cfu levels with asterisks indicating when mycelia proportion means are above the detection level. Error bars represent the standard error of the means.

Microscopic observations did not detect any GFP-fluorescent fungal structures for any subsample taken at any time. However, the proportion of the total cfu as mycelia at 3 dpi was 43.6% in the top section of the root system (**Figure 4.14**). Four weeks later, mycelia levels decreased in the top section to below the detection level and increased in the centre section to 29.0%. At 8 wpi, the overall mycelia proportion (mean of all sections) decreased below the detection level and increased rapidly at 12 wpi to 36.5%, 54.2% and 58.5% for the top, centre and bottom sections, respectively. At 16 wpi, the highest proportion of the total cfu as mycelia was recovered from the centre section of the root system with 89.0%. The proportion of cfu as mycelia in both the top and bottom section were 23.1% and 76.4%, respectively. The proportion of cfu as mycelia in the plantless system remained mostly below the detection level, except at 12 wpi, when a proportion in the top section of 31.8% was detected (**Table A4.7, Appendix 4**).

Similar to the trend in mycelia proportions, the proportion of LU592/C colonised root segments significantly increased after 12 weeks from 19.4% to 74.3% at 12 and 16 wpi, respectively (**Figure 4.15**). No internal root colonisation was observed at 3 dpi. At 12 wpi, the top (older) section was significantly stronger colonised compared with the centre and bottom (younger) sections. Across all sampling times, the top section of the root system was more extensively colonised with 40.1% compared with the centre and bottom sections both with 20.8%.

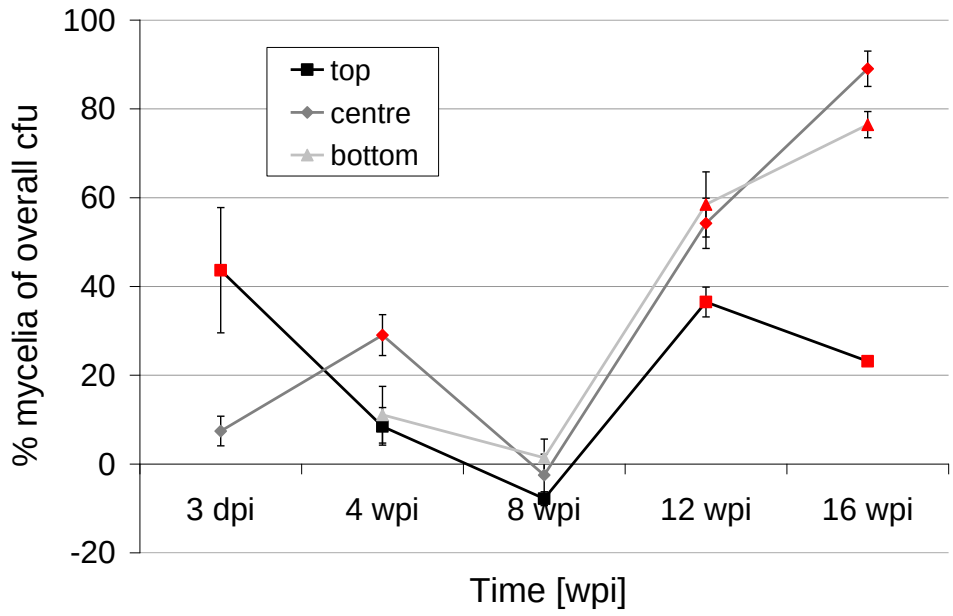


Figure 4.14 *Trichoderma hamatum* LU592/C mycelia proportions (% mycelia of overall colony forming unit (cfu)) recovered from potting mix at three different depths over time. Red values indicate means above the detection level. Error bars represent the standard error of the means (n = 8). Dpi/wpi = days/weeks post-inoculation.

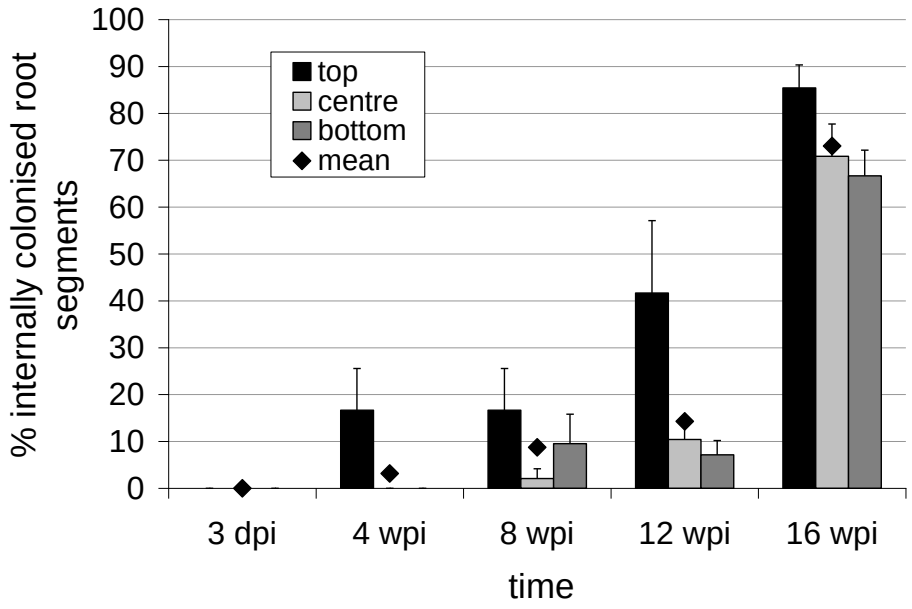


Figure 4.15 *Trichoderma hamatum* LU592/C population (% internally colonised root segments) recovered from surface-sterilised *P. radiata* root segments at three different depths (top: 1.5 - 2.5 cm, centre: 4.5 - 5.5 cm and bottom: 7.5 - 8.5 cm) over time. Diamond symbols represent means (n = 10) across all subsamples at each time. Error bars represent the standard error of the means (n = 8). Dpi/wpi = days/weeks post-inoculation.

4.4 Discussion

This study reinforced the value of genetic markers for use in ecological studies of filamentous fungi. Rhizosphere competence and internal colonisation of the root of *P. radiata* were confirmed for *T. hamatum* LU592. The ability to identify and track a specific isolate led to a better understanding of those qualities. A positive relationship was shown between the establishment of LU592/C in the root zone and its proximity to the root surface. The spatial and temporal proliferation of LU592/C was directly associated with *P. radiata* root development. With regard to root colonisation efficacy, there was an indication of an optimum inoculum concentration. Root penetration assessments and the ability to differentiate between propagule type provided additional knowledge about fungal activity.

Differentiation of LU592/C propagules

It is widely acknowledged that fungal mycelia are generally more active and, therefore, interact more strongly with their environment than resting structures such as spores. Many studies have focused on microscopic observations of hyphae to identify the potential of the agent to interact with the plant or pathogen (Freeman *et al.*, 2002; Lagopodi *et al.*, 2002; Etebarian *et al.*, 2005; Chacón *et al.*, 2007; Watanabe *et al.*, 2007). Further, biological activity (and fluorescence intensity) of genetically marked fungi was often associated with the presence of mycelia (such as Maor *et al.*, 1998; Lo *et al.*, 1998; Bao *et al.*, 2000; Green *et al.*, 2001; Orr & Knudsen, 2004; Bae & Knudsen, 2005; Papadopoulou *et al.*, 2005). This study attempted to differentiate mycelia from spores to provide a quantitative indicator of fungal activity.

The filtration dilution plating technique enabled the indirect enumeration of cfu originating from hyphal fragments. Filtration through three or four layers of miracloth removed sufficient hyphae to successfully determine the mycelial proportions. The detection of colonies originating from mycelia after filtration indicates that the smallest hyphal fragments were able to pass through the miracloth after shaking the hyphae-containing potting mix sample for 10 minutes.

This indicates that most hyphal fragments do not break up into individual compartments which would result in an underestimation of hyphal cfu.

Orr & Knudsen (2004) reported an innovative approach to estimate fungal activity of a *gfp*-tagged strain of *T. harzianum* in non-sterile soil using image analysis. Hyphal biomass was quantified by measuring the radii and lengths of visible fluorescent hyphae. This approach overcame many drawbacks of other techniques. However, a few limitations of this method include potential observer subjectivity and operator fatigue caused by the labour- and time-intensive acquisition and analysis of the images. Fungal fragments might also be underestimated when hyphae are hidden by soil particles or due to overlapping or when hyphae are out of focus. Moreover, the detection of fluorescent hyphae might be limited when masked by autofluorescence, particularly when the GFP-intensity varies. In comparison to the image analysis approach, the filtration dilution plating technique developed in this project had its own advantages and disadvantages. Hyphal cfu are determined indirectly which reduces sensitivity. It is also subject to the variability of the dilution plating method in general. Depending on the experimental set up and number of replicates the detection level varied between 7% and 20% of the overall cfu as mycelia. Therefore, an estimation of fungal activity of an abundant spore producer is compromised by this method. For instance, a mycelia proportion of 1%, or even 0.1%, of an overall cfu of 10^7 whilst below the detection level by this method might still be substantial in relation to active biomass able to interact with plants. As mentioned above (and by Ahmad & Baker, 1986), the general dilution plating method can potentially underestimate hyphal numbers. In contrast, the biological activity might be overestimated when dormant, but viable, hyphae are recovered. Nevertheless, it provides a better indicator of the fungal activity than overall cfu enumerations. In addition, the filtration dilution plating technique does not have any of the above mentioned visual-based drawbacks, it is simple to perform and less laborious than an image analysis approach.

Rhizosphere competence

The inoculum concentrations 10^5 and 10^7 spores/RSC were chosen as they are equivalent to the levels applied by seed coating (SC, ~10^5 spores/pot) and spray

application (SA, 5 x 10^6 spores/pot) in Chapter 2. Both seed coat and spray application resulted in similar growth promotion effects. In this study, results showed that the inoculum rate of 10^5 spores/pot resulted in the greatest establishment of LU592 in the rhizosphere and bulk potting mix with populations of the 10^3 and 10^7 application rates being similar but below that for the 10^5 application.

The subsample taken from 0-2 mm from the root surface was considered comparable to the subsample designated as rhizoplane in Chapter 2 and showed the highest establishment by LU592. The levels in subsamples further than 2 mm away from the roots were not different from that found in the bulk potting mix. However, the mycelial proportion data tells a different story. Total propagules as mycelia, that is to say, the proportion of active biomass was highest for both the 10^5 and 10^7 applications and with mycelia levels maintained at significantly higher levels within 10 mm of the root surface.

The indications given in Chapter 2 regarding the isolate's ability to colonise the rhizosphere were confirmed in more detail in this experiment. A plausible explanation for LU592's rhizosphere affinity is its preference for *P. radiata* root exudates such as sugars, amino acids and organics acids. Rhizosphere competence of *Trichoderma* spp. has been suggested to be associated with the secretion of root exudates (Aziz *et al.*, 1997; Mousseaux *et al.*, 1998; Green *et al.*, 2001; McLean *et al.*, 2005; Pinot *et al.*, 2006).

