Rhizosphere competence of selected *Trichoderma* species

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Trichoderma is a genus of fungi commonly used as biological control agents and growth promoters of plants. The ability of these fungi to become rhizosphere competent has been linked to their biological control capabilities. However, the extent of rhizosphere competence and factors that affect it are poorly defined in the literature. Rhizosphere competence (RC) is defined as the ability of an organism to colonise, grow and develop in the rhizosphere soil. The aim of this research was to determine RC across a range of *Trichoderma* species and isolates. A series of experiments were conducted to (1) rank RC, (2) determine if RC is plant species specific, (3) test RC under a range of abiotic conditions, and (4) evaluate RC over time. All experiments were carried out by coating seeds of test plants with *Trichoderma* spore suspensions and sowing seeds in non-sterile Wakanui silt-loam soil. The ranking of RC was assessed for 22 isolates of 11 of the most common *Trichoderma* species used for biological control in a non sterile sweetcorn expermental system in which plants were typically grown for 7 days (Chapter 2). Results showed that 82% of the *Trichoderma* isolates had populations that were significantly higher than the control indicating that rhizosphere competence was widespread within the selected species. The least and most rhizosphere competent isolates belonged to the same species, indicating that RC was not species specific. The three least and most RC isolates from the ranking experiment were tested on six plant species (sweetcorn, ryegrass, cauliflower, carrots, onion and white clover) in Chapter 3. Ryegrass and cauliflower were the most receptive plants to *Trichoderma* species, and clover was the least receptive. The least rhizosphere competent isolate on sweetcorn was the only one able to colonise onion roots and had significantly higher RC than all the other isolates, suggesting host plant specificity of RC. On average, *Trichoderma atroviride* LU132 was the most rhizosphere competent isolate across the six plant species. Rhizosphere competence of *T. atroviride* LU132 was assessed under a range of three soil moistures (16, 20 and 24% gravimetric water content, GWC), three pH levels (5.5, 6.5 and 7.5), and three soil available nitrogen concentrations (75, 150 and 300 kg N/ha) (Chapter4). This isolate was rhizosphere competent on sweetcorn and ryegrass, regardless of the abiotic conditions. *Trichoderma atroviride* LU132 also endophytically colonised sweetcorn and ryegrass shoots and roots in all test conditions.

Rhizosphere competence over time was assessed for a strong (*T. atroviride* LU132) and weak (*T. virens* LU556) sweetcorn coloniser at 7, 21, 35 and 56 days post planting (Chapter 5). The influence of both isolates on the rhizosphere microbial communities was assessed by denaturing gradient gel electrophoresis (DGGE). *Trichoderma atroviride* LU132 was more RC than *T. virens* LU556 at every assessment point. Whilst *T. virens* LU556 populations continued to increase over time, *T. atroviride* LU132 populations reached a maximum after 35 days. Shoots and roots of sweetcorn were endophytically colonised by both isolates regardless of harvest time. *Trichoderma atroviride* LU132 and *T. virens* LU556 changed the bacterial, fungal and arbuscular mycorrhizal fungal communities of the rhizosphere compared to untreated plants. Rhizosphere competence and endophytic colonisation of sweetcorn was also assessed at different root depths on 35 day old plants (Chapter 6). Results showed that regardless of the isolate, the top portion of sweetcorn roots had significantly higher *Trichoderma* populations than the middle and bottom parts. *Trichoderma atroviride* LU132 was more rhizosphere competent than *T. virens* LU556 regardless of the root portion. More endophytic *Trichoderma* colonies were isolated from plants treated with *T. atroviride* LU132, further indicating the greater RC of this isolate.

Overall, this research showed that the relationship between *Trichoderma* and the plant is dependant on the *Trichoderma* isolate and the plant species. However, some isolates are more broadly RC than others, and may have greater utility as plant protection agents. Greater RC appeared to be associated with enhanced endophytic colonisation.

Keywords: *Trichoderma* species, rhizosphere competence, maize, sweet corn, soil pH, soil GWC, soil available nitrogen, growth promotion, temporal and spatial distribution, endophyte, population dynamics, root depth.

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Literature review

The interaction between plants and microorganisms is recognized as key to the health and fecundity of the plant. Plants are increasingly viewed not as isolated individuals, but instead as microbiomes, playing host to, and interacting with thousands of microbial species. The effect of these interactions on the plant range from negative (pathogens causing disease) to positive (symbionts that assist with nutrient acquisition), with a wide range in between.

This project explored an area of particularly high microbial interaction and importance to plant health (the rhizosphere) and a genus of fungi renowned for beneficial interactions with plants: *Trichoderma.*

1.1 The rhizosphere

1.1.1 Definition

The term *rhizosphere* (from the Greek, rhizo meaning root, and sphere for zone of influence) was first used by Hiltner in 1904 to describe the region of interaction between microbes and legume roots (Lynch, 1990). At present, the term *rhizosphere* is defined as "the volume of soil influenced by the presence of growing roots". The size of the rhizosphere zone may vary with many factors including soil type, species, and plant age (Curl and Truelove 1986), but it is usually assumed to extend from the root surface (rhizoplane) out into the soil for up to a few millimetres, or possibly a few centimetres in the case of some desert and sand dune plants (Campbell and Greaves 1990). The rhizosphere is often divided into the ectorhizosphere, which is the soil portion, and the endorhizosphere, which includes the rhizoplane and the epidermal and cortical cells of the root that are invaded by microbes (Lynch 1990).

1.1.2 Microbial activity in the rhizosphere

Plants are constantly exuding a vast array of compounds both large and small from their roots (between 5 to 21% of photosynthetic product (Marschner 1995)). As a result the rhizosphere contains many times the microbial biomass of the root-free soil. Microorganisms make up the majority of organisms in the rhizosphere, including representatives of the major groups: bacteria, actinomycetes, fungi, protozoa and algae. Invertebrates are also found at elevated concentrations in the rhizosphere, compared to root-free soil (Bazin et al. 1990).

Microbes that can establish and proliferate in the rhizosphere as the seed germinates and the roots develop stand a good chance of forming a long-term relationship with the plant. Brimecombe and co-workers reported that the recruitment of microorganisms to the rhizosphere and root surface requires stimulation of the microbes by the growing root tip (Brimecombe et al. 2001). The stimulation of microbial activity arises as a result of the release of root exudates from plant roots. The ability of an organism to proliferate and function in the rhizosphere of developing roots is referred to as rhizosphere competence.

Root colonising microbes may cause plant disease (phytopathogenic) (Hawes 1990), or may confer beneficial effects (Gregory 2006), such as mycorrhiza and plant growth promoting rhizobacteria (PGPR). The interaction of the plant with the suite of phytopathogens and beneficial microorganisms it interacts with ultimately determines plant nutrition and health (Brimecombe et al. 2001). Rhizosphere organisms can release compounds (enzymes, toxins, growth regulators etc.) that cause diseases in plants by disrupting plant cell metabolism and by absorbing nutrients from host cells for their own use (Agrios 2005). In severe cases, pathogenic microorganisms can infect plant roots, causing the roots to rot and consequently stopping them from absorbing water and nutrients from the soil. Some examples of soil-borne phytopathogens that disrupt cultivation of the world's most important edible crop plants include *Rhizoctonia solani*, *Sclerotinia* species, several genera of the Oomycota group such as *Phytophthora* and *Pythium,* species of *Xanthomonas* bacteria and genera of nematodes such as *Meloidogyne* and *Heterodera* (Strange and Scott 2005).

A well-studied class of beneficial rhizosphere microorganisms are mycorrhizal fungi. These fungi form symbiotic associations with an estimated 80% of existant plants including most angiosperm, gymnosperm, fern and bryophyte families. Mycorrhizae aid the plant by assisting it in the absorption of nutrients from the soil, and are well known for increasing acquisition of soil phosphorus (Kuhad et al. 2004). For example, Stribley and co-workers grew leek seedlings in soils amended with various levels of added phosphorus and found that shoot growth and tissue phosphorus concentrations were increased by the mycorrhiza *Glomus mosseae* (Stribley et al. 1980). Many authors have reported that arbuscular mycorrhizal symbiosis can reduce root disease caused by several soil-borne pathogens. For instance, Pozo *et al.* (2002) studied the impact of colonisation by the arbuscular mycorrhizal fungus (AMF) *G. mosseae* on tomato root necrosis caused by the soil-borne pathogen *Phytophthora parasitica*. After 7 and 16 days of inoculation with zoospores of the pathogen, roots of plants colonised by the AMF had 39% and 30% (respectivel) fewer infection loci than those that were not colonised. At harvest, 61% of roots of non-colonised plants were necrotic compared with only 31% in AMF-colonised plants.

Plant growth promoting rhizobacteria (PGPR) is a collective term for beneficial plant-associated bacteria that are able to colonize and persist in the rhizosphere. Some PGPR provide the plant with fixed nitrogen and other compounds mobilised from the soil. Strains from the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium* and *Azorhizobium* are the most efficient nitrogen fixers, which form host-specific symbioses with leguminous plants (Paul and Clark 1998).

PGPR may also control plant diseases, promote growth and yield. For instance, Nayaka *et al.* (2009) applied different formulations of a *Pseudomonas fluorescens* to maize seeds. They observed that *P. fluorescens* was often effective in reducing the incidence of the phytopathogen *Fusarium verticillioides*. The same formulations were used under field conditions and the results demonstrated that *P. fluorescens* improved field emergence and grain yield.

Living organisms that reduce the impact of pests and pathogens on plants when introduced into an environment or agricultural setting are known as biocontrol agents. Fungi and bacteria in the rhizosphere that are able to control the growth or activity of plant pathogens and promote plant growth and development have the potential to be used as biocontrol agents of soil borne diseases.

Many strains belonging to the fungal genus *Trichoderma* are used as biocontrol agents for phytopathogens. Nearly all temperate and tropical soils contain 10^1–10^3 culturable *Trichoderma* propagules per gram (Harman et al. 2004a). The same authors have shown that a strain selected for its bioncontrol and plant growth promoting ability, *T. harzianum* T22, increased root development in maize and numerous other plants. Harman (2000) focused on this particular strain of *Trichoderma*, because *T. harzianum* T22, a protoplast fusant that had been proven to be the more rhizosphere competent than its parental strains. Harman (2000) grew maize plants in the field from *T. harzianum* T22 treated and untreated seeds. When plants were 2 m tall, the frequencies of root intercepts were determined. *T. harzianum* T22 induced twice as many deep-root intercepts (25-75 cm below the soil surface) resulting in increased drought tolerance for the plant. Furthermore, *T. harzianum* T22 was proven to be successful in the control of plant diseases in laboratory tests, caused by a range of pathogens that included *Fusarium graminearum*, *Rhizoctonia solani*, *Pythium ultimum* and *Sclerotium rolfsii* (Harman 2000). The strain *T. harzianum* T22 has subsequently been commercialized and is largely sold to the greenhouse, row crop, and turf industries.

1.2 Trichoderma

1.2.1 The genus *Trichoderma*

Trichoderma species are saprophytic, filamentous fungi belonging the Ascomycota division (Chaverri and Samuels 2003). Strains of *Trichoderma* are nearly ubiquitous in the environment, found in most ecosystems (farmed land, forests, grasslands, deserts, salt marshes, lake water and airborne particles) and have been isolated from a wide range of organic materials, including soils, dead plant matter, live roots (of most plant species) and seeds (Monte 2001). Stains of *Trichoderma* have been identified as opportunistic, avirulent plant symbionts, many are parasites of and compete against phytopathogenic fungi, and therefore can play an important role in suppressing plant diseases (Vinale et al. 2008).

The classification of *Trichoderma* species is complicated and confusing. Many isolates of *Trichoderma* that were initially reported in the literature as belonging to one species have now been reclassified into other species. Therefore, it is difficult to directly cross reference taxonomic information in the literature. The genus *Trichoderma* was originally introduced by Persoon in 1794 (Druzhinina *et al.*, 2006). Until the 1990's, morphological and physiological approaches were used to identify species of *Trichoderma*. From the late 1990s onwards, molecular taxonomy has been the main tool for identification. The use of phylogenetic markers (internal transcribed spacers ITS 1 and 2 of the rRNA, and a translation elongation factor *tef* 1) and the availability of tools to associate sequences with identified taxa (*TrichoKEY*, *Tricho*MARK, *Tricho*BLAST), have so far allowed scientists to distinguish more than a hundred different species of *Trichoderma* (Druzhinina et al. 2006).

1.2.2 *Trichoderma* as a biocontrol agent

The potential of *Trichoderma* species as biocontrol agents of plant diseases was first recognized in the early 1930's (Weindling 1932) and since then *Trichoderma* species have been reported to control many plant diseases of fruit and vegetable crops. Mechanisms of biological control include mycoparasitism and the production of lytic enzymes, competition with other fungi and the production of antifungal antibiotics, and the ability to induce systemic and localized resistance and promote growth (Harman 2000). These mechanisms are addressed in the next section.

According to Harman (2000), the most useful *Trichoderma* biocontrol isolates are those that are rhizosphere competent, meaning that they are able to survive and develop in the rhizosphere soil of developing roots. For example, Roberti et al. (2012) reported that the products Remedier (a mixture of *T. asperellum* ICC012 and *T.gamsii* ICC080) as well as Rootshield (*T. harzianum* T22) were able to control *Fusarium solani*, the agent causing foot and root rot in zucchini, enabled by strong rhizosphere competence.

Trichoderma species have been shown to control several soil borne plant pathogens in field and glass house conditions. Some of the pathogens *Trichoderma* species can control include for example *Pythium* sp., *Fusarium* sp., *Rhizoctonia solani*, and *Sclerotium rolfsii* (Benhamou and Chet 1993, Sivan and Chet 1993). McLean and Stewart (2000) demonstrated in glasshouse trials that fungal species such as *T. atroviride*, *T. koningii* and *T. virens* could control the pathogen *Sclerotium cepivorum* causing white rot in onions. The same authors showed that the application of T*. atroviride* LU132 as a soil additive decreased the disease incidence from 39.8% (control) to 7.7%. In a series of field experiments, Rabeendran et al. (2006) found that *T. hamatum* LU593 applied to lettuce plants in different ways (spore suspension, soil amendment or transplant treatement) could reduce the disease incidence of *Sclerotinia minor.*

Trichoderma is one the most studied genus of fungi. Species belonging to this genus are used worldwide as bio-pesticides, biofertilizers, growth enhancers and stimulants of resistance. Species such as *T. asperellum*, *T. atroviride, T. gamsii*, *T. hamatum*, *T. harzianum*, *T.polysporum*, *T. virens*, and *T.viride* are used as main ingredients, alone or in combination with other biological control agents, for commercial products across the world (Table 1-1). Example of *Trichoderma* biocontrol products commercialized in New Zealand include: Trichopel, Trichodry, Trichospray, Vinevax, Biodowel/Pruning, Sentinel, Tenet (Woo et al. 2014).

Table 1-1 Worlwide distribution of *Trichoderma* commercial products used in agriculture. Adapted from (Woo et al. 2014)

Region	Number of countries per region	Number of commercial products	Number of products registered
Africa	5	9	9
Asia	8	100	8
Europe	17	57	21
North America	2	29	19
Pacific	2	22	10
South and Central America	14	40	22

1.2.3 *Trichoderma* mechanisms of action

1.2.3.1 Mycoparasitism and lytic enzymes

A characteristic of some members of the genus *Trichoderma* is their ability to parasitize other fungi, most of which are plant pathogens and many instances of successful biocontrol with *Trichoderma* species have been ascribed to this mechanism. According to Harman and co-workers, mycoparasitism occurs in several steps. First, *Trichoderma* strains detect other fungi and grow trophically towards them (Chet et al. 1981). Then the diffusion of small concentrations of an extracellular exochitinase catalyses the release of cell-wall oligomers from target fungi, and this in turn induces the *Trichoderma* to produce fungitoxic endochitinases (Brunner et al. 2003), which diffuse to and begin to damage the target fungus before physical contact is made (Viterbo et al. 2002).

Once in contact, *Trichoderma* produces several fungi cell wall degrading enzymes (Chet et al. 1998). These enzymes function by breaking down the polysaccharides, chitin, and β-glucans which form fungal walls, thereby destroying the cell wall integrity of phytopathogenic fungi (Howell 2003). Howell (1982) observed *T. virens* (formerly *Gliocladium virens*) parasitizing *R. solani* by coiling around and penetrating the hyphae. When placed with seed sown in infested soil, the same *Trichoderma* isolate suppressed damping-off of cotton seedlings by *R. solani* and by *P. ultimum*. Cotton seedlings treatment with *T. virens* had double the survival rate compared to those in soil infested with either pathogen.

1.2.3.2 Antibiosis and secondary metabolites

Antibiosis is the process of secretion of anti-microbial compounds by antagonistic microbes to suppress and/or kill pathogenic microbes in the vicinity of their growth area (Schirmbock et al. 1994). *Trichoderma* produces a great number of secondary metabolites with biological activities that are strain dependent.

Ghisalberti and Sivasithamparam (1991) classified secondary metabolites into three categories: (i) volatile antibiotics, (ii) water-soluble compounds, and (iii) peptaibols. Lorito *et al.* (1996) investigated the activity of peptaibols and cell wall hydrolytic enzymes produced by *T. harzianum* in the antagonism of *B. cinerea*. Peptaibols trichorzianin TA and TB inhibited β-glucan synthase activity in the host fungus. The inhibition was synergistic with *T. harzianum* β-1, 3-glucanase and prevented the reconstruction of the phytopathogenic fungi's cell wall, which facilitated the action of the glucanase and enhanced the fungicidal activity.

1.2.3.3 Competition with pathogens and soil microbial community

Competition for nutrients, together with competition for space or specific infection sites, is a mechanism used by *Trichoderma* to control plant pathogens (Vinale et al. 2008). Gullino (1992) reported that *T. harzianum* was able to control *B. cinerea* on grapes by colonising blossom tissue and excluding the pathogen from its infection site. Competition for nutrients is the major mechanism used by *T. harzianum* to control *F. oxysporum* f. sp. *melonis* (Sivan and Chet 1989), and in Benitez *et al.* (2004)'s review, *Trichoderma* has a strong capacity to mobilize and take up soil nutrients which make it more efficient and competitive than other soil microbes.

1.2.3.4 Induced resistance

"The ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor" is defined as resistance (Agrios 2005). *Trichoderma* isolates have been reported to induce resistance in several plants.

Bigirimana *et al.* (1997) were the first to demonstrate that *Trichoderma* spp. could induce resistance in plants. They reported that bean plants grown in soil treated with *T. harzianum* T39 had significantly less disease symptoms after *B. cinerea* inoculation to the leaves compared with untreated control plants, even though T39 was only present on the roots and not on the foliage. Yedidia *et al.* (2003) presented compelling evidence for the induction of a systemic response against angular leaf spot of cucumber (*Pseudomonas syringae* pv. *lachrymans*) following application of *T. asperellum* to the root system. Symptoms of disease were reduced up to 80%, corresponding to a reduction of two orders of magnitude in bacterial cell densities in leaves of plants pretreated with *T. asperellum*. Similar studies have now been carried out with a wide range of plants, including both monocotyledons and dicotyledons, and with different *Trichoderma* species and strains (Harman et al. 2004a). The results have demonstrated that induced resistance can be affected by *Trichoderma* spp.

1.3 Rhizosphere competent *Trichoderma*

1.3.1 Rhizosphere competence

Early studies on rhizosphere competence of *Trichoderma* suggested that wild type strains were not able to colonise the rhizosphere of plants (Papavizas et al. 1982, Chao et al. 1986) but it was possible to induce rhizosphere competence by genetic mutation via UV irradiation, exposition to pesticides, or by protoplast fusion (Ahmad and Baker 1988b, Baker 1991b, Sivan and Harman 1991). In later studies, wild type *Trichoderma* species were shown to be rhizosphere competent and to colonise different plant species.

The difference in the ability of *Trichoderma* species to colonise the rhizosphere of plants in early and more recent studies, can be explained by the methods used to identify species. In former studies, the species-level identification of *Trichoderma*/*Hypocrea* isolates was based on morphological characteristics which would lead to incorrect diagnosis. In more recent studies, the difficulties of speices morphological characterisation were overcome by the use of biochemical and molecular methods, which are more reliable (Kredics et al. 2014). Currently, several isolates of *Trichoderma* have been shown to be rhizosphere competent.

Tsahouridou and Thanassoulopoulos (2002) treated tomato seeds with a *T. koningii* isolate and found that the isolate could colonise the rhizosphere of tomato in sterile potting compost and inhibit *Sclerotium rolfsii*. Rabeendran et al. (2006) showed that *T. hamatum* LU593 colonised the rhizosphere of lettuce in potting mix. Hohmann et al. (2011) studied the colonisation of pine roots by two *Trichoderma* isolates (*T. hamatum* LU592 and *T. atroviride* LU132). They discovered that both isolates could colonise pine roots grown in potting mix, however, *T. hamatum* LU592 was a better coloniser than LU132.

In other work Hohmann et al. (2012) showed that *T. hamatum* LU592 zone of activity went beyond the rhizosphere up to 1 cm away from pine roots in non-sterile potting mix. Bourguignon (2008) found that *Trichoderma* strains isolated from the rhizosphere of onion, potato, wheat and brassica, and belonging to *T. asperellum*, *T. atroviride, T. hamatum, T. harzianum* and *T. koningii* could colonise the roots of onion at different depths in gamma irradiated soil.

A well-known example of a rhizosphere competent *Trichoderma* strain is *T. harzianum* T22. *Trichoderma harzianum* T22 was extensively tested on a variety of crops including ferns, beans, corn and ornamental flowering plants and was found to colonise all parts of the root systems (Harman 2000). Moreover, high populations of *T. harzianum* T22 (around 10^5 CFU/g dry weight of roots) were found on rye grain cover crop roots six months after inoculation. When the cover crop was killed and sweet corn was planted in the same fields, it was noticed that plants in the plots treated with *T. harzianum* T22 were taller and had 1.7 times greater weight of ears compared to the plants grown on untreated plots. This demonstrated that *T. harzianum* T22 could persist and proliferate in the soils and on roots for a long time. Only a few studies in the literature have assessed rhizosphere competence of multiple isolates in non-sterile soil (Ahmad and Baker 1987a, Sivan and Harman 1991). In studies where multiple isolates were assessed, rhizosphere competence was addressed as an explanation for biological control but it was not the main focus of the study (Hoyos-Carvajal et al. 2009a).

Most studies addressing rhizosphere competence of *Trichoderma* species alone, were performed in sterile soil (Mehrabi-Koushki et al. 2012, Singh and Kumar 2012a), with the exception of Affokpon et al. (2011), who studied the ability of 17 *Trichoderma* isolates to colonise the roots of tomato plants in unsterilized potting soil. There is a lack of ecological studies on the ability of *Trichoderma* species to colonise the rhizosphere soil of different plant species in non sterile conditions and the interaction between *Trichoderma* species and other rhizosphere microorganims.

1.3.2 Rhizosphere competence and biocontrol activity

It is generally agreed that rhizosphere competence is important for optimal expression of biological activity by microbes antagonistic to root diseases or that stimulate induced resistance (Schmidt 1979, Baker 1991a). Among the most useful *Trichoderma* strains are those that are rhizosphere competent, i.e. able to colonise and grow with the root systems of most plants (Harman et al. 2004a). Several studies have demonstrated the importance of using rhizosphere competent strains for the biocontrol of plant pathogens, the increase of growth promotion and the induction of systemic resistance which benefit the plant. Ahmad & Baker (1988c) treated seeds of barley, cucumber, pea, radish and tomato with conidia of rhizosphere competent *T. harzianum* to reduce the incidence of pre-emergence damping-off caused by *P. ultimum*. They demonstrated that wild-type parents of these mutants were less effective at disease control. When rhizosphere competent mutants were applied to seed, plants produced higher fruit weight and higher dry weights than those treated with non-rhizosphere competent wild types and controls. Harman *et al.* (2004) observed the interactions between maize inbred line Mo17 with rhizosphere competent *T. harzianum* strain T22 and reported the effects of these interactions on the diseases caused by *P. ultimum* and *Colletotrichum graminicola*. The authors reported that shoots and roots of 10-day-old seedlings grown in the presence of *T. harzianum* T22 were larger than in its absence. Moreover, main and secondary roots increased in size and area in the presence of *T. harzianum* T22. The authors ascribed the increased growth to direct stimulation of plant growth and biological control of deleterious microflora. The authors also demonstrated that plants grown from *T. harzianum* T22 treated seed had reduced symptoms of anthracnose following inoculation of leaves with *C. graminicola,* which indicates that root colonisation by *T. harzianum* T22 induced systemic resistance.

Biological control and increased plant growth are potential benefits that may accrue from the induction of rhizosphere competence in *Trichoderma* strains (Baker 1991a).

1.3.3 Rhizosphere competence and environmental parameters

Microbial communities are influenced by soil parameters and abiotic factors, and so is root colonisation by *Trichoderma* species. Soil moisture content, as well as soil pH and soil nutrient concentrations, are known to be factors influencing the development of *Trichoderma* species in the soil. Palanna et al. (2005) reviewed the soil parameters affecting the development of *Trichoderma* species and concluded that pH and moisture had an influence on the abundance of *Trichoderma* species in the soil. Reports in the literature indicate that water availability is one of the most important limitations for *Trichoderma* root colonisation (Kredics et al. 2003). It has been shown to affect spore germination (Magan et al. 1989), mycelial growth (Schubert et al. 2008) the production of enzymes (Grajek and Gervais 1987), the saprophytic activity (Eastburn and Butler 1991) and the interaction with other fungi (Huang and Erickson 2008). Danielson and Davey (1973d) reported that the largest populations of *Trichoderma* in a variety of forest soils in the southeastern U.S. and Washington State were found in conditions of excessive moisture.

Soil moisture affects *Trichoderma* species development. Mycelial growth, spore germination, and germ tube growth are negatively affected by low soil water potentials (Eastburn and Butler 1991). In the unpublished work of Jones et al. (2014), the authors studied the ability of five strains of *Trichoderma* to parasitise sclerotia of *Sclerotinia sclerotiorum* by measuring their spore germination and mycelial growth under a range of water potentials in laboratory conditions. They found that all five strains mycelial growth and germination decreased when osmotic and matric potentials were decreased.

By affecting mycelial growth and spore germination, soil moisture content will ultimately have an effect on the ability of *Trichoderma* to degrade carbon and on their interaction with other microorganisms, especially for those that are antagonists of pathogens (Eastburn and Butler 1988, Elad et al. 1993, Kredics et al. 2003).

Schubert et al. (2010) studied the effect of different environmental parameters on conidial development of *T. atroviride* (T-15603.1) on agar and found that water activity, but not pH, had a significant effect on conidial growth. Germination decreased as water activity increased. Clarkson et al. (2004) showed that a high negative water potential affected the biocontrol activity of *Trichoderma* species against *Sclerotium cepivorum*. Paula Junior et al. (2007) found that soil moisture was important for the antagonist activity of *T. harzianum* against *R. solani* on bean plants grown in sterile soil. Meena and Paul (2008) studied the proliferation of *T. harzianum* (JMA-4) and observed that conditions for maximum development of the strain were 30% moisture and pH 6.6, with alkaline pH inhibiting growth of the isolate. Most *Trichoderma* isolates can proliferate at a pH range from 3.5 to 5.6 (Domsch et al. 1980).

Appropriate nutrition (including carbon and nitrogen) is essential for the development of microorganisms. Danielson and Davey (1973b) found that in buffered media *Trichoderma* grew better if the fungus was supplied with nitrogen in the form of ammonium compared to nitrate. However, other studies have demonstrated contrary results (Hacskaylo et al. 1954, Ward and Henry 1961).

Studies on nutrition in the literature have been mainly performed in buffered media and not in soil, where interactions are likely to be far more complex.

1.3.4 Relationship between rhizosphere competent *Trichoderma* and other soil microorganisms

1.3.4.1 Trichoderma and bacterial, fungal and arbuscular myciorrhizal populations in the rhizosphere

Brimmer and Boland (2003) reported that biological control agents could have negative effects on other rhizosphere microorganisms and potentially on other beneficial microorganisms such as growth promoting bacteria or mycorrhizal fungi. Given that *Trichoderma* species are known for their competitive saprophytic and parasitic abilities (Harman et al. 2004a), they could represent a risk for the diversity or abundance of other microorganisms in the rhizosphere. Cordier and Alabouvette (2009) showed that the introduction of a strain of *T. atroviride* in the soil modified the microbial diversity of soil communities. Summerbell (1987) studied the ability of several fungal strains isolated from the rhizosphere of *Picea mariana* to inhibit the formation of mycorrhiza by *L. bicolor* on seedlings grown in sterile conditions. Scientists found that two species of *Trichoderma* (*T. viride* and *T. polysporum*) were strong antagonists of mycorrhizal formation. Rousseau et al. (1996b) reported in a study on the interaction between *T. harzianum* and *G. intraradices* in axenic conditions that *T. harzianum* could penetrate the spores and hyphae of the AMF leading to burst of hyphae, loss of protoplasm and disorganisation of the AMF cells. In other studies, *Trichoderma* species were shown to act in synergy with other fungi (Datnoff et al. 1995, Nemec et al. 1996, Summerbell 2005).

Minchin et al. (2012) demonstrated that ectomycorrhizal fungi *Theolophora terrestris* and *T. ellisii* in *Pinus radiata* root tips were not negatively affected by the addition of ArborGuard™, a biopesticide formulated with *Trichoderma* species. Similarly McLean et al. (2014) found that a commercial product with *T. atroviride* did not have negative effects on the arbuscular mycorrhizal fungi (AMF) and *Pseudomonas* species microbial populations of a NZ native podocarp forest and grassland.

1.3.4.2 Methods to assess microbial diversity in the soil

Microbial diversity can be assessed using both culture dependent and culture independent methods. Culture dependant methods rely on recovering microorganisms on to a solid agar medium. Many micro-organisms grow under specific nutrient requirements and slow growing microbes can be easily outcompeted when mixtures of plant or soil material are plated. They are predominantly used to cultivate recovered microorganism on which functional assays are performed, for example, dual culture, siderophore production, enzyme production (chitinase, protease, cellulose etc).

Molecular methods are a common method for analysing microbial diversity as they detect both culturable and unculturable microorganisms. Most methods utilise universal primers for either taxonomically useful genes (such as rRNA, actin, tubulin etc) or functional genes (such as those involved iin the nitrogen cycle). Following amplification, PCR products are usually displayed on gels using methods such as denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) or run on genetic analysers eg T-RFLP (terminal restriction fragment length polymorphism). In recent years there has been the development of next generation sequencing technologies which allow thousands of DNA sequences generated from taxonomic genes or whole genomes to be analysed at once, producing large datasets.

1.3.5 Endophytic *Trichoderma*

Trichoderma isolates are known to endophytitcally colonise many plant species roots (Harman et al. 2004a). Reports on the colonisation of other plant tissues are rare, and address mainly the endophytic colonisation of seed pods and above ground parts of cocoa and woody trees (Bailey et al. 2008). Although the mechanisms of endophytic colonisation are not yet fully understood (Bailey and Melnick 2013), some reports showed root colonisation via the formation of penetrating structures (Viterbo and Chet 2006), the production of enzymes (Brotman et al. 2008) which in turn triggered the plant defense system and deposition of callose to stop *Trichoderma* strains from growing beyond the cortex cells (Yedidia et al. 2000).

Research on endophytic *Trichoderma* strains has become more important in the past years as they are thought to be more efficient for the control of pathogens and have been reported to produce beneficial compounds such as growth hormones and proteins that induce systemic resistance (Vinale et al. 2006, Woo et al. 2006), all beneficial for crops and agricultural systems. Although research has progressed, there are not many reports studying different strains and reports on the link between endophytic colonisation and rhizosphere competence.

Scope of PhD thesis

There are limited and conflicting reports of rhizosphere competence in the genus *Trichoderma*. Many authors suggest that rhizosphere competence is a trait associated with biocontrol ability. However, the literature is biased in that most accounts of rhizosphere competence are instances in which isolates have been selected and investigated for their biocontrol ability in the first instance. Demonstration of rhizosphere competence is then done on a cohort of isolates already selected for their biological activity. The aim of this project was to study rhizosphere competence in the genus *Trichoderma* using a representative range of species and isolates. The isolates were chosen to represent species from which known biocontrol agents have been derived (as these are the most abundantly described in the literature).

Objective 1: To determine whether rhizosphere competence is widespread in the *Trichoderma* genus.

Objective 2: To determine whether the rhizosphere competence status of a particular strain is host plant specific.

Objective 3: To determine whether key soil abiotic factors can influence the rhizosphere competence of a particular *Trichoderma* isolate

Objective 4: To determine whether rhizosphere competence changes over time

Objective 5: To assess the ability of rhizosphere competent *Trichoderma* isolates to colonise the rhizosphere and root cortex

2

Relative rhizosphere competence within the genus *Trichoderma* on the host plant *Zea mays*

2.1 Introduction

Among *Trichoderma* strains, those that are used for biological control of soil borne diseases are generally also rhizosphere competent. Indeed, there is an association in the literature between biocontrol ability and rhizosphere competence with several studies showing the importance of using rhizosphere competent strains for the control of plant pathogens, growth promotion and the induction of systemic resistance (Harman et al. 2004). Rhizosphere competence is often assessed to provide an explanation for observed growth promotion, induced resistance or biological control ability. However, few studies have objectively investigated the frequency of rhizosphere competence in the genus *Trichoderma* by looking at a range of isolates with variable biological control ability.

Many *Trichoderma* species and isolates have been proven to be rhizosphere competent on different plants, both dicot and monocot, and under different biotic and abiotic conditions. In studies where more than one *Trichoderma* species and/or isolate were assessed concurrently, the main goal was usually to determine if those species or isolates were potential biological control agents with rhizosphere competence, an additional trait that is associated to biological control potential. In most published studies assessment of rhizosphere competence is carried out in sterile conditions and assessed using marked strains or microscopy techniques. Thus, it is also difficult to understand the frequency and magnitude of this trait in the genus because there are many different ways by which it is assessed. For example, differences in classification of rhizosphere soil and root depths sampled, with many studies done under hydroponic and often sterile conditions which are difficult in the end to relate to the more natural soil environments. They are also done under different growth conditions including hydroponics and sterile systems which are difficult in the end to relate to the more natural soil environmental.

The aim of this chapter was to investigate the frequency of rhizosphere competence in the genus *Trichoderma* irrespective of the potential of an isolate to be a biological control agent. It aims to determine whether rhizosphere competence is an isolate or species specific trait. As the genus is large (in excess of 200 species), and there is limited information on many species, a choice was made to select *Trichoderma* isolates representative of species commonly used as biocontrol agents.

This was for three reasons i) these are the species for which the most literature on rhizosphere competence is available, ii) they are the species for which collections of *Trichoderma* have the most representation, and iii) there is a well characterised rhizosphere competent strain, T22, which can act as a positive control. The isolates used in this study were collected from different soils in New Zealand and overseas and will be ranked for relative rhizosphere competence using a plant bioassay in non-sterile conditions.

2.2 Materials and Methods

2.2.1 Selection of isolates

Twenty one *Trichoderma* isolates were selected from the Lincoln University Microbial Culture Collection to represent a range of different biological control species within the genus (Table 2-1). Most of the strains used in the experiments had been isolated from different types of New Zealand soils. A few strains had been imported from overseas (Malaysia and USA). A minority of the isolates had been isolated from plant parts such as petals or leaves and from sclerotia of fungi. Three representative isolates per species or sub-species group for six species were chosen. Species were chosen according to their availability in the Lincoln University Microbial Culture Collection. The nine species were *T. asperelloides*, *T. asperellum, T. atroviride, T. crissum, T. hamatum, T. harzianum, T. virens*, *T. viride* and *T. viridescens.*

Each isolate had been identified by sequence analysis of the ribosomal gene region (Druzhinina et al. 2005). For the *T. harzianum* isolates two groups of isolates were used according to their positions within a phylogenetic tree produced at the Bio-Protection Research Centre (BPRC). Isolate T22 was used as a positive control because it is a well-studied isolate, has been demonstrated to be rhizosphere competent, and has biological control activity (Sivan and Harman 1991, Harman 2000). *Trichoderma harzianum* T22 was provided by G.E. Harman (Cornell University, NY, USA). During the course of the experiment some of the isolates were re-identified using sequence analysis of the translation-elongation factor 1-α (tef1) according to new taxonomic criteria (Hoyos-Carvajal et al. 2009). Subsequently, isolates LU945, LU555 and LU761 were reclassified as shown in Table 2.1. After identification, a total of 11 species were represented by the 22 isolates.

Table 2-1 Details of the *Trichoderma* spp. isolates tested for rhizosphere competence

LU #	Species	Host and Origin
945	*T. asperelloides*[1]	Bamboo clump, Sarawak, Malaysia
150	*T. asperellum*	Soil, NZ
699	*T. asperellum*	*Pinus radiata*, Woodhill, Auckland, NZ
132	*T. atroviride*	Onion growing soil, Pukekohe, Auckland, NZ
140	*T. atroviride*	Onion growing soil, Pukekohe, Auckland, NZ
298	*T. atroviride*	Kiwifruit leaves, Tauranga, NZ
592	*T. hamatum*	Vegetable cropping soil, Preston Rd, Christchurch, NZ
595	*T. hamatum*	Vegetable cropping soil, Marshlands, NZ
740	*T. hamatum*	Unknown, Rylstone Orchards, Wainui, Auckland, NZ
T22	*T. harzianum*	USA
151	*T. harzianum*[2]	Unknown, NZ
571	*T. harzianum*[2]	*Ciborinia camelliae* infected Camellia petals, Wellington botanical garden, NZ
669	*T. harzianum*[2]	Kiwifruit leaves, Auckland, NZ
626	*T. harzianum*[3]	Unknown, Wombat lake track, Franz Josef National Parks, NZ
672	*T. harzianum*[3]	Unknown, Beltsville, USA
673	*T. harzianum*[3]	*Sclerotium rolfsii* sclerotia, Beltsville, USA
547	*T. virens*	Soil, Christchurch, NZ
556	*T. virens*	Onion growing soil, Pukekohe, Auckland, NZ
555	*T. crassum*[1]	Soil, Christchurch, NZ
570	*T. viride*	*Ciborinia camelliae* sclerotia, Wellington botanical gardens, NZ
817	*T. viride*	Soil, Kaingaroa pine plantation forest, NZ
761	*T. viridescens*[1]	Soil under grass, Arable and Forage Cropping farm, LU, NZ

1 Isolates reclassified by sequencing of the Tef1 gene: LU945 *T. asperellum* →*T. asperelloides*; LU555 *T. virens* → *T. crassum*; LU761 *T. viride* → *T. viridescens*
2 *T. harzianum* group I as defined by the BPRC phylogenetic tree
3 *T. harzianum* group II as defined by the BPRC phylogenetic tree

2.2.2 *Trichoderma* inoculum production

All isolates were derived from single spore cultures and had been maintained as spore suspensions in a 25% glycerol solution at -80°C. Three µL of each spore suspension were inoculated centrally onto each of 22 potato dextrose agar (PDA; Difco™, Becton, Dickinson and Company, USA) plates.

The plates were incubated in a locked cupboard under constant blue light (Sylvania F18w/blue) for 8 days to stimulate conidiation. The cupboard was in a laboratory at room temperature (between 18 to 23°C). Following conidiation, the plates were flooded with 8 mL of sterile distilled water (SDW) and rubbed with a sterile glass rod to free the conidia from the mycelium. The resultant conidial suspension was filtered through two layers of miracloth (Calbiochem, EMD Biosciences, Inc., La Jolla, California, USA) to remove hyphal fragments. The conidial concentration of the suspension was determined using a haemocytometer and adjusted to 1 x 10^8 conidia/mL using sterile water.