The ability to internally colonise the roots was determined as the second indicator of biological activity of LU592/C. Internal root colonisation, and presumably fungal activity, was shown at all three inoculum concentrations with the proportion of penetrated root segments being over 50%. The colonisation of the root system by LU592 was strongest when 10^5 spores were applied. This measure supports the dilution plating assessments of this experiment which showed that the 10^5 application resulted in the highest active biomass of LU592 associated with 6-month old *P. radiata* seedlings. Therefore, it can be assumed that the inoculum rate closest to the established level after 6 months provides an advantage in the effectiveness to colonise the root zone. The similar growth promotion effects of seed coat and spray application in Chapter 2 are best explained by the fact that

both the 10^5 and 10^7 application rates resulted in comparable biological activity of LU592 in the root zone.

Microscopical Examinations of LU592/C Root Colonisation

This study showed that microscopic observations can be subjective and reliant on the ability to visualise fungal structures. In addition, root disturbances cannot be avoided and complicate any assumptions about the undisturbed system. Microscopic observations are mainly of a qualitative nature and need to be accompanied by quantitative assessments. During the microcosm experiment, LU592 was found on all parts of the *P. radiata* root system including taproot, lateral roots, root tips and root hairs. The fungus was also found at all root depth (top, centre and bottom).

Waid (1956) concluded that root tips of perennial ryegrass are virtually free of fungal hyphae, including *Trichoderma* spp. However, this study showed qualitative evidence of the presence of *T. hamatum* LU592 on *P. radiata* root tips (external and internal), which are potential infection sites for root pathogens (Green *et al.*, 2001; Lagopodi *et al.*, 2002). More recently, root tip colonisation by *Trichoderma* spp. have been demonstrated in various annual crops and Douglas-fir (Mousseaux *et al.*, 1998; Yedidia *et al.*, 2000; Green *et al.*, 2001). Some of the microscopic observations suggest that mycelia of LU592 penetrated the outer root cortex colonising between epidermal cells. This is a well known feature reported for the genus *Trichoderma* in various plants (Waid *et al*., 1956; Harman *et al*., 2004a; Vinale *et al*., 2008) and is supported by the fact that LU592 was recovered from every surface sterilised root sample where fluorescent mycelia were visible beforehand. It has been suggested that the fungus occupies nutritional niches forming a symbiotic rather than parasitic relationship with the plant (Harman *et al.*, 2004a).

Although sufficient for qualitative interpretations, the search for fluorescent fungal structures was time-consuming and, despite *Trichoderma* propagules being recovered by dilution plating, often unsuccessful. In addition, no fluorescent fungal structures were observed in any root or rhizosphere sample in experiment 3. As discussed in Chapter 3, potential difficulties with visual assessments have been reported previously (Morgan *et al*., 1991; Orr & Knudsen 2004; Papadopoulou *et al.*, 2005). In this study, the inability to visualise LU592/C is most likely explained

by the fluorescence intensity being of insufficient brightness to be easily distinguishable from the background fluorescence. The fading of fluorescence in older mycelia was previously observed in Chapter 3 and fluctuating fluorescence intensities of mycelia were also seen in these experiments. In addition, the metabolic state of LU592 is likely to be less active in a potting mix environment than on a nutrient-rich PDA medium which would also account for a weaker fluorescence. It was noted in Chapter 3 that the *gfp* and *hph* expression levels of LU592/C might be lower than reported by other researchers (e.g. Thrane *et al.*, 1995). Although visualisation of LU592/C was limited in the present study, there are many reports of strong GFP-fluorescence of filamentous fungi in soil environments (Lagopodi *et al.*, 2002; Lu *et al.*, 2004; Rincón *et al.*, 2005; Chacón *et al.*, 2007) though mostly observed in axenic conditions, with few exceptions (Bae *et al.*, 2000; Lübeck *et al.*, 2002; Orr & Knudsen, 2004).

Conidiophore structures were easily detectable after 16 weeks which might partly be due to their relatively large and compact size, and would also be influenced by their high metabolic activity as discussed previously in Chapter 3.

The semi-quantitative approach to estimate preferred sites of internal colonisation revealed similar difficulties. What was appropriate for use in experiments 1 & 3, did not lead to meaningful results for experiment 2. The root segments for the experiment 2 assessment were up to 10 times shorter than those cut for the other two experiments to ensure root tips and intersections did not include straight parts of the root. This probably accounted for a lower likelihood of recovery of LU592/C as *Trichoderma* spp. are known to locally penetrate roots and only rarely show continuous, endophytic-like root colonisation (Harman *et al.*, 2004a). For future studies, it is suggested to determine an appropriate root segment length beforehand. Furthermore, the microcosms used in experiment 2 were designed to minimise disturbances, in which roots were growing against the lid and not entirely covered by potting mix and, therefore, might not have had an effective rhizosphere. Only those visible roots were sampled which could have resulted in an atypical colonisation pattern by LU592/C.

Spatio-Temporal Population Dynamics & Conclusions

The population dynamics of *T. hamatum* LU592 were examined in the root ecosystem of *P. radiata* seedlings. Overall cfu levels of LU592 significantly increased over time compared to the bulk potting mix (potting mix system without the plant). This establishment is supported by the mycelia and internal root colonisation data. An intitial rise in cfu levels after inoculation of *Trichoderma* was reported in annual crop systems (Green & Jensen, 1995; Lo *et al.*, 1998; Subhendu & Sitansu, 2007). It was shown that an abundant spore production can cause this increase (Lewis & Papavizas, 1984; Green & Jensen, 1995; Orr & Knudsen, 2004). The mycelia enumeration of this study indicated that almost half of the applied *Trichoderma* spores had germinated in the top section after 3 days. The nutrient-rich spermosphere probably initiated this spore germination.

A decrease in the number of propagules was observed 8 weeks after seedling emergence in experiment 1 of Chapter 2. The seedlings were transferred from the glasshouse to the outside at this time and, therefore, it was assumed that a change in environmental conditions caused a decrease in the *Trichoderma* population. However, a similar pattern was observed in this study where seedlings were grown under controlled conditions for the duration of the experiment. The decline in overall cfu and mycelia levels could have been caused by a depletion in nutrients, assuming that the maturing root did not provide enough nutrients yet. It is known that *Trichoderma* spp. are sensitive to various types of disturbances and stimuli (Lu *et al.*, 2004).

Maturing *P. radiata* roots experience physiological changes (Shi, 2010). Therefore, another possibility might be that changes in the composition of root exudates caused a decrease in overall *Trichoderma* populations. This assumption is supported by the fact that there was an increase in overall cfu and mycelia levels in the 4-week old root system in the centre section indicating sufficient nutrient availability. Propagule numbers increased after 12 weeks when an abundancy of roots was observed in the centre and bottom sections of the pot. Increasing mycelia levels also indicated high fungal activities at that time. Internal root colonisation was detected after 4 weeks with a sharp increase in the number of colonised root segments after 16 weeks. This steep increase is 4 weeks behind the increase in mycelia levels. Internal root colonisation may be an indication of

increased availability of nutrients released by the roots. In summary, a stronger spatial and temporal proliferation of LU592 was observed in the presence of the root compared to the bulk potting mix system. This reconfirms a positive relationship between the isolate's establishment and *P. radiata* root development.

The LU592 population stabilised at around 10^3 cfu in the bulk potting mix. Previous reports demonstrated long-term establishments of applied *Trichoderma* spp. at a similar level in natural soils and plant rhizosphere (Papavizas, 1982; Lewis & Papvizas, 1984; Leandro *et al.*, 2006; Longa *et al.*, 2008). However, mycelia ratios were below the detection level in the bulk potting mix after 16 weeks and after 6 months in experiments 3 and 1, respectively. A weak ability to proliferate in the bulk potting mix was observed. These results are in accordance with Fahima & Henis (1990) who concluded that *T. hamatum* is a poor competitor for dead organic substrates, but uses root exudates as a food source. In contrast, composted bark added to potting mix has recently been shown to stimulate *T. hamatum* activity, reducing disease incidence and enhancing plant vigour (Hoitink *et al.*, 2006). Further, addition of compost to potting mix resulted in the induction of systemic aquired resistance in cucumber by a strain of *T. hamatum* (Khan *et al.*, 2004). There seem to be a synergistic effect on the activity of *T. hamatum* when both composted organic substrates and plant roots are present.

Some reports have been published which indicate poor root colonisation ability by *Trichoderma* spp. *Trichoderma* spp. were detected only on the upper 3 cm of pea roots grown in non-sterile conditions and hyphae were only visible on the root surface when grown axenically (Chao *et al.,* 1986). Further, Thrane *et al.* (1995) and Green *et al.* (2001) reported that a *T. harzianum* isolate only superficially colonised the surface of healthy cucumber roots based on microscopic observations. However, the same report (Green *et al.*, 2001) stated that *Trichoderma* grew out of all parts of healthy pea and cucumber roots. Dense hyphae of another strain of *T. harzianum* was found on all parts of cucumber roots (Yedidia *et al.*, 2000).

The present study in contrast focused on detailed examinations of *Trichoderma*'s root colonisation behaviour in a perennial woody plant over months, rather than days or weeks (the timeframe of the previously mentioned studies). It was shown that root colonisation assessments need to be interpreted carefully, particularly

when observed visually (see also Green *et al.*, 2001). It was further demonstrated that conclusions based on overall cfu numbers can lead to misinterpretations. Firstly, relatively high overall cfu levels do not necessarily indicate high fungal activity as shown in the bulk potting mix of experiment 1 and 3 or for the 10^3 application of experiment 1. This issue with the traditional dilution plating method has been reported extensively (Ahmad & Baker, 1987; Parkinson & Coleman, 1991; Paulitz, 2000; Green *et al.*, 2001; Orr & Knudsen, 2004). Secondly, lower overall cfu levels do not necessarily indicate a low biological activity of *Trichoderma*. This was shown in experiment 3 when 43.6% of an overall cfu level of $<10^2$ was shown to be mycelia around the emerging radicle of a 3-day old seedling. Also, the mycelia data of experiment 1 indicated a bigger root zone under the influence of the root (up to 10 mm) than suggested by the overall cfu data (<2 mm). Mycelia proportions of the overall cfu level in experiment 3 reached up to 89%. The mycelia data of experiment 1 suggests that those proportions then stabilise at 20-25% in the rhizosphere. Future studies could observe mycelia levels in relation to change in root exudate quantity and composition over longer periods (years). The results of this study lead to the hypothesis that *Trichoderma* experiences cyclic patterns of higher and lower fungal activity.

The use of marker genes enabled monitoring of a specific isolate in non-sterile systems. Similar population dynamics of indigenous *Trichoderma* spp. did not compromise the assessments as was the case for Chapter 2 assessments, which has also been reported previously (Mousseaux *et al.*,1998; Lo *et al.*, 1998). Using the marked isolate the sensitivity of the dilution plating method was increased by at least a factor of ten compared to the detection levels achieved in Chapter 2. The exclusion of non-target microorganisms allowed the detection of isolate concentrations as low as 35 cfu/g dry potting mix. Furthermore, this study provides evidence to support previous statements that the biological activity associated with the distribution of an introduced isolate is more critical than the distribution itself. Therefore, in future ecological studies of *Trichoderma* bio-inoculants it is recommended to supplement traditional quantification techniques with root penetration and mycelia assessments as measures of fungal activity.

Chapter Five

Concluding Discussion

This PhD study is the first report of a detailed investigation of *Trichoderma* in the root ecosystem of *P. radiata* seedlings. The results of these experiments provided information on the ecology of *Trichoderma* spp. in relation to beneficial effects to the plant. *Trichoderma hamatum* LU592 emerged as being both a strong root coloniser and plant growth promoter. Although no definite conclusions can be drawn about the mechanisms by which LU592 elicits its benefits to the plant, this study provided some indications which will need further exploration. LU592 was found to colonise and penetrate the entire root system. Therefore, one possible mechanism is competition with minor root pathogens.

The fungus was shown to have substantial mycelial concentrations in the rhizosphere within a distance of at least 1 cm from the root surface. The rhizosphere of *P. radiata* seedlings was shown to be limited to within 5 mm of the root based on a study on phosphorus dynamics by Chen *et al.* (2002). LU592 clearly showed an affinity for *P. radiata* roots, but was also able to extend its zone of activity beyond the rhizosphere and, therefore, could have mobilised nutrients from the potting mix medium making them available to the plant. Previous reports support this assumption which demonstrated N and P mobilisation by *Trichoderma* spp. in the root zone of pine trees (Blakeman, 1978; Koide & Kabir, 2001; Wu *et al.*, 2003; Bae & Knudsen, 2005; Wu *et al.*, 2005).

Further, rhizosphere competence and the ability to penetrate roots are acknowledged to be crucial indicators of plant growth stimulation and activation of plant defences (Zeilinger *et al.*, 1999; Benhamou & Picard, 1999; Yedidia *et al.*, 2000; Shoresh *et al.*, 2005; Verma *et al.*, 2007). These attributes were also shown to indicate an improvement in the plant's ability to deal with abiotic stress and the ability of the agent to control root pathogens, particularly if competition was the mode of action (Kleifeld & Chet, 1992; Chet *et al.*, 1998; Yedidia *et al.*, 2001; Harman *et al.*, 2004a; Vinale *et al.*, 2008). These are all valuable areas to follow up on to better understand the mechanisms by which LU592 elicits its benefits to

P. radiata seedlings. Specific experiments are required to address each of the potential modes of action.

T. hamatum LU592 improved various health and growth factors of *P. radiata* seedlings in two commercial nursery experiments. The commercial *Trichoderma*-based product ArborGuard^{TM} was also shown to promote growth of *P. radiata* seedlings in the nursery situation and decreased *Armillaria* infection of *P. radiata* once these are outplanted into the plantation forest. Whether LU592 has any activity against *Armillaria* spp. or other root pathogens is not known, but needs to be determined. Protection of the seedlings/trees from root pathogens, as well as the already confirmed growth promotion effects would increase the value to the forestry industry of using LU592. Therefore, it is now desirable to further investigate the root colonisation behaviour of outplanted *P. radiata* trees along with ongoing health and growth assessments. Once outplanted, does LU592 maintain a population in the *P. radiata* root system? Is it dependent on the conditions in the outplanting site (soil type, climate, biotic factors etc.)? If it is maintained at a reasonable population in the rhizosphere, what effect does it have on the subsequent colonisation of ectomycorrhizae (ECM) types? Although this study indicated that ECM in *P. radiata* seedlings were not detrimentally affected by LU592 based on visible observations, it is not known whether LU592 influences ectomycorrhizal succession when outplanted.

The New Zealand Biotron Facility could be used to answer these questions using the genetically marked *Trichoderma* strain LU592/C. The Biotron Facility has been designed and constructed to study above/below-ground interactions of plants grown in soil under controlled Physical Containment 2 (PC2) conditions (BPRC, 2009). The use of the Biotron Facility would enable realistic simulation of the outplanting of *P. radiata* seedlings for long-term ecological studies of transgenic *Trichoderma* spp. Alternatively, isolate-specific markers can be developed to distinguish the target isolate from the microbial soil community (McDonald, 1997; Paulitz, 2000; Lübeck & Jensen, 2002; Dodd *et al.*, 2004a; Rubio *et al.*, 2005; Miranda *et al.*, 2006; Cordier *et al.*, 2007). Previous reports have demonstrated the successful quantification of introduced fungi in non-sterile soil environments via quantitative real-time PCR (qPCR) (Filion *et al.*, 2003; Landeweert *et al.*, 2003; Rubio *et al.*, 2005; Cordier *et al.*, 2007; Longa *et al.*, 2009). This approach allows high sample throughput at relatively affordable per-sample costs. Molecular markers enable the isolate-specific identification of wild type strains in nursery or field conditions without having to (i) carry out the transformation procedure to

insert foreign marker genes and (ii) use expensive and often more artificial PC2 conditions. qPCR can be used to enumerate gDNA copy numbers and, therefore, give an estimate of the total biomass of the target strain. In theory, the technique could also be used to determine fungal activity based on measurement of constitutively expressed genes via reverse-transcriptase qPCR (RT-qPCR). The number of transcribed mRNA per copy number of gDNA would provide an indicator of the metabolic state of the fungus.

Chapter 3 demonstrated that, 14 years after the first integration of the *gfp* gene in a filamentous fungus, the transformation procedure has not yet become routine practice. A major restriction for successful gene insertion remains the specificity of the transformation protocol at the species and possibly even isolate level (Cardoza *et al.*, 2006). More research is essential to identify and overcome influencing factors to enable the development of a standardised protocol for *Agrobacterium*-mediated transformation of *Trichoderma* spp. Some of these factors have been continually optimised over the last 10 years such as promoter modifications, base pair adjustments, and the use of acetosyringone to induce virulence of the *Agrobacterium*, (Lorang *et al.*, 2001; Zeilinger, 2004). However, little is known about preferred genome integration sites of the gene cassette by this technique. The gene location within the genome is, besides the promoter, one of the most important regulators of gene expression (Bickmore & Chupp, 2003; Remenyi *et al.*, 2004; Xiao *et al.*, 2009).

Other parameters requiring further investigations are the *Agrobacterium*-*Trichoderma* ratio, duration of virulence induction and transformation, and the selection pressure. The right antibiotic concentration will need to be high enough to exclude false positives, but also sensitive enough to recover the fragile transformants. Therefore, it is suggested that a lower concentration is used for the first transfer onto selection medium with increasing selection pressure during the purification steps. Once purified, it is important to compare physiological characteristics with the wild type to ensure that the ecological and biological behaviour of the transformants are not compromised. The soil sandwich assay (SSA) was shown to be particularly useful as it allowed the measurement and comparison of fungal growth rates in the same non-sterile potting mix system that was used for subsequent ecological studies. The transformed isolate of LU592 was then successfully visualised and recovered from non-sterile potting mix. However, there were indications of relatively low expression levels of the foreign genes. The low expression rate of the *gfp* gene is probably one of the reasons why

microscopic observations were not successful in identifying detailed colonisation patterns. In contrast, the use of the *hph* gene is independent of the gene expression intensity and proved to be a valuable tool in the Chapter 4 experiments.

The Allee effect is known in the field of animal ecology and describes the density dependency of a population on its ability to establish in a new environment (Kramer *et al.*, 2009; Tobin *et al.*, 2009). Etienne *et al.* (2002) reported for the insect *Drosophila melanogaster* that the Allee effect creates a lower and upper boundary to the population size. The initial population distribution and density determined whether a population established, while resource availability was responsible for the subsquent population persistence. These findings were recently confirmed within microbial communities (Bonde *et al.*, 2004; Aspray *et al.*, 2006; Kadam & Velicer, 2006) and highlight the importance of identifying the optimum inoculum concentration, that is to say, the optimum population densities within the new environment to result in establishment. In this context, application method is an important factor in delivering the optimum population level to a particular location to result in establishment. If the inoculum concentrations, as used in this study were evenly mixed with the potting mix, *Trichoderma* levels would have resulted in 4×10^3 spores/g and 5×10^4 spores/g potting mix for seed and spray inoculation, respectively. It is questionable whether those concentrations were above the threshold level for successful establishment. This threshold level is generally regarded to be $>10^5$ cfu/g substrate, in the absence of an additional food source (Lewis & Papavizas, 1984; Ahmad & Baker, 1986; Green *et al.*, 2001; Rabeendran *et al.*, 2006; Longa *et al.*, 2008; De Souza *et al.*, 2008). However, the application methods used in this study introduced the isolate to the upper part of the pot, where the fungus could first establish at a higher localised concentration and subsequently use nutrient resources to proliferate into the remaining parts of the potting mix as was observed in the experiments. It can, therefore, be concluded that less inoculum is needed when a higher concentration is applied to a small target area. This assumption is supported by the fact that the seed coat application, although applied at a 10-fold lower concentration than the spray inoculum, resulted in equal beneficial effects on *P. radiata* growth and health. In addition, the Allee effect (a lower and upper boundary to the population size) would explain why higher inoculum concentrations do not result in higher population sizes and do not increase the beneficial effects to the plant. As a consequence, determining the optimum *Trichoderma* inoculum concentration is

important for the success of a commercial bio-inoculant. As mentioned in Chapter 2, not only can overdosing result in detrimental side-effects to the plant (Ousley *et al.*, 1993; Rousseau *et al.*, 1996; Brimner & Boland, 2003), but can also unnecessarily increase the inoculum production costs of the agent. LU592 established at a higher level in the rhizosphere when inoculated with 10^5 spores/pot compared with a 100-fold higher inoculum concentration. This result provides valuable information about the optimum inoculum concentration of LU592 and also helps explain why previous reports did not show a positive correlation between plant growth promotion and *Trichoderma* inoculum rate (Arcia & Pérez-Pivat, 2008; GroChem NZ Ltd, commercial trial). Those optima would probably need to be re-assessed in the presence of a major root pathogen, where the main goal is not to establish a sustainable and balanced root ecosystem, but to alleviate disease symptoms of a disturbed system.

The simplicity of the application method together with relatively low application rates makes this fungus an ideal candidate to develop as a commercial product. LU592 can be applied/coated as spores without the addition of an external food source. A one-off application was shown to result in the establishment of the fungus in the root system of *P. radiata* seedlings. Both the production of sufficient inoculum and the involved costs are two major limitations for the use of bio-inoculants (Verma *et al.*, 2007). However, this is not necessarily true in confined greenhouse and nursery situations. In fact, LU592 is suitable for affordable mass production for application in tree nurseries. Although the species *T. hamatum* is considered amongst other *Trichoderma* spp. to be a poor sporulator (J. Steyaert, pers. comm.), it was possible to produce 5 x 10^{10} spores using 150 mL of an inexpensive peat/bran mix (50:50 V:V peat:bran) in a 1 L conical flask at room temperature and constant blue light. To apply 5 x 10^6 spores to each of the 2 million seedlings at the PF Olsen nursery site, ~10^{13} spores would be required. It can be calculated that 10 trays (0.15 m^2) filled with a pre-inoculated peat/bran mix (2 cm high) would provide enough inoculum to treat 2 million seedlings at a cost of less than $100. Those estimates are not comprehensive and do not include costs of labour and other equipment, however, they do illustrate the ease of use and potential for *Trichoderma*-based bio-inoculants in greenhouse and nursery situations.