2.2.3 Seed application of *Trichoderma* isolates

Seeds of Sweet corn (*Zea mays*) variety Chieftain were purchased from Corson Grain Ltd, New Zealand. Prior to inoculation, the seeds were surface sterilised for 10 min in a 1% sodium hypochlorite and 5% ethanol solution and washed three times (each rinse was 1 min long) with SDW. They were subsequently air-dried for 2 to 3 hours in a laminar flow cabinet. Seeds (6 to 18) for each *Trichoderma* isolate treatment were placed in a 50 mL plastic container and coated with a mixture of equal volumes of conidial suspension in water (1 x 10^8 conidia/mL) and a 2% (w/v) methyl cellulose (MC) solution (viscosity 400 cP, Sigma Aldrich Chemie GmbH, Steinheim, Germany) serving as sticker. The container was vigorously shaken by hand for 1 min to evenly distribute the *Trichoderma* conidia over the surface of the seed and then the seeds were left to dry overnight in a laminar flow cabinet. The control treatment, without *Trichoderma* conidia, was treated in the same way with SDW substituted for the conidial suspension.

2.2.4 Soil

Wakanui silt loam soil was collected from Lincoln University farm land (paddock H14, Christchurch, NZ) on the 20th January 2010 and sieved using a 4-mm-mesh screen. The soil was stored in sealed plastic containers at 4˚C in the dark until it was used for each of the three setups (May and June 2010).

Prior to each experimental setup, a comprehensive soil analysis was completed (Hills Laboratories, Hamilton, NZ) (Appendix A1) in order to characterise the soil and detect any potential significant changes caused by storage. The water holding capacity of the soil was 30%. The soil moisture content (SMC) was adjusted to 20% (w/w) before use. To adjust the SMC, the gravimetric water content (GWC) was calculated using the following formula:

$$GWC = (FS_g – DS_g) / DS_g * 100$$

FS_g is the weight in grams (g) of a fresh soil sample

DS_g is the weight in g of a dry soil sample after it has been dried in an oven at 105°C for 24 hours.

2.2.5 **Experimental design**

A modification of the method used by Ahmad and Baker (1987a) was used whereby plastic tubes (50 mL capacity) were sliced in half longitudinally. The two halves were held together with tape and wrapped with an extra-large latex glove finger. The joined tubes were filled with soil wetted to 20% moisture content and one sweet corn seed was sown 1 cm below the surface of the soil in each tube. Four randomised blocks were set up, with each block containing 28 tubes, one tube per treatment (isolate) and the control (bare seed-BS), and three tubes each for the MC only coated seed control (MC) and the positive control isolate T22. This resulted in four replicates per isolate and the untreated bare seed, and 12 replicates for both isolate T22 (positive control) and the MC only coated seed. The tubes were placed as randomised blocks into four polystyrene boxes (44.5 cm x 45 cm x 12 cm). Boxes were individually covered with a transparent plastic bag to maintain soil moisture (Figure 2-1).

Boxes were incubated in two different incubators (two boxes per incubator) under 16 hours light (average of 144 µmol photons/m^2/s) at 18˚C and 8 hours dark at 20˚C. No water was added to the growing systems after the seeds were sown. The experiment was set up on the 19th of May, and was repeated twice on the 1st and 14th of June 2010.

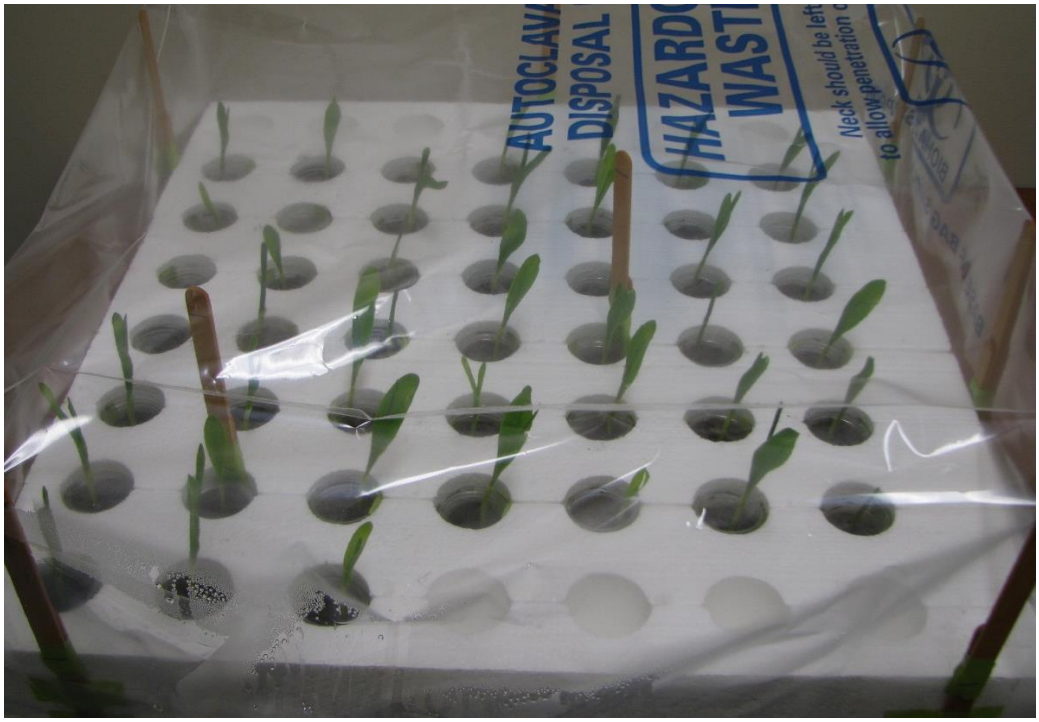

Figure 2-1 Polystyrene box (block) with 28 plastic tubes and 7 day old sweet corn seedlings before harvest. The polysterene box was covered with a plastic sheet to maintain soil moisture content.

2.2.6 Assessment

2.2.6.1 Rhizosphere competence

The assay was based on the method of Ahmad and Baker (1987a) and modified according to pilot studies. The seedling was gently shaken to remove any loosely adhering soil and the roots were excised from the crown. Roots with adhering soil were placed in a sterile 50 mL plastic tube, weighed and kept at 4˚C overnight until processed. The soil from each replicate was dried for 24 h in an oven at 105˚C to obtain the dry soil weight and enable the gravimetric water content GWC for each treatment to be calculated. The following day, 10 mL of a 0.5% Triton X 100 solution were added to each plastic tube containing the roots. Tubes were subsequently shaken for 30 min (94 rpm) in an orbital shaker (Ratek EOM5, Ratek Instruments PTY Ltd, Boronia, Australia). The suspensions were serially diluted to 10^{-1} and 10^{-2}. Aliquots of the initial suspensions and each dilution (200 µL) were plated onto each of three replicate Trichoderma selective medium (TSM, Appendix A2) plates. Plates were incubated at 20˚C in the dark. The number of Trichoderma colonies was counted at 10 to 13 days after incubation and the number of Trichoderma colony forming units (CFU)/g of dry rhizosphere soil (DRS) was calculated.

2.2.6.2 Growth promotion

Seedling emergence was recorded after 7 days. Shoot length was measured from the crown using a ruler. Shoots were detached from the roots at soil level and placed into paper bags. Shoots were subsequently dried in an oven at 65°C for 48 hours to a constant dry weight before being weighed.

2.2.6.3 Endophytic colonisation

After being used to assess rhizosphere competence, the roots were surface sterilised with chlorine gas. The methodology was adapted from (Hoyos-Carvajal et al. 2009a). The entire root system was placed in a plastic container perforated with holes to allow the passage of the gas. The plastic container was suspended in a desiccator over 100 mL commercial bleach (5.25% sodium hypochlorite) and 5 mL HCl (36%) for 30 min. The roots were then washed with 20 mL SDW for 5 min before being cut into 2 cm segments with a sterile scalpel blade in a laminar flow unit. After being cut, the segments were plated on Trichoderma selective media (one plant root per plate) and incubated in the dark at 20°C for 5 to 8 days. The presence or absence and the number of colonies on the root pieces were recorded for all the plates. A plate was recorded as positive for endophytic colonisation if ≥1 Trichoderma colony was present.

2.2.7 Statistical analysis

For each of the three experiments, the mean number of CFU/g DRS per isolate was calculated, $\log_{10}$ transformed and statistically analysed using a general analysis of variance (ANOVA). To combine the data for the three different experiments, the treatment means from each experiment were input into a second ANOVA. An unrestricted least significant difference (LSD) test (Fisher) was used to test for differences between the isolate means. To perform the species analysis, the mean number of $\log_{10}$ CFU/g DRS of individual isolates from each *Trichoderma* species were combined in another ANOVA. Isolates from nine different species were analysed. The *T. harzianum* species was subdivided into two categories corresponding to two different clusters in the *T. harzianum* tree designed at the BPRC. All analyses were carried out in GenStat 15th version (VSN International, Hemel Hempstead, UK). No statistical analysis was performed on the endophytic colonisation data as it was gathered with a qualitative goal.

2.3 Results

2.3.1 Rhizosphere competence

The number of CFU/g DRS for each of the 22 *Trichoderma* isolates was assessed on sweet corn, after 7 days, in non-sterile soil (Table 2-2). Although, the average CFU/g DRS for each isolate across the three experiments is presented (Table 2-2), results were generally consistent between the three experimental repeats (Appendix A3). For example, LU673 was in the top five isolates in each of the three experiments. Similarly, LU556 and LU672 populations were never significantly higher than those of the controls (CS and/or BS). There were significant differences (LSD comparisons) between the *Trichoderma* populations in the rhizosphere of the negative controls and those treated with different *Trichoderma* isolates. Populations ranged from 4.75 x 10^3 to 3.09 x 10^5 CFU/g DRS. Trichoderma population densities in the MC only coated seed (CS) and untreated seed (BS) were 4.75 x 10^3 and 8.43 x 10^3 CFU/g DRS, respectively and did not differ significantly from each other. *Trichoderma* population for T22 (positive control) was 6.14 x 10^4 CFU/g DRS being significantly higher than both CS and BS controls.

Eighteen isolates increased the population density of *Trichoderma* in the rhizosphere relative to the uncoated bare seed ($P<0.05$) (Table 2-2). Rhizosphere populations for the 18 isolates ranged from 3.12 x 10^4 CFU/g DRS (isolate LU626) to 3.09 x 10^5 (isolate LU673). Four isolates had rhizosphere populations that were not significantly different to BS control. These were isolates LU555, LU556, LU669 and LU672 whose populations were 1.63 x 10^4, 1.90 x 10^4, 1.92 x 10^4 and 1.73 x 10^4 CFU/g DRS, respectively. The 18 isolates that had significantly higher rhizosphere populations than observed in the BS could be placed into two groups based on their relativity to T22. Six isolates had populations significantly greater than T22 (the positive control). These were, in the table, isolates LU150 to LU673 whose populations ranged from 1.91 x 10^5 to 3.09 x 10^5 CFU/g DRS. Eleven isolates (LU626 to LU298) produced rhizosphere populations that were not different to T22. Those populations ranged from 3.12 x 10^4 to 1.76 x 10^5 CFU/g DRS. Of these isolates, all produced rhizosphere populations that were significantly different to the negative control (BS).

Table 2-2 ***Trichoderma*** **CFU/g dry rhizosphere soil (DRS) and log10 CFU/g DRS recovered from 7 day old sweet corn seedling roots grown in non sterile soil and treated with 22** ***Trichoderma*** **isolates. Results are means of three experiments. Untreated controls were bare seed (BS) and methyl cellulose coated seed (CS). Values with the same letters are not significantly different at P < 0.05.**

LU No.	*Trichoderma* species	CFU/g DRS*	Log_{10} CFU/g DRS	
673	*T. harzianum* group II	3.09×10^5	5.490	a
151	*T. harzianum* group I	2.45×10^5	5.389	a
132	*T. atroviride*	2.26×10^5	5.355	a
740	*T. hamatum*	1.96×10^5	5.292	ab
140	*T. atroviride*	1.92×10^5	5.283	ab
150	*T. asperellum*	1.91×10^5	5.281	ab
298	*T. atroviride*	1.76×10^5	5.245	abc
571	*T. harzianum* group I	1.73×10^5	5.238	abc
570	*T. viride*	1.67×10^5	5.223	abc
945	*T. asperelloides*	1.40×10^5	5.145	abcd
817	*T. viride*	1.14×10^5	5.057	abcde
592	*T. hamatum*	1.13×10^5	5.053	abcde
699	*T. asperellum*	7.21×10^4	4.858	bcdef
761	*T. viridescens*	6.78×10^4	4.831	bcdef
T22	*T. harzianum*	6.14×10^4	4.788	cdef
595	*T. hamatum*	4.86×10^4	4.687	defg
547	*T. virens*	3.91×10^4	4.592	efg
626	*T. harzianum* group II	3.12×10^4	4.494	fg
669	*T. harzianum* group I	1.92×10^4	4.284	gh
556	*T. virens*	1.90×10^4	4.279	gh
672	*T. harzianum* group II	1.73×10^4	4.238	gh
555	*T. crassum*	1.63×10^4	4.213	gh
BS	Bare seed	8.43×10^3	3.926	hi
CS	MC coated seed	4.75×10^3	3.677	i
LSD 5%			0.479	

*backtransformed mean

2.3.2 **Species specificity**

Because of the reclassification of the *Trichoderma* collection that was on going at the BPRC during and after this work took place, at the end of the three experiments, the 22 isolates belonged to nine species which were represented by either one, two or three isolates (Table 2-3). Rhizosphere populations for each of the nine species ranged from 1.63 x 10^4 (*T. crassum*) to 1.97 x 10^5 CFU/g DRS (*T. atroviride*). *Trichoderma atroviride* populations were the highest (1.97 x 10^5 CFU/g DRS) and were significantly greater than *T. virens* and *T. crassum* populations which were respectively 2.72 x 10^4 and 1.63 x 10^4.

Table 2-3 ***Trichoderma*** **CFU/g dry rhizosphere soil (DRS) and log_{10} CFU/g DRS for 11 species represented by 22 different isolates and recovered from 7 day old sweet corn seedling roots grown in non sterile soil. Values with the same letters are not significantly different at *P* < 0.05.**

Species	No. of isolates	Isolates LU No.	CFU/g DRS	Log_{10} CFU/g DRS	
T. atroviride	3	132, 140, 298	1.97 x 10^5	5.294	a
T. asperelloides	1	945	1.40 x 10^5	5.145	ab
T. viride	2	570, 817	1.38 x 10^5	5.140	ab
T. asperellum	2	150, 699	1.17 x 10^5	5.069	ab
T. hamatum	3	740, 592, 595	1.03 x 10^5	5.011	ab
T. harzianum group I	3	151, 571, 669	9.33 x 10^4	4.970	ab
T. viridescens	1	761	6.78 x 10^4	4.831	ab
T. harzianum T22	1	T22	6.14 x 10^4	4.788	ab
T. harzianum group II	3	673, 626, 672	5.51 x 10^4	4.741	ab
T. virens	2	547, 556	2.72 x 10^4	4.435	b
T. crassum	1	555	1.63 x 10^4	4.213	b
LSD 5% n=1 vs n=1				1.306	
LSD 5% n=1 vs n=2				1.131	
LSD 5% n=1 vs n=3				1.067	
LSD 5% n=2 vs n=2				0.924	
LSD 5% n=2 vs n=3				0.843	
LSD 5% n=3 vs n=3				0.754	

2.3.3 Growth promotion

Seeds treated with the isolates LU298, LU555 and LU673 had 100% emergence (Table 2-4). The untreated control BS had the lowest emergence (50%). Seeds coated with isolates LU298, LU55 and LU673 were significantly higher than those of seeds coated with isolates LU626, T22 and untreated control BS. Seeds treated with LU150, LU699, LU626, LU556 and T22 had emergence not significantly different from the untreated control BS. Seeds treated with the remaining isolates had significantly higher emergence than BS. Seedlings treated with isolate LU817 had significantly shorter shoots than 14 isolates but they were not significantly shorter than the untreated controls BS and CS. There were no significant differences in shoot dry weight between seedlings treated with the 22 different *Trichoderma* isolates and the two controls at 7 days (Table 2-4).

Table 2-4 Means of growth promotion parameters measured for sweet corn seedlings grown in non sterile soil for 7 days and treated with 22 *Trichoderma* isolates. Means were calculated across the three experiments. Controls = bare seed (BS) and MC coated seed (CS). Within a column, values with the same letters are not significantly different at $P < 0.05$.

LU No.	Species	Emergence (%)		Shoot Length (cm)		Shoot Dry Weight (mg)	
945	*T. asperelloides*	91.7	ab	5.1	ab	12.2	a
150	*T. asperellum*	75.0	abc	4.9	ab	11.5	a
699	*T. asperellum*	75.0	abc	6.3	a	16.0	a
132	*T. atroviride*	91.7	ab	6.9	a	17.9	a
140	*T. atroviride*	91.7	ab	6.2	a	13.3	a
298	*T. atroviride*	100.0	a	6.0	a	12.7	a
555	*T. crassum*	100.0	a	6.1	a	13.4	a
592	*T. hamatum*	83.3	ab	6.4	a	13.2	a
595	*T. hamatum*	91.7	ab	5.8	ab	12.9	a
740	*T. hamatum*	91.7	ab	5.7	ab	11.8	a
151	*T. harzianum* GI	83.3	ab	6.6	a	15.5	a
571	*T. harzianum* GI	83.3	ab	5.0	ab	10.7	a
669	*T. harzianum* GI	91.7	ab	6.9	a	17.7	a
672	*T. harzianum* GII	83.3	ab	6.3	a	16.1	a
673	*T. harzianum* GII	100.0	a	6.2	a	14.1	a
626	*T. harzianum* GII	66.7	bc	6.2	a	16.0	a
547	*T. virens*	83.3	ab	6.9	a	14.7	a
556	*T. virens*	75.0	abc	5.9	a	11.8	a
570	*T. viride*	91.7	ab	5.8	ab	15.4	a
817	*T. viride*	83.3	ab	3.8	b	9.8	a
761	*T. viridescens*	83.3	ab	5.5	ab	10.9	a
T22	*T. harzianum*	72.2	bc	6.1	a	15.7	a
BS		50.0	c	5.5	ab	15.7	a
CS		80.6	ab	5.1	ab	9.9	a
LSD 5%		25.55		2.12		8.53	

2.3.4 Endophytic colonisation

The data for each isolate and each of the three experiments is presented in Table 2-5. *Trichoderma* colonies were not recovered from any of the plants from the bare seed control treatments in any of the three experiments. In experiment 1, 50% of the isolates were found to be endophytic with 25 or 33% of plants positive for *Trichoderma* colonies. In experiment 2, 95% of the isolates were recovered from sweet corn plants. The percentage of plants positive for *Trichoderma* varied between 0 to 100%. *Trichoderma* colonies were isolated from 33% of the untreated CS control plants. In experiment 3, 36% of the isolates were found to be endophytic with 33 to 100% of plants positive for *Trichoderma* colonies. Five isolates were consistently isolated from sterilised roots across the three experiments: *T. harzianum* GI LU151, *T. atroviride* LU298, *T. viride* LU570, *T. harzianum* GII LU673 and *T. asperelloides* LU945.

The data for each isolate presented in Table 2-6 is the data collected across the three experiments. The number of plants assessed per isolate varied between six for the untreated control BS and 29 (untreated control CS). The number of plants colonised by *Trichoderma* was different amongst the 22 isolates. Isolates LU67 3and LU298 had the highest number compared to control BS and plants treated with LU626 which did not have *Trichoderma* colonies. For controls treated with MC, out of the 29 plants assessed, *Trichoderma* colonies were recovered from four of these. The number of plants colonised by *Trichoderma* also varied within a species (Table 2-6). For instance, in the *T. harzianum* GII group, out of the 12, 10 and 8 assessed plants inoculated respectively with isolates LU673, LU672 and LU626, seven, two and zero plants respectively were colonised by *Trichoderma*. The highest percentage of plants endophytically colonised by *Trichoderma* were those of plants treated with isolates *T. atroviride* LU298, *T. harzianum* GII LU673 and *T. hamatum* LU740. Plants colonised by these three isolates had similar numbers of colonies per root (3, 2, and 3, respectively).

Table 2-5 Percentage of 7 day old sweet corn plants positive for endophytic *Trichoderma* for each of the three experiments. Plants' seeds had been coated with one of 22 *Trichoderma* isolates and were grown in unsterilised Wakanui silt loam soil.

Isolate	Species	Experiment 1	Experiment 2	Experiment 3
132	*T. atroviride*	25	75	0
140	*T. atroviride*	0	67	0
150	*T. asperellum*	0	100	33
151*	*T. harzianum GI*	25	67	67
298	*T. atroviride*	25	100	50
547	*T. virens*	25	33	0
555	*T. crassum*	25	50	0
556	*T. virens*	0	67	0
570	*T. viride*	33	75	25
571	*T. harzianum GI*	33	100	0
592	*T. hamatum*	0	67	33
595	*T. hamatum*	0	100	0
626	*T. harzianum GII*	0	0	0
669	*T. harzianum GI*	0	50	0
672	*T. harzianum GII*	25	33	0
673	*T. harzianum GII*	25	75	75
699	*T. asperellum*	0	25	0
740	*T. hamatum*	0	75	100
761	*T. viridescens*	25	67	0
817	*T. viride*	0	33	0
945	*T. asperelloides*	33	50	50
T22	*T. harzianum*	0	40	0
Coated seed		0	33	0
Bare seed		0	0	0

*Shaded rows represent isolate treatments where endophytic *Trichoderma* colonies were recovered in all three experiments.

Table 2-6 Presence of *Trichoderma* colonies in the roots of 7 day old swet corn seedlings grown in non sterile soil and coated with 22 different *Trichoderma* isolates. The number of segments and *Trichoderma* colonies refer to the total number of segments and colonies counted for plants that were positive for *Trichoderma* isolations across the three experiments. No. = number.

LU No.	Species	No. of plants assessed	No. of plants positive for *Trichoderma*	No. of *Trichoderma* colonies	% of plants colonised by *Trichoderma*	*Trichoderma* colonies per root
945	*T. asperelloides*	11	5	16	45	3
150	*T. asperellum*	9	4	21	44	5
699	*T. asperellum*	9	1	8	11	8
298	*T. atroviride*	12	7	18	58	3
132	*T. atroviride*	11	4	10	36	3
140	*T. atroviride*	11	2	6	18	3
555	*T. crassum*	12	3	8	25	3
740	*T. hamatum*	11	6	18	55	3
595	*T. hamatum*	11	4	5	36	1
592	*T. hamatum*	10	3	10	30	3
T22	*T. harzianum*	26	4	8	15	2
151	*T. harzianum* GI	10	5	15	50	3
571	*T. harzianum* GI	10	5	12	50	2
669	*T. harzianum* GI	11	2	3	18	2
673	*T. harzianum* GII	12	7	17	58	2
672	*T. harzianum* GII	10	2	3	20	2
626	*T. harzianum* GII	8	0	0	0	0
556	*T. virens*	9	2	5	22	3
547	*T. virens*	10	2	8	20	4
570	*T. viride*	11	5	20	45	4
817	*T. viride*	10	1	6	10	6
761	*T. viridescens*	10	3	8	30	3
CS		29	4	9	14	2
BS		6	0	0	0	0

2.4 Discussion

2.4.1 Rhizosphere competence

This study examined the relative rhizosphere competence of *Trichoderma* species and isolates in non-sterile soil. Eighteen out of twenty two isolates were rhizosphere competent, representing 82% of the population tested. Of the 11 species, represented by the 22 isolates, all except *T. crassum* contained a rhizosphere competent strain. However, *T. crassum* was represented by a single individual as the result of a recent taxonomic revision of *T. virens*. Several isolates showed rhizosphere competence that was greater than T22, a commercial isolate that is well recognised for its biological control ability and rhizosphere competence. This study shows that rhizosphere competence is widespread within the genus *Trichoderma* with the best isolate (*T. harzianum* GII LU673) producing rhizosphere populations 37 fold higher than the methyl cellulose coated control.

The work presented here adds to the few reports in the literature of multiple isolates and species being tested for rhizosphere competence. Rhizosphere competence was determined by counting the colony forming units of *Trichoderma* that were recovered from roots. These were identified by typical morphology of *Trichoderma* species. It was assumed that the control roots subtracted from the inoculated treatments would give the CFU units of the applied isolate in the rhizosphere. This is a standard process used in the *Trichoderma* literature and previously published widely. For most published reports, investigation of relative rhizosphere competence was not the focus of the study but was assessed to explain biocontrol activity or growth promotion by *Trichoderma* species (Carvalho et al. 2011, Roberti et al. 2012). Thus, many of the isolates compared had already been selected for their biocontrol ability. For example, Mehrabi-Koushki et al. (2012) studied the ability of 13 *Trichoderma* isolates to colonize tomato roots in sterile soil. Isolates had previously been confirmed as having biological control ability with two belonging to unknown species while the rest represented the species *T. harzianum* (n=5), *T. koningii* (n1), *T. brevicompactum* (n=1) and *T. virens* (n=4). Two species were common with the present study. Fifteen days after planting, the authors found that the 13 *Trichoderma* isolates could colonize the rhizosphere of tomato roots with significant differences between the isolates and populations varying from 1.7×10^4 to 4.0×10^7 CFU/ g root. Similarly, with the aim to find a potential biological control agent against the pathogen causing the wilt of *Chrysanthemum* plants. Singh and Kumar (2012) screened seven *T. harzianum* strains for their rhizosphere competence ability in sterile soil. The authors found that 57% of their isolates were able to successfully colonize the entire length of the *Chrysanthemum* plant's roots. Despite the selection by these authors for biocontrol ability the same results are shown as presented here, in that rhizosphere competence is not restricted to particular *Trichoderma* species.

The two reports of (Mehrabi-Koushki et al. 2012) and (Singh and Kumar 2012a), differ substantially from the work described here as they assessed rhizosphere competence in sterile soil and with isolates that had been proven to be biological control agents. In these conditions, the assessment could have inflated the number of rhizosphere competent isolates beyond what would be seen in soil. In sterile soil, *Trichoderma* isolates would have not encountered other microorganisms that may have competed for the rhizosphere as a niche and, in the absence of these other microbial communities, they would have been able to develop without having to compete for space or nutrients exuded by the plant creating therefore a favourable and easy environment to colonise. Nevertheless, in Singh and Kumar (2012) study, the authors assessed rhizosphere competence by studying the growth of the selected *Trichoderma* species on the rhizoplane of *Chrysanthemum* plants. The seven isolates could colonise the rhizoplane of *Chrysanthemum* plants to different depths, showing a close relationship between the fungus and the plant and a form of rhizosphere competence.

In the literature, a strong link is made between biological control and rhizosphere competence (Harman et al. 2004a, Harman 2006, Sala et al. 2007, Roberti et al. 2012). Thus, it would be unsurprising that those studies that have chosen to test biocontrol isolates would contain a high number of rhizosphere competent isolates. However, the mechanism by which the isolate exerts biological control could explain the need for an isolate to be able to colonise the plant rhizosphere. In that sense for example, mycoparasitic isolates may not need to be as rhizosphere competent as those isolates whose primary mechanism for biocontrol is competitive exclusion. For example, in this study, T22 was in the middle of the list for rhizosphere competence and it is a well-known mycoparasite (Lo et al. 1998). This was illustrated in Singh and Kumar (2012a) where they showed that there was no correlation between the ability of the *Trichoderma* isolates to inhibit *Fusarium oxysporum* and to colonise the roots of *Chrysanthemum*. In the present study only 12 isolates (55% of the population tested) had some prior demonstration of biological control activity against different pathogens in glasshouse trials but 92% were rhizosphere competent, including three isolates each from the *T. atroviride* and *T. hamatum* species and one isolate from each of the *T. asperellum*, *T. harzianum* GI, *T. harzianum* GII and *T. virens* species. The ten remaining isolates that had no evidence of biocontrol activity despite being included in screening assays against pathogens, 70% were rhizosphere competent which still represented a large majority of the *Trichoderma* isolates.

Affokpon et al. (2011) is one of the few studies in which isolates were selected randomly and not because of their ability as biocontrol agents. In their study, the ability of 17 *Trichoderma* strains, encompassing five species, were assessed to colonize the roots of tomato plants in unsterilized potting soil.

The *Trichoderma* species had been isolated from root-knot nematode egg masses or from nematode infested vegetable fields located in Benin. The majority of their isolates were *T. asperellum* (n=9), with four other species, *T. harzianum*, *T. brevicompactum*, *T. hamatum* and *T. erinaceum* represented respectively by four, three and one individual isolates for each of the last two species. Three species were common with the current study. After 8 weeks, the authors recovered populations for the 18 isolates ranging from 7 x 10^3 to 6.8 x 10^4 CFU/gram root showing that all of the *Trichoderma* isolates could colonize the rhizosphere of tomato plants to different degrees. The saprophytic nature of *Trichoderma* species could explain the survival of *Trichoderma* isolates in the soil and especially in the rhizosphere soil where many organic compounds are exuded by plant roots. The present study agrees with this report demonstrating that rhizosphere competence is widespread within the genus.

In the present study, the best and the second least rhizosphere competent isolates (LU673 and LU672) belonged to the same species (*T. harzianum* GII) and isolates within other species also showed variable rhizosphere competence. This showed that rhizosphere competence, for a particular plant host, is variable within a *Trichoderma* species and is isolate dependant. This is in agreement with the literature. Similarly, in the Affokpon et al. (2011) study, nine isolates from the species *T. asperellum* showed different abilities to colonise the roots of 8 week old tomato plants in non-sterile potting soil. The researchers found that *Trichoderma* densities ranged from 10^6 to 5.6 x 10^8 CFU/mL of soil amongst the isolates and the nine isolates were classified in six statistically different groups according to their rhizosphere competence. Another *Trichoderma* species*, T. virens,* was studied by Mehrabi-Koushki et al. (2012). They compared the ability of four *T. virens* isolates to colonize the rhizosphere of 15 day old tomato seedlings in sterile soil. They found significant differences between the isolates' populations in the top of the root rhizosphere and these ranged from 1.6 to 3.6 x 10^7 CFU/g root, again dividing the isolates into statistically different groups. In another study, Sivan and Harman (1991) assessed the level of rhizosphere competence of three *Trichoderma harzianum* strains on 7 day old maize seedlings grown in unsterilized sandy loam soil. They found that T22, with populations varying from 10^3 to 10^5, was able to colonize the entire length of plant roots (22 cm), whereas, the other two isolates were unable to colonize the middle and bottom parts. The present study agrees with the literature in showing that isolates within the same *Trichoderma* species are different. Whether the rhizosphere competence of the isolates used in this study showed different colonization patterns along the length of the root is unknown as the entire root system was used for the assessment of rhizosphere competence. However, it would be interesting to know to what depth isolates with different rhizosphere competence ability can colonize young and old sweet corn roots.

In the present study, *T. harzianum* T22 was chosen as a positive control because it is a well-studied organism known to be rhizosphere competent, a mycoparasite, and a growth promoter. T22 is the main ingredient of commercial biopesticides such as RootShield®, T22 HC and Plant Shield®, used for the control of different pathogens on different crops (Harman 2000, Harman et al. 2004a, Harman 2006). Isolate T22 had been used as a control in other studies and is proven to be rhizosphere competent in many plant species especially sweet corn, hence the choice of host and control for this study. In the present study, *Trichoderma* populations of sweet corn plants inoculated with T22 reached 6.14 x 10^4 CFU/g DRS 7 days post planting therefore the isolate was considered to be rhizosphere competent. Similarly, (Sivan and Harman 1991) concluded that T22 was rhizosphere competent and the best among two other isolates when they coated it onto maize seeds and grew them for 7 days in unsterilized soil. In that study T22 averaged 1.17 x 10^4 CFU/g DRS (average between the 11 values found for different depths). In the present study, isolate T22 was more rhizosphere competent than four isolates from four different species, but it was not the most rhizosphere competent which was unexpected given its high profile as a biocontrol agent. Six isolates from five different species were more rhizosphere competent than T22.

The poorer performance of T22 than expected may be due to the soil used. Bennett and Whipps (2008) applied T22 to carrot and onion seeds and sowed them in three different unsterilized soil types comprising a light sandy loam, a peat soil and a sandy clay loam. The authors found that for onion plants, there were higher numbers of T22 recovered from the light sandy loam soil compared to the sandy clay loam soil. For carrot, they recovered higher populations from the light sandy loam and peat soils compared to the sandy clay loam soil. The physical and chemical properties of Wakanui silt loam soil may have had an influence on the colonisation by T22. It would have been interesting to assess rhizosphere competence of T22 on onion and carrot in Wakanui silt loam soil to see whether the soil was the main component influencing the fungus' development or the combination of plant and soil as demonstrated in the Bennett and Whipps (2008) study.

Another reason that may have explained why T22 did not perform as expected, may be the microorganisms present in the New Zealand unsterilized Wakanui silt loam soil which may have been suppressive to T22. Six isolates performed better than T22 in the present study, all of them except for LU672 had been isolated from New Zealand soils and may have had an advantage in being able to suppress microorganisms, leading to better colonisation of the sweet corn rhizosphere than T22.

The genetic background of the sweet corn hybrid used in this study could have also explained the lower level of T22 rhizosphere competence compared to the other six isolates. In a study of the interactions between T22 and maize inbred line Mo17, Harman et al. (2004) reported that the genetic component of the plant material influences the response of T22 and that beneficial activities of the fungus might be variable according to the type of plant (inbred or hybrid). Similarly, Tucci et al. (2011) reported that the beneficial effect of *Trichoderma* species are modulated by the plant genotype.

Although T22 was rhizosphere competent in the conditions of the present experiment, factors such as soil type, soil microbial communities and plant genetic material altered its ability to colonise sweet corn roots and perform as well as other isolates in this study.

2.4.2 Growth promotion

None of the plant growth parameters differed from those of the control seed coated only with methyl cellulose (MC). However, 77% of the isolates increased seedling emergence compared to the untreated control bare seed (no MC) and 14% were better than T22. The increase of seedling emergence induced by 77% of the isolates compared to the untreated bare seed control was not considered growth promotion in this study because the untreated control bare seed did not receive any MC. MC coated seeds (in the absence of inoculated *Trichoderma*) had 1.6 times better emergence than bare seed. The reason for the difference between both untreated controls may have been the formation of a viscous polymer at sowing time when the soil was at 20% moisture content that protected the MC coated seeds from the lack of water during germination.

The lack of an effect on seedling emergence in the present study is in agreement with Bell et al. (2000) study. The authors coated seeds of cucumber with seven *Trichoderma* isolates individually and in a mixture. Seeds were sown in a field and the number of healthy seedlings was recorded 8 days post planting. Results showed that the coating with *Trichoderma* isolates did not affect the seedling emergence compared with the untreated control. However, that paper was one of the few to show no effect and most of the literature indicates that certain isolates of *Trichoderma* can increase seedling emergence and plant growth in many plant species (Harman 2000, Mukhtar et al. 2012, Milanesi et al. 2013, Maag et al. 2014). Rhizosphere competent *Trichoderma* strains are suggested as more capable of promoting growth than non rhizosphere competent strains (Zachow et al. 2010, Roberti et al. 2012) and rhizosphere competence is among other mechanisms, such as inhibition of pathogenic microorganisms, solubilisation of nutrients, production of chemical compounds (antibiotics and phytohormones) and induction of systemic resistance, by which *Trichoderma* exerts growth promotion (Saba et al. 2012, Contreras-Cornejo et al. 2013, Stewart and Hill 2014).

One of the reasons why the isolates in this study did not promote growth may have been the experimental conditions. In the present study, sweet corn plants were grown at 20°C and in a soil with pH values varying between 5.3 and 5.7. When T22 was proven to be rhizosphere competent and a growth promoter of *Zea mays* (Sivan and Harman 1991), the temperature in the growth chamber was held at 25°C and the soil pH was 7.3 which may have improved the development of T22 and its bioactivity. Furthermore, in a study conducted by (Chang et al. 1986) where they assessed the potential of *T. harzianum* to promote growth in several horticultural and floricultural crops, growth responses were detected when *Trichoderma* populations were higher than 10^5 CFU/g of soil. In this study only half of the isolates achieved CFU numbers >10^5 and T22 only reached 6.14 x 10^4 CFU/g DRS.

Generally, plants grew well under the experimental conditions held for this study and did not seem to have been under any abiotic stress, and this may have been another reason why growth promotion was not detected, at least for isolate T22. Mastouri et al. (2010) examined seedling germination of tomato plants treated with T22 exposed to different abiotic stresses and observed that T22 had little or no effect on seeds that were not under stress.

In the present study, 66% of the isolates increased shoot length compared to isolate LU817. These results indicated that *Trichoderma* isolates differed in how they affected plant growth and is in agreement with various studies such as those of Clouston et al. (2010) and Hohmann et al. (2011). Clouston et al. (2010) tested the potential of 62 *Trichoderma* isolates to promote root growth in the ornamental plant *Impatiens walleriana*. The authors identified six individual isolates with the ability to improve growth of impatiens cuttings showing differences relative to growth promotion between isolates. Similarly, Hohmann et al. (2011) reported that two *Trichoderma* isolates differed in promoting growth of *Pinus radiata* seedlings.

Growth promotion was not observed for any of the isolates in this study, therefore, no correlation could be drawn between rhizosphere competence and growth promotion. However, *Trichoderma* isolates affected plant health differently and some were better at increasing seedling emergence and shoot length compared to others. Similarly to rhizosphere competence, *Trichoderma* isolates have different abilities to promote growth that are inherent to each isolate and depend on the interactions of the isolate with the plant and biotic and abiotic factors.

2.4.3 **Endophytic colonisation**

This study also examined the ability of multiple isolates to become endophytes in sweet corn plants. Results showed that across the three experiments, 95% of the isolates were able to internally colonise the roots of at least one sweet corn plant. Endophytic *Trichoderma* colonies were recovered from the top 3 cm from the crown root.

However, only five isolates (22%) were consistently recovered from surface sterilised roots in the three experiments. These isolates belonged to the species *T. harzianum* GI (LU151), *T. atroviride* (LU298), *T. viride* (LU570), *T. harzianum* GII (LU673) and *T. asperelloides* (LU945) and were in the top 10 most rhizosphere competent isolates. Two of these isolates were the most rhizosphere competent in the study. The present study's results were similar to Hoyos-Carvajal et al. (2009a) who assessed the ability of nine *Trichoderma* isolates to endophytically colonise the shoots and roots of bean seedlings. Using scanning electron microscopy, the authors observed two out of nine isolates (22%) in the cortex and vascular tissues of bean seedlings.

Trichoderma isolates are known to colonise internal root tissues of many plants species (Harman et al. 2004a) and several reports emphasize the importance of endophytism for biological control, growth promotion, induced systemic resistance and other beneficial effects (Shoresh et al. 2010), however, few studies have compared multiple isolates. Prisa et al. (2013) screened 162 *Trichoderma* isolates on the plant species *Limonium sinuatum* and *Cupressus sempervirens* and 202 isolates on *Camelia sinensis*. They found that, respectively, 6, 5.5 and 4% of the isolates were endophytic on the plants tested. Only three isolates were found to be endophytic in all three plant species. A larger number of *Trichoderma* isolates were found to be endophytic in the present study compared to Prisa et al. (2013) report. The reason for this difference may have been the plant. Perhaps sweet corn roots are more easily penetrated by *Trichoderma* species or the isolates from this study were better at colonising the cortex of sweet corn roots compared to the ability of the isolates at colonising *Limonium sinuatum*, *Cupressus sempervirens* and *Camelia sinensis* in Prisa et al. (2013) study. It would be interesting to test whether *Trichoderma* isolates are generalists for endophytic colonisation or whether this attribute is plant host specific. Many endophytes are not generalists and have evolved with the plant. In the case of opportunistic organisms such as *Trichoderma*, other factors such as the environmental conditions, the presence of a pathogen in the soil or the plant phenology could more significantly affect the establishment of this relationship with the host. It would be interesting to assess the endophytic colonisation of *Trichoderma* in a different host and under different environmental conditions.

The difference may have also been explained by the methodology for the assessment. In the present study, there was a difference between the results for the percentage of plants positive for *Trichoderma* across the three experiments and for each individual experiment. When assessing endophytic colonisation, it is important to take into consideration: the consistency of the isolate at being endophytic across a set of experiments, the percentage of plants positive for endophytic colonisation and the number of colonies per root or per cm of root, the latter illustrates if a large root depth is covered by the endophyte. In this study, most of the endophytic isolates were recovered from the same root zones and in similar quantities.

Nevertheless, all the reports agreed in showing that *Trichoderma* isolates differ in their relative ability to endophytically colonise plants. In the present study the most endophytic isolates were also the most rhizosphere competent suggesting a link between both attributes. This is in agreement with the literature (Harman 2006). However, endophytic colonisation in this study was measured only after 7 days. It would be interesting to know if the relationship between *Trichoderma* and its host, changes over time. Hoyos-Carvajal et al. (2009a) have shown that some isolates take more time to colonise the aerial parts of bean seedlings and become endophytic only at flowering stage.

In the last 26 years, there have been more than 64 publications addressing rhizosphere competence. Most of those papers agree in saying that rhizosphere competence is important for biological control. This study supports these published accounts that rhizosphere competence is widespread within the genus *Trichoderma* and is isolate specific. Although no growth promotion was detected, isolates showed different levels of endophytic colonisation for sweet corn. Some of the isolates tested in this study are the main ingredients of commercial biopesticides used to control various pathogens on multiple crops. Given that the performance of those isolates was variable for the three traits measured on sweet corn plants, whether changing the host plant alters their ability to colonise the rhizosphere would be interesting to know, and is investigated in the next chapter.

3

Relative rhizosphere competence of selected *Trichoderma* isolates on six different host plants

3.1 Introduction

There are many reports that a particular species or isolate of *Trichoderma* can colonise the rhizosphere of many different host plants, but the rhizosphere competence is seldom quantified or compared among a range of possible host plants in a single experiment. Moreover, only a few reports assess rhizosphere competence in non-sterile soil. Two of these reports are early research on rhizosphere competence when it was still believed that *Trichoderma* isolates needed to be mutated and resistant to benomyl to be able to colonise roots (Ahmad and Baker 1987a, Sivan and Harman 1991). It was then necessary to add benomyl to the soil at the same time as the isolate was applied. In those two studies, they investigated rhizosphere competence of the wild type and the mutant on five plant species, maize, cotton, cucumber, radish, bean and tomato. Results for three out of four isolates were not presented because the focus of the study was the mutant. Studies indicated, nevertheless, that *Trichoderma* isolates were variable in their colonisation of different plant species.

The question of plant host specificity has been addressed in the literature, but mainly for fungal species other than *Trichoderma*. For example, Al-Rawahi and Hancock (1997) studied the rhizosphere competence of *Pythium oligandrum* in association with 11 different plants species from seven families, and showed that the family and cultivar had an influence *on Pythium oligandrum*'s rhizosphere colonisation.

The objective of this chapter was to determine whether relative rhizosphere competence on sweet corn could extend across other plant species using six *Trichoderma* isolates that represented the most and least rhizosphere competence on sweet corn.

3.2 Materials and methods

3.2.1 Selection of isolates

The three most rhizosphere competent isolates (*T. harzianum* group II LU673, *T. harzianum* group I LU151 and *T. atroviride* LU132) and the three least rhizosphere competent isolates (*T. crassum* LU555, *T. virens* LU556 and *T. harzianum* group II LU672) from the previous experiment were selected to perform a plant host specificity test.

3.2.2 *Trichoderma* inoculum production

Inoculum for each isolate was produced following the method detailed in Chapter 2 (Section 2.2.2).

3.2.3 Application of Trichoderma isolates to the seeds

Seeds of sweet corn (*Zea mays* var. Chieftain), onion (*Allium cepa*), cauliflower (*Brassica oleracea* var. *botrytis cymosa*), perennial ryegrass (*Lolium perenne* var. Impact), white clover (*Trifolium repens* var. Nomad) and carrot (*Daucus carota*) were used. Prior to inoculation, the sweet corn seeds were sterilized for 10 minutes in a solution of 1% sodium hypochlorite and 5% ethanol and washed three times with SDW. The aim of the sterilisation was to remove surface contaminants. The seeds were subsequently air-dried in a laminar flow cabinet. For the other five plant species, the seeds were surface sterilized for 5 minutes in a solution with 1% sodium hypochlorite and 5% ethanol solution and rinsed three times with SDW.

For each of the six species, seeds were coated to obtain 10^6 spores per seed. A two-step coating method with SDW and methyl cellulose was adopted after trials were carried out with seeds of each of the plant species. For sweet corn, Petri dishes (90 mm diameter) with 40 seeds each received 2 mL each of a solution with equal volumes of one of the different *Trichoderma* conidial suspensions (1-6 x 10^8 spores/mL) and 2% MC solution to coat the seeds. The control seeds were treated the same way in a separate Petri dish, but the *Trichoderma* conidial suspension was replaced with SDW. For each of the other five plant species, 40-50 seeds were placed in Petri dishes.

Each Petri dish received 1 mL of a solution with equal volumes of one of the different *Trichoderma* conidial suspensions and 2% MC solution to coat the seeds. A pilot study had been previously set up to determine the amount of coating solution needed to achieve the average concentration of 10^6 spores per seed. Seeds were stirred with a pipette tip for 60 seconds to evenly distribute the *Trichoderma* conidia over the surface of the seeds and the seeds left to dry for 3 hours. Subsequently, a second coating with the same volumes was applied and the seeds were left to dry overnight in a laminar flow cabinet. Controls were treated the same way except the *Trichoderma* conidial suspension was replaced with water.

3.2.4 The growing system and experimental design

Seeds were sown in 10 cm diameter plastic pots (500 mL capacity) filled with moistened soil. Wakanui silt loam soil was used and prior to each experimental setup, a comprehensive soil analysis was completed (Hills Laboratories, Hamilton, NZ) (Appendix B1) in order to characterise the soil and detect any potentially significant changes caused by storage. Before each of the set ups, the soil was adjusted to 20% (w/w) moisture as previously described in Chapter 2 (section 2.2.4).

Each pot had 10 seeds coated with one of the *Trichoderma* isolates or control seeds. A total of one hundred and sixty two pots were set up in three blocks, such that each block had 54 pots with nine pots per plant species. Six out of the nine pots had 10 seeds coated with one of the six isolates, the three other pots had control seeds. The experiment was set up on the 1st November 2010 in a chamber where temperature and light were controlled. Temperature was maintained at 18°C (day time) and 20°C (night time). Light (average 216 µmol photons /m^2/s) was applied 16 h a day. This experiment was repeated on the 28th January 2011.

3.2.5 Growth promotion assessment

Seedling emergence was recorded at harvesting time which was different for each plant species. For each plant species and isolate, six seedlings were randomly harvested from the pots (two per block) and were used to assess growth promotion and rhizosphere competence. For the assessment of growth promotion, shoot and roots lengths were measured using a ruler to the nearest mm. Shoots were detached from the root, placed into paper bags and subsequently dried in an oven at 72°C for 48 hours to a constant dry weight before being weighed. After being used for the rhizosphere competence assessment (section 3.2.6), roots were washed, weighed and dried under the same conditions as shoots. Shoot and root lengths and dry weights were only assessed in experiment one.

3.2.6 Rhizosphere competence assessment

Prior to the experiment, a pilot assay was set up where seeds of the six plant species were grown in the same soil to select a harvest time. The harvest time for each species was chosen as the one that had a root surrounded by enough rhizosphere soil for the analysis.

Therefore, assessments were performed at different times according to the growing rate of plant species. Sweet corn was assessed 7 days, cauliflower 21 days, ryegrass 23 days, clover 28 days, carrots 30 days and onions 36 days post planting. For each plant species and isolate, six seedlings were harvested from the pots (two per block) and were used to to determine rhizosphere competence following the protocol previously described in Chapter 2 (section 2.2.6.1) to obtain CFU/g DRS.

3.2.7 Statistical analysis of growth promotion

Seedling emergence as well as shoot and root lengths were analysed using an analysis of variance (ANOVA) for a split plot design with six main-plot treatments being the six plant species and seven sub-plot treatments being six isolates and the untreated controls (three times replicated). Shoot and root dry weights were $\log_{10}$ transformed prior to being analysed with ANOVA.

Fisher's unrestricted least significant difference (LSD) was used to test for mean differences between seedling emergence, shoot and root lengths and shoot and root dry weights of treated plants with untreated controls. The analyses were performed in GenStat 15th edition (VSN International, Hertfordshire, UK).

3.2.8 Statistical analysis of rhizosphere competence

Trichoderma CFU/g DRS were $\log_{10}$ transformed and statistically analysed for each time replicate using an ANOVA for a split plot design with six main-plot treatments being the six plant species and six sub-plot treatments being six isolates. The 18 main plots were laid out in a randomised block design with three blocks. Controls had to be removed from the ANOVA for two different reasons. Firstly, the main goal of the experiment was to compare the performances of different isolates between each other on different plant species. Secondly, *Trichoderma* populations isolated from control plants of all species were very low and were violating the assumptions of normality for ANOVA. Fisher's unrestricted least significant difference (LSD) was used to test for differences between plant species means and between isolate means. The analysis was performed in GenStat 15th edition (VSN International, Hertfordshire, UK).

3.3 Results

3.3.1 Growth promotion results

Growth promotion parameters were compared within the same plant species between untreated controls and plants inoculated with each of the six *Trichoderma* isolates. Seedling emergence was measured in both experiments. The other parameters were only measured in experiment one.

3.3.1.1 Seedling emergence

In experiment 1, seedling emergence was significantly improved for the following host and *Trichoderma* strain combinations: carrot with *T. harzianum* LU672, onion with *T. harzianum* LU151 and sweet corn with *T. virens* LU556. *Trichoderma crassum* LU555 significantly decreased emergence of cauliflower when compared with control plants (Appendix B4).

In experiment 2, *T. crassum* LU555 and *T. virens* LU556 significantly increased emergence of carrot seedlings. Sweet corn seeds inoculated with *T. crassum* LU555 also had significantly better emergence than control seedlings'. *Trichoderma harzianum* LU673 decreased the emergence of clover and cauliflower seeds (Appendix B4).

3.3.1.2 Shoot and root lengths

Shoots of carrot plants inoculated with *T. harzianum* LU151 were significantly longer than the shoots of untreated plants. Shoots of ryegrass plants inoculated with *T. virens* LU556 were shorter compared to the untreated controls. Onion plants inoculated with *T. harzianum* LU151 had longer roots than control plants (Appendix B2).

3.3.1.3 Log_{10} of shoot and root dry weights

Carrot plants inoculated with *T. harzianum* LU151 had significantly heavier shoots than control plants. Shoots of clover plants inoculated with *T. crassum* LU555 were significantly heavier than untreated plants' shoots. *Trichoderma atroviride* LU132 significantly decreased the weight of clover plants' shoots and roots compared to the controls. All treated sweet corn plants had significantly higher root weights compared to untreated plants' roots (Appendix B4).

3.3.2 Rhizosphere competence of experiments one and two combined

Results for experiments one and two were similar (P = 0.395) (results for experiments one and two were added to appendices B2 and B3 respectively), therefore data from both experiments were combined and re-analysed.

Sweet corn plants inoculated with *T. atroviride* LU132, *T. harzianum* LU151 and *T. harzianum* LU673 had the highest *Trichoderma* populations and were significantly different to those of plants inoculated with *T. virens* LU556, *T. harzianum* LU672 and *T. crassum* LU555 (Table 3-1).

On ryegrass, the highest number of *Trichoderma* populations were isolated from plants inoculated with *T. atroviride* LU132 and was significantly higher than populations isolated from plants treated with *T. virens* LU556, *T. harzianum* LU672 and *T. crassum* LU555 (Table 3-1).

On onion, *Trichoderma* populations isolated from plants treated with *T. harzianum* LU672 had the highest *Trichoderma* populations and were significantly different to all other treated plants' *Trichoderma* populations (Table 3-1).

Trichoderma populations isolated from clover plants treated with different *Trichoderma* isolates were not significantly different (Table 3-1).

On cauliflower, the highest number of *Trichoderma* populations was recovered from plants treated with *T. atroviride* LU132. *Trichoderma* populations from all treated plants were significantly higher than those of *Trichoderma* populations isolated from plants treated with *T. harzianum* LU673 (Table 3-1).

On carrot, *Trichoderma* populations from plants inoculated with *T. virens* LU556 were significantly lower than those recovered from plants treated with *T. atroviride* LU132, *T. harzianum* LU151 and *T. harzianum* LU673 (Table 3-1).

Table 3-1 Combined analysis: Mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) (log_{10} values are indicated between brackets) from selected plant species treated with six different *Trichoderma* isolates. Mean CFU/g DRS followed by the same letter do not differ significantly within each column.

	Sweet corn	Ryegrass	Onion	Clover	Cauliflower	Carrot
***T. harzianum* LU673**	8.05×10^4 (4.906) a	2.06×10^5 (5.314) ab	2.47×10^4 (4.392) b	2.00×10^4 (4.3) a	1.78×10^4 (4.25) d	4.27×10^4 (4.63) a
***T. harzianum* LU151**	6.53×10^4 (4.815) a	2.61×10^5 (5.417) ab	3.37×10^4 (4.528) b	1.64×10^4 (4.214) a	2.24×10^5 (5.35) ab	3.56×10^4 (4.551) a
***T. atroviride* LU132**	1.42×10^5 (5.152) a	5.90×10^5 (5.771) a	2.01×10^4 (4.303) b	9.44×10^3 (3.975) a	3.62×10^5 (5.559) a	3.94×10^4 (4.596) a
T. virens LU556	8.32×10^3 (3.92) b	1.35×10^5 (5.13) b	1.62×10^4 (4.21) b	1.08×10^4 (4.035) a	6.73×10^4 (4.828) c	1.07×10^4 (4.031) b
T. harzianum LU672	6.75×10^3 (3.829) b	1.57×10^5 (5.196) b	1.22×10^5 (5.086) a	1.37×10^4 (4.136) a	1.08×10^5 (5.035) bc	1.94×10^4 (4.288) ab
T. crassum LU555	9.35×10^3 (3.971) b	1.11×10^5 (5.046) b	1.79×10^4 (4.254) b	1.89×10^4 (4.276) a	1.13×10^5 (5.052) bc	1.76×10^4 (4.246) ab
Control*	9.16×10^3 (3.962)	6.65×10^3 (3.823)	1.16×10^4 (4.065)	4.98×10^3 (3.697)	5.21×10^3 (3.717)	9.16×10^3 (3.962)
LSD 5%			0.4756			

* Control data was not included in the ANOVA analysis. Note-**LU132, LU673, LU151** selected as highly rhizosphere competent on sweet corn in chapter 2; LU556, LU555, LU672 selected as weakly rhizosphere competent on sweet corn in Chapter 2.

For all *Trichoderma* isolates except *T. harzianum* LU673, ryegrass and cauliflower had the highest *Trichoderma* populations (Table 3-2). In the case of plants treated with *T. harzianum* LU672, the highest *Trichoderma* populations were equally isolated from ryegrass, cauliflower and onion. For plants treated with *T. harzianum* LU673, *Trichoderma* populations isolated from cauliflower were the lowest (Table 3-2). For plants inoculated with isolates *T. virens* LU556, *T. harzianum* LU672 and *T.*

crassum LU555, *Trichoderma* populations recovered from sweet corn plants were significantly lower than for other isolates (Table 3-2).

Trichoderma populations isolated from clover plants treated with the six isolates were equivalent. *Trichoderma* populations isolated from onion plants treated with all isolates were equal except for those of plants treated with *T. harzianum* LU672 whose populations were significantly higher (Table 3-2).

Table 3-2 Combined analysis: Performance of six *Trichoderma* isolates across selected plant species. Mean CFU/g dry rhizosphere soil (DRS) followed by the same letter do not differ significantly within each column. Log_{10} values are indicated between brackets.

	***T. harzianum* LU673**	***T. harzianum* LU151**	***T. atroviride* LU132**	*T. virens* LU556	*T. harzianum* LU672	*T.crassum* LU555
Sweet corn	8.05×10^4	6.53×10^4	1.42×10^5	8.32×10^3	6.75×10^3	9.35×10^3
	(4.906) ab	(4.815) b	(5.152) bc	(3.92) b	(3.829) b	(3.971) b
Ryegrass	2.06×10^5	2.61×10^5	5.90×10^5	1.35×10^5	1.57×10^5	1.11×10^5
	(5.314) a	(5.417) a	(5.771) a	(5.13) a	(5.196) a	(5.046) a
Onion	2.47×10^4	3.37×10^4	2.01×10^4	1.62×10^4	1.22×10^5	1.79×10^4
	(4.392) bc	(4.528) bc	(4.303) de	(4.21) b	(5.086) a	(4.254) b
Clover	2.00×10^4	1.64×10^4	9.44×10^3	1.08×10^4	1.37×10^4	1.89×10^4
	(4.3) c	(4.214) c	(3.975) e	(4.035) b	(4.136) b	(4.276) b
Cauliflower	1.78×10^4	2.24×10^5	3.62×10^5	6.73×10^4	1.08×10^5	1.13×10^5
	(4.25) c	(5.35) a	(5.559) b	(4.828) a	(5.035) a	(5.052) a
Carrot	4.27×10^4	3.56×10^4	3.94×10^4	1.07×10^4	1.94×10^4	1.76×10^4
	(4.63) bc	(4.551) bc	(4.596) cd	(4.031) b	(4.288) b	(4.246) b
LSD 5%			0.5219			

Note – **LU132, LU673, LU151** selected as highly rhizosphere competent on sweet corn in chapter 2; LU556, LU555, LU672 selected as weakly rhizosphere competent on sweet corn in chapter 2.

Ryegrass and cauliflower were the plant species with the highest *Trichoderma* populations regardless of the isolate that had been used to treat plants. *Trichoderma* populations reached 2.05×10^5 and 1.03×10^5 CFU/g DRS for ryegrass and cauliflower respectively. Clover plants had the lowest populations (1.43×10^4 CFU/g DRS) and were similar to those of carrot, sweet corn and onion plants (Table 3-3).

Table 3-3 Combined analysis: Log $_{10}$ values and mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) of six plant species. Mean CFU/g DRS followed by the same letter do not differ significantly within the column.

Plant	Log_{10} CFU/g DRS	Mean CFU/g DRS	
Ryegrass	5.312	2.05×10^5	a
Cauliflower	5.012	1.03×10^5	a
Onion	4.462	2.90×10^4	b
Sweet corn	4.432	2.70×10^4	b
Carrot	4.39	2.45×10^4	b
Clover	4.156	1.43×10^4	b
LSD 5%	0.5219		

Irrespective of plant species, the highest *Trichoderma* populations were isolated from plants treated with *T. atroviride* LU132 and *T. harzianum* LU151 equally (Table 3-4). Both were significantly higher than the populations isolated from all plants species treated with *T. crassum* LU555 and *T. virens* LU556. The lowest *Trichoderma* populations were isolated from plants treated with *T. virens* LU556 (Table 3-4).

Table 3-4 Combined analysis: Log $_{10}$ values and mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) recovered from six plant species for six different isolates. Mean CFU / g DRS followed by the same letter do not differ significantly within the column.

Isolate	Log10 CFU / g DRS	Mean CFU / g DRS	
***T. atroviride* LU132**	4.892	7.80×10^4	a
***T. harzianum* LU151**	4.813	6.50×10^4	ab
***T. harzianum* LU673**	4.632	4.29×10^4	bc
T. harzianum LU672	4.595	3.94×10^4	c
T. crassum LU555	4.474	2.98×10^4	cd
T. virens LU556	4.359	2.29×10^4	d
LSD 5%	0.5219		

3.4 Discussion

3.4.1 Rhizosphere competence

The aim of this chapter was to assess the rhizosphere competence ability of several *Trichoderma* isolates on different plant species. The isolates tested in this chapter were chosen according to their ability to colonise the rhizosphere of sweet corn seedlings in chapter two. In chapter two, *T. atroviride* LU132, *T. harzianum* LU151 and *T. harzianum* LU673 colonised sweet corn better than *T. virens* LU556, *T. harzianum* LU672 and *T. crassum* LU555. The results of this study showed the same pattern of colonisation for sweet corn plants in both experiments. However, this pattern did not repeat itself for the other plant species. Poor rhizosphere colonisers of sweet corn such as *T. virens* LU556, *T. harzianum* LU672 and *T. crassum* LU555 were good rhizosphere colonisers of ryegrass and cauliflower. In contrast, *T. harzianum* LU673 which was a good rhizosphere coloniser of sweet corn showed a particularly poor ability to colonise cauliflower roots. Moreover, isolate *T. harzianum* LU672 which was the worst rhizosphere coloniser of sweet corn was the only isolate capable of colonising onion roots at the same level on ryegrass and cauliflower. The results showed that some plants, in this case ryegrass and cauliflower, were more receptive to *Trichoderma* colonisation than other plants such as onion, sweet corn, carrot and clover. Also, some isolates were capable of colonising and developing in the rhizosphere of several plants compared to others which were more host specific. These results indicated, therefore, that rhizosphere colonisation by *Trichoderma* isolates is plant host specific. However, it should be noted that this was for a single time point and may not reflect colonisation over time.

In this study some isolates were more rhizosphere competent than others for one plant species. For example, *T. atroviride* LU132, *T. harzianum* LU151 and *T. harzianum* LU673 were better at colonising sweet corn roots compared to *T. virens* LU556, *T. harzianum* LU672 and *T. crassum* LU555. These results are in agreement with the previous chapter of this thesis and the studies of (Sivan and Harman 1991) and (Ahmad and Baker 1987a). Sivan and Harman (1991) tested the ability of three *Trichoderma harzianum* isolates to colonise the roots of maize and cotton in unsterilized field soil. Seven days post planting for maize and 8 days post planting for cotton, they found that the three *T. harzianum* isolates, T12, T95 and T22 were better colonisers of maize rhizosphere and rhizoplane compared to cotton as they could recover more CFU/g DRS for each of the isolates from the rhizosphere of maize and up to 6 cm depth. Moreover, the authors found that for maize alone and at a depth of 3 cm to 6 cm, CFU/g DRS for isolates T22 and T95 were significantly higher than those of T12, indicating that T22 and T95 were better than T12 at colonising deeper roots of maize. They also found that for cotton, isolates T95 and T22 could colonise the rhizosphere of the first 4 cm but were unable to colonise the plant below 5 cm.

The present study agrees with these findings in that isolates will behave differently according to the plant species and their inherent ability to colonise roots. However, it would be interesting to know, as per (Sivan and Harman 1991), to what depths isolates can colonise roots of each plant species. Perhaps for plant species with pivot roots, *Trichoderma* isolates can grow vertically alongside the root penetrating deeper in the soil, whereas for plant species forming early branching roots, *Trichoderma* isolates follow new roots spreading more horizontally rather than vertically suggesting that root structure could be playing a role in *Trichoderma* colonisation.

Similar work, showing differences among the root colonisation of plant species by *Trichoderma* isolates, was done by Ahmad and Baker (1987a). In unsterilized soil, they compared the ability of four *Trichoderma* isolates belonging to the species *T. koningii*, *T. viride* and *T. harzianum* (n = 2), to colonise the roots of cucumber, bean, tomato and maize plants. Eight days post planting, they found that *T. harzianum* T95 could colonise the upper and middle parts of maize rhizosphere soil better than the same parts in cucumber plants. They also found that *T. harzianum* T95 colonized bean and tomato roots similarly. As the main focus of their study was isolate *T. harzianum* T95, the results for the other three isolates on all four plant species were not presented. Nevertheless, the results did show that isolate T-8 was isolated more frequently from the upper parts of cucumber roots (until 3 cm depth) than isolate T-S-1 which was, in turn, better than T12. These results demonstrated the propensity for isolate level variation amongst *Trichoderma harzianum* as has been shown here.

Isolate variation in root colonisation is not unique to *Trichoderma* and has been shown for other soil borne fungi such as the biocontrol agent *Pythium oligandrum*. Al-Rawahi and Hancock (1997) studied rhizosphere competence of *P. oligandrum* on 11 plant species representing seven different plant families and found that not only the plant family, but also the plant cultivar were different in their receptivity to the fungus.

Root exudates may explain the differences found in root colonisation patterns across the isolates in this study. *Trichoderma* isolates are genetically different from each other and as saprophytes can degrade different carbon sources. *Trichoderma* strains are known to secrete several enzymes and the ability to produce these enzymes is variable and inherent to each strain. For example, Ahmad and Baker (1987b) measured the production of cellulase for six different *Trichoderma* isolates (species *T. harzianum*, *T. koningii* and *T. viride*) in a broth with different sources of carbon. They found that T-12 could not produce cellulase in the presence of carboxymethyl cellulose whereas T-95 had produced high amounts. The authors found that the most rhizosphere competent isolates were those that had the highest production of cellulase.

Cumagun et al. (2009) reported that the cellulose adequacy indices (CAI) of the 41 isolates tested, varied between 0.091 and 0.921 showing 10 fold differences in the decomposition of the straw's cellulose. The authors linked the CAI with the saprophytic ability of *Trichoderma* strains and showed that strains with higher CAI colonised and degraded more straw. These results indicated that *Trichoderma* isolates are different in their ability to metabolize different chains of carbon making them different in their ability to survive in the rhizosphere soil of different plant species. However plants, by modifying the secretion of root exudates and by naturally producing different compounds intra and inter specifically can also select microbial communities in the rhizosphere which may further affect *Trichoderma* populations.

There is some literature on similar studies with *Trichoderma* isolates on the plant hosts used in this chapter. Celar (2002) studied the influence of several plant root exudates on four *Trichoderma* isolates: *T. longibrachiatum*, *T. harzianum*, *T. viride* and *T. koningii* by collecting the root exudates after growing the plants in sterile distilled water for 10 to 20 days and using this liquid to make growing media to which the isolates were added in the form of an agar plug. The results of Celar's (2002) study showed that onion root exudates inhibited *T. longibrachiatum* and *T. harzianum* and those of cabbage inhibited *T. longibrachiatum* and *T. harzianum* as well as *T. viride*. Maize root exudates stimulated *T. longibrachiatum* and *T. harzianum*. None of the root exudates had an effect on isolate *T. koningii*. These findings could be applied to the present study and explain for example the preference of *T. harzianum* LU672 for onion roots. It would have been interesting to assess the production of root exudates for each of the plant species in this study and relate it to the colonisation by different *Trichoderma* isolates however collecting the root exudates would have represented a significant challenge (Neumann et al. 2009). In the rhizosphere, not only exudates but also other microorganisms that are attracted to those same compounds exuded by the plant will influence how a *Trichoderma* isolate colonises roots.

In this study, because plants were harvested at different times, they may have been at different vegetative or phenological stages, influencing the *Trichoderma* populations found in the rhizosphere. For example, Gransee and Wittenmayer (2000) found that younger maize plants exuded higher amounts of organic substances per g root dry weight matter than older ones. They also found that during plant development the amounts of exuded sugars decreased. Similarly, Groleau-Renaud et al. (1998) studied the root exudation of maize plants in axenic conditions at 4, 8, 12 and 16 days and found that after a peak at 8 days, exudation decreased regularly from 8 to 16 days. Butler et al. (2004) used ^{13}C pulse-chase labelling to assess the temporal flow of photosynthetically fixed ^{13}C into the rhizosphere soil microbial biomass of ryegrass plants. They found that the flow of ^{13}C through microbial biomass was faster in rhizosphere soil than in bulk soil and that it was also faster at the first stage of growth development during the transition between active root growth and rapid shoot growth.

Another reason why *Trichoderma* isolates might have increased in the rhizosphere of some plants more than others is root morphology and architecture. For example, ryegrass plants were more receptive to *Trichoderma* isolates overall than sweet corn. When ryegrass roots were harvested, 23 days after planting, they had many lateral roots as well as areas where new root tips were branching to form more secondary roots. These would have formed a larger surface area to support a higher number of microbial populations, including *Trichoderma*. In contrast, 7 day old sweet corn roots' structures were formed by a single tap root with a few secondary roots branching from the top part of the pivot root. Groleau-Renaud et al. (1998) studied the effect of root morphology on the release of exudates by 4 to 16 day old maize seedlings grown in axenic conditions. The authors found that the quantity of carbon exudates increased with shoot and root biomass and root surface area. They showed that root exudation was correlated with morphological parameters and more especially with the number of roots compared to dry matter or root surface. Therefore, as more roots were releasing exudates, more *Trichoderma* would have been able to live off the multiple sources of exudates along the roots of ryegrass.

Clover was harvested 28 days post planting and had more secondary roots than sweet corn. In this study, ryegrass was however more receptive to *Trichoderma* species than clover despite of age and structure of roots. According to Skipp and Christensen (1989) who conducted large studies to characterize the internal micro flora colonising roots of the main pasture plants in New Zealand, fungi colonising white clover and ryegrass are host specific. When Skipp and Christensen (1989) studied the presence of Deuteromycetes in segments of surface sterilised ryegrass roots, they found that out of 18 species, *Trichoderma* spp. were the fifth most frequent colonisers at 3.1% of all sites included. In that study they could also isolate *Trichoderma* species from both young and old ryegrass roots. However, when similar studies were conducted with white clover roots, *Trichoderma* species were rarely isolated from 14 day old seedlings inoculated with a mixture of fungi (including *Trichoderma* species) that had previously been isolated from clover (Skipp and Christensen 1982 a) and were not isolated at all from 10 to 30 day old clover roots (Skipp and Christensen 1981).

3.4.2 Growth promotion

Seedling emergence differed to the untreated control methyl cellulose coated seed for some of the plant isolate combinations, indicating that some isolates could improve seedling emergence for some plant species and also improve growth. Some isolates also increased or decreased shoot and root lengths and shoot and root dry weights in some plant species. These indicated that isolates are different in their ability to promote growth in different plant species and agrees with the literature.

For example Clouston et al. (2010) screened 62 *Trichoderma* isolates for their potential to promote growth in the species *Impatiens walleriana*. They found that LU556 (IT160) increased root length and root dry weight compared to the control, but isolate IT167 was better at increasing shoot dry weight. LU132 was also included in their study but did not show any significant growth effect on impatiens cuttings. In the present study, LU555 and LU556 which were the least rhizosphere competent isolates on sweet corn increased seedling emergence. In contrast, Hohmann et al. (2011) showed that two Trichoderma isolates with different root colonisation behaviour on *Pinus radiata* had different abilities to promote growth. They showed that the most rhizosphere competent isolate LU592 could reduce seed mortality by up to 29%, promoted shoot height up to 16% and increased dry root weights by 31%. LU132, the least rhizosphere competent one did not show any growth promotion.

Interestingly, in the present study, sweet corn seedlings' emergence was improved by the application of isolates LU555 and LU556. In chapter two, none of the isolates, including LU555 and LU556 had measurable plant growth effects. The plant growing conditions may have explained the differences in growth promotion between the two experiments. In chapter two, each sweet corn seedling was grown alone in a 50 mL plastic tube, whereas in this study up to ten seedlings were grown together in a 500 mL pot. Plants may have been stressed by other plants growing in these conditions. Plants can produce allelochemicals in the exudates that can either be beneficial or detrimental to the neighbouring plants (Bertin et al. 2003). *Trichoderma* isolates have been shown to have a positive effect when plants are stressed (Mastouri et al. 2010), therefore, the growth promotion found in this experiment would have to be confirmed in an experiment where stress conditions could be ruled out.

In the previous chapter, it was shown that *Trichoderma* isolates have different ability to colonise the rhizosphere of sweet corn plants. The aim of this chapter was to assess the rhizosphere competence ability of *Trichoderma* isolates on six different plant species. The colonisation of sweet corn was similar in both chapters, which reinforces the robustness of the assays and sweet corn as a model plant for the study, however isolates colonised other plant hosts differently. This indicated that rhizosphere competence is plant host specific at least for the isolates tested in this study. The rhizosphere competence assessment was performed with the entire root system and at different harvest times for each of to the plant species. As plants select microorganisms by exuding carbon compounds, it would be interesting to know whether rhizosphere competent *Trichoderma* isolates interact with other microbial populations selected by the plant roots.

4

Influence of selected abiotic factors on the colonisation of sweet corn and ryegrass roots by isolate *Trichoderma atroviride* LU132

4.1 Introduction

The use of *Trichoderma* strains as biological control agents for several crops and many pathogens is widespread around the world. Variability in the control efficacy has been a major limitation in the further widespread use of commercial *Trichoderma* products (Stewart 2010). Abiotic factors are known to affect the activity of biocontrol agents and have potential to compromise the control of the pathogen.

Various abiotic and soil biotic factors in the soil such as temperature, water potential, pH, the presence of pesticides, metal ions and antagonistic microorganisms influence the efficiency of *Trichoderma* biocontrol activity (Kredics et al. 2003). Soil moisture, pH and temperature are known to be important factors influencing the development, activity and survival of fungi (Meena and Paul 2008). Badham (1991) reported that hyphal extension rates and conidial germination of *Trichoderma harzianum* decreased as water potential decreased from -0.7 to -14 MPa. Eastburn and Butler (1988) suggested that long-term changes in soil moisture can affect both the distribution and density of *Trichoderma* populations in the soil. *Trichoderma* species are also strongly influenced by pH. Jeong et al. (1997) Rhizosphere competence of *T. harzianum* inoculated on cucumber seeds was reported to be higher in soils of pH 5 and 6 compared to soils at pH 7 (Jeong et al. 1997). Mondal et al. (1996) reported that acidic conditions favoured the development of the species *T. koningii* and *T. viride*. Further, *Trichoderma* species are known to absorb and utilise several sources of nitrogen (Danielson and Davey 1973b), but the influence of soil nitrogen concentration on the colonisation of growing plant roots has never been studied despite that combined applications of fertilizer and antagonistic microbes are used in agriculture improve seedling establishment and provide early control of pathogens

Many of the studies investigating the influence of abiotic factors on the bioactivity of *Trichoderma* isolates have been carried out under in vitro conditions, and it is therefore difficult to relate these results to effects in soil and, more importantly, the rhizosphere. Saprophytic ability has been assessed in soil but populations in the rhizosphere were not analysed and usually these studies have investigated the influence of abiotic factors in the presence of a pathogen.

Other work in soil has been done where multiple factors are analysed from soil in the absence of a growing plant. It is likely that abiotic factors will affect the relationship between plant and fungus, particularly at the rhizosphere level, but the magnitude of this effect is unknown.

Two studies from the late 80s and 90s have addressed rhizosphere competence of a few isolates in soil in the presence of growing plants, but only the influence of pH was evaluated in both and temperature in one (Ahmad and Baker 1987a, Lo et al. 1996)

The objectives of this chapter were to investigate the influence of three abiotic factors on the most rhizosphere competent isolate, and to ascertain if the effect was consistent in two plant species, ryegrass and sweet corn.

4.2 Materials and methods

Rhizosphere competence of isolate *Trichoderma atroviride* LU132 was assessed on sweet corn and ryegrass in non-sterile soil under different experimental conditions in six different experiments. Two experiments were carried out per abiotic factor, one experiment and its repeat. The abiotic factors tested were three different soil moisture content levels, three soil pH levels and three soil available nitrogen concentrations.

4.2.1 Trichoderma inoculum production

A conidial suspension of *T. atroviride* LU132 (3 µL of 10^8 conidia/mL) previously derived from a single spore culture and that had been maintained in a 25% glycerol solution at -80°C was inoculated centrally onto eight potato dextrose agar (PDA) plates. The plates were incubated in a closed cupboard under constant blue light (Sylvania F18w/blue) for 8 days to stimulate conidiation. The cupboard was located in a laboratory at room temperature (18 to 23°C). Following conidiation the plates were flooded with 8 mL of sterile distilled water (SDW) and rubbed with a sterile hockey stick to free the conidia from the mycelium. The conidial suspension was filtered through two layers of miracloth (Calbiochem, EMD Biosciences Inc., La Jolla, California, USA) to remove hyphal fragments. The conidial concentration of the suspension was determined using a haemocytometer and adjusted to required concentration using SDW.

4.2.2 Application of Trichoderma isolates to the seeds

Seeds of sweet corn (*Zea mays*) variety Chieftain (Corson Grain Ltd, Gisborne, New Zealand) and seeds of ryegrass variety Impac (Agriseeds) were used.

Prior to inoculation, sweet corn seeds were surface sterilized for 10 min in a 1% v/v sodium hypochlorite (Advance liquid bleach, 5g/100 mL sodium hypochlorite) and 5% ethanol solution and washed three times with SDW. Ryegrass seeds were surface sterilized in a 1% v/v sodium hypochlorite and 5% ethanol solution for 20 min. The seeds were subsequently air-dried in a laminar flow cabinet. For sweet corn, five standard size (9cm diam.) Petri dishes with forty seeds each were prepared.

Each of the Petri dishes received 2 mL of a solution with equal volumes of *T. atroviride* LU132 conidial suspension and 2% methyl cellulose (MC) solution to coat the seeds. Petri dishes were manually shaken for 1 min to distribute the conidia. For ryegrass, forty to fifty seeds were placed in each of five small size (6 cm diameter) Petri dishes. Each Petri dish received 1 mL of a solution with equal volumes of *T. atroviride* LU132 conidial suspensions and 2% MC solution to coat the seeds. Seeds were stirred with a pipette tip to evenly distribute the *Trichoderma* conidia over the surface of the seeds. Seeds were left to dry for 3 hours in the laminar flow cabinet before a second coating with the same volumes was applied. Control seeds were treated the same way and were coated with a solution of equal volumes of SDW and 2% MC solution. After the second coating, seeds were left to dry overnight in a laminar flow cabinet. When dry, seeds from the different Petri dishes were combined in batches for sweet corn treated and untreated and ryegrass treated and untreated. Thirty seeds from the combined treated batches were randomly selected and used to assess the number of spores coated per seed.

4.2.3 The growing system and experimental design

Similar growing systems and experimental designs were used accross the six experiments. The growing system consisted of 10 cm diameter plastic pots (500 mL capacity) filled with 20% (w/w) moistened Wakanui silt loam soil (except for the gravimetric water content (GWC) experiments where pots were filled with soil at different GWC levels), the same soil that was used in previous chapters. Each pot had either 10 seeds coated with *T. atroviride* LU132 or control seeds (Section 2.2.3). Sweet corn seeds were evenly spaced and planted 1 cm deep, and covered with a thin (0.5 cm) layer of soil. Ryegrass seeds were spaced in the same fashion as sweet corn, but they were placed on the soil surface and covered with a thin (0.5 cm) layer of soil. For the experimental design of each of the six experiments, 60 pots were laid out in five randomized blocks. Each block was divided into two subplots, one for ryegrass and the other one for sweet corn. Each subplot had six pots corresponding to the three treatments (abiotic factor and seeds coated with *T. atroviride* LU132) and their controls (abiotic factor with seeds coated with SDW and MC).

4.2.3.1 Soil moisture content experiments

The effect of soil moisture content on rhizosphere competence of *T. atroviride* LU132 was assessed in Wakanui silt loam soil with three different gravimetric water content (GWC) levels: 16, 20 and 24% (w/w). Soil with different GWC was obtained by adding SDW until the desired GWC was reached.

To calculate the amount of water needed, the GWC formula was used in the same way as in Chapter 2 (Section 2.2.4). Unlike for experiment 1, the soil moisture content of pots for experiment 2 was checked daily for the first five days of the experiment and every three to four days the last weeks of the experiment.

The pots were weighed before the set-up of the experiment and reweighed at regular intervals with the pots watered as required to maintain the correct weight and therefore soil moisture content over the experimental period. The experiments were carried out in a growth room. Experimental conditions were as follows: 16 hour day at 18°C and 8 hour night at 20°C. Light intensity was measured with a quantum radiometer (Li 188B, Li-Cor Biosciences, Inc., Lincoln, Nebraska, USA) and averaged 216 µmol photons /m^2/s across the growth room.