The number of *Trichoderma*-based commercial products which target greenhouse plants is increasing worldwide (Verma *et al.*, 2007). Simultaneously, the demand for sustainable production systems is increasing, which will constrain the future

use of pesticides (UNFCCC, 2009). Integrated pest management (IPM) practices attempt to address these changes (IPM CRSP, 2009). Current IPM approaches do not necessarily replace pesticides, but rather integrate complementary strategies such as bio-inoculation with the aim of reducing pesticide use. Future research is required to investigate the sensitivity of *T. hamatum* LU592 to some of the common fungicides used in the nursery system (such as probineb, thiram, carbendazim, mancozeb). This would provide the industry with guidelines on the compatibility of the bio-inoculant with fungicides to ensure maximum efficacy. A similar study has been carried out for the commercial biological control agent (BCA) *T. atroviride* LU132 which is registered for use to protect onions from *Sclerotium cepivorum* and grapes from *Botrytis cinerea*, and enabled guidelines to be provided on which fungicides were able to be used in conjunction with the BCA and which had detrimental effects on colonisation and biocontrol efficacy (McLean *et al.*, 2001).

Another field of IPM research could look at the bioremediation potential of beneficial *Trichoderma* spp. It has been shown that *Trichoderma* spp. are able to degrade various soil pollutants including synthetic herbicides (Verma *et al.*, 2007). Herbicides are often mandatory and usually difficult to replace in agricultural systems. Therefore, it is desirable to identify *Trichoderma* strains with herbicide degradation potential to reduce herbicide residues and maintain a healthy soil and plant environment.

The outcomes of this study highlight the value of relating plant performance assessments with comprehensive ecological studies to successfully identify *Trichoderma*-based bio-inoculants. Chapter 2 provided valuable lessons to consider in future evaluations of bio-inoculants. The growth promotion effects observed in the commercial experiment in 2004 could not be reproduced in the first experiment. Answering many different questions in a single experiment inevitably results in a large amount of work which often limits the number of replicates that can be assessed. In addition, the limitations of the dilution plating technique need to be considered. The differentiation of the introduced isolate from the microbial community in non-sterile potting mix (or soil) systems was shown to be challenging, particularly in the presence of fungi with indistinguishable morphological characteristics and similar population dynamics. The quantification of fungal propagules provides valuable information about the agent's establishment in the root system, however, might not necessarily correlate with its

biological activity. Therefore, we need to exercise caution when drawing conclusions about the plant-microbe interactions observed in Chapter 2.

Both the reproducibility problem and the limitations of the dilution plating technique were overcome in this project by addressing growth promotion and population dynamics of LU592 in separate experiments. The two main drawbacks of the standard dilution plating technique were alleviated with the methodology used in Chapter 4. The use of a genetically marked strain allowed the specific identification of the introduced isolate. Further, it is proposed that endorhizosphere competence and mycelia enumeration as measures of biological activity of the fungus be included in future studies. The described techniques have been shown to enable valuable information about spatio-temporal population dynamics to be determined and will be useful in future studies.

In summary, there are a number of potential future research areas based on the findings presented in this study. First and foremost, the influences of biotic and abiotic factors on the agent's performance need to be better understood. Variation in efficacy is known to be one of the major constraints for the successful use of bio-inoculants and will only be overcome by identifying the environmental parameters that influence its performance (Stewart, 2009; Stewart *et al.*, 2009). In addition, nutrient mobilisation, induced systemic resistance and competition are potential mechanisms by which the fungal agent elicits its benefits to the plant and need further investigation. An understanding of both the agent's colonisation behaviour and its mode(s) of action will help to identify the key factors which could restrict its successful integration into the nursery practice. This study improved the understanding of the ecology of *Trichoderma* bio-inoculants in the root ecosystem of *P. radiata* seedlings. LU592 was shown to consistently enhance various growth and health factors in two different large-scale experiments under commercial growing conditions. Furthermore, the importance of an optimum inoculum application was highlighted which will be helpful not only to enhance root colonisation and plant vitality, but will also minimise inoculum production costs. It is proposed to determine the establishment and population dynamics of an introduced *Trichoderma* isolate to identify more predictable and effective bio-inoculants for use in plant ecosystems.

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Appendices

Appendix 1

Media and Buffer

***Trichoderma* Selective Medium - Lincoln University (TSM-LU)**

Bacteriological agar (Danisco® New Zealand Ltd)	20.0 g/L
Glucose	3.00 g/L
Ammonium nitrate (NH_4NO_3)	1.00 g/L
Dipotassium hydrogen orthophosphate trihydrate ($K_2HPO_4 \cdot 7H_2O$)	0.90 g/L
Magnesium sulphate 7 hydrate ($MgSO_4 \cdot 7H_2O$)	0.20 g/L
Potassium chloride (KCL)	0.15 g/L
Terrachlor® 75 WP (quintozene 750 g/kg a.i.)	0.20 g/L
Rose Bengal	0.15 g/L
Iron sulphate (Ferrous sulphate) 7 hydrate ($FeSO_4 \cdot 7H_2O$)	1.00 g/L
Manganous sulphate tetrahydrate ($MnSO_4 \cdot 4H_2O$)	0.65 g/L
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	0.90 g/L

in sterile distilled water (SDW), autoclaved.

Add:

Chloramphenicol	2.50 mg/L

The above recipe is a modified version of the medium initially developed by Elad *et al.* (1981), modified by Elad & Chet (1983) and modified again by Askew and Laing (1993).

Soil Extract Agar (SEA)

Soil	10.0 g/L

in SDW, left overnight, filtered through Whatman® 105 paper.

Filtrate	20 %
Bacteriological agar	12.0 g/L
Dipotassium hydrogen orthophosphate trihydrate ($K_2HPO_4 \cdot 7H_2O$)	0.50 g/L

in SDW, pH 6.8, autoclaved.

50x Tris-acetate EDTA buffer (TAE)

Tris-base (J.T. Baker®, USA)	242.0 g/L
Glacial acetic acid (Sigma-Aldrich® Inc., USA)	5.11 %
Ethylenediamine tetraacetic acid (EDTA; pH 8)	50 mM

in SDW, autoclaved.

5x Tris-borate EDTA buffer (TBE)

Tris-base (J.T. Baker®, USA)	54.0 g/L
Boric acid (Sigma-Aldrich® Inc., USA)	27.5 g/L
EDTA (pH 8)	10 mM

in SDW, autoclaved.

6x Loading buffer

Bromophenol blue	0.25 %
Xylene cyanol FF	0.25 %
Sucrose	40% (w/v)

in SDW, stored at 4ºC.

20x sodium chloride sodium citrate buffer (SSC)

sodium chloride (NaCl)	3 M
tri-sodium citrate	0.3 M

in SDW, autoclaved.

Methods

Standard Polymerase Chain Reaction

All PCR amplifications were performed in an Eppendorf Mastercycler® Gradient PCR machine (Eppendorf-Netherler-Hinz Ltd, Hamburg, Germany). Each 25 µL PCR reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP (Advanced Biotechnologies Ltd), 5 pmol of each primer, 10 ng of *Trichoderma* DNA and 1.25 U Taq DNA polymerase (Roche Diagnostics Ltd, Mannheim, Germany). Unless otherwise stated, amplification consisted of an initial denaturation of 6 min at 95ºC, followed by 30 cycles of 30 s denaturation at 95ºC, 30 s primer annealing at 50ºC and 30 s extention at 72ºC, and a final extension of 7 min at 72ºC.

Single-Spore Isolation

Isolates were grown on hygB-amended PDA at 22ºC under constant light. After 14 days, sporulating plates were flooded with 5 mL of sterile distilled water (SDW) and spores were scraped off with a sterile hockey stick and filtered through a double layer of sterile miracloth (an aliquot of each spore-suspension was stored in 25% glycerol at -80ºC). Serial dilutions were made from the spore-suspension and 100 µL of each dilution were spread onto three plates of hygB-amended PDA. Plates were then incubated at 20ºC in the dark. Plates were assessed daily for germinating spores using a stereo microscope and three emerging colonies transferred to new plates of hygB-amended PDA and kept in the dark at 20ºC until further use.

Agarose Gel Electrophoresis

A 1% agarose gel was prepared by dissolving 1 g of agarose (Agarose MP, Roche Diagnostics Ltd) in 100 mL of 1 x TAE buffer by boiling. The dissolved agarose was cooled to 60ºC, poured into a gel casting tray containing a well-forming comb and allowed to set for ~1 h. The comb was removed and the casting tray containing the solidified gel transferred to an electrophoresis tank (E-C Apparatus Corp.) containing 1 x TAE buffer. For each well, 5 µL of DNA solution was mix with 1 µL of 6x loading buffer. Electrophoresis was carried out at 10 V/cm for 1 h at RT,

depending on the desired separation. After electrophoresis, DNA fragments were stained with Ethidium Bromide (12.5 µL of 1% Ethidium Bromide in 500 mL SDW) for 10 min and visualised under UV light (300 nm) using VersaDoc™ Imaging System (Bio-Rad® Model 3000, Bio-Rad Laboratories, USA)

Appendix 2: Chapter Two

Table A2.1 Plant performance assessments of *P. radiata* seedlings treated with three different *Trichoderma* isolates (LU132, LU592 and LU686) as either seed coat (SC) or spray application (SA).

	% seedling emergence	Health score		% dead seedlings[A]	
Treatment	6 wpi	3 mpi	7 mpi	3 mpi	7 mpi
SC132	91.7	2.69	2.65	3.3 (10.5)	5.7 (13.8)
SA132	92.9	2.81	2.67	1.4 (6.89)	4.3 (12.0)
SC592	93.5	2.87	2.68	**0.19** (2.47)	5.5 (13.5)
SA592	93.5	2.90	2.91	0.19 (2.51)	0.97 (5.66)
SC686	94.6	2.68	2.80	0.36 (3.46)	1.8 (7.70)
SA686	94.6	2.85	2.77	1.4 (6.82)	4.5 (12.2)
SCcontrol	91.1	2.74	2.65	5.2 (13.2)	5.8 (13.9)
SAcontrol	93.5	2.79	2.68	2.6 (9.30)	3.8 (11.3)
F pr. (isolate)	0.556	0.198	0.746	0.074	0.910
F pr. (application)	0.522	0.074	0.611	0.674	0.642
F pr. (isolate.application)	0.915	0.773	0.856	0.676	0.769
LSD (*P* = 0.05)	5.63	0.203	0.465	(9.84)	(17.0)

Bold values indicate significant differences to the respective control. LSD = least significant difference (n = 4). Wpi/mpi = weeks/months post-inoculation; F pr. = F probability of a general unblocked ANOVA; SC = seed coating; SA = spray application.
[A] shown are back transformed means with arcsin-transformed values in parentheses.

Table A2.2 Plant performance assessments of *P. radiata* seedlings treated with three different *Trichoderma* isolates (LU132, LU592 and LU686) as either seed coat (SC) or spray application (SA).

	Shoot height [cm]		Shoot diameter [mm]	Dry weight (7 mpi) [g/10 plants]		Root/shoot ratio
Treatment	3 mpi	7 mpi	7 mpi	roots	shoots	
SC132	7.29	12.74	2.82	4.95	10.9	0.450
SA132	7.23	14.86	3.08	6.51	14.6	0.445
SC592	8.28	14.18	3.05	5.46	14.3	0.383
SA592	7.35	13.85	3.08	4.97	13.8	0.358
SC686	7.85	14.10	3.03	4.95	13.3	0.371
SA686	7.75	13.92	3.07	5.45	13.5	0.403
SCcontrol	6.91	12.39	2.97	5.09	12.9	0.403
SAcontrol	7.07	13.64	3.07	5.47	13.5	0.404
F pr. (isolate)	0.483	0.682	0.749	0.732	0.801	0.001**
F pr. (application)	0.611	0.293	0.201	0.220	0.275	0.884
F pr. (isolate.application)	0.830	0.516	0.742	0.330	0.408	0.423
LSD (*P* = 0.05)	1.83	2.75	0.332	1.58	3.8	0.0523

Bold values indicate significant differences to the respective control. LSD = least significant difference (n = 4). Mpi = months post-inoculation; F pr. = F probability of a general unblocked ANOVA; SC = seed coating; SA = spray application.
** = significant at *P* <0.01.

Table A2.3 Summary of *Trichoderma* populations in the **top bulk**, **centre bulk**, **bottom bulk**, **rhizosphere** and **rhizoplane** potting mix of *P. radiata* seedlings for each sampling time and treatment.

	Trichoderma population [cfu/g dry potting mix][A]					
Treatment	0 wpi	4 wpi	8 wpi	12 wpi	20 wpi	28 wpi
Top bulk						
SC132	9.2×10^3 (3.97)	1.1×10^4 (4.03)	2.5×10^4 (4.40)	2.4×10^4 (4.38)	1.1×10^4 (4.06)	6.3×10^3 (3.80)
SA132	**1.7×10^5 (5.22)**	**1.2×10^5 (5.09)**	**1.2×10^5 (5.09)**	**7.3×10^4 (4.87)**	**2.8×10^4 (4.45)**	**1.4×10^4 (4.14)**
SC592	2.7×10^4 (4.43)	**2.8×10^3 (3.45)**	2.3×10^4 (4.37)	9.8×10^3 (3.99)	1.1×10^4 (4.02)	5.9×10^3 (3.77)
SA592	**3.3×10^5 (5.52)**	**4.0×10^5 (5.60)**	**3.3×10^5 (5.52)**	**3.1×10^5 (5.49)**	**1.7×10^5 (5.22)**	**4.6×10^4 (4.66)**
SC686	8.6×10^3 (3.94)	1.1×10^4 (4.04)	2.4×10^4 (4.38)	1.6×10^4 (4.19)	4.6×10^3 (3.66)	3.1×10^3 (3.49)
SA686	**1.6×10^5 (5.22)**	**1.2×10^5 (5.07)**	**9.9×10^4 (5.00)**	**5.0×10^4 (4.70)**	**1.9×10^4 (4.27)**	4.1×10^3 (3.61)
SCcontrol	9.3×10^3 (3.97)	1.5×10^4 (4.17)	8.4×10^3 (3.92)	1.5×10^4 (4.18)	9.1×10^3 (3.96)	4.7×10^3 (3.67)
SAcontrol	5.1×10^3 (3.71)	1.1×10^4 (4.04)	4.8×10^3 (3.68)	7.6×10^3 (3.88)	7.5×10^3 (3.88)	2.0×10^3 (3.31)
F pr. (isolate)	<.001***	0.008**	<.001***	0.004**	<.001***	0.012*
F pr. (application)	<.001***	<.001***	<.001***	<.001***	<.001***	0.143
F pr. (isolate.application)	0.003**	<.001***	0.018*	<.001***	<.001***	0.080
LSD (*P* <0.05)	(0.616)	(0.409)	(0.594)	(0.539)	(0.310)	(0.676)

	0 wpi	4 wpi			0 wpi	4 wpi
Centre bulk				**Bottom bulk**		
SC132	9.8×10^3 (3.99)	7.3×10^3 (3.86)			9.2×10^3 (3.97)	5.1×10^3 (3.70)
SA132	**5.1×10^4 (4.71)**	3.0×10^4 (4.47)			**2.6×10^4 (4.42)**	2.7×10^3 (3.44)
SC592	8.3×10^3 (3.92)	5.7×10^3 (3.76)			1.8×10^4 (4.25)	1.6×10^4 (4.22)
SA592	7.3×10^3 (3.86)	1.5×10^4 (4.13)			5.7×10^3 (3.76)	5.2×10^3 (3.71)
SC686	6.9×10^3 (3.84)	2.6×10^4 (4.41)			8.9×10^3 (3.95)	6.9×10^3 (3.84)
SA686	**3.1×10^4 (4.49)**	3.4×10^4 (4.54)			1.0×10^4 (4.01)	1.1×10^4 (4.06)
SCcontrol	1.1×10^4 (4.03)	6.7×10^3 (3.82)			1.3×10^4 (4.11)	5.3×10^3 (3.73)
SAcontrol	6.5×10^3 (3.81)	1.0×10^4 (4.02)			5.4×10^3 (3.73)	8.9×10^3 (3.95)
F pr. (isolate)	0.001**	0.115			0.423	0.436
F pr. (application)	0.003**	0.056			0.469	0.654
F pr. (isolate.application)	<.001***	0.731			0.041*	0.433
LSD (*P* <0.05)	(0.338)	(0.697)			(0.500)	(0.772)

Bold values indicate significant difference to the control. LSD = least significant difference (n = 4). Wpi = weeks post-inoculation; F pr. = F probability of a general unblocked ANOVA; * = significant at F <0.05; ** = significant at F<0.01; *** = significant at F <0.001.

[A] shown are back transformed means with $\log_{10}$-transformed means in parentheses.

Table A2.3 Continued.

Treatment	*Trichoderma* population [cfu/g dry potting mix][A]					
	0 wpi	4 wpi	8 wpi	12 wpi	20 wpi	28 wpi
Rhizosphere						
SC132		6.4×10^3 (3.81)	4.2×10^4 (4.62)	4.8×10^4 (4.68)	7.4×10^4 (4.87)	1.3×10^5 (5.13)
SA132		4.2×10^4 (4.63)	4.3×10^4 (4.63)	1.8×10^4 (4.26)	1.2×10^5 (5.09)	1.1×10^5 (5.06)
SC592		5.7×10^3 (3.76)	1.9×10^4 (4.28)	2.2×10^4 (4.34)	5.1×10^4 (4.71)	8.4×10^4 (4.92)
SA592		**7.1×10^4 (4.85)**	4.4×10^4 (4.64)	2.2×10^4 (4.34)	1.9×10^5 (5.29)	**3.8×10^5 (5.57)**
SC686		7.7×10^4 (3.89)	5.8×10^4 (4.76)	2.0×10^4 (4.31)	1.3×10^5 (5.12)	5.4×10^4 (4.73)
SA686		**8.2×10^4 (4.92)**	8.5×10^4 (4.93)	**4.2×10^4 (4.62)**	1.3×10^5 (5.10)	6.9×10^4 (4.84)
SCcontrol		9.7×10^3 (3.99)	2.3×10^4 (4.36)	1.9×10^4 (4.29)	1.2×10^5 (5.08)	1.0×10^5 (5.01)
SAcontrol		1.3×10^4 (4.11)	3.3×10^4 (4.52)	1.2×10^4 (4.08)	7.2×10^4 (4.86)	3.7×10^4 (4.56)
F pr. (isolate)		0.545	0.156	0.176	0.859	0.101
F pr. (application)		<.001***	0.209	0.435	0.281	0.697
F pr. (isolate.application)		0.218	0.833	0.090	0.178	0.105
LSD (*P* <0.05)		(0.728)	(0.559)	(0.415)	(0.533)	(0.625)
Rhizoplane						
SC132			2.2×10^5 (5.35)	8.9×10^4 (4.95)	7.8×10^5 (5.89)	2.1×10^5 (5.32)
SA132			**5.9×10^4 (4.77)**	4.7×10^4 (4.68)	2.7×10^5 (5.42)	3.4×10^5 (5.54)
SC592			6.5×10^4 (4.81)	4.1×10^4 (4.61)	4.8×10^5 (5.68)	1.7×10^5 (5.24)
SA592			9.9×10^4 (5.00)	**8.2×10^4 (4.91)**	7.1×10^5 (5.85)	8.7×10^5 (5.94)
SC686			1.3×10^5 (5.12)	1.8×10^4 (4.26)	3.2×10^5 (5.51)	2.1×10^5 (5.32)
SA686			1.4×10^5 (5.14)	4.1×10^4 (4.61)	2.6×10^5 (5.42)	**1.8×10^5 (5.25)**
SCcontrol			1.3×10^5 (5.13)	5.5×10^4 (4.74)	8.5×10^5 (5.93)	4.3×10^5 (5.63)
SAcontrol			2.2×10^5 (5.35)	1.4×10^4 (4.13)	4.6×10^5 (5.66)	1.3×10^6 (6.10)
F pr. (isolate)			0.204	0.131	0.196	0.231
F pr. (application)			0.731	0.700	0.166	0.117
F pr. (isolate.application)			0.056	0.070	0.276	0.571
LSD (*P* <0.05)			(0.451)	(0.584)	(0.475)	(0.832)

Bold values indicate significant difference to the control. LSD = least significant difference (n = 4). Wpi = weeks post-inoculation; F pr. = F probability of a general unblocked ANOVA; * = significant at F <0.05; ** = significant at F<0.01; *** = significant at F <0.001.

[A] shown are back transformed means with $\log_{10}$-transformed means in parentheses.