4.2.3.2 **Soil pH experiments**

Three pH levels were tested: 5.5, 6.5 and 7.5. For both pH experiments, the Wakanui silt loam soil used was initially at pH 5.5. The pH of the soil was increased to 6.5 and 7.5 by adding sodium carbonate (Na_2CO_3). A soil titration curve was calculated to predict the Na_2CO_3 quantities required to increase the soil pH (Appendix C1). A pilot study was carried out to determine whether the pH of soil modified by the addition of Na_2CO_3 would remain stable for the duration of the experiment (Appendix C2). Soil for each of the experiments was prepared four weeks prior to each set up. After the set up of experiment 1, a sample was sent to Hill Laboratories for analysis (Appendix C3). After filling the pots with moistened soil at different pH levels and sowing the seeds, pots were completely sealed in a plastic bag to maintain the soil moisture content and placed in an incubator. Experimental conditions were the same as in previous experiments: 16 hour day at 18°C and 8 hour night at 20°C. Light intensity in the top half on the incubator was on average 124 µmol photons /m2/s and light intensity in the bottom half of the incubator was 136 µmol photons /m2/s.

4.2.3.3 **Soil available nitrogen experiments**

Three levels of soil available nitogen (AN) were tested: 75, 150 and 300 kg N/ha. For both experiments, the Wakanui silt loam soil used had 75kg N/ha available nitrogen. The soil AN was increased by adding different quantities of ammonium nitrate (NH_4NO_3) to the same weight of soil. Two options were available to increase the concentration of nitrogen in the soil. The first option was to add nitrogen in the form of organic matter, however as nitrogen in this form would have required many months to move into the mineralisable pool, the second option which consisted in the addition of nitrogen fertiliser was preferred. Soil for each of the experiments was prepared the week before each set up. After the set up of experiment 1, a sample was sent to Hill Laboratories for analysis (Appendix C5). After filling the pots with the same amount of moistened soil at the different AN levels, pots were completely sealed in a plastic bag to maintain the soil moisture content and placed in an incubator.

Experimental conditions were the same as in previous experiments: 16 hour day at 18°C and 8 hour night at 20°C. Light intensity in the top half on the incubator was on average 124 µmol photons /m2/s and light intensity in the bottom half of the incubator was 136 µmol photons /m2/s.

4.2.4 Rhizosphere competence assessment

Assessments were done at 7 days after sowing for sweet corn and 21 days after sowing for ryegrass. For each plant species and treatment, 10 seedlings were harvested from the pots (two seedlings per block and plant species). Roots were cut from the crown of each plant, weighed and kept in a 50 mL capacity plastic tube until they were processed.

Tubes were stored at 4˚C in the dark overnight. The following day, 10 mL of a 0.5% v/v Triton X 100 solution was added to each 50 mL plastic tube containing the roots. Tubes were then shaken for 30 min (94 rpm) in an orbital shaker (Ratek EOM5, Ratek Instruments PTY Ltd, Boronia, Australia) to suspend the *Trichoderma* propagules. The suspensions were serially diluted to 10^{-1} and 10^{-2}. Aliquots of the initial suspensions and each dilution (200 µL) were plated onto each of three *Trichoderma* selective medium (TSM) plates. Plates were incubated at 20˚C in the dark. The number of *Trichoderma* colony forming units (CFU) was counted 10 to 13 days after incubation and the number of *Trichoderma* CFU/g of DRS was calculated.

4.2.5 Growth promotion assessment

The number of seedlings which emerged was recorded after 7 and 21 days for sweet corn and ryegrass, respectively. The length of the shoots and roots of the plants used for the rhizosphere competence assessment were measured using a tape measurer after being excised from the seed. Shoots and roots (after being used for rhizosphere assessments) were dried to constant weight in an oven at 65˚C for two days to obtain their dry weight. Roots dry weights of ryegrass plants for the soil moisture content experiments were too small to be recorded. The use of a scale with precision to 1 mg was not enough to detect a difference between root weights.

4.2.6 Endophytic assessment

Two plants per pot and treatment were randomly picked to assess endophytic colonization. The roots and shoots were first washed under tap water. Sweet corn plant material was surface sterilized by immersion for 4 min in a 1% AI sodium hypochlorite solution followed by two 2 min rinses in SDW. Ryegrass plant material was surface sterilized by immersion for 1.5 min in a 1% sodium hypochlorite solution followed by two 2 min rinses in SDW. The entire shoots and roots were then cut into 2 cm segments with a sterile scalpel blade in a laminar flow unit. Shoot and root segments were plated on TSM. Sweet corn shoots and roots were plated onto one Petri plate.

Ryegrass shoots and roots were plated onto 1-2 Petri plates each depending on the length. The multiple plates per root or shoot system were sequentially numbered. The plates were incubated in the dark at 20°C for 5 to 8 days. The presence or absence of colonies on the shoot and root pieces was recorded for all the plates. A plate was recorded as positive for endophytic colonisation if ≥1 *Trichoderma* colony was present.

4.2.7 Statistical analysis of rhizosphere competence

In the 6 experiments, for both plant species, the control seedlings often had zero *Trichoderma* CFUs in the rhizosphere, so these data were summarized as means only and were omitted from the ANOVA.

In addition, for the *T. atroviride* LU132 inoculated seedlings, the sweet corn (log_{10}) data were considerably more variable than the ryegrass data, violating the ANOVA assumption of homogeneity of variance. Therefore two randomized block ANOVAs were carried out for each experiment, one for ryegrass and one for sweet corn. Before the ANOVA, the mean number of CFU per isolate was calculated and log_{10} transformed. The ANOVA analyses were performed in GenStat 15th edition (VSN International, Hertfordshire, UK) with treatment means compared using Fishers unprotected LSD at $P = 0.05$.

4.2.8 Statistical analysis of growth promotion

Dry weights were log transformed (log_{10}) when the distribution was not normal. For each parameter, a mean was calculated with the two plants harvested per pot. Means were then analysed using ANOVA and Genstat 15th edition (VSN International, Hertfordshire, UK) with treatment means compared using Fishers unprotected LSD at $P = 0.05$.

4.3 Results

4.3.1 Soil moisture content experiments

4.3.1.1 Rhizosphere competence

The number of *Trichoderma* CFUs in the rhizosphere of ryegrass and sweet corn plants was assessed for each of the three GWC levels (16%, 20% and 24% w/w) across the duplicate experiments (Tables 4-1 and 4-2).

In experiment 1 (Table 4-1), the *Trichoderma* populations in the control for ryegrass and sweet corn were low (10^2 - 10^3 CFU/g DRS) and much lower than those of *T. atroviride* LU132 inoculated seeds.

For sweet corn, inoculated plants grown at 24% GWC had significantly higher *Trichoderma* populations than plants grown at 16% GWC (3.24×10^4 compared to 2.24×10^5 CFU/g DRS, respectively) with the *Trichoderma* population level of plants grown at 20% GWC not differing significantly from either that at 16 or 24% GWC. For ryegrass, plants grown at 16% GWC did not emerge. The highest *Trichoderma* populations were also found in plants grown at 24% GWC, however these were not different to the populations recovered from plants grown at 20% GWC.

Similarly to experiment 1, in experiment 2 (Table 4-2), control populations for both plant species were lower than those of *Trichoderma* inoculated seeds. For ryegrass seeds grown at 16%, *Trichoderma* rhizosphere colonisation was not included in the analysis as emergence was low. As was seen in experiment 1, the highest *Trichoderma* populations for both plant species was found for plants grown at 24%, however in this second experiment there were no significant differences between *Trichoderma* populations for sweet corn plants grown at 16 and 24%.

Table 4-1 Moisture content experiment 1: Colony forming units CFU (Log_{10})/g dry rhizosphere soil (DRS) counts for sweet corn and ryegrass grown in non-sterile soil at three different Gravimetric Water Content levels (16, 20 and 24% w/w) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Log_{10} values followed by the same letter in a consecutive series (column) are not significantly different.

	Sweet corn		**Ryegrass**	
	CFU/ g DRS (log_{10})		CFU/ g DRS (log_{10})	
Gravimetric Water Content	Control	LU132	Control	LU132
16%	1.36×10^3	3.24×10^4	#	#
	(3.133)*	(4.51) b	#	#
20%	6.35×10^2	1.05×10^5	1.75×10^3	6.32×10^5
	(2.803)*	(5.02) ab	(3.243)*	(5.801) a
24%	1.03×10^3	2.24×10^5	3.94×10^3	7.08×10^5
	(3.014)*	(5.35) a	(3.596)*	(5.85) a
LSD (5%)	-	0.703	-	0.337

* data for untreated controls not included in the analysis, # no plant emergence

Table 4-2 Moisture content experiment 2: Colony forming units CFU (Log_{10})/g DRS counts for sweet corn and ryegrass grown in non-sterile soil at three different Gravimetric Water Content levels (16, 20 and 24% w/w) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132 in experiment 2. Log_{10} values followed by the same letter in a consecutive series (column) are not significantly different.

	Sweet corn		Ryegrass	
	CFU/g DRS (log_{10})		CFU/g DRS (log_{10})	
Gravimetric Water Content	Control	LU132	Control	LU132
16%	8.61×10^2	2.29×10^5	#	#
	(2.935)*	(5.36) a	#	#
20%	4.37×10^2	3.52×10^5	8.91×10^2	5.37×10^5
	(2.64)*	(5.546) a	(2.95)*	(5.73) a
24%	1.33×10^2	2.56×10^5	9.77×10^2	4.79×10^5
	(2.124)*	(5.409) a	(2.99)*	(5.68) a
LSD (5%)	-	0.388	-	0.299

* data for untreated controls not included in the analysis; # not assessed due to low plant emergence

4.3.1.2 Growth promotion

In experiment 1, treated and untreated sweet corn plants grown at 20 and 24% GWC had higher emergence than all plants grown at 16% (Table 4-3). *Trichoderma atroviride* LU132 treatment had no significant effect on seedling emergence compared with the untreated control at any of the GWC tested. All sweet corn plants grown at 24% GWC had significantly longer shoots compared with those grown at 16%, but no significant differences were found between untreated and *T. atroviride* LU132 treated sweet corn plants at any of the GWC (Table 4-3). Root lengths of sweet corn plants were not significantly different between treatments at any GWC. Shoot dry weights of treated and untreated plants grown at 24% GWC were significantly higher than treated and untreated plants grown at 16% GWC, but no differences were detected between treatments at any GWC (Table 4-3). Root dry weights of sweet corn plants treated with *T. atroviride* LU132 were twice those of the untreated controls at 16% GWC (Table 4-3). Similar results were observed in experiment 2 (Table 4-4) with regards to seedling emergence, with their being no significant difference in shoot or root length between *T. atroviride* LU132 and control treatments at each GWC. At 24% GWC, shoot and root dry weights of sweet corn plants treated with *T. atroviride* LU132 were respectively 1.4 and 1.7 times greater than the controls. The same differences as in experiment 1 were found in experiment 2 for shoot dry weights of treated plants.

In experiment 1, ryegrass plants did not emerge at 16% GWC (Table 4-3). No significant differences between treatments were detected either for any of the parameters analysed, however, for *T. atroviride* LU132 treatments, ryegrass plants at 24% GWC had taller shoots than those grown at 20% GWC (Table 4-3). In experiment 2, field emergence of ryegrass treated and untreated plants was significantly higher at 20 and 24% GWC compared to 16% GWC. In general ryegrass plants grown at 20% GWC had higher growth parameters excluding seelding emergence than plants grown at 16 and 24% GWC but no differences were detected between treated and untreated plants. For *T. atroviride* LU132 treated ryegrass plants however, root lengths and shoot dry weights were significantly higher at 20% GWC compared to 24%. No root dry weights were carried out for ryegrass plants due to the small size of the plants.

Table 4-3 Moisture content experiment 1: growth parameters including seedling emergence, shoot and root length and shoot and root dry weight measured for sweet corn and ryegrass seedlings grown in non-sterile soil at three different Gravimetric Water Content levels (16%, 20% and 24%) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Means for seedling emergence determined for 10 seeds. Values followed by the same letter in a consecutive series (row) are not significantly different.

Sweet corn							
Parameters	GWC 16 %		GWC 20 %		GWC 24 %		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	2.8 b	1.6 b	6.0 a	5.6 a	6.6 a	7.8 a	2.214
Shoot Length (cm)	4.07 c	5.22 bc	7.57 ab	8.36 a	8.35 a	8.30 a	2.462
Root Length (cm)	13.20 b	15.20 ab	16.56 ab	18.6 a	14.95 b	15.75 ab	3.605
Shoot Dry Weight (mg)	7.34 b	9.5 b	13.7 ab	18.8 a	17.8 a	19.0 a	6.689
Root Dry Weight (mg)	5.9 b	12.7 a	11.1 ab	14.7 a	9.7 ab	13.6 a	6.258
Ryegrass							
Parameters	GWC 16 %		GWC 20 %		GWC 24 %		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	#	#	8.0 a	6.6 ab	6.4 b	6.8 ab	1.482
Shoot Length (cm)	#	#	18.01 ab	17.74 b	18.64 ab	19.66 a	1.659
Root Length (cm)	#	#	16.20 a	17.05 a	15.25 a	15.35 a	4.726
Shoot Dry Weight (mg)	#	#	6.1 a	5.7 a	6.9 a	6.2 a	2.309
Root Dry Weight (mg)	#	#	-*	-	-	-	-

no plant emergence for ryegrass at 16% GWC; * roots too small to be recorded

Table 4-4 Moisture content experiment 2: growth parameters including seedling emergence, shoot and root length and shoot and root dry weight measured for sweet corn and ryegrass seedlings grown in non-sterile soil at three different Gravimetric Water Content levels (16%, 20% and 24% w/w) from seeds either uncoated (control) or coated with *T. atroviride* LU132. Means for seedling emergence determined for 10 seeds. Values followed by the same letter in a consecutive series (row) are not significantly different.

Sweet corn							
Parameters	GWC 16 %		GWC 20 %		GWC 24 %		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	4.2 a	4.6 a	5.2 a	4.2 a	3.4 a	4.6 a	2.376
Shoot Length (cm)	7.15 a	6.28 a	6.80 a	7.72 a	6.44 a	7.33 a	1.612
Root Length (cm)	14.45 a	12.55 a	11.8 ab	13.55 a	7.60 b	11.10 ab	4.937
Shoot Dry Weight (mg)	13.3 ab	11.8 b	13.3 ab	16.4 a	11.6 b	16.8 a	4.398
Root Dry Weight (mg)	7.3 ab	5.6 ab	6 ab	7.9 a	4.8 b	8.1 a	2.759
Ryegrass							
Parameters	GWC 16 %		GWC 20 %		GWC 24 %		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	1.8 c	1.7 c	4.0 b	4.0 b	6.0 ab	6.2 a	2.045
Shoot Length (cm)	16.90 a	13.66 a	17.70 a	19.00 a	17.85 a	17.05 a	5.452
Root Length (cm)	12.90 ab	12.49 ab	13.42 ab	15.80 a	12.40 ab	11.40 b	4.209
Shoot Dry Weight (mg)	#	#	6.2 ab	8.2 a	7.3 ab	5.6 b	2.221
Root Dry Weight (mg)	#	#	-*	-	-	-	-

no shoot dry weight due to low plant weights for ryegrass at 16% GWC; * roots too small to be recorded

4.3.1.3 Endophytic colonisation

In experiment 1, a total of 44 sweet corn plants were assessed for endophytic *Trichoderma* colonisation (Table 4-5). *Trichoderma* colonies were not recovered from untreated plants except on one occasion. *Trichoderma* colonies were equally recovered from shoots and roots of treated plants regardless of the GWC, although only one plant was assessed for 16% GWC. *Trichoderma* was isolated from the base of the shoots (in the first 0.5 cm attached to the root) and the first 2 cm of the roots. For two roots only, all the pieces contained *Trichoderma* species. Endophytic fungi with morphological characteristics of the genera *Fusarium* and *Verticillium* were observed (Dr Kirstin McLean) in treated shoots and roots. Forty ryegrass plants were assessed for endophytic colonisation in experiment 1 (Table 4-5). *Trichoderma* colonies were equally recovered from shoots and roots irrespective of the GWC. For 14 (out of 18) ryegrass shoots and all (20) roots, all pieces assessed contained endophytic *Trichoderma* colonies.

In other ryegrass shoots and roots, *Trichoderma* colonies were isolated from the base of the shoot and sparsely from roots but from no specific region. In experiment 2, no *Trichoderma* colonies were isolated from the control plants. *Trichoderma* colonies were more often isolated from sweet corn shoots compared to roots (Table 4-6). *Trichoderma* colonies were also equally isolated from plants grown at 16% GWC.

Other fungal species than *Trichoderma* were isolated from shoots and roots in higher numbers than for experiment 1. In the case of ryegrass plants, fewer plants were endophytically colonised at 24% GWC compared to the first experiment with *Trichoderma* colonies isolated from 50% of shoots and 20% of roots (Table 4-6). Ryegrass shoots and roots pieces were not all colonised by *Trichoderma* species as in experiment 1. *Trichoderma* colonies were isolated from the base of shoots and the top 3 cm of roots.

Table 4-5 Soil moisture experiment 1: Isolation of *Trichoderma* from shoot and root pieces (2 cm pieces) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or untreated assessed 7 or 21 days, respectively after sowing. Plants were grown in non-sterile soil at three Gravimetric Water Content levels: 16, 20 and 24% w/w. Percentage colonisation indicated in brackets.

Gravimetric Water Content	Number of plants assessed		Number of plants positive for *Trichoderma*			
			Control		LU132	
Sweet corn	Control	LU132	Shoot	Root	Shoot	Root
16%	3*	1*	0	0	1 (100)	1 (100)
20%	10	10	0	1	9 (90)	7 (70)
24%	10	10	0	0	8 (80)	8 (80)
Ryegrass	Control	LU132	Shoot	Root	Shoot	Root
20%	10	10	0	0	8 (80)	10 (100)
24%	10	10	0	0	10 (100)	10 (100)

* Low emergence

Table 4-6 Soil moisture experiment 2: Isolation of *Trichoderma* from shoot and root pieces (2 cm pieces) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or untreated assessed 7 or 21 days, respectively after sowing. Plants were grown in non-sterile soil at three Gravimetric Water Content levels: 16, 20 and 24% w/w. Percentage colonisation indicated in brackets.

Gravimetric Water Content	Number of plants assessed		Number of plants positive for *Trichoderma*			
			Control		LU132	
Sweet corn	Control	LU132	Shoot	Root	Shoot	Root
16%	8	9	0	0	8 (89)	4 (44)
20%	8	8	0	0	8 (100)	6 (75)
24%	5	8	0	0	8 (100)	5 (63)
Ryegrass	Control	LU132	Shoot	Root	Shoot	Root
16%	1	1	0	0	0	1 (100)
20%	5	7	0	0	6 (86)	5 (71)
24%	10	10	0	0	5 (50)	2 (20)

4.3.2 Soil pH experiments

The soil pH of each of the three soils for each experiment was measured in the Lincoln University laboratory with a pH-meter before set up and had all achieved the desired pH values (5.5, 6.5 and 7.5). As a control, for the first experiment, a sample of each of the three soils were sent for analysis to Hill Laboratories (Appendix C3). As the results from both measurements differed greatly, the soil pH for the set up of experiment 2 was only measured in the laboratory. The pH was also measured at the end of the experiments (harvest time) by collecting bulk soil from each treatment pot (Appendix C4).

4.3.2.1 Rhizosphere competence

The number of *Trichoderma* CFUs in the rhizosphere of ryegrass and sweet corn plants was assessed for each of the three soil pH levels (5.5, 6.5 and 7.5) across duplicate experiments (Tables 4-7 and 4-8). *Trichoderma* CFU for control treatments and for both plant species were extremely low (10^1 – 10^2 CFU/g DRS) in both experiments and were not included in the analysis. In both experiments, results showed that *Trichoderma* CFU in the rhizosphere of sweet corn and ryegrass plants did not differ between soils at different pH levels (Tables 4-7 and 4-8).

Table 4-7 pH experiment 1: CFU (Log_{10})/g DRS counts for sweet corn and ryegrass grown in soil at three different pH levels (5.5, 6.5 and 7.5) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Log_{10} values followed by the same letter in a consecutive series (column) are not significantly different.

	Sweet corn CFU/ g DRS (log_{10})		**Ryegrass** CFU/ g DRS (log_{10})	
pH	Control	LU132	Control	LU132
5.5	6.03×10^2	4.17×10^4	5.36×10^1	7.26×10^5
	(2.78)*	(4.62) a	(1.729)*	(5.861) a
6.5	6.92×10^2	7.24×10^4	5.31×10^1	5.89×10^5
	(2.84)*	(4.86) a	(1.725)	(5.77) a
7.5	7.59×10^1	1.86×10^5	1.22×10^2	1.22×10^6
	(1.88)*	(5.27) a	(2.085)	(6.087) a
LSD (5%)	-	0.902	-	0.346

* data for untreated controls not included in the analysis

Table 4-8 pH experiment 2: CFU (Log_{10})/g DRS counts for sweet corn and ryegrass grown in soil at three different pH levels (5.5, 6.5 and 7.5) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Log_{10} values followed by the same letter in a consecutive series (column) are not significantly different.

	Sweet corn CFU/ g DRS (log_{10})		**Ryegrass** CFU/ g DRS (log_{10})	
pH	Control	LU132	Control	LU132
5.5	1.07×10^2	1.36×10^5	1.07×10^2	1.13×10^6
	(2.03)*	(5.133) a	(2.03*)	(6.052) a
6.5	1.23×10^2	2.60×10^5	1.07×10^2	1.47×10^6
	(2.09)*	(5.415) a	(2.03)	(6.167) a
7.5	1.07×10^2	3.50×10^5	1.07×10^2	1.22×10^6
	(2.03)*	(5.544) a	(2.03)	(6.087) a
LSD (5%)	-	1.101	-	0.382

* data for untreated controls not included in the analysis

4.3.2.2 **Growth promotion**

In experiment 1, sweet corn plants inoculated with *T. atroviride* LU132 had higher emergence compared to untreated controls at pH 5.5 and 6.5 (Table 4-9). At pH 5.5, control plants had significantly longer shoots than *T. atroviride* LU132 treated sweet corn plants (Table 4-9).

In general, growth parameters of untreated and *T. atroviride* LU132 treated sweet corn plants were higher at pH 5.5 compared to pH 7.5, although not significantly different for shoot length and dry weight.

For ryegrass plants, no significant differences were detected between treatments at any of the soil pH tested (Table 4-9). Treated and untreated plants grown at pH 5.5 and 6.5 had longer shoots than those grown at pH 7.5. In general plants grown at pH 5.5 and 6.5 had higher values for the growth parameters assessed compared to the plants grown at pH 7.5 although no significant differences were detected.

In experiment 2, sweet corn plants inoculated with *T. atroviride* LU132 had higher emergence compared to the untreated control at pH 7.5 (Table 4-10). At pH 6.5 control plants had longer shoot length and greater root dry weight compared with *T. atroviride* LU132 treated plants. At pH 6.5, untreated sweet corn plants had longer shoots and heavier roots than *T. atroviride* LU132 treated plants. Untreated and *T. atroviride* LU132 treated plants at pH 5.5 as well as untreated plants grown at pH 6.5 had significantly longer and heavier shoots and roots than untreated and *T. atroviride* LU132 treated plants grown at pH 7.5 (Table 4-10).

For ryegrass plants in experiment 2, *T. atroviride* LU132 treated plants grown at pH 7.5 had longer roots than the untreated controls. No other differences between treatments were detected. In general, plants grown at pH 5.5 and 6.5 had higher values for the growth parameters assessed compared to the plants grown at pH 7.5, although these were not all significantly different (Table 4-10).

Table 4-9 pH experiment 1: growth parameters including seedling emergence, shoot and root length and shoot and root dry weight measured for sweet corn and ryegrass seedlings grown in non-sterile soil at three different pH levels (5.5, 6.5 and 7.5) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Values followed by the same letter in a consecutive series (row) are not significantly different.

Sweet corn							
Parameters	pH 5.5		pH 6.5		pH 7.5		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	4.4 bc	6.6 a	3.4 c	5.8 ab	4.6 bc	5.2 bc	1.739
Shoot Length (cm)	6.43 a	4.76 b	4.39 b	5.25 ab	4.08 b	4.29 b	1.524
Root Length (cm)	14.40 a	13.40 a	11.40 ab	13.20 ab	8.20 c	10.25bc	3.031
Shoot Dry Weight (mg)	14.1 a	10.4 ab	8.1 b	10.7 ab	9.9 ab	8.0 b	4.633
Root Dry Weight (mg)	8.2 a	6.9 ab	4.9 bc	5.6 abc	3.8 bc	3.6 c	3.173
Ryegrass							
Parameters	pH 5.5		pH 6.5		pH 7.5		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	7.4 a	6.4 a	7.4 a	7.4 a	6.8 a	5.8 a	2.167
Shoot Length (cm)	18.00 a	16.35 a	17.41 a	16.50 a	10.30 b	9.08 b	2.455
Root Length (cm)	13.35 ab	12.85 ab	14.41 a	11.9 abc	11.7 bc	9.55 c	2.611
Shoot Dry Weight (mg)	7.1 a	5.3 ab	7.4 a	5.7 ab	4.8 b	3.8 b	2.212
Emergence /10 seeds	2.8 a	2.4 a	2.1 a	2.4 a	3.0 a	2.8 a	1.203

Table 4-10 pH experiment 2: growth parameters including seedling emergence (SE), shoot and root length and shoot and root dry weight (DW) measured for sweet corn and ryegrass seedlings grown in non-sterile soil at three different pH levels (5.5, 6.5 and 7.5) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Values followed by the same letter in a consecutive series (row) are not significantly different.

Sweet corn							
Parameters	pH 5.5		pH 6.5		pH 7.5		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	4.6 a	5.8 a	6.0 a	4.4 a	2.0 b	4.8 a	2.13
Shoot Length (cm)	5.44 ab	5.53 ab	6.86 a	4.31 bc	2.97 c	3.46 c	1.816
Root Length (cm)	12.85 ab	13.85 a	15.05 a	12.15 abc	9.25 bc	8.48 c	3.719
Shoot Dry Weight (mg)	12.2 ab	13.4 a	13.6 a	8.9 abc	5.0 c	8.0 bc	5.251
Root Dry Weight (mg)	8.0 ab	8.4 ab	8.9 a	5.3 bc	2.8 c	3.3 c	3.574
Ryegrass							
Parameters	pH 5.5		pH 6.5		pH 7.5		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	7.4 a	6.2 ab	6.8 ab	4.6 b	5.2 ab	6.8 ab	2.49
Shoot Length (cm)	16.1 a	15.6 a	17.1 a	16.4 a	11.4 b	11.0 b	2.114
Root Length (cm)	11.7 a	12.7 a	11.0 a	12.1 a	6.5 b	10.9 a	3.771
Shoot Dry Weight (mg)	4.5 b	4.6 b	6.6 a	6.8 a	3.9 b	3.4 b	1.976
Root Dry Weight (mg)	2.0 a	2.5 a	2.2 a	2.9 a	2.4 a	2.7 a	1.301

4.3.2.3 Endophytic colonisation

In experiment 1, *Trichoderma* colonies were recovered from shoots and roots of treated sweet corn plants regardless of the soil pH level (Table 4-11). Control plants did not have *Trichoderma* colonies. *Trichoderma* colonies were recovered from the shoot base and sporadically across root lengths of sweet corn plants. Unlike for sweet corn, fewer *Trichoderma* colonies were recovered from ryegrass shoots compared with ryegrass roots regardless of the pH level. *Trichoderma* colonies were isolated from all root segments in 12 out of the 29 *T. atroviride* LU132 treated plants that were assessed. A large proportion of untreated shoots but mainly roots of ryegrass plants had *Fusarium species* colonies growing endophytically. *T. atroviride* LU132 treated ryegrass plants had *Fusarium* and *Trichoderma* species growing endophytically. Similar results were found in experiment 2. *Trichoderma* colonies were recovered from shoots and roots of treated sweet corn and ryegrass plants regardless of the soil pH level. Control plants for both species did not have *Trichoderma* colonies however a large part of the plant material was endophytically colonised by *Fusarium* species.

Fewer shoots and roots of sweet corn plants were endophytically colonised by *Trichoderma* species compared to experiment 1. Ryegrass shoots in experiment 2 had more *Trichoderma* colonies than in experiment 1.

Table 4-11 pH experiment 1: Isolation of *Trichoderma* from shoot and root pieces (2 cm) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or untreated assessed 7 or 21 days respectively, after sowing. Plants were grown in non-sterile soil at three soil pH levels: 5.5, 6.5 and 7.5. Percentages colonisation indicated in brackets.

pH	Number of plants assessed		Number of plants positive for *Trichoderma*			
			Control		LU132	
Sweet corn	Control	LU132	Shoot	Root	Shoot	Root
5.5	9	9	0	0	7 (78)	5 (56)
6.5	6	10	0	0	8 (80)	6 (60)
7.5	10	7	0	0	6 (86)	6 (86)
Ryegrass	Control	LU132	Shoot	Root	Shoot	Root
5.5	10	9	0	2 (20)	3 (33)	9 (100)
6.5	8	10	0	0	1 (10)	8 (80)
7.5	10	10	0	0	4 (40)	10 (100)

Table 4-12 pH experiment 2: Isolation of *Trichoderma* from shoot and root pieces (2 cm) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or untreated assessed 7 or 21 days respectively, after sowing. Plants were grown in non-sterile soil at three soil pH levels: 5.5, 6.5 and 7.5. Percentage colonisation indicated in brackets.

pH	Number of plants assessed		Number of plants positive for *Trichoderma*			
			Control		LU132	
Sweet corn	Control	LU132	Shoot	Root	Shoot	Root
5.5	8	8	0	0	4 (50)	3 (38)
6.5	10	9	0	0	3 (33)	3 (33)
7.5	2	9	0	0	4 (44)	5 (56)
Ryegrass	Control	LU132	Shoot	Root	Shoot	Root
5.5	10	9	0	0	7 (78)	8 (89)
6.5	9	8	0	0	6 (75)	6 (75)
7.5	9	10	0	0	8 (80)	9 (90)

4.3.3 Soil nitrogen experiments

4.3.3.1 Rhizosphere competence

The number of *Trichoderma* CFU in the rhizosphere of ryegrass and sweet corn plants was assessed for each of three soil available nitrogen (AN) levels (75, 150 and 300 kg N/ha) across duplicate experiments (Tables 4-13 and 4-14). In experiment 1 (Table 4-13), the *Trichoderma* populations for the untreated ryegrass and sweet corn controls were low (10^2 – 10^3 CFU/g DRS) and significantly lower than those of *T. atroviride* LU132 inoculated seeds. *Trichoderma* populations recovered from sweet corn treated plants grown at different AN levels varied between 1.27 x 10^5 CFU/g DRS (300 kg N/ha) and 1.65 x 10^5 CFU/g DRS (75 kg N/ha) and were not significantly different to each other. For ryegrass *T. atroviride* LU132 treated plants, *Trichoderma* populations isolated from plants grown at 150 and 300 kg N/ha (1.22 x 10^6 and 1.42 x 10^6 CFU/g DRS, respectively) were significantly higher than those grown at 75 kg N/ha (5.12 x 10^5 CFU/g DRS). Results for sweet corn were similar in experiment 2 (Table 4-14). However, for ryegrass, no significant differences were found in *Trichoderma* rhizosphere colonisation between plants grown at different AN levels.

Table 4-13 Nitrogen experiment 1: CFU (Log_{10})/g dry rhizosphere soil (DRS) counts for sweet corn and ryegrass grown in soil at three different nitrogen concentrations (75, 150 and 300 kg N/ha) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Log_{10} values followed by the same letter in a consecutive series (column) are not significantly different.

	Sweet corn CFU/g DRS (log_{10})		**Ryegrass** CFU/g DRS (log_{10})	
Nitrogen	Control	LU132	Control	LU132
75 kg/ha	9.77×10^2	1.65×10^5	2.06×10^3	5.12×10^5
	(2.99)*	(5.218) a	(3.313)*	(5.709) b
150 kg/ha	9.64×10^2	1.41×10^5	3.61×10^3	1.22×10^6
	(2.984)*	(5.149) a	(3.557)*	(6.087) a
300 kg/ha	7.06×10^2	1.27×10^5	2.79×10^3	1.42×10^6
	(2.849)*	(5.103) a	(3.446)*	(6.152) a
LSD (5%)	-	0.764	-	0.325

* data for untreated controls not included in the analysis

Table 4-14 Nitrogen experiment 2: CFU (Log_{10})/g dry rhizosphere soil (DRS) counts for sweet corn and ryegrass grown in soil at three different nitrogen concentrations (75, 150 and 300 kg N/ha) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Log_{10} values followed by the same letter in a consecutive series (column) are not significantly different.

	Sweet corn CFU/g DRS (log_{10})		**Ryegrass** CFU/g DRS (log_{10})	
Nitrogen	Control	LU132	Control	LU132
75 kg/ha	3.18×10^2	3.36×10^5	6.41×10^1	2.32×10^6
	(2.502)*	(5.526) a	(1.81)*	(6.366) a
150 kg/ha	3.18×10^2	4.26×10^5	2.88×10^1	1.75×10^6
	(2.502)*	(5.629) a	(1.46)*	(6.243) a
300 kg/ha	7.82×10^2	2.11×10^5	2.88×10^1	2.97×10^6
	(2.893)*	(5.325) a	(1.46)*	(6.473) a
LSD (5%)	-	0.803	-	0.559

* data for untreated controls not included in the analysis

4.3.3.2 Growth promotion

In experiment 1, there were no significant differences between the untreated control and *T. atroviride* LU132 treated plants at any of the available nitrogen (AN) concentrations tested (Table 4-15). Similar results were found for ryegrass plants. Untreated sweet corn plants grown at 300 kg N/ha AN concentration had higher emergence than untreated sweet corn plants grown at 75 kg N/ha AN. In general, growth parameters for untreated and *T. atroviride* LU132 treated sweet corn plants were higher in AN concentrations 75 and 150 kg N/ha. At 150 kg N/ha, shoot and root lengths of sweet corn plants treated with *T. atroviride* LU132 were significantly greater than shoot and root lengths at 300 kg N/ha.

Untreated and *T. atroviride* LU132 treated ryegrass plants grew well at all of the AN concentrations tested, with there being no significant difference in any of the growth parameters assessed. In experiment 2 (Table 4-16), shoots of untreated sweet corn plants grown at 150 kg N/ha AN, were significantly longer than those of the *T. atroviride* LU132 treated plants grown in the same conditions. In general, untreated sweet corn plants grown at 75 kg N/ha had significantly higher growth parameters (except for emergence) than untreated plants grown at 300 kg N/ha. Similarly to experiment 1, there were no differences between treatments for ryegrass plants grown at the three AN concentrations, apart for shoots length, where at 300 kg N/ha AN untreated plants had longer shoots than *T. atroviride* LU132treated plants.

Table 4-15 Nitrogen experiment 1: growth parameters including seedling emergence, shoot and root length and shoot and root dry weight measured for sweet corn and ryegrass seedlings grown in non-sterile soil at three different nitrogen concentrations (75, 150 and 300 kg N/ha) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Values followed by the same letter in a consecutive series (row) are not significantly different.

Sweet corn							
Parameters	75 kg N/ha		150 kg N/ha		300 kg N/ha		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	6.4 b	7.8 ab	7.8 ab	8.6 a	8.8 a	8.4 a	1.539
Shoot Length (cm)	7.90 a	7.52 ab	7.50 ab	8.25 a	6.64 ab	5.82 b	1.724
Root Length (cm)	16.55 a	16.35 a	16.15 a	16.05 a	13.75 ab	12.20 b	2.82
Log_{10} Shoot Dry Weight	1.25 a	1.16 a	1.18 a	1.28 a	1.13 a	1.09 a	0.187
Log_{10} Root Dry Weight	1.04 a	1.04 a	0.99 a	1.03 a	0.85 a	0.88 a	0.092
Ryegrass							
Parameters	75 kg N/ha		150 kg N/ha		300 kg N/ha		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	6.2 a	6.0 a	6.4 a	7.0 a	7.4 a	6.8 a	1.931
Shoot Length (cm)	15.80 a	16.80 a	16.41 a	16.30 a	16.75 a	15.05 a	2.367
Root Length (cm)	13.15 a	13.05 a	12.75 a	10.90 a	12.60 a	12.40 a	2.262
Log_{10} Shoot Dry Weight	0.73 a	0.80 a	0.83 a	0.75 a	0.80 a	0.72 a	0.12
Log_{10} Root Dry Weight	0.33 a	0.40 a	0.39 a	0.36 a	0.48 a	0.41 a	0.216

Table 4-16 Nitrogen experiment 2: growth parameters including seedling emergence, shoot and root length and shoot and root dry weight measured for sweet corn and ryegrass seedlings grown in non-sterile soil at three different nitrogen concentrations (75, 150 and 300 kg N/ha) from seeds either uncoated (control) or coated with *Trichoderma atroviride* LU132. Values followed by the same letter in a consecutive series (row) are not significantly different.

Sweet corn							
Parameters	75 kg N/ha		150 kg N/ha		300 kg N/ha		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	8.2 a	7.2 a	7.2 a	8.4 a	7 a	8.4 a	2.035
Shoot Length (cm)	8.15 a	7.3 abc	7.51 ab	6.05 cd	5.45 d	6.4 bcd	1.319
Root Length (cm)	19.4 a	16.9 ab	15.3 bc	13.85 cd	11.75 d	13.35 cd	2.999
Log_{10} Shoot Dry Weight	1.33 a	1.20 ab	1.29 ab	1.16 b	1.15 b	1.27 ab	0.159
Log_{10} Root Dry Weight	1.11 a	1.02 ab	1.00 ab	0.87 ab	0.80 b	1.02 ab	0.246
Ryegrass							
Parameters	75 kg N/ha		150 kg N/ha		300 kg N/ha		
	Control	LU132	Control	LU132	Control	LU132	LSD 5%
Emergence /10 seeds	6 a	6.4 a	7.2 a	5.8 a	6.8 a	5.6 a	2.583
Shoot Length (cm)	16 ab	16 ab	16.35 a	16.8 a	17.05 a	12.95 b	3.275
Root Length (cm)	11.7 a	12.4 a	11.5 a	12.55 a	9.9 a	10.6 a	3.081
Log_{10} Shoot Dry Weight	0.79 a	0.68 a	0.75 a	0.71 a	0.67 a	0.65 a	0.148
Log_{10} Root Dry Weight	0.25 a	0.32 a	0.17 a	0.34 a	0.35 a	0.32 a	0.226

4.3.3.3 Endophytic colonisation

In experiment 1, *Trichoderma* colonies were isolated from shoots and roots of both plant species regardless of the AN concentration in the soil (Table 4-17). In the case of sweet corn, *Trichoderma* colonies were isolated equally from the shoots (from the base and up to 1 cm distance) or roots (upper parts up to 3 cm distance) of plants. For ryegrass plants, *Trichoderma* colonies were more often isolated from roots compared to shoots. Three roots of *T. atroviride* LU132 treated plants across the three AN levels had *Trichoderma* colonies growing out of all the segments analysed. Similar results were observed in experiment 2 (Table 4-18) although *Trichoderma* colonies were more often isolated from shoots and roots of both plants compared to experiment 1. In contrast to experiment 1, *Trichoderma* colonies were equally isolated from shoots and roots of ryegrass plants. In both experiments, untreated and treated ryegrass and sweet corn plants were colonised by other endophytic fungi such as *Fusarium* species. Those species were mostly observed in roots of untreated plants, however they were also seen recovered with *Trichoderma* colonies from *T. atroviride* LU132 treated plants.

Table 4-17 Nitrogen experiment 1: Isolation of *Trichoderma* from shoot and root pieces (2 cm) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or untreated assessed 7 or 21 days, respectively after sowing. Plants were grown in non-sterile soil at three nitrogen concentrations: 75, 150 and 300 kg N/ha. Percentage colonisation indicated in brackets.

Nitrogen concentration	Number of plants assessed		Number of plants positive for *Trichoderma*			
			Control		LU132	
Sweet corn	Control	LU132	Shoot	Root	Shoot	Root
75 kg N/ha	10	10	0	1 (10)	7 (70)	5 (50)
150 kg N/ha	10	10	0	4 (40)	7 (70)	6 (60)
300 kg N/ha	10	10	0	0	6 (60)	3 (30)
Ryegrass	Control	LU132	Shoot	Root	Shoot	Root
75 kg N/ha	10	10	0	0	5 (50)	10 (100)
150 kg N/ha	10	10	0	2 (20)	4 (40)	9 (90)
300 kg N/ha	10	10	0	0	6 (60)	9 (90)

Table 4-18 Nitrogen experiment 2: Isolation of *Trichoderma* from shoot and root pieces (2 cm) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or untreated assessed 7 or 21 days, respectively after sowing. Plants were grown in non-sterile soil at three nitrogen concentrations: 75, 150 and 300 kg N/ha. Percentage colonisation indicated in between brackets.

Nitrogen concentration	Number of plants assessed		Number of plants positive for *Trichoderma*			
			Control		LU132	
Sweet corn	Control	LU132	Shoot	Root	Shoot	Root
75 kg N/ha	10	10	0	0	9 (90)	8 (80)
150 kg N/ha	10	10	0	0	8 (80)	6 (60)
300 kg N/ha	10	10	0	0	6 (60)	5 (50)
Ryegrass	Control	LU132	Shoot	Root	Shoot	Root
75 kg N/ha	9	10	0	0	10 (100)	10 (100)
150 kg N/ha	10	10	1 (10)	2 (20)	10 (100)	10 (100)
300 kg N/ha	10	7	0	2 (20)	7 (70)	7 (100)

4.4 Discussion

4.4.1 Influence of soil moisture content on rhizosphere competence of *Trichoderma atroviride* LU132

The results showed that *T. atroviride* LU132 was rhizosphere competent on sweet corn and ryegrass regardless of the soil moisture content level. There were differences in rhizosphere competence observed in experiment 1, where *Trichoderma* populations in sweet corn were higher with increasing soil moisture levels, however, in the first experiment humidity was not well maintained and checked regularly, therefore the soil became dry and affected plant growth in the first week of the experiment. When the gravimetric water contents were measured at harvest times for sweet corn plants in experiment 1, they were 13, 15 and 18% for both untreated and *T. atroviride* LU132 treated plants when they should have been respectively 16, 20 and 24%. When the humidity was maintained at a constant level (experiment 2) there were no differences in rhizosphere competence of *T. atroviride* LU132 on sweet corn at 16, 20 and 24% GWC.

This result is in agreement with (Huang and Erickson 2008) who studied the effects of soil moisture (9, 16, and 24% w/w) on the biological control of *Sclerotinia sclerotiorum* by *T. virens* (LRC 2425) in sterile and unsterile soils. The authors found that *T. virens* reduced the viability of the pathogen's sclerotia in sterile soil at 9 and 24%, however in unsterile soil *T. virens* was ineffective. They concluded that in the presence of other microorganisms soil moisture was not the main factor influencing the bioactivity of *T. virens*. Similarly, Wong et al. (2002) tested the effect of moisture on the survival of *Fusarium pseudograminearum* in buried wheat straw sprayed with the isolate *T. koningii* (BM1) in a red duplex soil (pH 5.2). They found that in wet (-0.03 MPa) and moist (-0.3 MPa) soils the survival of the pathogen was significantly reduced compared to the non-*Trichoderma* control at 25°C. *Trichoderma* populations were not directly assessed by Wong and coworkers, indeed the authors concluded that *T. koningii* (BM1) was developing because they isolated less pathogen from the sprayed straws compared to the controls. Moreover, the temperatures at which the experiment was conducted were different compared to this study which makes comparison with this work difficult, as temperature is an important parameter for fungal development (Palanna et al. 2005). Eastburn and Butler (1991) measured the saprophytic ability of *Trichoderma harzianum* on alfalfa stem pieces in non-sterile soil (Rincon silty clay loam) with matric potentials varying from 0.0 (flooding) to -15 bar. Results showed that *Trichoderma* was more active at soil matric potentials from -0.5 to -1.0 bar corresponding to soil moistures of 18 to 24% and at temperatures ranging from 15 to 21°C. They showed that the saprophytic ability of *Trichoderma* declined at matric potentials of 0.0 (flooded soil), -7.5 (14% soil moisture), and -15.0 bars (13% soil moisture). The water holding capacity of the Wakanui silt loam soil used for this work was 30%. A Wakanui silt loam soil at 15% and 28% GWC corresponded to soil moistures of -1.0 and -0.1 bar, respectively. Although there was an indication in the present study, from the results of experiment 1, that rhizosphere colonisation by *T. atroviride* LU132 declined at soil moisture content less than 15%, the isolate was still able to colonise the rhizosphere to appreciable and greater levels at 13% GWC than in the corresponding control. In general the results are similar to those in Eastburn and Butler's study, however their assessment was done on alfalfa stems which will differ in terms of composition and exudates to growing roots of sweet corn or ryegrass.

Only one isolate of one species, *T. atroviride* LU132, was assessed in this study. Different *Trichoderma* species and isolates within and across species are likely to vary in their response to soil moisture. Two *Trichoderm*a isolates (*T. asperellum* LU687 and *T. harzianum* LU698) were reported to vary in the soil water potential range at which they parastised sclerotia of *S. sclerotiorum* in non-sterile soil (Jones et al. 2014).

Although no colonisation of sclerotia was observed by either isolate in dry soils (-5.0 MPa), *T. asperellum* LU697 was active over a wider water potential range (-0.01 – -1.5 MPa) compared with *T. harzianum* LU698 (-0.1 – -0.3 MPa).

These assays were conducted in the absence of plants, and whether the effect of soil moisture on sclerotial colonisation ability would also translate to differences in ability to colonise the rhizosphere was not investigated.

The results also showed that both plant species supported high numbers of *T. atroviride* LU132 and similar populations to those found in Chapters 2 and 3 for sweet corn and Chapter 3 for ryegrass at 20% GWC. This indicated that under the experimental conditions used in this study, soil moisture level over the range tested did not have a strong influence on rhizosphere competence of *T. atroviride* LU132. However, rhizosphere competence was measured at one time only and did not reflect the variations in the system throughout the experiment and their implications on the development of *T. atroviride* LU132. For example, Eastburn and Butler (1988) studied 54 factors affecting *Trichoderma harzianum* population density in natural soil by analysing multiple 50 mg soil samples. They found that *Trichoderma* populations increased with increases in soil moisture but soil moisture was not associated with the presence or absence of *Trichoderma* at the microsite level. They inferred that soil moisture content had an effect on the development once the fungus was established but it was not determining the sites occupied by *Trichoderma* and as their measurements were also done at one time they concluded that long term changes in soil moisture regimes influenced the distribution and density of *Trichoderma* populations. Thus, populations of *T. atroviride* LU132 may have changed in the present experiment over the one or three weeks growing period for sweet corn and ryegrass, respectively but changes were not detected in the rhizosphere competence assessment at the harvest times chosen and for the entire plant root. The ability of *T. atroviride* LU132 to colonise the rhizosphere of sweet corn and ryegrass at different moisture levels would contribute to its ability as a biological control agent. Pathogens have different tolerance to dry conditions and mostly develop in wet conditions, consequently *T. atroviride* LU132 has potential as an antagonist across a wide range of soil moisture contents.

4.4.2 Growth promotion by *T. atroviride* LU132 at different soil moisture contents

Trichoderma atroviride LU132 increased root dry weights of sweet corn plants at 16% GWC (actual GWC at harvest time was 13%) in the first experiment. It has been shown in previous studies that certain isolates of *Trichoderma* can alleviate stress induced by abiotic factors by helping with water uptake (Mastouri et al. 2012). This indicates that *T. atroviride* LU132 may help with water absorption in stress conditions however the experiment would need to be repeated with growth promotion being the main purpose of the study, as the current study was designed to determine the effect on rhizosphere competence.

The results however showed that *T. atroviride* LU132 could promote growth in dry conditions. Mechanisms could include a linkage to rhizosphere competence as the establishment of the fungus in the rhizosphere may have helped sweet corn with water absorption, particularly as this isolate was shown to be endophytic. Bae et al. (2009) studied the impact of the endophytic isolate *Trichoderma hamatum* DIS 219b on the responses of cacao to drought and found that colonisation by the fungus delayed changes in stomatal conductance, net photosynthesis, and green fluorescence emissions expected when drought is induced. Moreover, they showed that colonisation by *T. hamatum* DIS 219b promoted seedling growth by increasing root growth parameters such as root fresh weight, root dry weight, and root water content. Although not significant in the present study *T. atroviride* LU132 also increased sweet corn root length and weight.

Growth promotion by *T. atroviride* LU132 on sweet corn was not shown in any other experiments of this thesis. In the second experiment *T. atroviride* LU132 promoted growth of shoots and roots of sweet corn plants grown at 24% GWC indicating that *T. atroviride* LU132 may have helped to alleviate water logging effects on sweet corn. In this experiment untreated sweet corn plants grew better at 20% and were stunted at 24% demonstrating a waterlogging effect. This may also explained why growth promotion was not detected in previous chapters as the plants were always grown at 20% GWC, which maybe close to the optimum level for plant growth, indicating that *T. atroviride* LU132 may only able to induce growth promotion when plants are grown under suboptimal conditions (Mastouri et al. 2010). This also demonstrated the consistency of the rhizosphere competence and growth promotion results across experiments in the thesis.

4.4.3 Influence of soil pH on rhizosphere competence of *T. atroviride* LU132

Results showed that *T. atroviride* LU132 colonised sweet corn and ryegrass rhizosphere soils regardless of the soil pH over the range pH 5.5 to pH 7.5, in agreement with the work of (Schubert et al. 2010). They determined the effect of combined environmental parameters on the conidial development of *T. atroviride* (T-15603.1) using a statistical approach and found that whilst water activity and temperature affected the development of the fungus conidia pH however had no significant effect. Many *in vitro* studies have shown that *Trichoderma* strains can grow at a range of pH, but rhizosphere competence in soils with different pH has seldom been addressed. Kolli et al. (2012) studied the pH level tolerance and biomass production of 26 *Trichoderma* isolates from seven species. They found that at pH levels between 4.5, 5.5, 6.5 and 7.5, isolates varied in biomass production. Sixteen isolates had the highest biomass production at pH 7.5 and none of the isolates had a high biomass production at pH 4.5. They also found that isolate *T. reesei* (TR) and *T. longibrachiatum* (M1Ps) were less affected by pH change. In contrast, Kredics et al. (2004) analysed the effect of pH *in vitro* on mycelial growth and extracellular enzymes activity of five *Trichoderma* strains belonging to three species.

They found that all strains had the highest mycelial growth at pH 4, but enzymes were active at a wider pH range varying from 3 to 7 depending on the enzyme. Begoude et al. (2007) also showed that mycelial growth of four strains belonging to the species *T. asperellum* was highest at pH between 4.5 and 6.5 but possible at pH 8.5. Unlike the previous reports, Ahmad and Baker (1987a) assessed rhizosphere competence of two *T. harzianum* isolates (T-95 and WT) on cucumber and radish plants in unsterilized Nunn sandy loam soil. They found that T-95 and WT could, respectively, colonise the entire roots of cucumber and radish at pH levels from 5 to 7. They found that with increasing pH, higher populations were isolated from roots at greater soil depths. In this work, rhizosphere competence was not measured at different root depths, but population numbers obtained for the entire roots of sweet corn and ryegrass were similar to those obtained for cucumber and radish. Thus, this study agrees with literature which indicates that *Trichoderma* species are able to grow at pH values from 5.5 to 7.5. Lo et al. (1996) demonstrated that *T. harzianum* T22 populations were maintained in soil at pH 8 for eight months at levels between 10^5 and 10^6 CFU/g dry weight sample. In this experiment the soil pH for both plant species varied between 5.5 and 7.5 which is inside the range expected for *Trichoderma* to grow and explains why changing the pH did not affect *T. atroviride* LU132's colonisation of sweet corn and ryegrass. Moreover, the colonisation pattern of *T. atroviride* LU132 on sweet corn and ryegrass was similar to that of Chapter 2 with higher populations in ryegrass roots compared to sweet corn, confirming that a change in soil pH does not affect *T. atroviride* LU132 growth on the two plant species grown in soil at pH between 5.5 and 7.5. The pH range used in this study represents the normal range of New Zealand agricultural soils, and indicates that soil pH is not going to be a limiting factor for *T. atroviride* LU132 colonisation in agricultural systems.

4.4.4 Growth promotion by *T. atroviride* LU132 at different soil pH levels

Results showed that growth parameters for sweet corn and ryegrass plants regardless of the treatment were better at pH 5.5 and 6.5. This could be explained by the addition of sodium carbonate which was used to increase the soil pH resulting in increased amounts of sodium in the soil. At pH 6.5 and 7.5, the quantities of sodium were respectively, 15 and 32 times higher than at pH 5.5. Tavakkoli et al. (2010) studied the responses to salt addition of faba bean in a soil based system and found that high Na^+ ions' concentration in the soil reduced growth by impairing photosynthesis.

No growth promotion was detected for ryegrass which was in agreement with results from Chapter 3. However, *T. atroviride* LU132 improved seedling emergence of sweet corn plants at all soil pH values tested across both experiments. The improvement in sweet corn seedling emergence at pH 7.5 in experiment 2 could be explained by the fact that the emergence in the control treatment was low even compared to controls at other pH levels.

The same reason would explain the improvement in seedling emergence in experiment 1 at pH 6.5. *Trichoderma atroviride* LU132 increased seedling emergence of sweet corn at pH 5.5 in experiment 1, however this result was not replicated in experiment 2 which was more in agreement with previous results from Chapters 2 and 3. *Trichoderma atroviride* LU132 decreased shoot length at pH 5.5 in experiment 1. This result was not replicated in experiment 2 for the same pH, but *T. atroviride* LU132 treated plants at pH 6.5 had shorter shoots and lighter roots than control plants. Although *T. atroviride* LU132 had not been shown in this thesis to negatively affect sweet corn at pH 5.5 (regular pH for Wakanui silt loam soil used in all the experiments) it decreased the weight of clover plants' shoots and roots in Chapter 3. The negative effect on shoots and roots of *T. atroviride* LU132 at pH 6.5 could be explained by the fact that Na^+ ions in the soil interfere with K^+ and Ca_2^+ nutrition, therefore *Trichoderma* and the plant may have been competing for nutrients. However, this was not the case in experiment 1, but control plants were also smaller compared with at pH 5.5 and experiment 2. Consequently, the experiment would need to be repeated to enable any conclusions regarding growth promotion by *T. atroviride* LU132 at different pH levels to be made.

4.4.5 Influence of soil available nitrogen quantities on the rhizosphere competence of *T. atroviride* LU132

Results showed that *T. atroviride* LU132 colonised sweet corn and ryegrass rhizosphere soils regardless of the nitrogen quantity added to the soil.

A difference was found for *Trichoderma* populations developing in ryegrass in the first experiment, where more *Trichoderma* populations were isolated from rhizosphere soils with higher nitrogen quantities. But the pattern did not repeat itself in the second experiment, therefore it was concluded that available nitrogen concentrations in the soil did not affect rhizosphere competence of *T. atroviride* LU132 on sweet corn and ryegrass under the conditions of this experiment.

This is in agreement with Anis et al. (2013) who investigated the effect of fertilizers in combination with two *Trichoderma* isolates, one each of *T. viride* and *T. reesei,* on the control of the pathogen *Macrophomina phaseolina* which causes dry root rot, stem canker, stalk rot and charcoal rot in several plant species. They coated sunflower (*Helianthus annuus* L.) seeds with the antagonists which they sowed in a soil amended with different fertilizers (urea, diammonium phosphate, potassium sulphate and ammonium nitrate) and naturally infested with *M. phaseolina* sclerotia. Neither of the isolates were found to have any effect on plant growth in combination with ammonium nitrate, with no increase in factors such as percentage germination, or plant length and weight compared to controls without *Trichoderma* and fertilizers. Since the authors did not assess *Trichoderma* populations or the presence of the pathogen in the soil a direct comparison with this study is difficult, as the presence of the pathogen may have had an effect on *Trichoderma* colonisation.

In contrast McLean et al. (2012) reported that the same isolate as used in the current study, *T. atroviride* LU132, was sensitive to higher doses of nitrogen in the form of urea, with urea concentration equivalent to 75 kg N/ha significantly reducing conidial germination *in vitro*. Similarly urea at 75 kg N/ha was seen to significantly reduce *T. atroviride* LU132 population in soil over the 3 week incubation period compared with the unamended control, whilst field rate of 37.5 kg N/ha had no effect. The form in which nitrogen is applied to soil is likely to vary in their effect on *Trichoderma* growth and proliferation, with nitrogen as ammonium sulphate increasing saprophytic growth of a *T. koningii* isolate whilst growth was suppressed by nitrate (Wakelin et al. 1999). Whether different nitrogen sources influence rhizosphere colonisation has not been investigated.

Trichoderma atroviride LU132 reduced shoot lengths of sweet corn and ryegrass at different nitrogen concentrations but this effect was inconsistent across both plant species and both experiments. Therefore, it was inferred that *T. atroviride* LU132 did not promote growth nor did it hinder plant growth parameters.

4.4.6 Influence of abiotic factors on endophytic colonisation of isolate *T. atroviride* LU132

The results from this chapter added further weight to observations that *T. atroviride* LU132 is an endophyte. For all three abiotic factors *T. atroviride* LU132 was recovered as an endophyte from both plant species however *T. atroviride* LU132 had different colonisation patterns on sweet corn and ryegrass shoots and roots. Ryegrass roots and shoots appeared to be completely colonised endophytically by *T. atroviride* LU132, whereas, in sweet corn the colonisation was generally localised at the base of the shoot, the top part of the root and sporadically along the root length. Nonetheless, endophytic colonisation did not depend on the GWC, pH or nitrogen quantities available in the soil. This is the first study on the influence of abiotic factors on the endophytic colonisation by *Trichoderma* strains.

Druzhinina et al. (2011) suggested that the ability of *Trichoderma* to endophytically colonise plants is a recently acquired trait with evolutionary data indicating that *Trichoderma* species progressed from a saprophytic lifestyle to being parasites and plant endophyte.

Trichoderma atroviride LU132 was demonstrated to be rhizosphere competent and endophytic on sweet corn and ryegrass regardless of soil moisture content, soil pH and soil available nitrogen concentrations within the range tested in the experiments. The results of the current study also suggest that *T. atroviride* LU132 is able to alleviate water logging or water deficit for sweet corn, but had no effect on ryegrass with regards to growth promotion.

The experiments were carried out in conditions where both the plants chosen and the fungus could survive, which constrained the ranges for each of the abiotic factors tested. Future work could include the study of rhizosphere competence of *T. atroviride* LU132 on plants that are more tolerant to a wider range of water stress levels and soil pH for example.

5

Temporal dynamics of *Trichoderma* rhizosphere competence and the influence of a high and low rhizosphere competent isolates on other rhizosphere microbial communities

5.1 Introduction

Many studies investigate rhizosphere competence at a single time point (Sivan and Harman 1991, Ahmad and Baker 1987a). This varies between papers, host plants and *Trichoderma* species making it hard to compare between studies. In most of these studies changes in rhizosphere competence of different isolates over time has not been determined. For those studies which did investigate the temporal dynamics of rhizosphere colonisation, many have only used one *Trichoderma* isolate, which in many cases had previously been identified as having biocontrol or growth promotion activity. For long term suppression of root pathogens maintaining the rhizosphere populations over prolonged periods may be an important trait.

Trichoderma species are well recognised rhizosphere colonisers (Brimmer and Boland, 2003). In the rhizosphere they exert pathogen suppressive activity but they may also inhibit other rhizosphere microbes including beneficials such as symbiotic arbuscular mycorrhizal fungi. Brimmer and Boland (2003) showed that biocontrol organisms have a negative impact on the microbial communities in the soil. Similarly to this Cordier and Alabouvette (2009) observed a decrease in diversity in the presence of *T. atroviride*. In contrast, McLean et al. (2014) demonstrated that there were no negative effects with the application of a commercial product on the arbuscular mycorrhizal communities of two New Zealand native ecosystem.

The aim of this chapter was to compare survival over time of two *Trichoderma* isolates with contrasting rhizosphere competence as measured at 7 days on sweet corn. In previous chapters it was shown that *T. atroviride* LU132 was more rhizosphere competent than *T. virens* LU556 at seven days, however no other point in time was examined. Therefore, it was unknown if LU132 remained more rhizosphere competent than *T. virens* LU556 over time and if the 7 day assay was appropriate to distinguish relative differences in rhizosphere. Although two different species were chosen in this chapter, previous results (Chapter 2) showed that rhizosphere competence is isolate dependant and not species specific.

Another aim of the chapter was to investigate how a high and low rhizosphere competent isolates, colonise the internal parts of sweet corn and whether there is a link between rhizosphere competence and endophytic ability. Finally, this chapter also aimed at assessing the influence of a high and low rhizosphere competent isolates on other rhizosphere microbial communities.

5.2 Materials and methods

5.2.1 Selection of isolates and preparation of inocula

Two isolates, *T. atroviride* LU132 and *T. virens* LU556 were used in this experiment. *Trichoderma atroviride* LU132 was the most rhizosphere competent isolate in previous experiments (Chapters 2 and 3), whereas *T. virens* LU556 was amongst the least rhizosphere competent isolates. Both isolates' inocula were prepared as described in previous chapters (Section 2.2.2).

5.2.2 Seed application of *Trichoderma* isolates

Seeds of sweet corn (*Zea mays*) were purchased from Kings Seeds. Prior to inoculation, the seeds were surface sterilised for 20 min in a 1% a.i. sodium hypochlorite and 5% ethanol solution and washed three times with sterile distilled water (SDW). They were subsequently air-dried for 2 hours in a laminar flow cabinet. Seeds (in batches of 40) for each *Trichoderma* isolate treatment were placed in a standard size Petri dish (six Petri dishes per isolate were prepared) and coated with a mixture of equal volumes of conidial suspension in water (1 x 10^8 conidia/mL) and a 2% (w/v) methyl cellulose (MC) solution serving as sticker. The final concentration of MC in the coating applied to the seeds was 1%. The Petri dishes were vigorously shaken by hand for 1 min to evenly distribute the *Trichoderma* conidia over the surface of the seed. Seeds were left to dry for a few hours (typically 2 to 3) before applying a second coating following the same procedure. The seeds were then left to dry overnight in a laminar flow cabinet. The control treatment, without *Trichoderma* conidia was treated in the same way with sterile water substituted for the conidial suspension.

5.2.3 Soil

Wakanui silt loam soil was collected from Lincoln University farm land (paddock H14, Christchurch, NZ). The soil was pulverised through a soil shredder to obtain particles of size between 1 and 5 mm. The soil was stored in a 600 L bag made out of woven plastic mesh. Before being used in the experiment the soil was adjusted to 20% soil moisture content (SMC) by adding the required quantity of water. To adjust the SMC, the gravimetric water content (GWC) was calculated using the same formula as in previous chapters (Section 2.2.4). Prior to the experimental setup, a basic soil analysis was completed (Hills Laboratories, Hamilton, NZ; Appendix D1).

5.2.4 Experimental design

Seventy two, 4 L plastic pots were filled with moistened soil and were placed in six randomised blocks. Each block contained 12 pots, four pots per isolate and control. Each of those four pots was harvested at four different times: 7, 21, 35 and 56 days after sowing. Seven seeds of sweet corn previously surfaced sterilised and coated with either *T. atroviride* LU132 or *T. virens* LU556 or with a solution of methyl cellulose (control) were sown 1 cm deep in each pot (Figure 5.1 A). After emergence, the plants were thinned to four plants per pot. Pots were wrapped in a plastic bag and placed in a growth room (Figure 5.1 B) under the following experimental conditions: 16 hours light (average of 144 µmol) photons/m^2/s) at 18˚C and 8 hours dark at 20˚C. No water was added to the growing systems during the first 7 days after which, water was added as required.

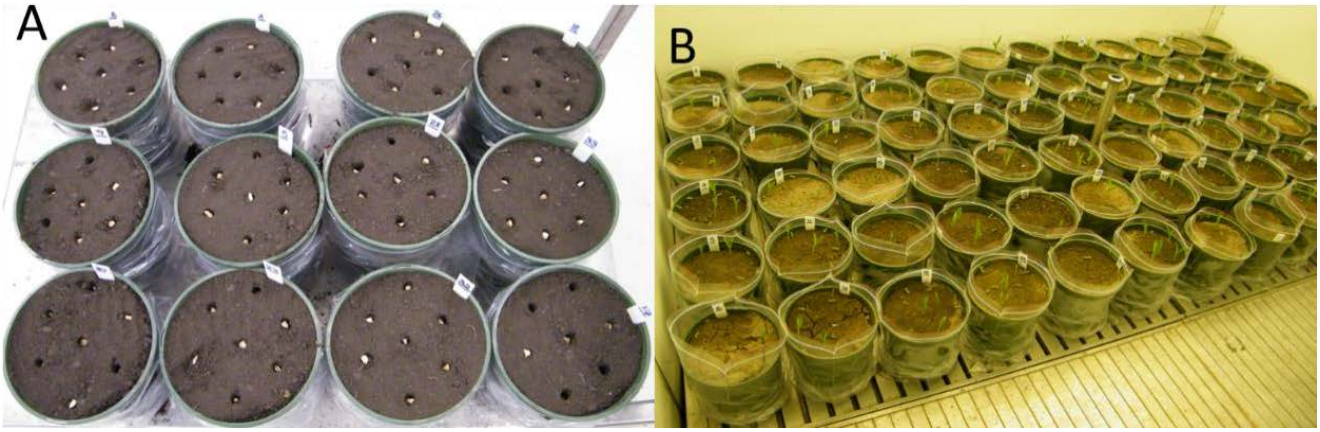

Figure 5-1 A: Sweet corn seeds sown in 4 L plastic pots. B: Set up of the experiment in the growth room.

5.2.5 Rhizosphere competence assessment

The assay was based on the method of Ahmad and Baker (1987a) and adjusted according to the size of the plants. The seedlings were gently shaken to remove any loosely adhering soil and the roots were excised from the crown. Seven day old roots with adhering soil were placed in a sterile 50 mL plastic tube and kept at 4˚C overnight until processed. Conical flasks (250 mL volume) were used for 21 and 35 day old plants and 500 mL volume conical flasks were used for 56 day old plants. All plant material was kept at 4˚C overnight before being processed.

The following day, between 10 and 80 mL of a 0.5% v/v Triton X 100 solution was added to the flasks containing the roots. The quantity of Triton X 100 solution was determined on the day of the analysis depending on the size of the root. The 7, 21, 35 and 56 day old roots were washed respectively with 10, 30, 60 and 70 mL of Triton X 100 solution.

Tubes and conical flasks were subsequently shaken for 30 min (94 rpm for 50 mL plastic tubes and 180 rpm for conical flasks) at room temperature in an orbital shaker (Ratek EOM5, Ratek Instruments PTY Ltd, Boronia, Australia). The suspensions were serially diluted to 10^{-1}, 10^{-2} and 10^{-3}. Aliquots of the initial suspensions and each dilution (200 µL) were plated onto each of three replicate *Trichoderma* selective medium plates (Appendix A2). Plates were incubated at 20˚C in the dark. The number of *Trichoderma* colonies was counted at 10 to 13 days after incubation and the number of *Trichoderma* CFU/g of DRS was calculated. The original soil dilution was stored at -80˚C until used for DNA extraction (Section 5.2.8.1).

5.2.6 Growth promotion assessment

The number of seedlings which emerged was recorded after 7 days. After 7, 21, 35 and 56 days, two plants per pot and treatment were randomly selected and harvested to assess growth promotion and rhizosphere competence. The remaining two plants were used for the assessment of endophytic colonisation. Shoot and roots lengths were measured using a ruler to the nearest mm. Shoots were detached from the root, placed into paper bags and subsequently dried in an oven at 72°C for 48 hours to a constant dry weight before being weighed. After being used for the rhizosphere competence assessment (Section 5.2.5), roots were washed, weighed and dried under the same conditions as shoots. Part of the root system broke and was lost during harvest, therefore, in order to enable comparisons between treatment weights, ratios for shoot (shoot dry weight/shoot length = SDW/SL) and root (RDW/RL) were calculated to estimate the weight per cm of plant material.

5.2.7 Endophytic assessment

Two plants per pot and treatment (when possible) were used to assess endophytic colonization. The roots and shoots were first washed under tap water. Subsequently, they were surface sterilised in a 50% a.i. sodium hypochlorite (5.25 g/L available chlorine) bath for 3.5 min followed by two 2 min SDW baths. For shoots, only the first 10 to 12 cm from the crown to the first leaf collar was assessed, whereas for the roots the whole root was assessed. Roots and shoots were then cut into 2 cm segments with a sterile scalpel blade in a laminar flow unit. For 7, 21 and 35 day old plants, the entire root system was cut and plated. For 56 day old plants, two representative roots were randomly chosen for processing. Shoot and root segments were plated on *Trichoderma* selective media. Each shoot was plated onto one Petri plate. Each root was plated onto 1-3 Petri plates depending on the length of the root system. The multiple plates per root system were sequentially numbered. The plates were incubated in the dark at 20°C for 5 to 8 days.

The presence or absence of colonies on the shoot and root pieces was recorded for all the plates. A plate was recorded as positive for endophytic colonisation if ≥1 *Trichoderma* colony was present.

5.2.8 Bulk and rhizosphere soil microbial community analysis

5.2.8.1 DNA extraction

After plating aliquots of rhizosphere soil dilutions (Section 5.2.5) for each plant, the remaining solutions were stored at -80°C until used for DNA extractions. For each treatment, the bulk soil from all the pots where the plants had grown was collected in a bag, homogenised and two samples were taken for DNA extraction. For the rhizosphere soil DNA extractions, samples were thawed and centrifuged to pellet the soil. For each sample, 0.25 g of rhizosphere soil was weighed and DNA extracted with the PowerSoil™ DNA Extraction Kit (MOBIO Laboratories Inc.). The extraction was done following the manufacturer's instructions. DNA was stored at -20°C until use.

5.2.8.2 PCR

Primer pair 357F GC (5'-Gcclamp-CCT ACG GGA GGC AGC AG-3') (Muyzer et al. 1993) and 518R (5'- ATT ACC GCG GCT GCT GG-3') (Van der Gucht et al. 2005) was used to amplify bacterial DNA (16S rRNA). For fungal DNA, a nested PCR approach was used to amplify the 18S rRNA gene DNA. Primers used were: step 1 AU2 (5'-TTT CGA TGG TAG GAT AGD GG-3') and AU4 (5'-RTC TCA CTA AGC CAT TC-3') (Muyzer et al. 1993); step 2 FF390 (5'-CGA TAA CGA ACG AGA CCT-3') and FR1-GC (5'-Gcclamp-GCA CGG GCC GAI CCA TTC AAT CGG TAI T-3') (Vainio and Hantula 2000). For arbuscular mycorrhizal fungi (AMF), DNA was amplified using a nested approach. Primers used were: step one AML1 and AML2 (Lee et al. 2008); step two Glo1 and NS31GC (Simon et al. 1992). PCR were performed with reagents from FastStart DNA amplification kit (Roche) using a Veriti® thermal cycler (Applied Biosystems). Each reaction had a volume of 25 µL and contained 1 X buffer with 1.5 mM $MgCl_2$, 200 µM dNTPs, 5 pmol each of the forward and reverse primers, 1U of Taq DNA polymerase and 1 µL of DNA template. For amplification of DNA from AMF and fungi, the PCR master mix for the nested PCR contained 1 µL of diluted (1/10) DNA template from the primary PCR. Bacterial DNA was amplified using the following thermal cycle: Denaturation at 95°C for 3 min, then 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min with a final extension at 72°C for 7 min. AMF and fungi were amplified using a nested PCR approach. The thermal cycle for the primary PCR for fungi and AMF was the same as for bacteria except the annealing temperature was 50°C. The thermal cycle for the secondary PCR for AMF consisted of 95°C for 3 min, then 35 cycles at 94°C for 45 s, 52°C for 45 s and 72°C for 1 min, final extension at 72°C for 7 min.

The conditions for the fungal secondary PCR were as follows: Amplification was done at 95°C for 2 min, then 8 cycles at 95°C for 30 s, 55°C for 30 s (touchdown of 1°C per cycle) and 72 °C for 1 min, thereafter 27 cycles at 95°C for 30 s, 47°C for 30 s and 72°C for 1 min, final extension at 72°C for 7.5 min. PCR products were separated by electrophoresis at 10 V/cm for 45 minutes in a 1% agarose gel in 1 x TAE (tris-acetate-EDTA). Gel was then stained with ethidium bromide (0.5 µg/ml) for 20 min and destained in tap water for 10 min before observation under UV light.

5.2.8.3 DGGE

Different denaturing gradients of an 8% polyacrylamide gel (37.5:1 acrylamide: bis-acrylamide ratio; BioRad) were prepared. Gel gradients were 30-45% for AMF, 45-65% for bacteria and 25-55% for fungi. Twelve µL of PCR product per sample and an equal volume of dye were loaded in each well. The gels were run at 58°C at 90 V for 15 hours in a Cipher DGGE Electrophoresis System (CBS Scientific).

At the end of the run, each gel was fixed for 3 min in 250 mL of Cairn's solution (ethanol, acetic acid and H_2O; 4: 0.25: 0.75) before being stained for 10 min in 250 mL of silver stain (250 mL of Cairn's solution + 0.5 g of silver nitrate). After being stained, each gel was rinsed and washed with Millipore water for 2 min after which they were bathed in developer for 40 min (500 mL of Millipore water, 15g sodium hydroxide and 0.5 mL formaldehyde). Prior to the development, each gel was fixed again for 5 min, washed in Millipore water for 2 min for a second time and preserved in Cairn's preservation solution for 7 min before being wrapped in a plastic sheet. Gels were dried at 60°C overnight.
A pdf image was obtained by scanning each gel in a copier/printer (image RUNNER ADV C2020, Canon, New Zealand). The pdf image was used in Phoretix (Totallab, New Castle, UK) to manually detect bands and produce a presence/absence matrix for each species which was analysed in PRIMER 6 (PRIMER-E Ltd, Ivybridge, UK).

5.2.9 Statistical analysis

5.2.9.1 Rhizosphere competence

The mean number of CFU/g DRS per isolate was calculated, $\log_{10}$ transformed and statistically analysed using a general analysis of variance (ANOVA). Controls had to be removed from the ANOVA for two reasons. Firstly, the main goal of the experiment was to compare the performances of the two isolates over time. Secondly, *Trichoderma* populations isolated from control plants were very low and violated the assumptions of normality for ANOVA. Fisher's unrestricted least significant difference (LSD) was used to test for differences between isolates across four harvest times. The analysis was performed in GenStat 15th edition (VSN International, Hertfordshire, UK).

5.2.9.2 Growth promotion

Seedling emergence, shoot and root lengths were analysed using an analysis of variance (ANOVA). Shoot and root dry weights as well as ratios (SDW/SL and RDW/RL) were $\log_{10}$ transformed previously to being analysed with ANOVA. Fisher's unrestricted least significant difference (LSD) was used to test for mean differences between seedling emergence, shoot and root lengths, shoot and root dry weights and ratios of treated plants with untreated controls. The analyses were performed in GenStat 15th edition (VSN International, Hertfordshire, UK).

5.2.9.3 Microbial community analysis

Treatment impacts on bulk and rhizosphere soil communities (AMF, bacteria and fungi) were compared in PRIMER by performing a permutational analysis of variance (PERMANOVA) of Bray-Curtis dissimilarity. Differences were visualised with nonmetric multidimensional scaling (NMDS). Treatment impacts on different microbial communities (AMF, bacterial and fungal) was assessed in PRIMER using PERMANOVA of Jaccard similarity indices, these were based on presence/absence of bands in the DGGE gels.

5.3 Results

5.3.1 Rhizosphere competence assessment

Control populations were low and varied between 1.71 x 10^3 CFU/g DRS at 7 days and 2.79 x 10^4 CFU/g DRS 56 days post planting (Figure 5-2). *Trichoderma* populations recovered from plants treated with *T. atroviride* LU132 increased significantly from day 7 (1.29 x 10^5 CFU/g DRS) to day 35 (6.11 x 10^6 CFU/g DRS). After 35 days, the population declined, but not significantly, to 3.55 x 10^6 CFU/g DRS at 56 days. *Trichoderma* populations recovered from plants treated with *T. virens* LU556 increased significantly from day 7 (3.79 x 10^4 CFU/g DRS) to days 35 and 56 where they reached respectively 1.71 x 10^5 and 5.30 x 10^5 CFU/g DRS. At days 21, 35 and 56, populations in the rhizosphere of plants treated with *T. atroviride* LU132 were significantly higher than for plants treated with *T. virens* LU556.

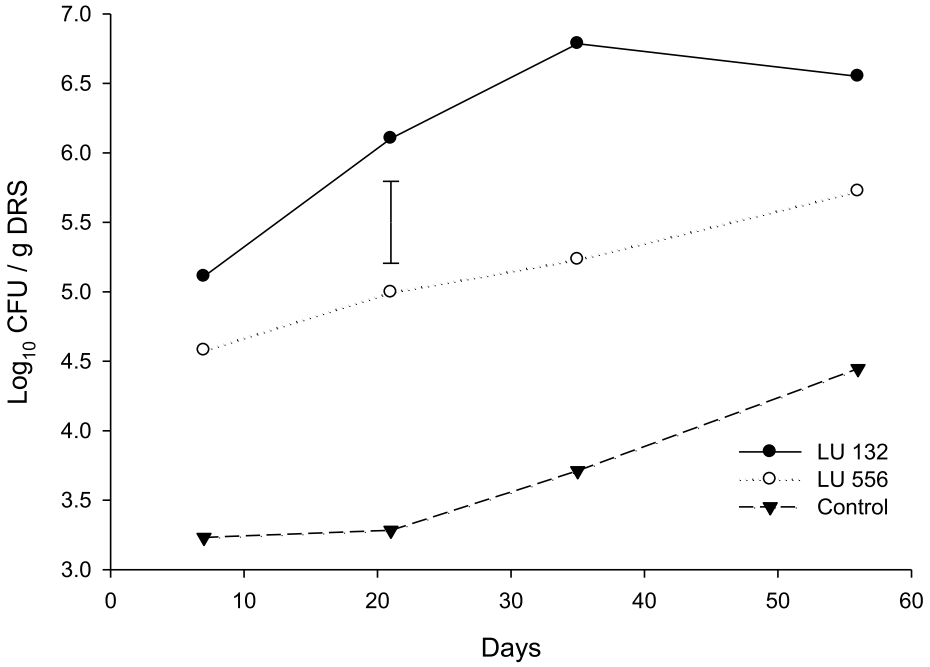

Figure 5-2 Log10 CFU/g DRS counts for *Trichoderma* populations recovered from the rhizosphere soil of sweet corn plants at 7, 21, 35 and 56 days after sowing and coated with *T. atroviride* LU132 and *T. virens* LU556. Values for the controls are presented to provide an indication of the indigenous *Trichoderma* populations recovered, but were not included in the analysis of variance. The vertical bar represents the LSD 5%= 0.588

5.3.2 Growth promotion

Field emergence of untreated and treated seeds was measured after 7 days. There were no significant differences between the untreated controls and the seedlings treated with either *T. atroviride* LU132 or *T. virens* LU556. For untreated and treated plants, shoot length increased significantly from day 7 to day 56 (Figure 5-3A). At days 35 and 56, the shoots of *T. atroviride* LU132 treated plants were significantly shorter than the shoots of the control and *T. virens* LU556 treated plants, which were not different from each other. At 56 days, roots of untreated plants, *T. virens* LU556 and *T. atroviride* LU132 treated plants were 5, 3 and 2 times longer, respectively than at 7 days (Figure 5-3B). At day 56, plant roots treated with *T. atroviride* LU132 were significantly shorter compared with control and *T. virens* LU556 treated plant roots. Shoot and root dry weights increased significantly for untreated and treated plants from day 7 to day 56 (Figure 5-3C and D). At 35 and 56 days, *T. atroviride* LU132 treated plants had shoot dry weights significantly lower than the shoot dry weights of plants treated with *T. virens* LU556. At day 35, *T. atroviride* LU132 treated plants had significantly smaller root dry weights than plants treated with *T. virens* LU556 (Figure 5-3D).