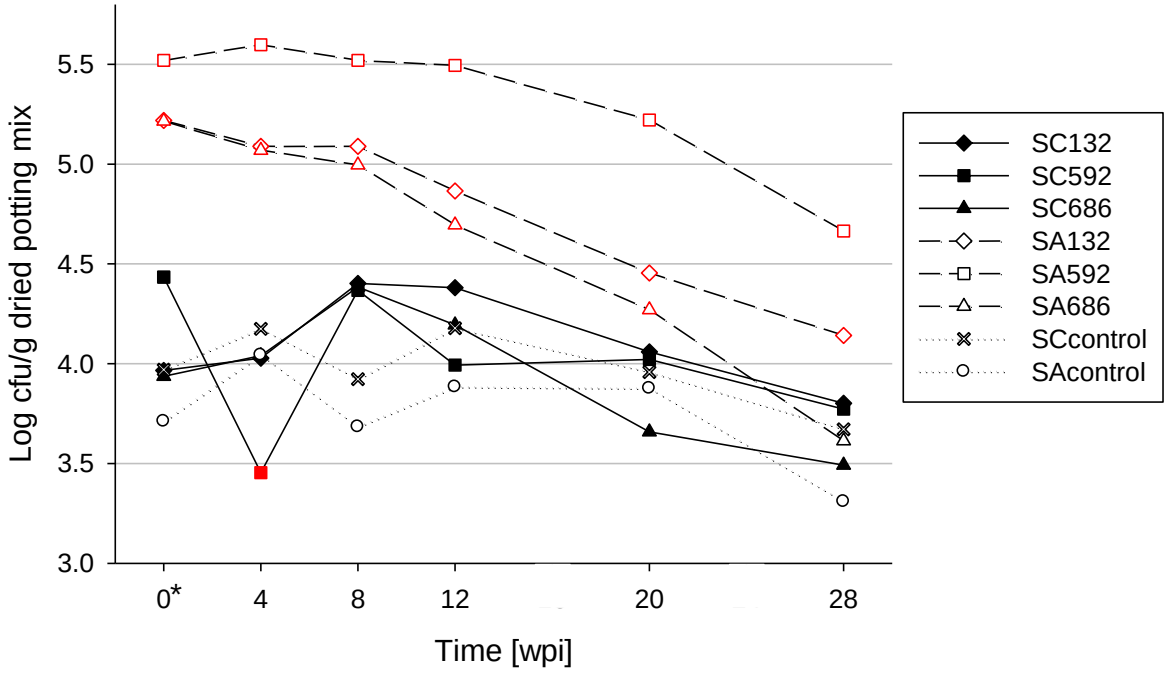

Figure A2.1 *Trichoderma* population (log_{10} colony-forming unit (cfu)/g dry potting mix) recovered from **top bulk** potting mix treated with three different *Trichoderma* isolates (LU132, LU592, LU686) either as a seed coat (SC) or spray application (SA) over time. Red values indicate significant differences to the respective control within each sampling time (n = 4). Wpi = weeks post-inoculation (*: first sampling was 24 h post-inoculation).

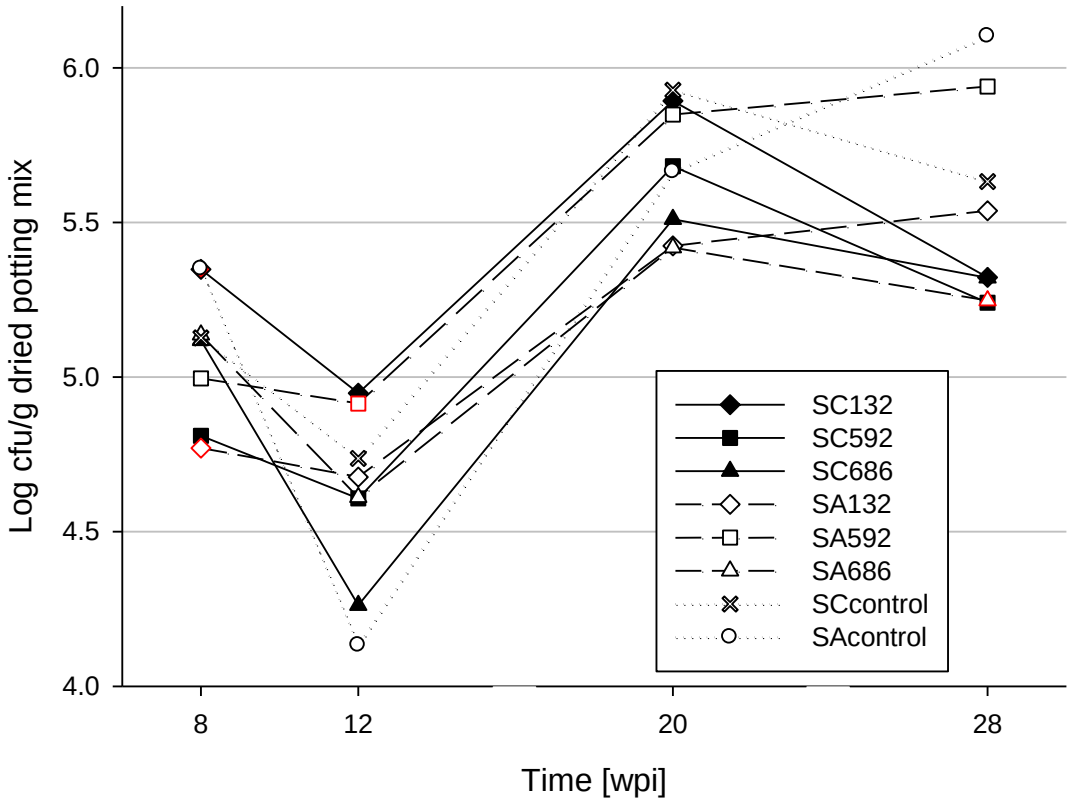

Figure A2.2 *Trichoderma* population (log_{10} colony-forming unit (cfu)/g dry potting mix) recovered from the rhizoplane treated with three different *Trichoderma* isolates (LU132, LU592, LU686) either as a seed coat (SC) or spray application (SA) over time. Red values indicate significant differences to the respective control within each sampling time (n = 4). Wpi = weeks post-inoculation.

Table A2.4 Estimated proportion of each morphology group recovered from the **top bulk**, **rhizosphere**, **rhizoplane** and **endorhizosphere** of *Pinus radiata* seedlings treated with three different *Trichoderma* isolates (*T atroviride* LU132, *T. hamatum* LU592 and *T. harzianum* LU686) as either a seed coat (SC) or spray application (SA) treatments 20 weeks post inoculation.

	% *Trichoderma* spp. (morphology group)					
Treatment	*T. atroviride* (A)	*T. hamatum* (B)	*T. harzianum* (C)	*T. asperellum* (D)	*T. viride* (E2)	*T. viride or hamatum* (E1)
Top bulk						
SC132	17	0	0	57	22	4
SA132	90	0	0	10	0	0
SC592	0	30	0	61	4	4
SA592	0	100	0	0	0	0
SC686	0	0	0	67	29	5
SA686	0	0	29	24	5	43
SCcontrol	4	0	0	42	33	21
Control	0	0	0	79	13	8
Rhizosphere						
SC132	0	0	0	76	24	0
SA132	20	0	0	52	16	12
SC592	0	17	0	43	0	39
SA592	0	67	0	13	21	0
SC686	0	0	0	32	14	55
SA686	0	0	0	46	29	25
SCcontrol	0	0	0	72	12	16
Control	0	0	0	55	23	23
Rhizoplane						
SC132	4	0	0	39	39	17
SA132	25	0	0	21	33	21
SC592	0	23	0	0	41	36
SA592	0	58	0	4	25	13
SC686	0	0	0	48	19	33
SA686	0	0	4	29	8	58
SCcontrol	0	0	0	17	26	57
Control	0	0	0	8	54	38

Table A2.4 Continued.

Treatment	% *Trichoderma* spp.					
	T. atroviride (A)	*T. hamatum* (B)	*T. harzianum* (C)	*T. asperellum* (D)	*T. viride* (E2)	*T. viride or hamatum* (E1)
Endorhizosphere						
SC132	0	0	0	0	40	60
SA132	0	0	0	33	33	33
SC592	0	0	0	11	67	22
SA592	0	92	0	0	0	8
SC686	0	0	0	17	83	0
SA686	0	0	0	38	38	25
SCcontrol	0	0	0	17	50	33
Control	0	0	0	13	50	38

Table A2.5 Plant performance assessments of *P. radiata* seedlings treated with LU592 as either seed coat (SC) or spray application (SA).

	% seedling emergence		Shoot height [cm]			Shoot diameter [mm]	
Treatment	2 mpi	4 mpi	2 mpi	4 mpi	6 mpi	2 mpi	4 mpi
SC592	83.4	94.1	**3.70**	**16.1**	**28.8**	**1.18**	**3.15**
SA592	83.1	93.2	**3.78**	**16.5**	**29.4**	**1.18**	**3.16**
Control	84.4	93.8	3.59	15.2	26.8	1.16	2.89
F pr.	0.665	0.686	<.001***	<.001***	<.001***	0.002**	<.001***
LSD (*P* = 0.05)	3.17	2.15	0.0695	0.51	0.91	0.0146	*0.108*

	Shoot dry weight [mg/shoot]		Root dry weight [mg/root]		Root/shoot ratio		Root tips count [root tips/root]
Treatment	2 mpi	4 mpi	2 mpi	4 mpi	2 mpi	4 mpi	2 mpi
SC592	**41.6**	**604**	**82.4**	**1419**	**0.51**	**0.43**	**36.4**
SA592	**42.3**	**613**	**85.3**	**1466**	**0.50**	0.42	35.0
Control	38.3	493	81.0	1215	0.48	0.41	32.8
F pr.	0.088	<.001***	0.004**	<.001***	0.048*	0.023*	0.032
LSD (*P* = 0.05)	3.96	*87.7*	2.42	*37.6*	0.0246	*0.015*	*2.72*

	ECM root coverage [0 - 3]	Health score [0 - 3]	% severe infected shoot tips		% dead seedlings	% marketable seedlings
Treatment	4 mpi	4 mpi	4 mpi	6 mpi	9 mpi	9 mpi
SC592	**1.78**	2.26	8.89	5.33	**9.30**	**79.2**
SA592	**1.88**	2.40	4.44	**3.11**	**8.70**	**78.3**
Control	1.20	2.26	12.2	6.89	12.2	74.2
F pr.	<.001***	0.222	0.155	0.035*	0.013*	0.021*
LSD (*P* = 0.05)	*0.244*	*0.191*	*7.950*	*2.857*	2.456	3.76

Bold values indicate significant differences to the control. LSD = least significant difference (n = 60; *n = 30*). Mpi = months post-inoculation; F pr. = F probability of a standard ANOVA with randomised block (15) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.

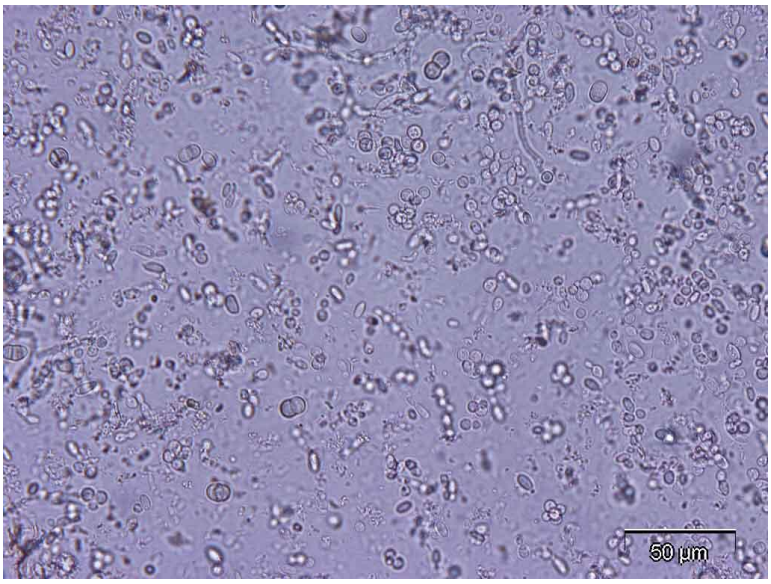

Figure A2.3 Microscopic observation of the washed-off seed surface of SC132.