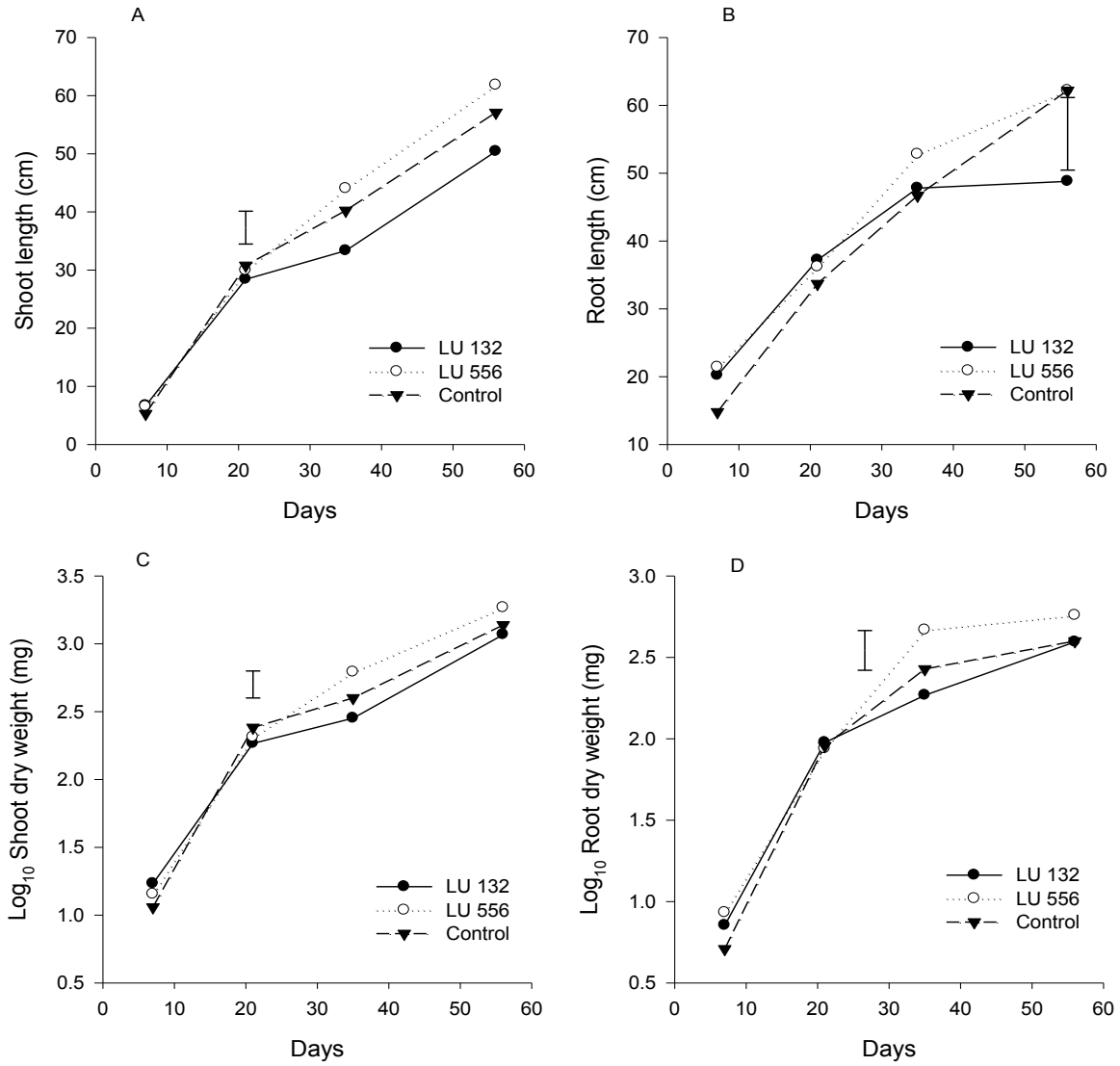

Figure 5-3 Growth promotion parameters assessed on sweet corn plants untreated and treated with *Trichoderma atroviride* LU132 and *Trichoderma virens* LU556 harvested at 7, 21, 35 and 56 days after sowing. (A) Shoot length. LSD 5% = 5.666 (B) Root length. LSD 5% = 10.7 (C) Log_{10} Shoot dry weight LSD 5% = 0.198 (D) Log_{10} Root dry weight. LSD 5% = 0.245

The shoot ratio increased significantly throughout the whole experiment (Figure 5-4A). At 35 days, the shoot ratio for plants treated with *T. atroviride* LU132 was significantly lower than the ratio of plants treated with LU556. At 35 days the shoot ratio of plants treated with *T. virens* LU556 was significantly higher than the ratio for untreated plants. For all plants, the root ratio increased significantly from day 7 to day 56 (Figure 5-4B). From day 35 to 56, the root ratio of control and *T. virens* LU556 plants did not increase significantly. In contrast, the root ratio of *T. atroviride* LU132 treated plants increased significantly from 35 to 56 days. At day 35, *T. virens* LU556 plants' root ratios were significantly different to root ratios of control and *T. atroviride* LU132 treated plants. At 56 days, *T. virens* LU556

plants' root ratios were significantly different to the ratio of control plants but did not differ from that for *T. atroviride* LU132 treated plants.

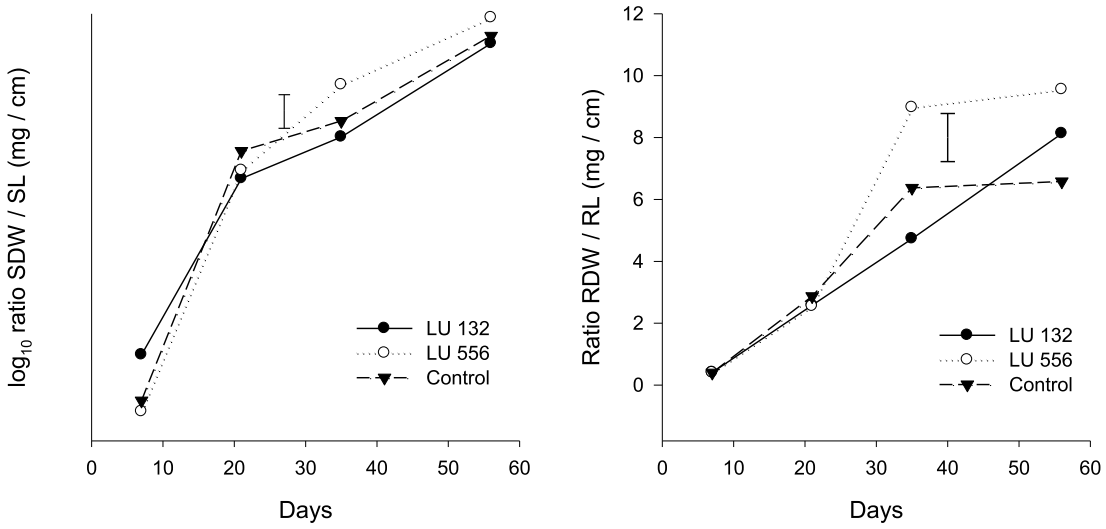

Figure 5-4 Ratios shoot dry weight (SDW)/shoot length (SL) (A) and root dry weight (RDW)/root length (RL) (B) for untreated and treated sweet corn plants harvested at 7, 21, 35 and 56 days. Treatments were T. atroviride LU132 and T. virens LU556. Vertical bars are LSD 5% = 4.238 (A) LSD 5% = 1.552 (B)

5.3.3 Assessment of endophytism

Colonies characteristic of *Trichoderma* species were recovered as endophytes from untreated shoots and roots at 7 days and from roots only at 35 and 56 days (Table 5-2 and Figures 5-5 and 5-6). For *T. atroviride* LU132 treated plants, endophytic *Trichoderma* was isolated at all harvest times and from both plant parts. For 21 and 35 day old *T. atroviride* LU132 treated plants a greater percentage of root fragments compared to shoots contained endophytic *Trichoderma*. At 56 days, 100% of shoots and roots of *T. atroviride* LU132 treated plants had endophytic *Trichoderma*. For *T. virens* LU556 treated plants, there was a greater frequency of *Trichoderma* recovered from roots than shoots at all harvests times (Table 5-2 and Figures 5-5 and 5-6). At 7 days 100% of shoots and roots treated with *T. virens* LU556 had endophytic *Trichoderma*.

Table 5-1 Number of *Trichoderma atroviride* LU132 and *Trichoderma virens* LU556 treated and untreated sweet corn plants assessed at different times for endophytic colonisation, and the number and percentage (in brackets) of shoots and roots from which endophytic *Trichoderma* was isolated.

	Number of plants assessed			Number of plants positive for *Trichoderma* (%)					
				Control		LU132		LU556	
Days	Control	LU132	LU556	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**
7	2	5	2	1 (50)	1 (50)	4 (80)	3 (60)	2 (100)	2 (100)
21	2	7	6	0 (0)	0 (0)	6 (86)	7 (100)	2 (33)	4 (67)
35	4	5	5	0 (0)	3 (75)	2 (40)	5 (100)	1 (20)	4 (80)
56	4	6	9	0 (0)	3 (75)	6 (100)	6 (100)	5 (56)	6 (67)

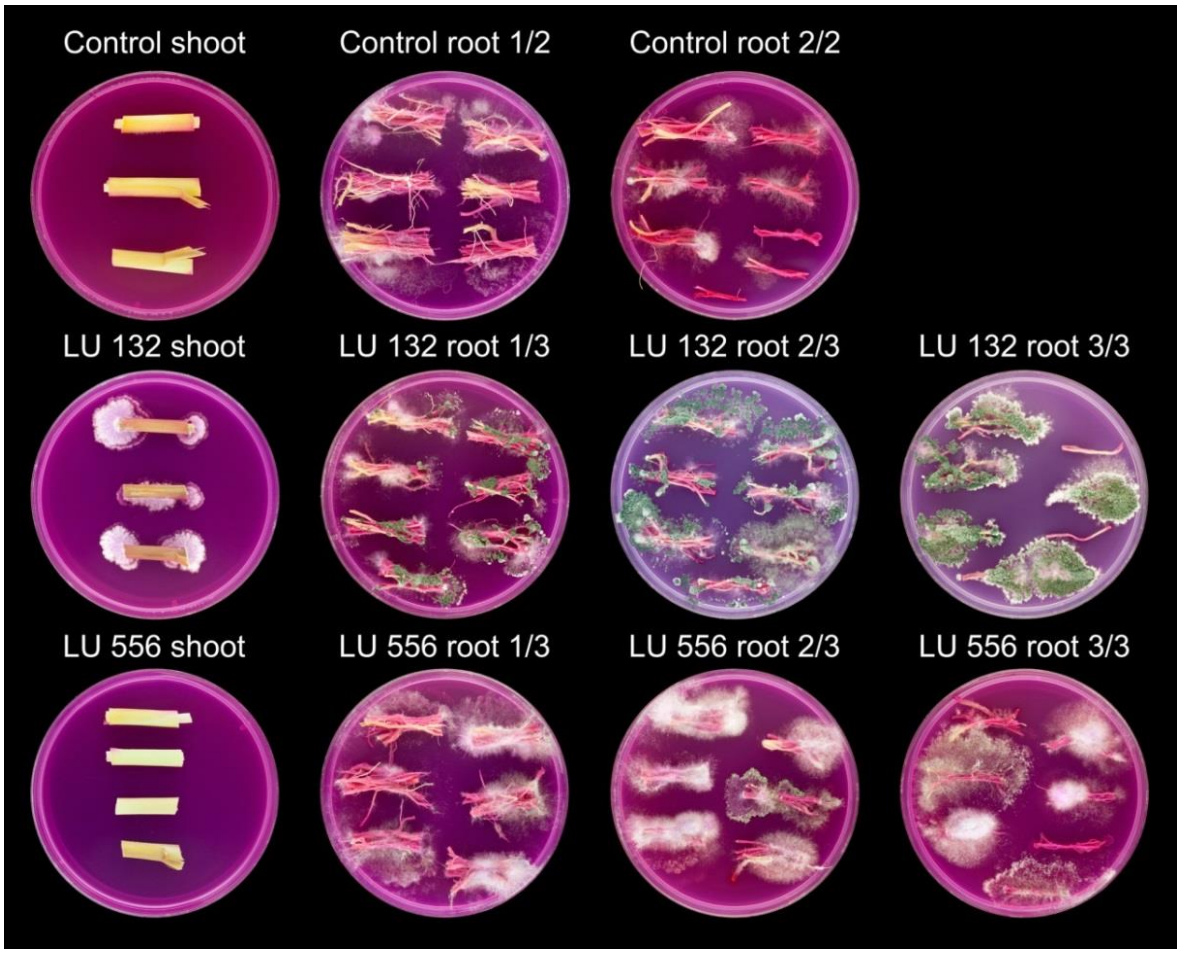

Figure 5-5 Endophytic *Trichoderma* recovered from 35 day old sweet corn plants untreated or treated with *Trichoderma atroviride* LU132 and *Trichoderma virens* LU556.

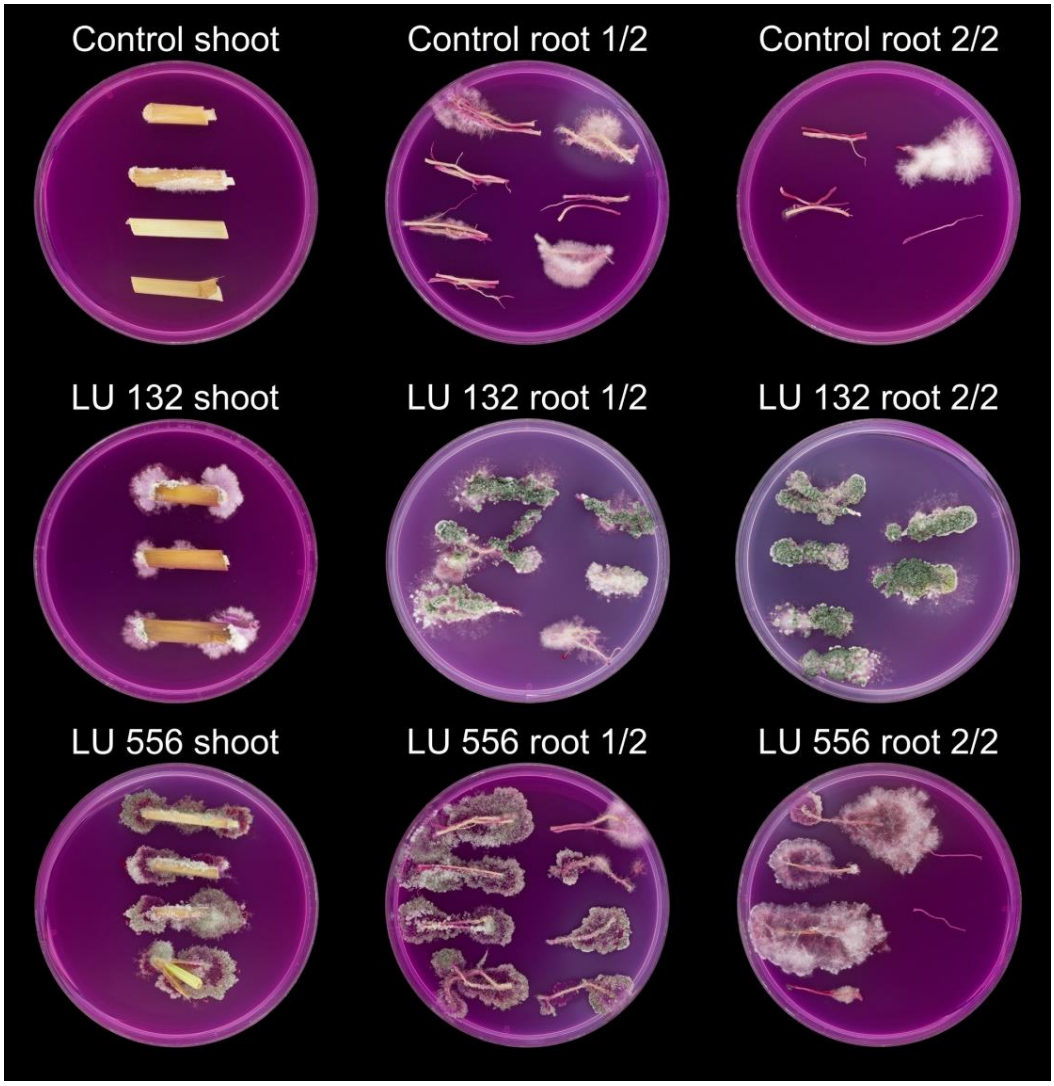

Figure 5-6 Endophytic *Trichoderma* recovered from 56 day old sweet corn plants untreated and treated with *Trichoderma atroviride* LU132 and *Trichoderma virens* LU556.

5.3.4 Impact of Trichoderma isolates on microbial communities in the rhizosphere

Time (from 7 to 56 days) had no effect on community composition of AMF, fungi or bacteria in the rhizosphere soil of sweet corn plants untreated and treated with *T. atroviride* LU132 or *T. virens* LU556.

5.3.4.1 AMF communities

AMF species composition differed significantly between the set-up, bulk and rhizosphere soils across all treatments and assessment times (PERMANOVA Table Appendix D2). An example of DGGE gel is presented in Figure 5-6. There were no differences between *Trichoderma* treatments and the untreated soil or between the two *Trichoderma* treatments across 56 days in the composition of AMF populations in the bulk soil. Both *T. atroviride* LU132 (P = 0.0007) and *T. virens* LU556 (P= 0.0003) affected the AMF composition in the rhizosphere soil compared with the untreated soil.

The rhizosphere soil of plants treated with *T. atroviride* LU132 and *T. virens* LU556 were no different from each other in their AMF composition (*P* = 0.1452) (PERMANOVA Table Appendix D2).

A two dimensional representation of the distribution of populations is presented in the form of a non-metric multidimensional scaling (MDS) plot in Figure 5-7.

Richness was significantly different between rhizosphere soil and bulk soil across all treatments for the 56 days period. The richness of the bulk soil (16 species) was higher than the rhizosphere soil (11 species) and set up soils (13 species) (*P* = 0.001). The rhizosphere and set up soils did not differ in their richness. When rhizosphere soil was analysed alone, treatments had an effect but time did not have any effect on the soil AMF composition. Rhizosphere soil of plants inoculated with *Trichoderma* isolates had significantly lower richness (10 species for each *Trichoderma* treatment) than the rhizosphere soil of untreated plants (16 species) (*P* = 0.001). In the bulk soil, AMF richness differed significantly between plants treated with *T. atroviride* LU132 and plants either treated with *T. virens* LU556 or untreated. Richness of untreated plants (14 species) and those treated with *T. virens* LU556 (15 species) was lower than in the bulk soil of plants treated with *T. atroviride* LU132 (19 species) (*P* = 0.006).

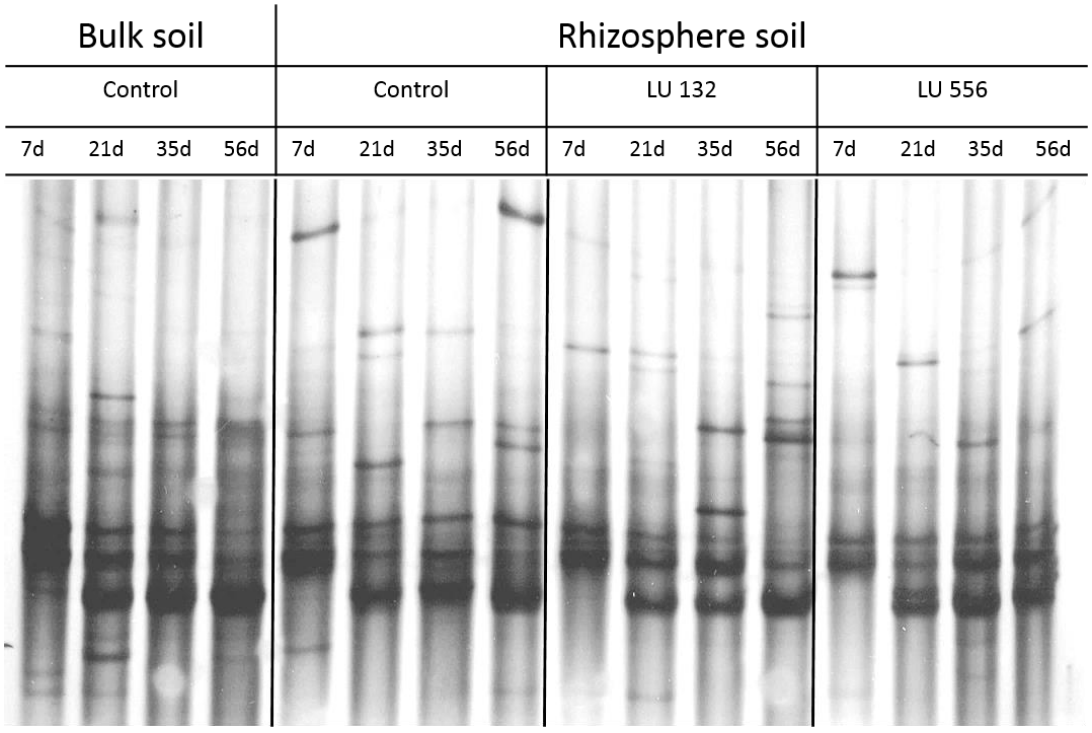

Figure 5-7 Arbuscular mycorrhizal fungi 18S rRNA-denaturing gradient gel electrophoresis (DGGE) profile in bulk and rhizosphere soils of 7, 21, 35 and 56 day old sweet corn plants untreated (control) and treated with *T. atroviride* LU132 and *T. virens* LU556.

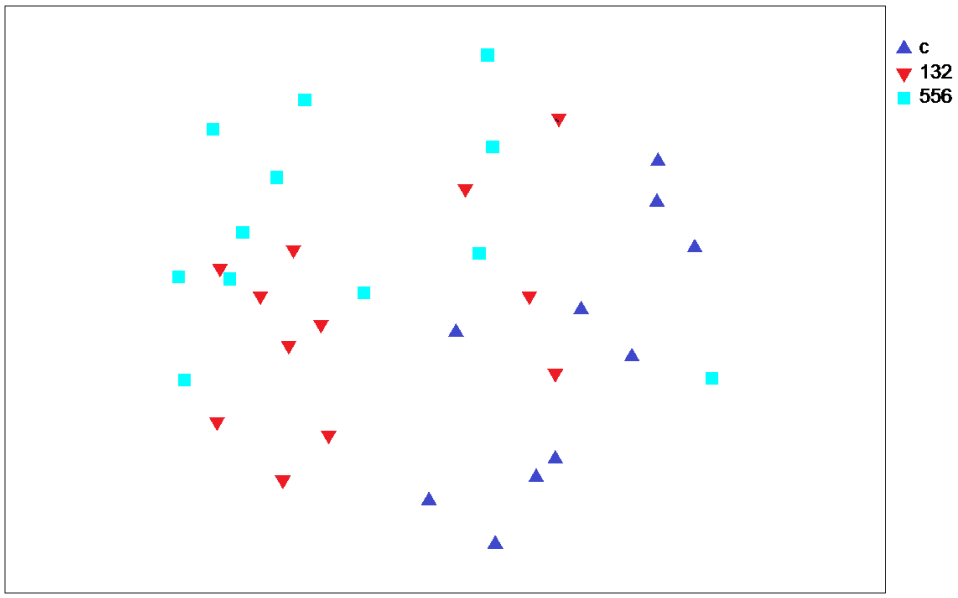

Figure 5-8 Non-metric multidimensional scaling (MDS) plot of arbuscular mycorrhizal fungal communities in the rhizosphere soil of 7, 21, 35 and 56 day old sweet corn plants untreated (c) and treated with *T. atroviride* LU132 (132) and *T. virens* LU556 (556). Ordination was performed with twelve and ten samples per treatment and control, respectively.

5.3.4.2 Bacterial communities

Bacterial composition differed significantly between the set-up, bulk and rhizosphere soils across all treatments and assessment times (PERMANOVA Table Appendix D3; Figure 5-8). *Trichoderma atroviride* LU132 affected bacterial communities in the rhizosphere compared with untreated control (P = 0.0047). Similarly, *T. virens* LU556 affected (P = 0.0666) rhizosphere bacterial communities compared with the control. The rhizosphere soil of plants treated with *T. atroviride* LU132 and *T. virens* LU556 were not different from each other in their bacterial composition (P = 0.2259) (PERMANOVA Table Appendix D3; Figure 5-9). There were no differences between *Trichoderma* treatments or untreated controls in the bulk soil. There were 71, 64 and 62 species in the setup, rhizosphere and bulk soils, respectively with species richness not significantly different to each other.

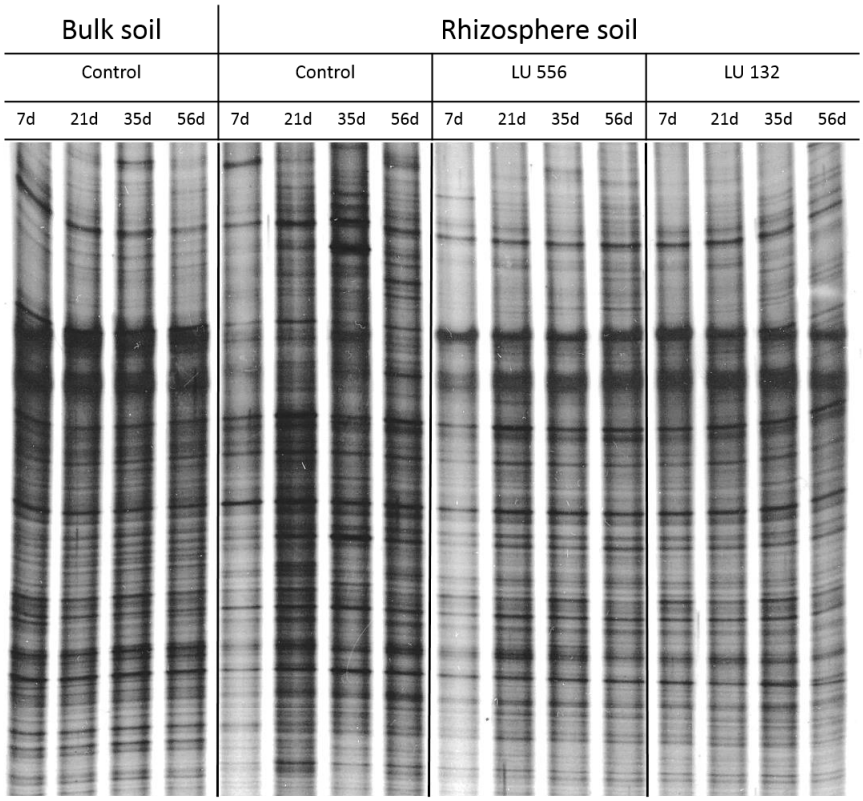

Figure 5-9 **Bacterial 16S rRNA-denaturing gradient gel electrophoresis (DGGE) profile in bulk and rhizosphere soils of 7, 21, 35 and 56 day old sweet corn plants untreated (control) and treated with *T. atroviride* LU132 and *T. virens* LU556.**

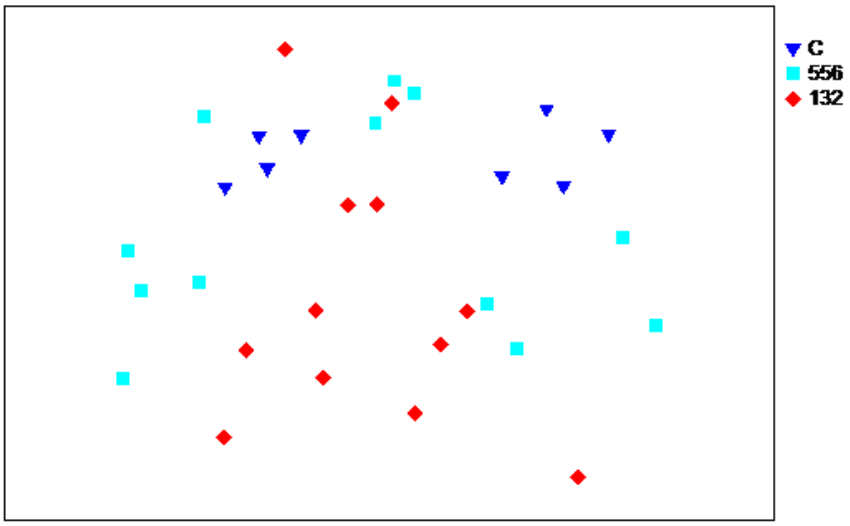

Figure 5-10 **Non-metric multidimensional scaling (MDS) plot of bacterial communities in rhizosphere soil of 7, 21, 35 and 56 day old sweet corn plants untreated (c) and treated with *T. atroviride* LU132 (132) and *T. virens* LU556 (556). Ordination was performed with twelve and ten samples per treatment and control, respectively.**

5.3.4.3 **Fungal communities**

Fungal community composition differed significantly between the set-up, bulk and rhizosphere soils across all treatments and assessment times (PERMANOVA Appendix D4; Figure 5-10). In the rhizosphere soil, *T. atroviride* LU132 affected the fungal community compared with the untreated control (*P* = 0.0038). *Trichoderma virens* LU556 also affected the rhizosphere community (*P* = 0.0919) compared with the control. The fungal community in the rhizosphere of *T. atroviride* LU132 and *T. virens* LU556 treated plants differed between each other (*P* = 0.0856) (PERMANOVA Appendix D4; Figure 5-11). There were no differences between treatments or controls in the bulk soil. Richness of the set-up, bulk and rhizosphere soils were respectively 33, 38 and 41 and were not significantly different to each other (*P = 0.81*).

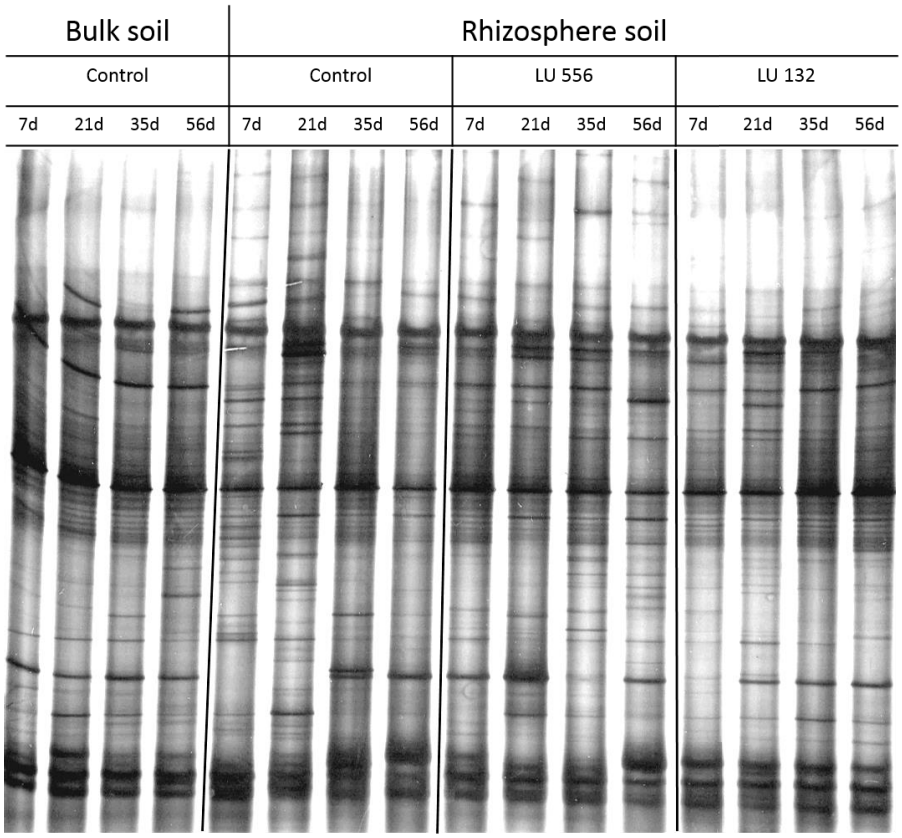

Figure 5-11 Fungal 18S rRNA-denaturing gradient gel electrophoresis (DGGE) profile in bulk and rhizosphere soils of 7, 21, 35 and 56 day old sweet corn plants untreated (control) and treated with *T. atroviride* LU132 and *T. virens* LU556.

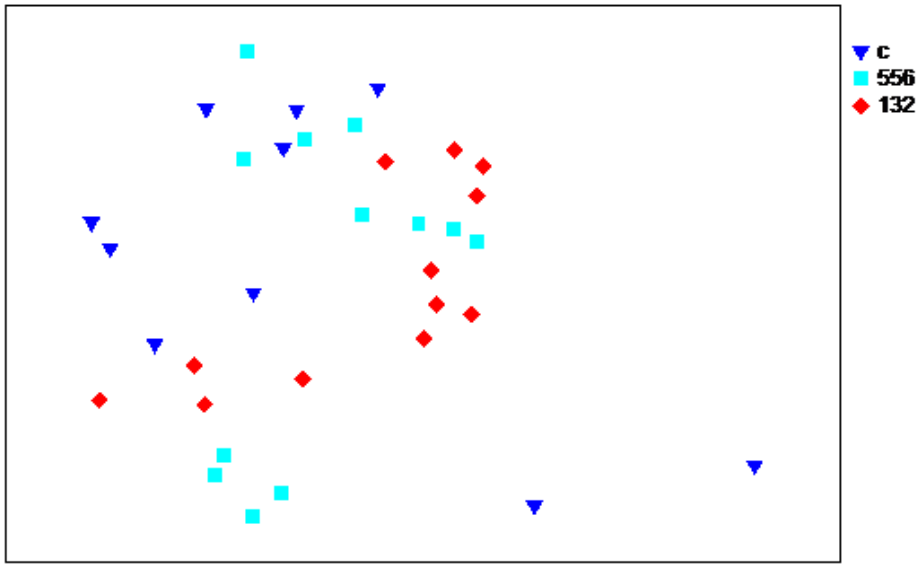

Figure 5-12 Non-metric multidimensional scaling (MDS) plot of fungal communities in rhizosphere soil of 7, 21, 35 and 56 day old sweet corn plants untreated and treated with *T. atroviride* LU132 and *T. virens* LU556. Ordination was performed with twelve and ten samples per treatment and control, respectively.

5.4 Discussion

5.4.1 Rhizosphere competence

The aim of this chapter was to compare the ability of two isolates of *Trichoderma*, *T. atroviride* LU132 and *T. virens* LU556, to colonise sweet corn roots over time. The isolates were chosen on the basis of the rhizosphere colonisation that they had demonstrated in Chapter 2. *Trichoderma atroviride* LU132 was a strongly rhizosphere competent isolate, whereas, *T. virens* LU556 was a weakly rhizosphere competent isolate. However, these measurements were based on assessments done at 7 days post planting only. LU132 also has a distinctive morphology and it was often noted that this morphology was the only one recovered from plants inoculated with this isolate. Thus, the number of LU132 recovered from the rhizosphere is likely to be higher than what is presented here. The results presented in this chapter confirmed that *T. atroviride* LU132 is more rhizosphere competent than *T. virens* LU556 over the 56 day experimental period, however, both isolates showed variation in their colonisation patterns over time. *Trichoderma virens* LU556 populations slowly increased throughout the 56 days, whereas *T. atroviride* LU132 populations increased until 35 days and then stabilised.

For all treatments, including the control, *Trichoderma* colonisation was shown to increase over time. This was expected as *Trichoderma* species are known to have many ecological niches but are mainly known to be biotrophic and saprotrophic (Atanasova 2014). *Trichoderma* species are also stimulated by the presence of plants, due to the release of exudates as previously discussed. Thus, it is likely that as the plants grew and produced a greater surface area, this ecological niche was then occupied by *Trichoderma* species.

In the early stages of plant growth there was isolate variability, with *T. atroviride* LU132 demonstrating more rhizosphere competence than *T. virens* LU556. This was consistent with previous experiments. *Trichoderma atroviride* LU132 populations rapidly increased in the rhizosphere compared to *T. virens* LU556 which showed a much slower colonisation pattern. *Trichoderma atroviride* LU132 is known to be an early and abundant coloniser of the rhizosphere and in doing so is likely to displace the native microbial population both by utilising soil nutrients/plant exudates making them unavailable to other microorganisms and also by secreting compounds that influence soil microbial communities. *Trichoderma* species and isolates are known to produce different chemical compounds, such as pyrones, polyketides and mycotoxins with antimicrobial and antifungal properties.

The Wakanui silt loam soil used for the assessment in this chapter was collected from the same paddock where soil for previous experiments had been collected. However, soil collection for this experiment was done in winter time compared to summer time for other work in this thesis.
Soils collected in different seasons may have harboured different populations of microorganisms that could have affected the two *Trichoderma* isolates. Elnaghy et al. (2014) reported seasonality for *Pythium* species with higher number of isolates recorded in spring followed by winter and summer. Nevertheless, *T. atroviride* LU132 was more rhizosphere competent than *T. virens* LU556 and both had populations similar to those found in previous experiments. This showed that *T. atroviride* LU132 was consistently more rhizosphere competent than *T. virens* LU556 on sweet corn at 7 days, up to 56 days, and in soils collected at different times of the year.

One of the interesting observations was that populations of *T. virens* LU556 increased in the rhizosphere over time. It is possible that growing plants inoculated with *T. atroviride* LU132 and *T. virens* LU556 for longer periods (>56 days) may have produced different results. Although still significantly different at 56 days it was clear that *T. virens* LU556 populations were still growing and may have eventually matched *T. atroviride* LU132 populations.

The rhizosphere populations of *T. atroviride* LU132 appeared to plateau after 35 days. The known bioactivity of *T. atroviride* LU132 may allow it to be an early coloniser, which may be important for biological control against some pathogens, and *T. virens* LU556 may be a late coloniser. Alternatively, both isolates would have ultimately reached a population number that they could have maintained for several months. Harman (2000) showed that *T. harzianum* T22 can persist in the rhizosphere of maize roots at populations of 10^5/g dry weight of roots for over 6 months. In contrast, McLean et al. (2005) followed the proliferation of *T. atroviride* LU132 (C52) introduced as a pellet or solid substrate in an onion field trial and found that populations were variable and experienced successive increases and decreases during the 19 weeks assessment period of the field trial.

5.4.2 Growth promotion

Growth promotion was measured for plants treated as seed with *T. atroviride* LU132 and *T. virens* LU556 over 56 days to determine if rhizosphere competence correlated with growth promotion and whether there was a difference over time between the isolates. Results showed that none of the isolates promoted growth in the early stages of the experiment up to 21 days. This is in agreement with the measurements of growth promotion at 7 days in the previous chapters. *Trichoderma atroviride* LU132 did not promote growth and appeared to have reduced shoot and root lengths. This is not in agreement with Maag et al. (2014) who showed that oilseed rape plants inoculated with *T. atroviride* LU132 had significantly larger shoot and root biomass than controls 49 days after sowing. However, this was in potting mix, and not in soil as in the current experiment, and it is likely that the microbial community was substantially different from normal soil communities and additionally in a different plant system. In addition, no growth promotion of another brassica species, cabbage, by *T. atroviride* LU132 was seen in Chapter 3.

Nevertheless, although in the current experiment *T. atroviride* LU132 reduced shoot and root lengths, this did not necessarily prove that the isolate could negatively affect plant health for two reasons. Firstly, shoot and root dry weights were not negatively affected by the inoculation of *T. atroviride* LU132 and these are better indicators of plant growth promotion than lengths alone. Indeed measuring only length does not provide any indication of diameter or water content in the plant material and in some cases aerial parts may be tall but thin. Secondly, shoot and root ratios were not negatively affected by *T. atroviride* LU132. The root ratio for plants inoculated with *T. atroviride* LU132 suggested heavier root weights indicating perhaps wider roots or presence of more lateral roots despite the shorter length compared to untreated controls and plants inoculated with *T. virens* LU556. It was concluded therefore that *T. atroviride* LU132 did not affect plant health.

Results showed that plants inoculated with *T. virens* LU556 had better growth response than those inoculated with *T. atroviride* LU132. Moreover, *T. virens* LU556 increased the root ratio. This is promising for isolate *T. virens* LU556, however this would need to be confirmed in an experimental set up where growth promotion is the main focus of the research and where only one seed is sown per pot. In this work, each pot had four seedlings growing together and while roots did not touch each other in the first 21 days, at the latter assessment times, roots were entangled indicating that there may have been competition between the plants affecting each others development.

Results of the present work show no positive correlation between rhizosphere competence and growth promotion in sweet corn as the more rhizosphere competent isolate did not promote growth whilst the least rhizosphere competent one did. However, only two isolates were used and to determine whether this was the case the experiment would need to be repeated using more isolates, or isolates varying in rhizosphere competence. It is likely that some isolates are capable of promoting growth under certain conditions whereas others are not. This is likely to be affected by plant host, edaphic, environmental factors and the presence of microbial communities in the rhizosphere. The results here demonstrate that those *Trichoderma* isolates which are capable of promoting growth do not need to be highly rhizosphere competent and those isolates which are rhizosphere competent are not necessarily able to promote growth.

5.4.3 Endophytic assessment

Results from the plating of surface sterilised material suggested that *T. atroviride* LU132 is able to endophytically colonise sweet corn to a higher level than *T. virens* LU556. Nonetheless, both *T. atroviride* LU132 and *T. virens* LU556 demonstrated endophytic ability in shoots and roots of sweet corn up to 56 days. *Trichoderma* species were also found as endophytes in control plants, but this was expected as there are naturally occurring strains capable of becoming endophytes in non-sterile soil. Moreover, *Trichoderma* species are proven to be endophytes on a wide range of plants (Harman 2006).

Trichoderma colonies were more often isolated from shoots and roots of plants treated with *T. atroviride* LU132 and *T. virens* LU556 than from control plants. This is likely to have been affected by both rhizosphere competence of the isolates and the quantity of *Trichoderma* applied to the seed. These aspects could be tested by applying *Trichoderma* at different quantities to the seed coat. Inoculating a seed with high numbers of a competitive *Trichoderma* strain would form a physical barrier and also increase the number of potential penetration points for that particular *Trichoderma* isolate to gain access to the internal portions of the plant (internal niche).

It would be interesting to determine the colonisation pattern of *T. atroviride* LU132 versus *T. virens* LU556 *in planta*. *Trichoderma atroviride* LU132 may produce more colonisation as hyphal networks internally and have a continuous distribution throughout the tissue. This would allow it to travel throughout the root and emerge into the soil at different sites. This intimate connection with the plant may improve its access to nutrients. In contrast the less frequent colonisation by *T. virens* LU556 may be demonstrated by a more discontinuous presence within the plant. *Trichoderma atroviride* LU132 may also have an advantage over *T. virens* LU556 in occupying the rhizosphere by being an endophyte hence higher rhizosphere competence.

Colonies of *Fusarium* species were observed in roots of plants inoculated with both isolates. *Fusarium* species are known to be highly competitive with *Trichoderma* species (El-Hassan et al. 2013, Kim and Knudsen 2013, Marzano et al. 2013). In this work, it was observed that more *Fusarium* colonies were isolated from plant roots that had been treated with *T. virens* LU556 and fewer colonies were isolated from those that had been treated with *T. atroviride* LU132. The improved rhizosphere and endophytic colonisation by *T. atroviride* LU132 may have reduced internal root colonisation by *Fusarium* soil inoculum. Alternatively, there is the possibility that *T. atroviride* LU132 has mycoparasitic activity compared with *T. virens* LU556 and is able to coil around and feed on the endophytic *Fusarium* as both species were colonising the internal parts of roots.

This could also explain why more *Trichoderma* colonies were isolated from the shoots of plants treated with *T. atroviride* LU132 compared to shoots of plants that had been treated with *T. virens* LU556. In a review, Bailey and Melnick (2013) indicated that, with respect to stem colonisation, endophytic *Trichoderma* species preferred to colonise woody trees and rarely had *Trichoderma* been isolated from shoots or stems of other plants under non-sterile conditions. Nevertheless, *Trichoderma* has been shown to be able to colonise shoots and stems. For example, Harvey and Hunt (2006) showed that *T. harzianum* penetrated pruning-wounded grape canes being recovered 23.4 mm down the cane 4 days after inoculation. Similarly, Sobowale et al. (2011) studied the ability of 10 *Trichoderma* isolates from four species (*T. pseudokoningii*, *T. harzianum*, *T. hamatum* and *T. longibrachiatum*) to persist within maize stems after inoculation using a toothpick method. Results showed that all species moved up and down from the inoculation point after 2, 3, 4, 5 and 6 weeks of inoculation. Among those strains, two were more endophytic than the others. In another study Sobowale et al. (2007) examined the ability of four *Trichoderma* isolates (species *T. pseudokoningii* and *T. harzianum*) to colonize maize stem and persist in the presence of *Fusarium verticilloides*. The work showed that all strains could endophytically colonise the stem of maize when inoculated on their own, however simultaneous inoculations of both pathogen and antagonist improved endophytic growth by *Trichoderma*.

Penetration of the plant roots by the pathogen, in this case *Fusarium*, may have also facilitated the penetration by the more rhizosphere competent *T. atroviride* LU132. Thus, high rhizosphere competence of *T. atroviride* LU132 and its competition with *Fusarium* species may have allowed *T. atroviride* LU132 to become endophytic to a greater degree than *T. virens* LU556.

The results suggest that the more rhizosphere competent isolate (*T. atroviride* LU132) was a better endophyte than the least rhizosphere competent (*T. virens* LU556). This relationship between endophytic ability and rhizosphere competence is unclear and warrants more investigation. It is probable that *T. atroviride* LU132 is a better rhizosphere coloniser and that this may be in part due to its ability to become an endophyte. However, the number of plants and isolates is too few to make conclusions about the relationship between rhizosphere competence and endophytism. Moreover, although it is stated in the literature that epiphytic growth is required to form endophytic associations, there is a lack of studies addressing the relationship between rhizosphere competence and endophytic colonisation.

5.4.4 Impact of *Trichoderma* inoculations on the rhizosphere soil microbial populations

Results showed that bacterial, fungal and arbuscular mycorrhizal fungal populations in the rhizosphere soil of sweet corn plants inoculated with *T. atroviride* LU132 and *T. virens* LU556 were different compared to in the rhizosphere of untreated plants. In contrast, bulk soils of treated and untreated plants were similar in terms of microbial populations. This is probably expected if the *Trichoderma* inoculants are predominantly colonising the rhizosphere, and therefore exerting more effect on the rhizosphere microbial community compared with the bulk soil. Sweet corn seeds were coated with *Trichoderma* spores (10^6 spores/seed) which may have contributed to rhizosphere colonisation and therefore the differences found between treated and untreated rhizosphere soils. This indicated that coating the seed with a particular isolate will have an influence on microbial populations present in the rhizosphere. In contrast the study by McLean et al. (2014) showed that application of the commercial product Tenet (*T. atroviride* LU132) to the root zone of transplanted native seedlings did not result in the non-target impacts on the rhizosphere microbial community. The difference in the method by which *T. atroviride* LU132 was applied might have influenced their interaction with the rhizosphere microbial community through differences in rhizosphere colonisation. Additionally, differences in the plant species used may have also contributed to the differences seen. Gupta et al. (2014) reported that coating seeds of the legume *Cajanus cajan* with different bio-inoculants, including *Trichoderma harzianum* MTCC 801, differentially influenced the soil microbial community assessed using T-RFLP analysis.

However, *T. harzianum* inoculation had no effect on the total population size of two specific microbial groups, actinomycetes or β-proteobacteria. In the current study, the rhizosphere microorganisms' populations were not sequenced, therefore it is difficult to interpret the result and conclude on a positive or negative influence of *Trichoderma*. Depending on the microorganisms living in the sweet corn rhizosphere, synergistic, mycoparasitic or competitive relationships may have been established by the *Trichoderma* isolates.

The results also showed that the inoculum rather than time had an influence on rhizosphere microbial populations. Although for both *Trichoderma* isolates, populations increased in the rhizosphere with time, their effect on the rhizosphere microbial community was seen after the first assessment (7 days) and did not change thereafter. In contrast, incorporation of a *Trichoderma atroviride* isolate into two soils was reported by Cordier and Alabouvette (2009) to slightly alter the soil microbial community initially, but microbial communities did not differ at later sampling dates. However, the assessments were not based on changes in rhizosphere community as no plants were included in their study. The results of the current study indicated that the minimum antagonist level resulting in influences on the rhizosphere microbial community was achieved early in the growth of sweet corn plants for both *Trichoderma* isolates.

Richness of bacterial and fungal populations in the bulk and rhizosphere soils of untreated and treated plants did not differ between each other. In contrast the richness of arbuscular mycorrhizal populations in bulk and rhizosphere soils of untreated and treated plants were different. This result suggested competition between arbuscular mycorrhizal fungi and *Trichoderma* isolates. Arbuscular mycorrhizal fungi colonise the space in and around the rhizosphere producing hyphae which radiate out into the surrounding soil. They also penetrate the plant through infection sites in the root surface in the same way as *Trichoderma* isolates are believed to do. Both *Trichoderma* and arbuscular mycorrhizal fungi occupy the same niche in the rhizosphere and it is expected that they would compete for nutrients. There are reports of synergistic and negative interactions between the two fungi (Brimmer and Boland 2003, McLean et al. 2014, Cordier and Alabouvette 2009).

There were more arbuscular mycorrhizal species in the bulk soil of *T. atroviride* LU132 treated plants than in the bulk soil of untreated and *T. virens* LU556 treated plants. This is likely to be linked to the higher rhizosphere colonisation by *T. atroviride* LU132 compared to *T. virens* LU556 which limited colonisation of the roots by arbuscular mycorrhizal excluding these to the bulk soil.

The results in this chapter indicated that temporal colonisation dynamics of *T. atroviride* LU132 and *T. virens* LU556 in the rhizosphere of sweetcorn differed, with *T. atroviride* population levels plateauing after 35 days whilst *T. viride* population appeared to be increasing. Whether this was related to differences in spatial colonisation by the two isolates, with *T. viride* preferentially colonising more mature roots or roots at different soil depths compared with *T. atroviride* was not investigated and will be determined in the next chapter.

6

Rhizosphere competence and endophytic colonisation by *Trichoderma* isolates *T. atroviride* LU132 and *T. virens* LU556 at different root depths on *Zea mays*

6.1 Introduction

Petrini (1991) defined endophytes as "all organisms inhabiting plant organs which, at some time in their life, can colonize internal plant tissues without causing apparent harm to their host". Some strains of Trichoderma are capable of establishing a close relationship with plants, live on the rhizoplane and penetrate it to a few cells below that level and become endophytes (Harman et al. 2004a). The biological control activity of Trichoderma species has been linked to their ability to endophytically colonise plants and their rhizosphere. Endophytic *Trichoderma* are thought to be able to promote growth and induce resistance (Shoresh et al. 2010) and therefore have been the object of several recent studies. For example, Hoyos-Carvajal et al. (2009a) assessed rhizosphere competence and endophytic colonisation of Trichoderma isolates from Colombia and evaluated the relationship of these to growth promotion of bean seedlings at an early stage. The authors found that rhizosphere competence and endophytic colonisation was linked to growth promotion, however this study did not address root depth and few actually do.

Studies where rhizosphere competence at different root depths was assessed in non-sterile conditions have shown that Trichoderma isolates colonise roots in a C shape fashion where middle segments rhizosphere soil harbour less CFU/g of rhizosphere soil than top and tip segments (Ahmad and Baker 1987a, Sivan and Chet 1989). However these studies assessed rhizosphere competence at depths up to 12 cm maximum. Other researchers have found Trichoderma species in the rhizosphere of plants up to 15 and 22 cm depth (Sivan and Harman 1991, McLean et al. 2005). Other work in sterile conditions has shown that *Trichoderma* can grow in the rhizoplane up to 12 cm (Singh and Kumar 2012a). Bourguignon (2008) reported that one isolate of each *T. atroviride*, *T. hamatum*, *T. harzianum* and *T. koningii* varied in their ability to colonise the rhizosphere of 6 week old onion at different depth (top 1-3cm and 6-9 cm) when applied as a mixed inoculum. *Trihcoderma hamatum* was seen to preferentially colonise the top section whilst *T. atroviride* was more dominant in the lower section. *Trichoderma koningii* and *T. harzianum* were found to equally colonise the rhizosphere at both depths.

In previous chapters, rhizosphere competence had been analysed taking into account the whole root system. Endophytic colonisation had been assessed and considered positive when more than one colony was found in a root without recording the specific location along the root. In the literature, some isolates were shown to be different at colonising root depths. Therefore, in order to complete the study of rhizosphere competence and endophytic colonisation, a 35 day long sweet corn assay was designed.

The aim of this experiment was to compare the rhizosphere colonisation and the endophytic ability of two *Trichoderma* species with different degrees of rhizosphere competence at different root depths. Another aim, was to analyse the effect of seed inoculation with *Trichoderma* isolates on the rhizosphere AMF communities at different depths, since these were the most affected communities in the previous experiment.

6.2 Materials and methods

6.2.1 Experimental design

The assay was carried out using 70 cm high PVC pipes (10.5 cm diameter). The pipes were cut lengthwise to facilitate the access to plant roots and rhizosphere soil at harvest time after 35 days post planting. The length of the assay was chosen according to the results from the previous experiment. In the temporal study of rhizosphere competence, the highest difference in root colonisation (log_{10}CFU/g DRS) between a high rhizosphere competent isolate (*T.atroviride* LU132) and a low rhizosphere competent one (*T. virens* LU556) was recorded after 35 days. The pipe halves were joined together by applying two strips of duct tape longitudinally. A double layer of cheese cloth was taped across the outside of one end to form the bottom and to retain the soil. 18 pipes were filled to the rim with Wakanui silt loam soil (passed through a shredder as described in Section 5.2.3) previously moistened to 20% soil moisture content. The soil was analysed by Hills laboratories prior to the experimental set up (Appendix E1). The pipes were placed in three randomised blocks in a growth room under the experimental conditions used for previous experiments (Section 5.2.4; Figure 6-1A). Each block contained six pipes, two pipes per isolate and control. Two isolates, *Trichoderma atroviride* LU132 and *Trichoderma virens* LU556 were used in this experiment. Inocula were prepared as described in the previous chapter (Section 5.2.1). Isolates were applied to sweet corn seeds (Chieftain F1) following the same procedure described in the previous chapter (Section 5.2.2). Untreated control seeds consisted of sweet corn seeds coated with a solution of methyl-cellulose as described in previous chapters (Section 5.2.2). Five seeds per pipe were sown. A plastic bag with a zip lock system was placed over the top of each pipe to maintain the soil moisture for one week. After emergence, the plants were thinned to one plant per pipe.

Three pipes per block, one per isolate and one untreated control treatment, were harvested 35 days after sowing and used for rhizosphere competence and endophytic colonisation assessments. Four plants were also harvested to assess the effect of rhizosphere soil processing method on subsequent endophytic colonisation. The remaining plants were harvested at 40 days after sowing and used for microscopic observations.

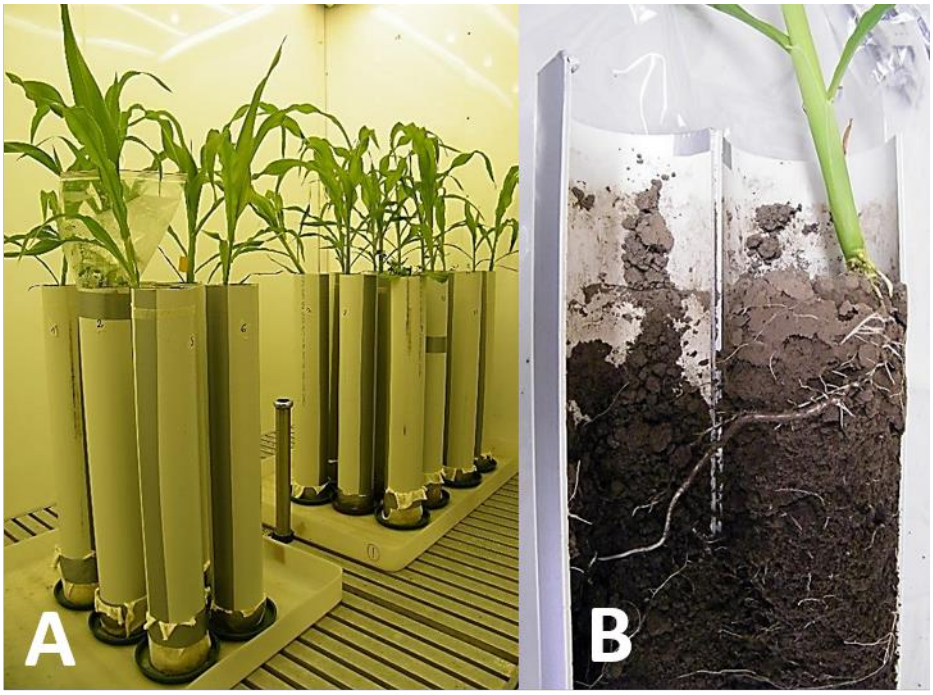

Figure 6-1 A: Pipes (70 cm long) with 35 days old sweet corn plants grown in non-sterile soil in a growth room. B: Cut open pipe showing the top part of a 35 day old sweet corn root system.

Two roots per plant were cut from the crown (Figure 6-1B). Adhering soil was shaken off. Each root was divided into three segments of equal length irrespective of the root length. The majority of plants' roots used for this assessment were 60 cm long. A 16 to 17 cm long portion from the top of each segment was collected, weighed and placed in a sterile 50 mL plastic tube and stored at 4°C until further processing. The following day, 10 to 20 mL of a 0.5% Triton X 100 solution was added to each plastic tube. Tubes were subsequently shaken for 30 min at 94 rpm in an orbital shaker (Ratek EOM5, Ratek Intruments PTY Ltd, Boronia, Australia). As in previous experiments, the suspensions were serially diluted to 10^{-1}, 10^{-2} and 10^{-3}. Aliquots (200 µL) of the initial suspension and each dilution were plated onto each of three replicate *Trichoderma* selective medium (TSM) plates. After the plating, the left over Triton X 100 solution containing the rhizosphere soil was stored at -80°C for DGGE analysis. Plates were incubated at 20˚C in the dark. The number of *Trichoderma* colonies was counted at 10 to 13 days after incubation and the number of *Trichoderma* colony forming units (CFU)/gram of dry rhizosphere soil (DRS) was calculated.

6.2.2 Rhizosphere soil arbuscular micorrhizal fungi community analysis

Rhizosphere soil solutions previously stored at -80˚C after the rhizosphere competence assessment were thawed. Nine samples in total were analysed for microbial communities. Samples from all top root samples per treatment (n=6) were mixed together in a tube and centrifuged to obtain the rhizosphere soil. The same procedure was followed to obtain the total rhizosphere soil of all middle (n=6) and bottom (n=6) samples. DNA extraction, PCR and DGGE was performed as outlined in Chapter 5 (Section 5.2.8).

6.2.3 Endophytic assessement

Shoot and roots were analysed to assess Trichoderma endophytic colonisation in plants treated with either of the two Trichoderma isolates or untreated. Following the soil dilution plating to assess rhizosphere competence, the same root segments were used to assess endophytic colonisation. Root segments were first washed under tap water to remove any rhizosphere soil adhering after the Triton wash. Root segments were subsequently sterilised in a 50% a.i. bleach (5.25 g/L available chlorine) bath for 3.5 min followed by two 2 min SDW baths. Root segments were then cut into 1 cm pieces with a sterile scalpel blade in a laminar flow unit. After being cut, the segment pieces were plated on Trichoderma selective media and incubated in the dark at 20°C for 5 to 8 d. Shoots were processed in the same way, however, only the first 10 to 12 cm from the crown to the first leaf collar were plated onto one Petri dish. Each segment piece was given a number (1 to 16) starting from the crown of the root to the tip. The presence of colonies was recorded for each segment.

6.2.4 Microscopic observation

Microscopic observations of shoots and roots of 35 day old untreated and treated sweet corn plants were made in order to understand the way *Trichoderma* isolates colonised roots. The aim was to investigate the morphologies present on the rhizoplane.

Three plants in total (one per treatment) were used for the microscopic observation. These were harvested 40 days after being sown. Shoots were cut from the roots at the crown level. Both parts were washed under tap water. Cross and longitudinal sections were cut and prepared to be observed under the fluorescent microscope following the methods of (Tate 1981, Williamson et al. 1995, Hood and Shew 1996) and modified by Anusara Herath Mudiyanselage (PhD student). Specimens were first fixed in Carnoy solution (ethanol, chloroform and glacial acetic acid, 6:3:1, v/v/v) for 24 hours for shoots and 40 min for roots. Following fixation, specimens were softened and cleared by adding them to individual 1.7 mL tubes containing 0.6 mL of a 1 M NaOH (sodium hydroxide) solution. Tubes were left in a water bath at 60°C for 1 hour. Samples were subsequently rinsed with SDW.

Prior to observation under the microscope, samples were mounted on a slide, excess water was blotted with a tissue paper and a 150 µL of 0.1% aniline blue (freshly prepared in 0.1 M K_2HPO_4) solution was pipetted on top of each sample. To improve the contrast, slides with samples were kept at 4°C overnight before observation.

Aniline blue has been used in the observation of plant fungal interactions. The dye has a fluorochrome which binds with various glucans and polysaccharides present in plants (callose) and fungi from different subdivisions such as Ascomycotina and Basidiomycotina.

6.2.5 Statistical analysis

6.2.5.1 Rhizosphere competence

The mean number of *Trichoderma* CFU per treatment and segment was averaged for each plant. These means were log_{10} transformed and statistically analysed using a general analysis of variance (ANOVA) in GenStat 15th edition (VSN International, Hertfordshire, UK) with treatment means compared using Fishers unprotected LSD at $P = 0.05$.

6.2.5.2 Microbial community analysis

The presence of bands on the DGGE gel images were manually scored in a matrix using Phoretix (TotalLab Ltd, Newcastle upon Tyne, UK). A presence absence matrix was generated with the same software. The low number of samples did not allow for an abundance analysis of microbial communities in each root segment. However, an UPGMA (Jaccard Unweighted Pair Group Method with Arithmetic Mean) dendogram showing clustering of samples was obtained in Phoretix. Species richness of samples was analysed with ANOVA performed in Genstat 15th edition (VSN International, Hertfordshire, UK).

6.3 Results

6.3.1 Rhizosphere competence of *T. atroviride* LU132 and *T. virens* LU556

Trichoderma populations in the rhizosphere soil recovered from top, middle and bottom sections of the roots in the untreated control were 1.07 x 10^4, 4.79 x 10^3 and 1.41 x 10^3 CFU/g DRS, respectively (Table 6-1; Figure 6-2). These were lower than the populations recovered from the *Trichoderma* treated plant roots but were not included in the ANOVA analysis. *Trichoderma* populations in the top, middle and bottom root portions from *T. atroviride* LU132 treated plants were 2.45 x 10^6, 2.14 x 10^4 and 9.77 x 10^4 CFU/g DRS, respectively. The *Trichoderma* population in the top root section were significantly higher than those from the middle and bottom sections. There were no differences in the *Trichoderma* population in the middle and bottom root sections for *T. atroviride* LU132 treated plants.

For LU556 treated plants, the highest number of Trichoderma CFU/g DRS was recovered from the rhizosphere of the top part of roots (1.62 x 10^5 CFU/g DRS) and was significantly higher than the middle (1.58 x 10^3 CFU/g DRS) and bottom (2.09 x 10^3 CFU/g DRS). Significantly higher *Trichoderma* populations were recovered from the rhizosphere soil at all root depths for *T. atroviride* LU132 treated plants compared with LU556.

Table 6-1 ***Trichoderma*** **population (log10 CFU/g dry rhizosphere soil (DRS)) recovered from the top, middle and bottom root portions of 35 days old sweet corn plants untreated and treated with *T. atroviride* LU132 and *T. virens* LU556. Values with the same letter are not significantly different within the same column. *T. atroviride* LU132 and *T. virens* LU556 populations were significantly different at all depths.**

	CFU/g DRS		
Segment	Control	LU132	LU556
Top	1.07 x 10^4	2.45 x 10^6	1.62 x 10^5
	(4.03)	(6.39) a	(5.21) a
Middle	4.79 x 10^3	2.14 x 10^4	1.58 x 10^3
	(3.68)	(4.33) b	(3.20) b
Bottom	1.41 x 10^3	9.77 x 10^4	2.09 x 10^3
	(3.15)	(4.99) b	(3.32) b
LSD 5%	1.082		

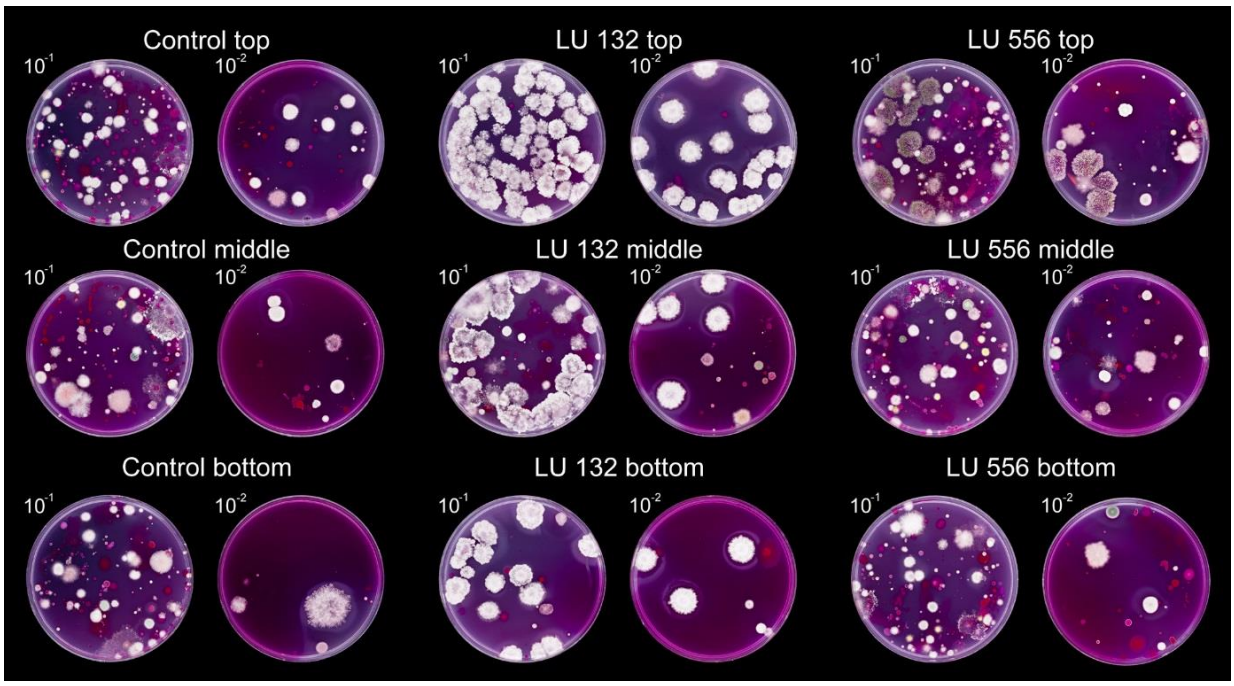

Figure 6-2 Agar plates from top, middle and bottom segments of 35 day old sweet corn plant roots grown individually in pipes. Dilutions 10^{-1} and 10^{-2} are presented for control and plants treated with *T. atrovirirde* LU132 and *T. virens* LU556 isolates.

6.3.2 Arbuscular mycorrhizal fungi community analysis

Four main groups (I to IV) were distinguished at ~60% similarity (Figure 6-3A and B). The AMF community in the rhizosphere associated with control plants clustered together in group II with the AMF population from the top of the root of plants treated with *T. virens* LU556. The AMF populations in the bottom of the *Trichoderma* treated plants clustered together (Group I). The AMF populations from the middle of *Trichoderma* treated roots and the top of the *T. atroviride* LU132 treated roots were clustered in groups III and IV. No AMF communities were >80% similar to each other. There were no significant differences in species richness according to root depth for arbuscular mycorrhizal fungi.

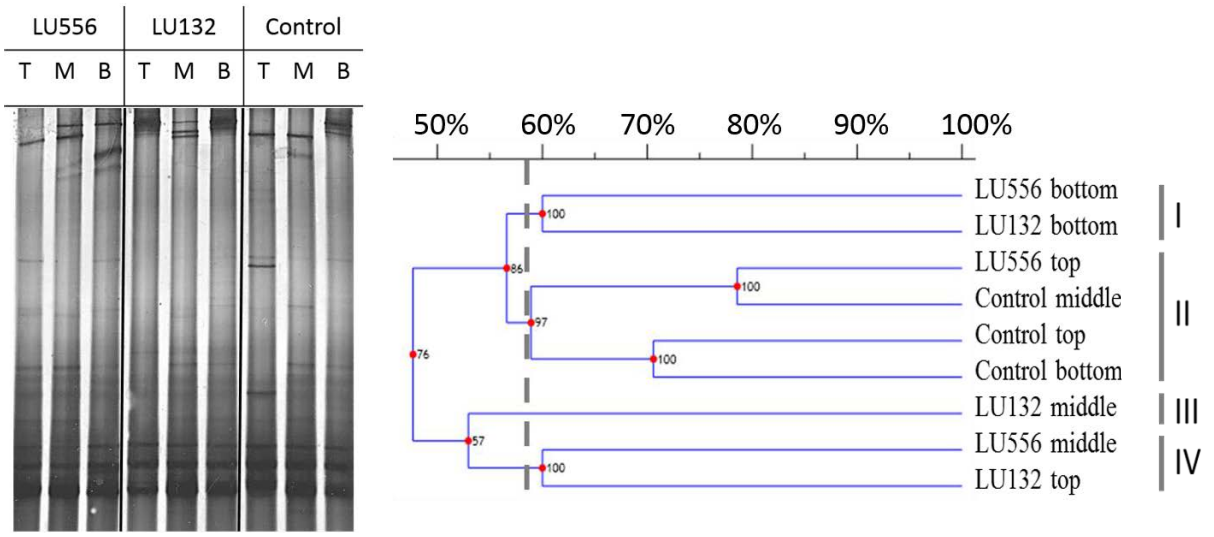

Figure 6-3 A: Arbuscular mycorrhizal fungal 18S rRNA-denaturing gradient gel electrophoresis (DGGE) profile in the top (T), middle (M) and bottom (B) rhizosphere soils of 35 day old sweet corn plants, untreated (Control) and treated with *T. atroviride* LU132 and *T. virens* LU556. B: Arbuscular mycorrhizal fungal unweighted pair group method of analysis (UPGMA-Jaccard coefficient) dendogram from different depths of rhizosphere soil collected from 35 days old sweet corn plants' roots untreated and treated with *T. atroviride* LU132 and *T. virens* LU556.

6.3.3 Endophytic colonisation by *T. atroviride* LU132 and *T. virens* LU556

No *Trichoderma* colonies or colonies from other fungal species were isolated from the shoots. Data from roots is presented below in the form of tables for each root portion. No *Trichoderma* colonies were recovered from the top portion of control plants' roots (Table 6-2). Colonies morphologically identified as *Fusarium* species (Mark Braithwaite) were isolated from two plants. Control plant 3, had *Fusarium* species in all root pieces (14). From the top part of *T. atroviride* LU132 treated plants' roots, *Trichoderma* colonies were isolated from 39 out of the 90 root pieces plated and were recovered from five of the six plants assessed. Fusarium colonies were also isolated from two plants. *Fusarium* and *Trichoderma* species were isolated from the same root piece in plant 3. For *T. virens* LU556 treated plants, *Trichoderma* colonies were isolated from 8 root pieces of the 92 pieces sampled and were distributed across three plants. *Fusarium* colonies were isolated from 22 pieces across four different plants with *Fusarium* and *Trichoderma* colonies isolated together from one root piece for plant 1. Endophytic *Trichoderma* and *Fusarium* colonies are shown in Figure 6-4.

For the middle root portions (Table 6-3), *Trichoderma* species were isolated from two control plants (a total of 6 root pieces) and one plant treated with *T. virens* LU556 (one root piece). *Fusarium* colonies were isolated from two control and three *T. virens* LU556 treated plants. *Fusarium* and *Trichoderma* species were isolated from one root piece from one control plant. No *Trichoderma* or *Fusarium* colonies were isolated from *T. atroviride* LU132 treated plants (82 pieces examined across 6 plants).

For the bottom root portion for control and plants treated with *T. atroviride* LU132 and *T. virens* LU556 (Appendix E2), *Trichoderma* colonies were isolated from two root pieces in one control plant. Fusarium colonies were isolated from two pieces of one control plant. No *Trichoderma* colonies were isolated from the roots of either *T. atroviride* LU132 or *T. virens* LU556 treated plants. Colonies of *Fusarium* were isolated from one plant inoculated with *T. virens* LU556 (one piece only).

Table 6-2 Isolation of *Trichoderma* (green), *Fusarium* (yellow) and both (blue) from root pieces (1 cm pieces) from the top root segment from plants treated with *T. atroviride* LU132, *T. virens* LU556 or untreated assessed 35 days after sowing. Six plants per treatment were assessed with a maximum 16 root pieces per plant with piece 1 corresponding to the top portion of the root at the crown level. x = 0 colony recovered.

Isolate	Plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Control	1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	3																	
	4				x	x	x	x	x		x	x	x	x	x	x	x	
	5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
LU 132	1		x	x		x	x		x	x	x	x		x	x		x	
	2	x		x		x	x	x								x	x	
	3		x	x	x													
	4	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	5			x	x	x	x	x		x			x		x			
	6		x				x		x	x								
LU 556	1		x					x	x		x		x					
	2		x		x	x	x	x	x	x								
	3		x	x	x			x	x	x	x	x	x	x	x	x	x	x
	4	x		x		x	x		x	x		x	x					
	5	x	x	x	x	x	x	x	x	x		x			x		x	
	6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	

Table 6-3 **Isolation of *Trichoderma* (green), *Fusarium* (yellow) and both (blue) from root pieces (1 cm pieces) from the middle root segment from plants treated with *T. atroviride* LU132, *T. virens* LU556 or untreated assessed 35 days after sowing. Six plants per treatment were assessed with a maximum 16 root pieces per plant with piece 1 corresponding to the top portion of the root segment. x = 0 colony recovered.**

Isolate	Plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Control	1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	3		x	x	x	x	x	x	x	x	x	x	x	x	x		
	4	x	x	x	x	x	x	x	x		x			x	x	x	x
	5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	6						x	x	x		x	x	x	x	x	x	x
LU 132	1	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	2	x	x	x	x	x	x	x	x	x	x	x	x	x			
	3	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	4	x	x	x	x	x	x	x	x	x	x	x	x				
	5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	6	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
LU 556	1		x	x	x	x		x	x			x	x	x	x		
	2		x	x	x	x	x	x	x	x	x		x	x	x	x	
	3	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
	4	x	x	x	x	x	x	x	x		x	x	x	x	x		x
	5	x		x	x	x	x	x	x	x	x	x	x	x	x	x	
	6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

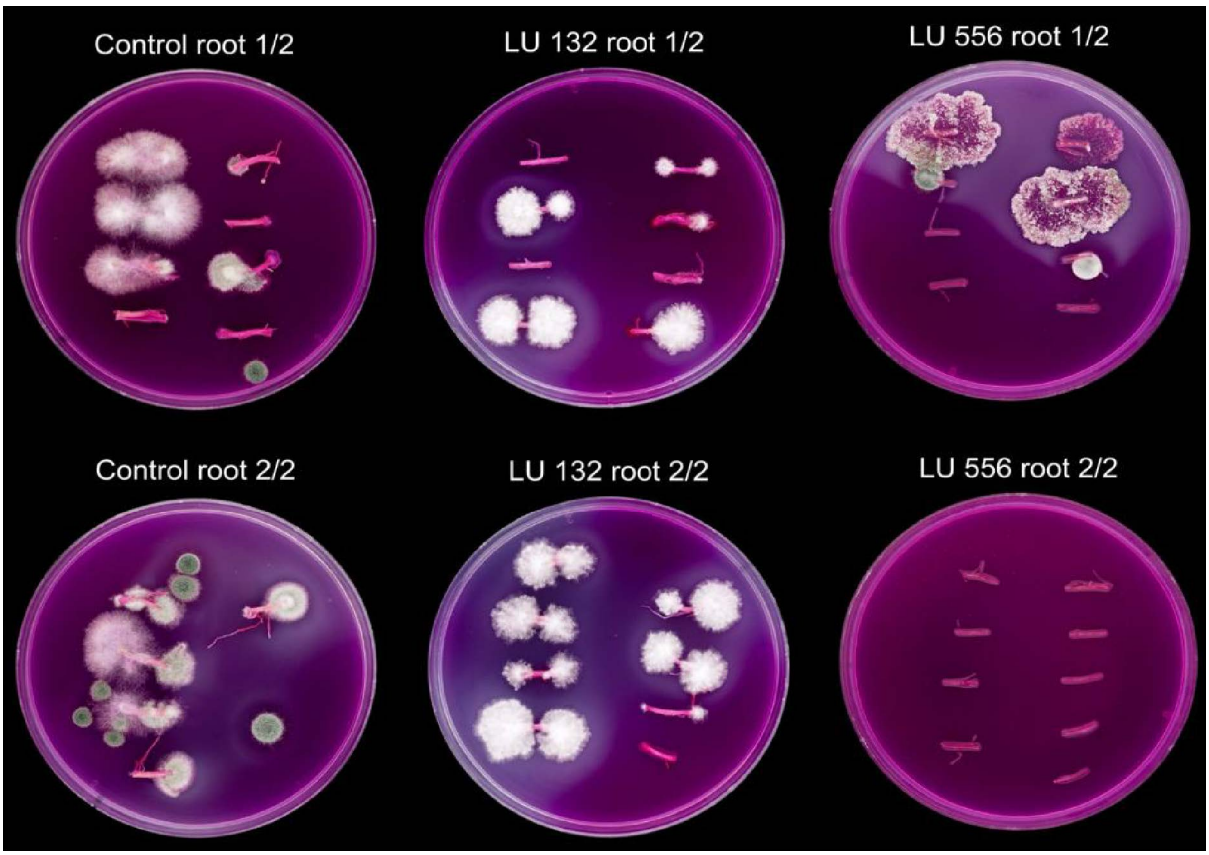

Figure 6-4 Untreated (control) and treated (with isolates *Trichoderma atroviride* LU132 or *Trichoderma virens* LU556) 35 day old sweet corn top root portion segments with endophytic *Trichoderma* and *Fusarium* colonies.

6.3.4 Microscopic observations

Three slides were mounted per treatment and per root portion. The presence of hyphae was recorded (Table 6-4). The hyphae appeared to be discontinuous and in short pieces. It was sparsely seen as only one per slide was seen at best. No branching structures were observed, hyphae grew parallel along the cortex cells and were never observed inside the cells (Figure 6-5). Mycelium was seen in six slides, and when present was always located in the outer layers of the root. Mycelium was mostly seen in the top portion of roots of plants treated with *T. atroviride* LU132. Fluorescent mycelia were observed in three out of three slides. Fluorescent mycelium was also seen in the two middle portions belonging to a control and one *T. virens* LU556 treated plant. Fluorescent mycelium was observed in a root piece from one bottom portion of one *T. virens* LU556 treated plant. Plant strucutres and other root colonisers (AMF) were observed (Figure 6-6).