Appendix 3: Chapter Three

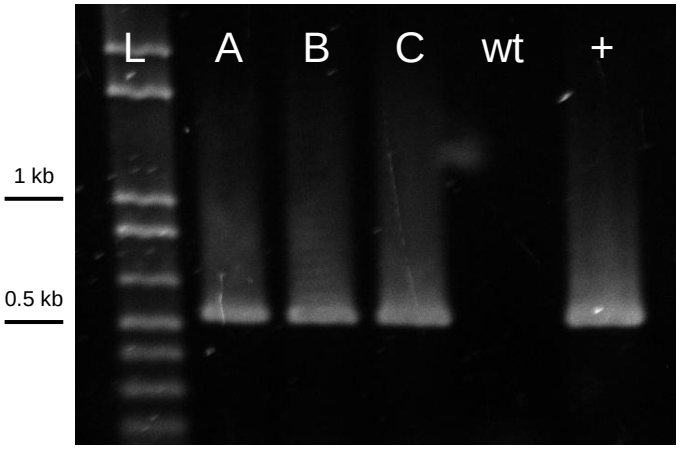

Figure A3.1 PCR analysis showing the presence of the *gfp/hph* cassette in DNA extracted from the three transformants LU592/A (A), LU592/B (B) and LU592/C (C). Ladder (L): 1kb plus ladder (Fermentas, USA), wt = LU592 wild type, + = p0300GFP plasmid as positive control.

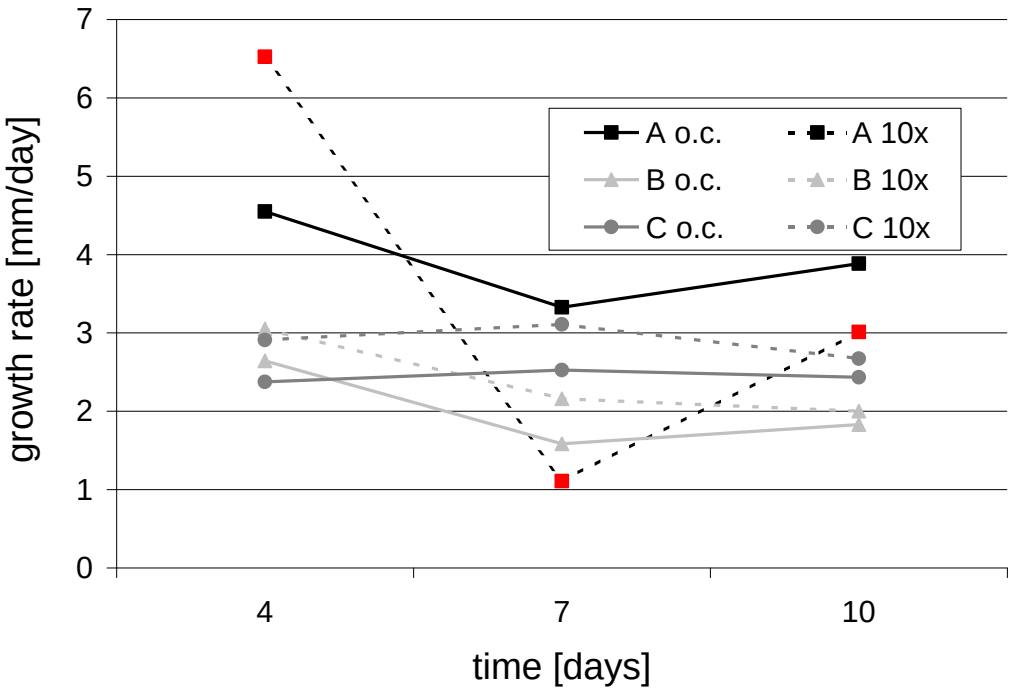

Figure A3.2 Mycelial growth of the three transformants LU592/A (A), LU592/B (B) and LU592/C (C) on hygB-amended PDA originating from either the original culture (o.c.) or after ten subcultures (10x) on non-selective PDA (n = 3). Red values indicate significant difference to respective original culture.

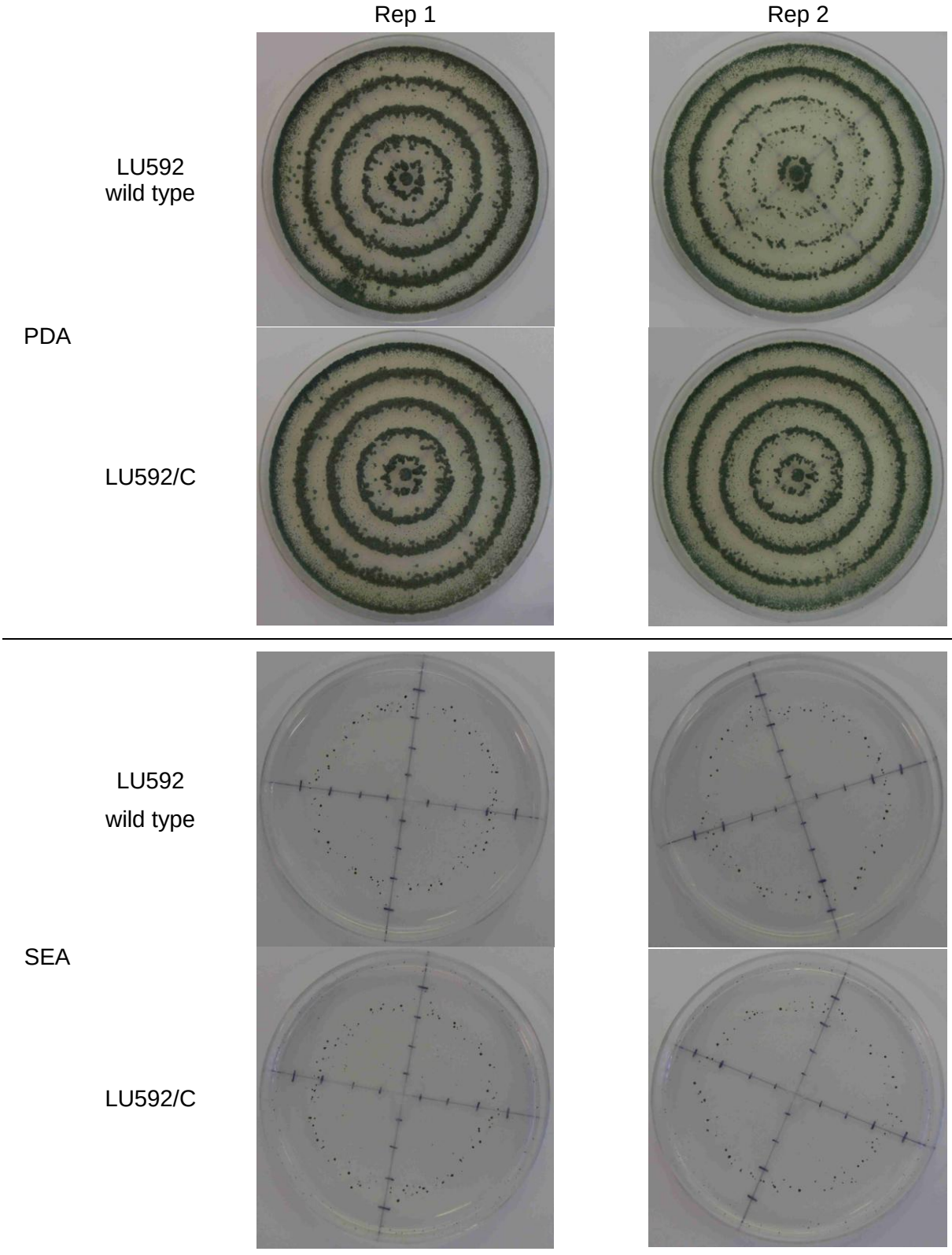

Figure A3.3 Photographs of representative sporulation patterns on either potato dextrose agar (PDA) or soil extract agar (SEA) between LU592 wild type and LU592/C grown for 14 days at 20ºC under 12 h/12 h light/dark conditions.

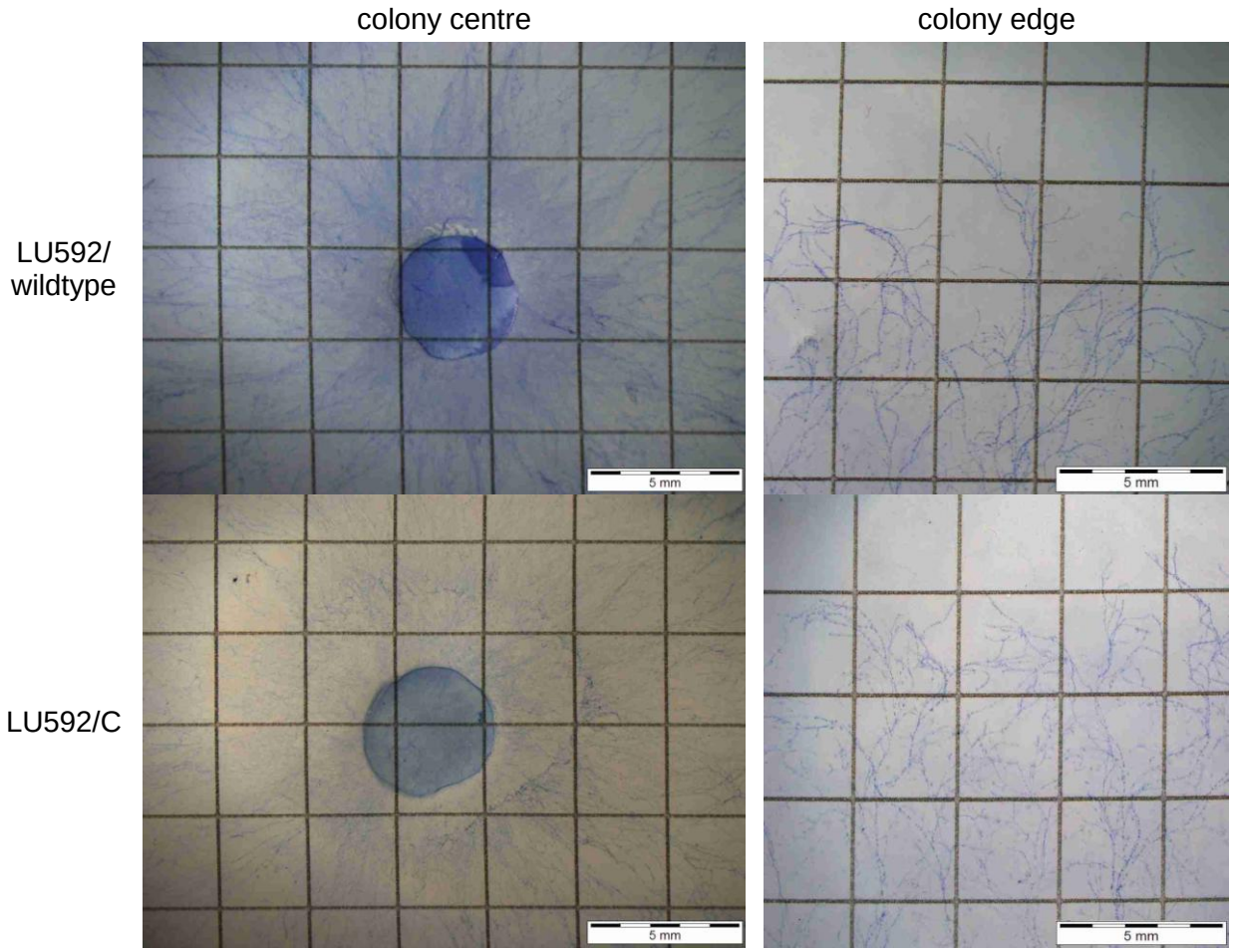

Figure A3.4 Stained mycelia of LU592 wild type and transformant C grown on nitrocellulose filter-membrane after 6 days of incubation in non-sterile potting mix.

Appendix 4: Chapter Four

Table A4.1 Summary of the LU592/C population recovered from non-sterile potting mix after inoculation with two concentrations of either mycelia or spores and filtered through no, 2, 3 and 4 layers of mira cloth.

	LU592/C population [cfu/g dry potting mix][A]	
Treatment	Mycelia	Spores
10^4		
no layers	1.6×10^4 (4.19)	2.3×10^4 (4.36)
2 layers	**1.7×10^3 (3.22)**	1.8×10^4 (4.25)
3 layers	**1.9×10^2 (2.28)**	2.9×10^4 (4.46)
4 layers	**3.1×10^2 (2.45)**	2.0×10^4 (4.30)
10^6		
no layers	2.3×10^6 (6.36)	1.3×10^6 (6.11)
2 layers	**3.3×10^4 (4.51)**	2.0×10^6 (6.30)
3 layers	**4.7×10^3 (3.67)**	1.8×10^6 (6.23)
4 layers	**2.8×10^3 (3.45)**	1.4×10^6 (6.16)
F pr. (concentration)	<.001***	0.814
F pr. (layers)	<.001***	<.001***
F pr. (concentration.layers)	0.070	0.762
LSD (*P* = 0.05)	0.640	0.458

Bold values indicate significant difference to the no-layer control. LSD = least significant difference (n = 3). F pr. = F probability of a general unblocked ANOVA; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.

[A] shown are back transformed means with $\log_{10}$-transformed means in parentheses.

Table A4.2 Summary of the LU592/C population recovered from potting mix of *P. radiata* seedlings 6 months after inoculation with three concentrations (10^3, 10^5 and 10^7 spores/RSC) of LU592/C.

	LU592/C population [cfu/g dry potting mix][A]			
Treatment	0-2 mm[B]	4-6 mm[B]	8-10 mm[B]	bulk
10^3	4.8×10^3 (3.68)	3.3×10^3 (3.52)	3.3×10^3 (3.52)	1.8×10^3 (3.26)
10^5	4.6×10^4 (4.66)	1.2×10^4 (4.07)	1.0×10^4 (4.01)	9.1×10^3 (3.96)
10^7	9.8×10^3 (3.99)	3.6×10^3 (3.56)	2.8×10^3 (3.45)	3.0×10^3 (3.48)
F pr.	0.006**	0.014*	0.094	0.038*
LSD (*P* = 0.05)	0.577	0.395	0.553	0.542

LSD = least significant difference (n = 10). F pr. = F probability of a standard ANOVA with block (2) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.
[A] shown are back transformed means with $\log_{10}$-transformed means in parentheses.
[B] distance from the root surface.

Table A4.3 Summary of the proportion of the total LU592/C cfu (colony-forming unit) as mycelia recovered from potting mix of *P. radiata* seedlings 6 months after inoculation with three concentrations (10^3, 10^5 and 10^7 spores/RSC) of LU592/C.

	% of total cfu as mycelia[A]			
Treatment	0-2 mm[B]	4-6 mm[B]	8-10 mm[B]	bulk
10^3	5.3 (0.02)	3.5 (0.02)	17.5 (0.02)	-28.7 (-0.11)
10^5	*31.7 (0.17)*	*32.3 (0.17)*	*27.5 (0.17)*	3.6 (0.02)
10^7	*35.1 (0.19)*	*24.4 (0.12)*	*12.6 (0.19)*	20.7 (0.10)
F pr.	0.003**	<.001***	0.176	0.274
LSD (*P* = 0.05)	0.081	0.071	0.089	0.264
LSE (*P* = 0.05)	0.057	0.050	0.063	0.186

Values in *italics* are significantly different from zero. LSD = least significant difference (n = 10). LSE = least significant effect. F pr. = F probability of a standard ANOVA with block (2) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.
[A] shown are back transformed means with $\log_{10}$-transformed ratio means (overall cfu/conidial cfu) in parentheses.
[B] distance from the root surface.

Table A4.4 Summary of the proportion of internally colonised root segments of 6-month old *P. radiata* seedlings by LU592/C after inoculation with three concentrations (10^3, 10^5 and 10^7 spores/RSC) of LU592/C.

	% internally colonised root segments		
Treatment	upper section	middle section	lower section
10^3	55.0	51.7	46.7
10^5	96.7	76.7	68.3
10^7	88.3	60.0	51.7
F pr.	<.001***	0.001**	0.033*
LSD (*P* = 0.05)	10.27	12.53	16.69

LSD = least significant difference (n = 10). F pr. = F probability of a standard ANOVA with block (2) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.

Table A4.5 Summary of the proportion of internally colonised root segments of 16-week old *P. radiata* seedlings by LU592/C after inoculation with three concentrations (10^3, 10^5 and 10^7 spores/RSC) of LU592/C.

	% internally colonised root segments		
Treatment	root tip	straight section	intersection
10^3	0	0	0
10^5	5.6	0	0
10^7	11.1	5.6	5.6
F pr.	0.296	0.422	0.630
LSD (*P* = 0.05)	15.70	11.10	15.70

LSD = least significant difference (n = 3). F pr. = F probability of a standard ANOVA with block (2) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.

Table A4.6 Summary of the LU592/C population recovered from potting mix in either presence (+) or absence (-) of *P. radiata* seedlings at three depths (top: 1.5-2.5 cm, centre: 4.5-5.5 cm, bottom: 7.5-8.5 cm) over time.

	Trichoderma population [cfu/g dry potting mix][A]				
Treatment	3 dpi	4 wpi	8 wpi	12 wpi	16 wpi
top					
+	6.8×10^2 (2.83)	4.1×10^4 (4.61)	6.3×10^3 (3.80)	4.7×10^4 (4.67)	1.7×10^5 (5.22)
-	4.0×10^2 (2.61)	1.4×10^3 (3.15)	2.2×10^3 (3.35)	7.6×10^3 (3.88)	6.7×10^3 (3.83)
F pr.	0.755	<.001***	0.073	0.018*	<.001***
LSD (*P* = 0.05)	1.53	0.424	0.492	0.631	0.555
centre					
+	5.5×10^0 (0.82)	4.2×10^2 (2.63)	1.7×10^2 (2.22)	6.2×10^3 (3.79)	3.3×10^4 (4.51)
-	3.4×10^1 (1.55)	3.2×10^2 (2.51)	8.1×10^1 (1.91)	6.8×10^2 (2.84)	6.3×10^2 (2.80)
F pr.	0.306	0.781	0.323	0.014*	<.001***
LSD (*P* = 0.05)	1.480	0.914	0.657	0.734	0.622
bottom					
+	0.0 (0.00)	5.2×10^1 (1.73)	1.2×10^2 (2.07)	1.2×10^3 (3.09)	1.4×10^4 (4.15)
-	0.0 (0.00)	7.3×10^0 (0.24)	7.4×10^0 (0.24)	1.3×10^2 (2.11)	3.5×10^1 (1.56)
F pr.		0.003**	<.001***	0.026*	<.001***
LSD (*P* = 0.05)		0.875	0.899	0.850	1.070

LSD = least significant difference (n = 8). F pr. = F probability of a standard ANOVA with block (2) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.

[A] shown are back transformed means with $\log_{10}$-transformed means in parentheses.

Table A4.7 Summary of the proportion of total LU592/C cfu as mycelia recovered from potting mix in either presence (+) or absence (-) of *P. radiata* seedlings at three depths (top: 1.5-2.5 cm, centre: 4.5-5.5 cm, bottom: 7.5-8.5 cm) over time.

	% of total cfu as mycelia[A]				
Treatment	3 dpi	4 wpi	8 wpi	12 wpi	16 wpi
top					
with plant	*43.6 (0.249)*	8.5 (0.038)	-7.8 (-0.033)	*36.5 (0.197)*	*23.1 (0.114)*
without plant	20.5 (0.099)	11.3 (0.052)	-9.3 (-0.039)	*31.8 (0.166)*	0.7 (0.003)
F pr.	0.230	0.866	0.911	0.654	<.001***
LSD (*P* = 0.05)	0.2820	0.1713	0.1159	0.1459	0.0118
LSE (*P* = 0.05)	0.1994	0.1211	0.0820	0.1032	0.0084
centre					
with plant	7.4 (0.033)	*29.0 (0.149)*	-2.5 (-0.011)	*54.2 (0.339)*	*89.0 (0.960)*
without plant	-30.1 (-0.114)	17.7 (0.085)	11.8 (0.055)	9.3 (0.043)	-0.8 (-0.004)
F pr.	0.097	0.354	0.337	0.002	<.001***
LSD (*P* = 0.05)	0.2349	0.1272	0.1460	0.1558	0.1258
LSE (*P* = 0.05)	0.1661	0.0899	0.1032	0.1102	0.0890
bottom					
with plant		11.1 (0.051)	1.4 (0.006)	*58.5 (0.382)*	*76.4 (0.628)*
without plant				18.0 (0.086)	4.3 (0.019)
F pr.				0.002**	<.001***
LSD (*P* = 0.05)				0.1551	0.0799
LSE (*P* = 0.05)		0.1246	0.1046	0.1097	0.0565

Values in *italics* are significantly different from zero. LSD = least significant difference (n = 8). LSE = least significant effect. Dpi/wpi = days/weeks post-inoculation. F pr. = F probability of a standard ANOVA with block (2) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.

[A] shown are back transformed means with $\log_{10}$-transformed ratio means (overall cfu/conidial cfu) in parentheses.

Table A4.8 Summary of the proportion of internally colonised root segments of *P. radiata* seedlings by LU592/C at three depths (top: 1.5-2.5 cm, centre: 4.5-5.5 cm, bottom: 7.5-8.5 cm) over time.

	% internally colonised root segments				
Treatment	3 dpi	4 wpi	8 wpi	12 wpi	16 wpi
top	0.0	16.7	16.7	41.7	85.4
centre	0.0	0.0	2.1	10.4	70.8
bottom	0.0	0.0	9.5	7.1	66.7
F pr.		0.056	0.311	0.024*	0.088
LSD (*P* = 0.05)		15.55	19.37	26.81	17.52

LSD = least significant difference (n = 8). F pr. = F probability of a standard ANOVA with block (2) design; * = significant at F <0.05; ** = significant at F <0.01; *** = significant at F <0.001.