Table 6-4 **Presence of fluorescent hyphae in the outer cells of top, middle and bottom portions of sweet corn plants grown for 35 days in unsterilized soil and untreated or treated with *Trichoderma atroviride* LU132 and *Trichoderma virens* LU556. Three slides per root portion and treatment were observed.**

Portion	Control	LU 132	LU 556
Top	0/3	3/3	0/3
Middle	1/3	0/3	1/3
Bottom	0/3	0/3	1/3

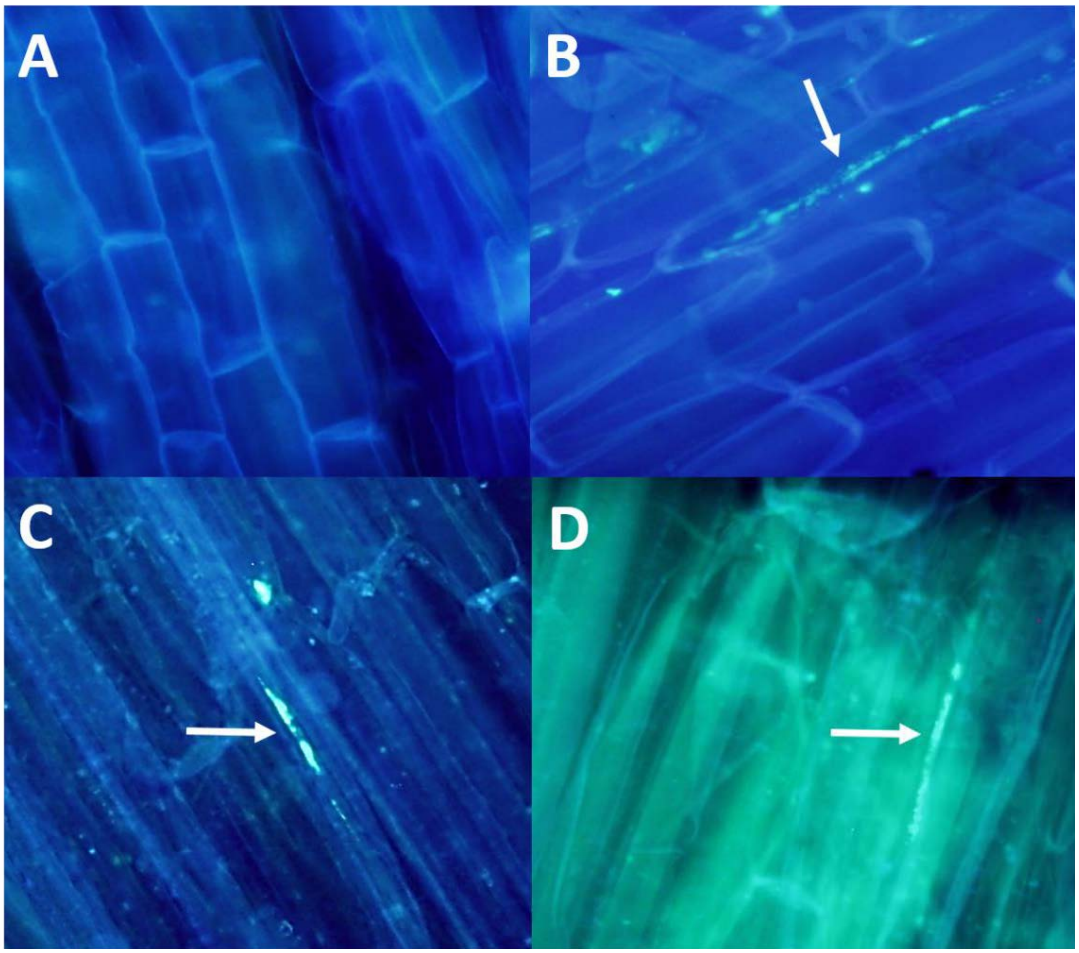

Figure 6-5 **Top and middle portion root cortex cells of 35 day old sweet corn plants inoculated with *T. atroviride* LU132 or *T. virens* LU556. A: Top root portion plant inoculated with LU132 showing no fungal structures. B: Top root portion plant inoculated with LU132. C: Bottom root portion plant inoculated with LU556. D: Middle root portion inoculated with LU556. White arrows indicate hyphal structures.**

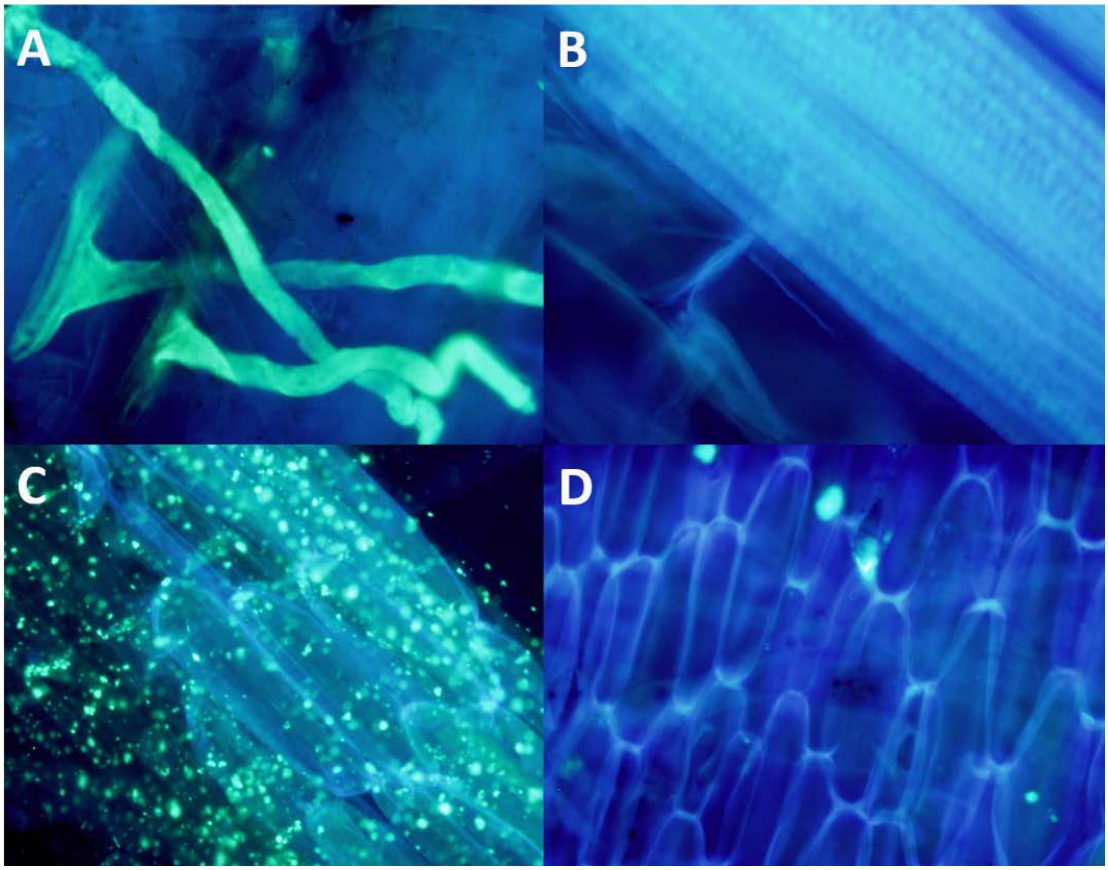

Figure 6-6 Plant structures seen with aniline blue staining during microscopic observations. A: AMF appressorium and hyphae. B: root cylinder. C: apex mucilage and outer cells. D: apex cells. Pictures were taken at 40X.

6.4 Discussion

6.4.1 Rhizosphere competence

This experiment compared the way two *Trichoderma* isolates, *T. atroviride* LU132 (rhizosphere competent) and *T. virens* LU556 (non rhizosphere competent) colonised different root depths of 35 day old sweet corn plants. The results indicated that *Trichoderma* populations were significantly higher in the upper parts of sweet corn roots compared to the middle and bottom root portions regardless of the isolate that had been applied to the seeds.

This result agrees with (Mehrabi-Koushki et al. 2012) who conducted an assay on the ability of different species of *Trichoderma* to colonise the rhizosphere of 15 day old tomato seedlings. They showed that, of the 13 isolates tested, higher *Trichoderma* populations were isolated from the rhizosphere of upper root parts compared to the middle and bottom parts with populations ranging from 1.6×10^7 to 4×10^7 CFU/g root for top-root, 1.7×10^5 to 5.7×10^6 CFU/g root for middle-root and 1.7×10^4 to 2.5×10^6 CFU/g root for tip-root. However, this work was carried out in sterile soil where there would not have been any competition from other microorganism to stop *Trichoderma* isolates to grow and proliferate in the rhizosphere of plants. The lack of competition might have explained the higher populations numbers for the upper, middle and tip root segments compared to this study where *Trichoderma* populations isolated from top, middle and bottom segments averaged respectively: 1.62×10^5 to 2.45×10^6, 1.58×10^3 to 2.14×10^4 and 2.09×10^3 to 9.77×10^4 CFU/g DRS.

In this experiment, both *Trichoderma* isolates responded similarly to increasing root depth. In other work some researchers have shown that there is isolate variation in the depth to which *Trichoderma* isolates can colonise. For example Ahmad and Baker (1987a) studied rhizosphere competence of four isolates on cucumber plants and found that three of the isolates (*T. koningii* T-8, *T. viride* T-S-1 and *T. harzianum* WT) were able to colonise the first 3 cm of cucumber roots compared to isolate *T. harzianum* T-95 which colonised the entire cucumber root. Similarly, Sivan and Harman (1991) showed that isolate *T. harzianum* T22 was better than isolates *T. harzianum* T-95 and T-12 at colonising the entire rhizosphere of 7 day old maize plants. McLean et al. (2005) assessed the ability of *T. atroviride* LU132 (C52) to colonise the rhizosphere of onion plants in a mix of Wakanui silt loam soil and potting mix. They found that after 4, 8, 12 and 16 weeks, *T. atroviride* LU132 could equally colonise the proximal and distal segments of onion roots with populations reaching 7.8×10^1 (proximal segment at 16 weeks) and 1.3×10^2 (distal segment at 16 weeks). However, the root length of plants analysed in these assays varied between 9, 15 and 22 cm which would correspond to the top part only of the sweet corn roots in this work which were up to 60 cm long. Isolate variability was also shown by Singh and Kumar (2012a) who found that only four out of seven *T. harzianum* could colonize the entire rhizoplane (12 cm) of 15 day old *Chrysanthemum* plants while the three other *Trichoderma* isolates were only able to colonise the first four cm from the crown of examined roots. There are no similar assays comparing the ability of different *Trichoderma* isolates to colonise roots to 60 cm depth.

The root structure could explain the vertical distribution of both *Trichoderma* isolates *T. atroviride* LU132 and *T. virens* LU556 on sweet corn. *Trichoderma* species are known to be recruited by the plant to assist with water uptake and help with water deficit (Mastouri et al. 2010, 2012).

During harvest, it was noticed that top, middle and bottom roots had different structures that may have served different purposes during plant development and therefore supported different microbial populations. When the pipes were cut open, the top soil (first 40 cm) had nodal and seminal roots. Nodal roots were thick and woody and seminal roots were numerous, thin, woody and had multiple thin lateral roots. There were no nodal roots in the middle soil and the lateral roots were less abundant than in the top soil. In the bottom soil, fewer roots were present, these were seminal roots that had become thick and turgescent and did not have lateral roots. The watering was done from the bottom mainly and caused the soil in the pipes to be dryer and lighter at the top, humid in the middle and wet and compact at the bottom. Sweet corn plants may have recruited *Trichoderma* to help with water uptake in the top and middle parts where the soil was dryer, by producing multiple lateral roots exuding carbon sources for microorganisms. However at the bottom parts of the pipes, the soil was compacted and wet and resulted in low oxygen availability. Therefore, the few roots present that were gorged with water may have been searching for oxygen instead (most of the plants' turgescent roots had pierced the cheesecloth and were growing in spirals against it outside the pipe) which may have explained why *Trichoderma* was not as present in the bottom roots as it was in the top and middle root parts.

Nevertheless, studies addressing the vertical distribution of *Trichoderma* in soil without the presence of plants have shown that the colonisation pattern is similar to the colonisation of roots presented here. For example, Sariah et al. (2005) assessed the abundance of *Trichoderma* species from different fields in different oil palm ecosystems. They found that across all fields and ecosystems, the two upper soil horizons (A1 and Be, 0-30 cm and 30 to 60 cm, respectively) harboured the highest *Trichoderma* populations ranging from 2.1 x 10^3 to 10.2 x 10^3 CFU/g DS between 0-30 cm and 0.8 x 10^3 to 13.4 x 10^3 CFU/g DS between 30 to 60 cm depth. Similarly, Longa et al. (2009) studied the horizontal and vertical distribution of *Trichoderma* in Italian vineyards and found that the population numbers decreased with depth. This means that depth wise, not only the root and its exudates and competition with other microorganisms for nutrients will have an effect on *Trichoderma* colonisation but the physical properties of the soil will also play a role and factors affected by soil depth such as CO_2 levels, oxygen and pH influence the spore germination and hyphal growth of *Trichoderma* species (Danielson and Davey 1973a, Verma et al. 2006).

Results indicated that *T. atroviride* LU132 was a better rhizosphere coloniser at all depths than *T. virens* LU556 as more *Trichoderma* CFU/g DRS were isolated from the top, middle and bottom portions of 35 day old sweet corn plants treated with *T. atroviride* LU132 compared to plant segments' inoculated with *T. virens* LU556. This is consistent with the previous experiment (Chapter 5), where at 35 days plants inoculated with *T. atroviride* LU132 had higher *Trichoderma* populations than plants inoculated with *T. virens* LU556. This is also consistent with previous chapters in this thesis (Chapters 1 and 2) and confirms the robustness of the model plant chosen for the assays.

6.4.2 Endophytic colonisation

It was possible to isolate more endophytic *Trichoderma* colonies from plants inoculated with *T. atroviride* LU132 compared to plants inoculated with *T. virens* LU556. This was supported by microscopy images that showed more hyphal structures observed in preparations with top part roots of plants inoculated with *T. atroviride* LU132. Hyphal structures were also observed in middle root parts (control and *T. virens* LU556 treated plants) and bottom root parts (*T. virens* LU556 treated plants). Although it was easier to find fungal structures in preparations with plant material inoculated with the most endophytic isolate *T. atroviride* LU132, the plants were grown in unsterilized soil and could have had other endophytes than *Trichoderma*. Endophytic *Fusarium* species were in fact isolated in 30% of the plants assessed including plants inoculated with *Trichoderma* isolates. *Trichoderma* species have been shown to be able to grow endophytically in plant shoots and roots but the studies were performed in sterile conditions or with transformed strains. This also agreed with the previous chapter where *T. atroviride* LU132 had also been endophytically isolated more often than *T. virens* LU556. This could mean that rhizosphere competence is improved by endophytism as the most rhizosphere competent isolate was also the best endophytic coloniser.

Surprisingly no endophytic *Trichoderma* or other species' colonies were isolated from the shoots of 35 day old sweet corn plants. Although this is in agreement with the review on endophytic *Trichoderma* published by Bailey and Melnick (2013) these results were different from the endophytic colonisation recorded in Chapter 5. Endophytic colonisation of shoots at 35 days in Chapter 5 was the lowest compared to other harvest times but was still evident in plants inoculated with both isolates. This difference in shoot endophytic colonisation may have been caused by the experimental conditions and the influence of these on plant health and fitness. In Chapter 5, four plants were grown in 4 L plastic pots. In this experiment, the pipes contained approximately the same volume of soil but one plant was grown in each pipe. Shoots in this experiment reached higher leaf stages (7 to 8) compared to shoots in the previous experiment (4 to 5 leaf stages).

Roots were also more voluminous. Plants in Chapter 5 may have been more stressed by neighbouring plants, lack of nutrients, water and it is possible that the defence mechanisms were overwhelmed and both *Fusarium* and *Trichoderma* could access the shoot as well as the root. Root endophytic colonisation of plants inoculated with the two isolates were also higher in Chapter 5 (100% for *T. atroviride* LU132 and 80% for *T. virens* LU556) compared to Chapter 6 (80% for *T. atroviride* LU132 and 50% for *T. virens* LU556) which supports that endophytic *Trichoderma* populations (shoot and root) might be regulated by plant fitness.

In this work *T. atroviride* LU132 has been shown to be an endophyte through recovery onto agar, however, this would be improved by molecular tools such as isolate specific PCR which could confirm the presence internally. The study could also be repeated with strains containing an antibiotic resistance genes or that have an isolate specific marker to track the isolate in the rhizosphere in unsterile conditions. It would be useful to determine whether the *T. atroviride* LU132 grows as mycelium through the plant tissue or is localised at the site of infection. Microscopic images showed fluorescent hypha in plant tissue. These could not be conclusively identified as *T. atroviride* LU132 but there were more of these hyphae identified in areas with high levels of endophytic *T. atroviride* LU132.

Results of this experiment showed that endophytic *Trichoderma* colonies were isolated more frequently from the upper parts of sweet corn roots compared to the middle and bottom root portions regardless of the isolate. This would suggest again a link between rhizosphere competence and endophytic colonisation as more endophytic colonies were isolated from root portions with high levels of rhizosphere competence.

6.4.3 Impact of *Trichoderma* isolates on arbuscular micorrhizal fungi (AMF) communities

Another objective of this experiment was to study the influence of *T. atroviride* LU132 and *T. virens* LU556 on the rhizosphere AMF communities at different root depths. To do this the populations were analysed by denaturing gradient gel electrophoresis (DGGE) and this data used to construct a resemblance tree. An arbitrary line was placed on the dendogram and the samples placed into groups to aid interpretation. The tree was constructed with one sample per treatment. Given the low replication, the use of nested PCR to generate amplimers, the lack of an internal standard and the variable amount of soil used for DNA extraction, the relative abundance of bands were not assessed.

Trichoderma affected soil populations of AMF. Arbuscular mycorrhizal fungi populations in the control treatment were most similar to each other or the top horizon of soil inoculated with *Trichoderma* isolate *T. virens* LU556.

The AMF populations of the remaining samples from the two *Trichoderma* treatments were more similar to each other than to the control. This indicated that inoculation with *Trichoderma* was able to change AMF populations in the rhizosphere. Wyss et al. (1992) investigated the effect of a *Trichoderma harzianum* strain on the formation of vesicular-arbuscular mycorrhizae in soybean. Their results showed that the presence of *Trichoderma* significantly reduced the formation of *Glomus mosseae* mycorrhizal formation. In contrast, McLean et al. (2014) analysed the effect of a commercial *Trichoderma* bio pesticide on the AMF populations of two ecosystems (a podocarp forest and grassland) and found that native AMF populations were not affected by the strain in either of the ecosystems. There are other reports showing positive and negative effects (Paulitz and Linderman 1991, Rousseau et al. 1996a). In this experiment, AMF populations were changed but it is unknown if the effect of *Trichoderma* was positive or negative. Arbuscular mycorrhizal fungi are known to be beneficial to plants. The relationship between other beneficial microorganism and the antagonist should be tested before it is applied as a biological control agent.

Overall inoculation with *Trichoderma* does affect microbial communities. This was irrespective of differences in the CFU at each depth. Although Brimmer and Boland (2003)indicated that it is important to consider the potential non target impacts of *Trichoderma* use, there has been relatively few studies in the literature regarding the impacts of these antagonists on non-target species (Purin and Rillig 2008). Additionally none have compared the effect of different *Trichoderma* isolates on soil microbial communities at different depths.

7

General discussion

The aim of this thesis was to investigate the rhizosphere competence of selected *Trichoderma* species. Rhizosphere competence is the ability of a microorganism to colonise, grow and reproduce in the rhizosphere of growing roots. The study of rhizosphere competence has been suggested as an important trait for growth promotion, induction of systemic resistance and biocontrol activity of *Trichoderma* strains. However, in the literature, there is a lack of ecological studies addressing the ability of *Trichoderma* as a genus to establish a relationship with the plant in the rhizosphere. Understanding this relationship and the factors affecting it, will generate knowledge that may improve the selection, application and establishment of *Trichoderma* in agricultural systems.

This is the first study to assess the rhizosphere competence of multiple *Trichoderma* isolates (22) from 11 species in non-sterile soil in a single experiment. The results showed that rhizosphere competence is widespread within *Trichoderma* species and is isolate specific, confirming the reports in the literature on biological control by *Trichoderma* species. Of the isolates tested, six had greater populations than the well described positive control (*T. harzianum* isolate T22) and 18 had greater populations than the negative controls, representing the majority (82%) of the isolates tested. The 18 isolates exhibited significant variation in the degree of rhizosphere competence ranging from 3.12×10^4 to 3.09×10^5 CFU/g dry rhizosphere soil and the relative levels did not correlate with particular species nor with biocontrol potential.

The majority of previous work that examined rhizosphere competence in this genus was carried out in sterile soil or inert sterile media, such as vermiculite, sand, potting mix and hydroponics, and usually with fewer isolates. Non-sterile soil has presented a challenge because there is the need to assess the background levels of *Trichoderma* in order to evaluate the effectiveness of inoculation. An accurate assessment of background levels requires a high number of controls and high replication for treatments which is labour intensive and time consuming. As highlighted in Chapter 2, comparison with the control may also underestimate numbers for highly competent isolates which are likely to displace the natural soil population. Most often the goal has been to test rhizosphere competence to understand the mechanism by which an isolate is exerting biological control or promoting growth and was not therefore assessed in comparison with isolates that did not show these biological activities (Sivan and Harman 1991, Carvalho et al. 2011, Mehrabi-Koushki et al. 2012, Roberti et al. 2012). The measurement of rhizosphere competence in sterile soil is also misleading as it does not take into account the impact other rhizosphere microorganisms could have on *Trichoderma* species.

Non-sterile soil is the most appropriate environment in which to evaluate the effectiveness of *Trichoderma* especially if this trait is required by a biocontrol agent. The present research is one of four published accounts in non-sterile soil. The research further demonstrated that relative rank for rhizosphere competence obtained in one plant system was not necessarily applicable to another plant system. For example, *T. harzianum* LU672 unlike other isolates, colonised onion roots indicating that rhizosphere competence is plant host specific. This was supported by two previous studies that investigated the relative rhizosphere competence of *Trichoderma* isolate on more than one host in a single experimental system. The results on sweet corn were consistent, demonstrating the robustness of the assay. Moreover results showed that some isolates were capable of colonising most of the plant species, while others were more host selective, and some plants were more receptive to *Trichoderma* than others.

It was also demonstrated in Chapter 5 that *Trichoderma* populations can change over time. Although this was only monitored on two strains it suggested that the rhizosphere is a dynamic environment and can play a strong role in recruiting *Trichoderma* isolates. This might be due to variation in compatibility with changing root exudates and/or root architecture. As different lengths of time were required for each plant species to reach a sufficient size for harvest and the recovery of enough rhizosphere soil for assessment, each plant species was harvested at different times and maturity. Previous research has shown that the ability of *Trichoderma* to produce carbon degrading enzymes is inherent to each isolate and, therefore, would determine the type of root exudates a specific isolate can assimilate. The compatibility between the suite of carbon degrading enzymes and the specific composition of plant exudates will ultimately determine the success of the isolate in the rhizosphere at any given point in time. Although the different plant species were of similar size at harvest they will have produced different root exudates and had different root structures which may provide variable surface area and harbour different quantities of *Trichoderma*. Root structure has been shown to have an effect on natural populations of *Trichoderma* (Bourguignon 2008).

The consistency of the results for rhizosphere competence across Chapters 2, 3, 5 and 6 demonstrated that the sweet corn plant assay (7 days) was robust and a good method to detect early rhizosphere colonisers. However, it was demonstrated that rhizosphere competence changed overtime for two isolates. Although the results showed that *T. atroviride* LU132 was consistently more rhizosphere competent, the *T. atroviride* LU132 growth curve had reached an asymptote at 35 days which would suggest that it had reached maximum establishment potential in the rhizosphere. It is possible that the amount of rhizoplane available for it to colonise became saturated. Thus, it would be interesting to determine the maximum load of *Trichoderma* that a plant root can host and what the time required for this to be attained is. In contrast *T. virens* LU556 did not reach an asymptote at 56 days and it is unclear whether it would have reached the same saturation point.

This also raises the relationship between early and late rhizosphere colonisers. In this work it was clear that these two isolates had contrasting colonisation patterns but what the outcome of co-inoculating these two species may be is unknown. In many other fungal communities such as ectomycorrhizae and leaf litter degradation (Deacon 2006, Walbert 2008) succession is observed. There are no reports of succession for *Trichoderma* and this concept would be interesting to investigate. It is possible that if co-inoculated *T.virens* LU556 could displace or match *T. atroviride* LU132 as root exudates changed to become more optimal for the slower colonising isolate. In the genus this may be at a species or isolate level. This would have major implications for the long term success of a biological control isolate following soil application.

Trichoderma atroviride LU132 stood out as the best isolate as it could colonise most of the plant species. Thus it was chosen as the isolate on which to test abiotic factors with the reasoning that differences would most easily be observed using this isolate. In the present study, the rhizosphere competence of *T. atroviride* LU132 was assessed on two plants under a range of different soil moisture content (16, 20 and 24% gravimetric water content), soil pH (5.5, 6.5 and 7.5) and available nitrogen concentrations (75, 150 and 300 kg N/ha). These particular factors were chosen because they are the most important factors for plant growth. The range for each factor was set at extremes that would still allow plant growth. None of these conditions affected the rhizosphere competence of *T. atroviride* LU132 indicating that the host plant was a much more significant factor (Chapter 3) than these. A reason for the lack of differences might have been that the conditions were not extreme enough but moving beyond this range severely compromised plant growth and, therefore, the experiment. Indeed, when the soil became very dry in the first soil moisture content experiment, a difference in root colonisation was observed with higher populations at higher moisture levels. However, there was no emergence of ryegrass at this level and there was a high mortality of sweet corn, lowering the statistical power. Collectively, these results showed that *T. atroviride* LU132 remained highly rhizosphere competent under a wide range of conditions. Further investigation could look at the interaction between these factors rather than single iterations such as is presented here. More data could also be generated using systems that investigate respiration changes, such as MicroResp™, with carbon sources and these could be linked to exudates, however this would need to be adapted to a sterile soil system.

In Chapters 5 and 6, rhizosphere competence as well as endophytic colonisation were examined. Results consistently showed that more endophytic *Trichoderma* colonies were isolated from plant shoots and roots that had been treated with the most rhizosphere competent isolate (*T. atroviride* LU132). This suggested a link between rhizosphere competence and endophytism, however, only a single highly competent and poorly competent isolates were examined in great detail.

This link was investigated further in Chapter 6. Chapter 6 was the first study to have analysed rhizosphere competence and endophytic colonisation of *Trichoderma* at a root depth below 60 cm. Results showed that the rhizosphere and the internal cortex cells of the top parts of the roots were more colonised by *Trichoderma* than middle and bottom parts regardless of the isolate. This and the fact that the most rhizosphere competent isolate was more endophytic than the least rhizosphere competent one suggested that endophytic colonisation is related to rhizosphere competence. To establish a strong link between the two, a greater number of isolates with contrasting rhizosphere competence should be compared. This should be accompanied by molecular tools such as isolates transformed with fluorescent markers or isolate specific PCR and qPCR techniques which can confirm the presence of a particular isolate, visualise the internal colonisation (by hyphae or conidia) and quantify the biomass. Confocal and scanning electron microscopy could also add support to these observations.

The exact molecular mechanisms by which *Trichoderma* becomes endophytic is poorly understood but is an area of intense research. It has also been strongly linked with beneficial traits such as growth promotion, induction of systemic resistance, biocontrol efficacy and alleviation of abiotic stress. Strongly rhizosphere competent isolates may have an advantage as they are able to colonise two niches, both the rhizosphere and the interior of the plant. By being able to develop and grow inside the plant, an isolate will be protected from competition from soil microorganisms and potentially be able to emerge to "seed" new colonies along the length of the root. As endophytism is known to trigger defence pathways in the plant, it would be interesting to know exactly which genetic features of an isolate make it most compatible with plants. As seen in Chapter 5 it is unclear whether stressed plants are simply more susceptible to colonisation by *Trichoderma* or whether they actively secrete molecules to recruit *Trichoderma* during times of need. To investigate this more closely endophytism in stressed and non-stressed plants should be examined separately with a consistent cohort of *Trichoderma* with variable colonisation abilities.

In Chapter 5 plant shoots were more colonised by both *Trichoderma* isolates than in Chapter 6 where no shoot colonisation was observed. Assessment of shoot colonisation is not commonly done in experiments looking at rhizosphere competence and it was an unexpected result. Plants in Chapter 5 were much smaller than (~50%) plants in Chapter 6. The reasons may have been that in Chapter 5 plants may have been under the stress due to the close proximity of other plants. The shoots were less woody in Chapter 5 and this may have made them more susceptible to colonisation by *Trichoderma* especially if they are actively attempting to recruit *Trichoderma* due to a stress response. It is well established that stressed plants attracted more *Trichoderma*.

Given the lack of effect of abiotic factors on rhizosphere competence some exploration of the effect on biotic parameters was undertaken. DGGE was used to investigate the influence of *Trichoderma* application on the microbial populations in the rhizosphere. Although only the presence/absence of species was assessed the results showed that bacterial, fungal and arbuscular mycorrhizal populations in the rhizosphere of plants treated with *Trichoderma* isolates changed, compared to the control plants. Of these populations, AMF were the most affected. Interestingly, AMF richness in the rhizosphere was decreased and was increased in the bulk soil in the presence of the most rhizosphere competent isolate. This suggests there is some exclusion of AMF proliferation in the rhizosphere by *T. atroviride* LU132. The increase in the bulk soil could be the result of AMF seeking unexploited niches in the bulk soil. However, because sequencing of bands was not done for any of the microbial groups there is no way to know specifically what species and functions were affected. It is likely given the results of Chapter 3 that these effects may also be plant host and soil specific. Overall, this facet of rhizosphere competence and its relationship to the success or failure of an isolate to colonise the rhizosphere warrants more investigation. There are several new technologies that may allow greater exploration of these microbial relationships in the rhizosphere. DNA technology such as next generation sequencing and GeoChip can identify and determine the relative functions of microbial communities in the rhizosphere. The application of these to understanding the effect of *Trichoderma* on the ecology of the rhizosphere could generate significant new understanding of all of the factors addressed in this thesis.

Perhaps multiple points of colonisation are necessary for effective protection against a pathogen or to promote some of the effects *Trichoderma* is known to cause through endophytism such as growth promotion or induced systemic resistance. Perhaps the entire root needs to be colonised to benefit from *Trichoderma* actions. Also it would be easy to think that for an isolate it would be less difficult to colonise the plant if it travels inside and it is protected from the attack of other microorganisms in the rhizosphere, but the roots are also colonised by other fungi like micorrhizae or other organism that can compete with *Trichoderma* for the niche.

This thesis has not addressed the linkage between rhizosphere competence and biocontrol. Rhizosphere competence is clearly not a prerequisite for a biological control agent, for example, the well characterised biocontrol agent *T. harzianum* T22 did not perform as well as many other isolates that have not demonstrated any biocontrol ability. Although some good biocontrol agents like *T. atroviride* LU132 appear to be highly rhizosphere competenct. Perhaps it is this aggressive early colonisation by *T. atroviride* LU132 on multiple plant hosts (ie. it is not fussy) that makes it good. It has been used against *Rhizoctonia solani* damping off (early plant disease) in ryegrass and other pasture species, Sclerotium cepivorum (onion white rot; infects through soil mycelia) and some foliar diseases.

The mechanism by which these soil borne pathogens infect could be suppressed by a strongly *Trichoderma* presence in the rhizosphere especially early in seedling establishment.

In summary, this research has generated data showing that rhizosphere competence is widespread in *Trichoderma* species, is isolate specific and does not correlate with biocontrol ability. The results showed that *Trichoderma* isolates and the plant species appeared to be the main drivers of the establishment of the relationship between *Trichoderma* and the host plant. There was some indication that microbial communities in the soil may also play a role but this was not fully explored. In this study, soil moisture content, soil pH and soil nitrogen concentrations did not have a significant effect on rhizosphere competence. Further work that more closely examines C sources, their relationship to root exudates and the breadth and quantity of enzymes and antimicrobial substances secreted by isolates is likely to be useful to extend the work presented here. Rhizosphere competence changes over time although *Trichoderma* isolates regardless of their ability to colonise roots will colonise the rhizosphere of the upper root portion better. These results suggested a link between rhizosphere competence and endophytism but that should be more fully explored with an accompanying suite of molecular tools. Although these findings are separate from the biocontrol ability of an isolate they do have some implications for a better understanding of the mechanisms underlying root colonisation

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Appendix A: **Appendices for Chapter 2**

A.1 Soil characteristics for each of the three experiments

	Level found		
	19th May	**1st June**	**14th June**
pH (pH Units)	5.3	5.7	5.6
Olsen Phosphorus (mg/L)	30	35	38
Potassium (me/100g)	0.38	0.49	0.51
Calcium (me/100g)	6	6.4	7.3
Magnesium (me/100g)	0.82	0.87	0.92
Sodium (me/100g)	0.2	0.19	0.2
CEC (me/100g)	12	12	14
Total Base Saturation (%)	60	67	66
Volume Weight (g/mL)	1.01	1.02	1.09
Available Nitrogen (15cm Depth) (kg/ha)	44	63	55
Anaerobically Mineralisable N (μg/g)	29	42	33

A.2 *Trichoderma* selective medium recipe

For 1 L

20.0 g Agar

3.0 g Glucose

0.2 g Terrachlor 75WP fungicide

0.15 g Rose Bengal

1 mL iron/manganous/zinc solution

1 mL magnesium sulphate solution

5 mL ammonium/dipotassium/potassium solution

Make up to 1 L and autoclave at 121°C for 15 minutes. Cool to about 60°C then add

1 mL chloramphenicol stock solution

***Trichoderma* selective media (TSM stock solutions)**

- Ammonium/dipotassium/potassium solution:

In 500 mL of nanopure H_2O dissolve 100 g ammonium nitrate; 90 g dipotassium hydrogen orthophosphate trihydrate ($K_2HPO_4.3H_2O$); 15 g potassium chloride (KCl).

- Magnesium sulphate solution:

20 g magnesium sulphate 7 hydrate ($MgSO_4.7H_2O$) in 100 mL nanopure H_2O.

- Iron/manganous/zinc solution:

In 100 mL of nanopure H_2O dissolve 0.01 g iron sulphate (Ferrous sulphate) 7 hydrate ($FeSO_4.7H_2O$); 0.065 g manganous sulphate tetrahydrate ($MnSO_4.4H_2O$); 0.09 g zinc sulphate ($ZnSO_4.7H_2O$).

- Chloramphenicol stock solution:

Weigh 2.5 g of chloramphenicol into a 100 mL volumetric flask. Add approx. 80 mL absolute (96%) ethanol, and swirl to dissolve. Make up the volume in the flask to 100 mL with absolute ethanol. Store at -20°C.

A.3 ***Trichoderma*** **CFU/g dry rhizosphere soil (DRS) and log_{10} values obtained in three experiments for 7 old sweet corn seedlings grown in non-sterile soil. Min = minimum replication (isolates). Max = maximum replication (methyl-cellulose coated seed and** ***T. harzianum*** **T22)**

Experiment 1				Experiment 2				Experiment3			
LU No.	Log_{10} CFU	CFU/g DRS		LU No.	Log_{10} CFU	CFU/g DRS		LU No.	Log_{10} CFU	CFU/g DRS	
132	5.7	4.81×10^5	a	150	5.6	4.36×10^5	a	740	5.9	7.11×10^5	a
298	5.5	3.52×10^5	ab	151	5.6	3.60×10^5	ab	673	5.8	5.71×10^5	ab
673	5.5	3.51×10^5	ab	132	5.5	3.28×10^5	ab	151	5.7	5.25×10^5	abc
571	5.4	2.64×10^5	abc	817	5.3	2.07×10^5	abc	140	5.6	3.70×10^5	abcd
570	5.2	1.58×10^5	abcd	673	5.2	1.75×10^5	abc	595	5.5	2.94×10^5	abcde
740	5.2	1.45×10^5	abcd	140	5.2	1.74×10^5	abc	570	5.4	2.52×10^5	abcde
150	5.1	1.29×10^5	abcde	571	5.2	1.67×10^5	abc	298	5.4	2.40×10^5	abcde
817	5.1	1.28×10^5	abcde	945	5.1	1.35×10^5	abcd	945	5.4	2.29×10^5	abcde
140	5.1	1.15×10^5	abcde	592	5.1	1.24×10^5	abcde	699	5.3	2.20×10^5	abcde
592	5.0	1.02×10^5	abcde	570	5.1	1.18×10^5	abcde	150	5.2	1.60×10^5	abcdef
945	5.0	9.12×10^4	abcde	761	5.1	1.16×10^5	abcde	592	5.1	1.18×10^5	bcdefg
761	4.9	8.61×10^4	abcde	740	5.0	9.06×10^4	abcdef	571	5.0	1.11×10^5	cdefg
626	4.9	8.36×10^4	abcde	T22	4.9	7.24×10^4	cdefg	817	4.9	7.93×10^4	defgh
151	4.9	7.76×10^4	abcde	298	4.8	6.78×10^4	bcdefgh	132	4.8	6.92×10^4	efghi
T22	4.8	6.35×10^4	abcde	699	4.8	6.52×10^4	bcdefgh	T22	4.8	5.86×10^4	fghij
547	4.6	3.78×10^4	abcdef	547	4.6	4.46×10^4	cdefghi	547	4.6	3.61×10^4	fghijk
669	4.6	3.68×10^4	bcdef	672	4.4	2.69×10^4	defghi	761	4.5	3.54×10^4	fghijk
555	4.5	2.91×10^4	bcdef	626	4.4	2.32×10^4	efghi	556	4.4	2.77×10^4	ghijkl
699	4.4	2.81×10^4	bcdef	595	4.4	2.29×10^4	efghi	669	4.4	2.71×10^4	ghijkl
595	4.3	2.07×10^4	cdef	669	4.3	1.87×10^4	fghij	672	4.2	1.74×10^4	hijkl
556	4.2	1.70×10^4	def	555	4.2	1.76×10^4	fhij	626	4.2	1.51×10^4	ikl
672	4.0	1.09×10^4	ef	556	4.2	1.63×10^4	fhij	BS	4.1	1.30×10^4	kl
CS	3.8	6.98×10^3	f	BS	4.0	9.16×10^3	ij	555	4.1	1.19×10^4	kl
BS	3.7	5.04×10^3	f	CS	3.7	5.51×10^3	j	CS	3.9	8.75×10^3	l
LSD 5%	Min-min	Max-min	Max-max	LSD 5%	Min-min	Max-min	Max-max	LSD 5%	Min-min	Max-min	Max-max
	1.114	0.909	0.643		0.747	0.610	0.431		0.695	0.568	0.401

Appendix B: **Appendices for Chapter 3**

B.1 Soil analysis for experiments 1 and 2

Analysis	Level found	
	Experiment 1	Experiment 2
pH (pH Units)	5.8	6
Olsen Phosphorus	47	52
Potassium (me / 100g)	0.51	0.62
Calcium (me / 100g)	7.4	8
Magnesium (me / 100g)	0.87	1.02
Sodium (me / 100g)	0.18	0.22
CEC (me / 100g)	13	14
Total Base Saturation (%)	68	73
Volume Weight (g / mL)	1.08	0.93
Available Nitrogen (15cm Depth) (kg / ha)	43	62
Anaerobically Mineralisable N (µg / g)	27	45

B.2 Rhizosphere competence result tables for experiment 1

B2 1 **Mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) (log_{10} values are indicated between brackets) from selected plant species treated with six different *Trichoderma* isolates. Mean log_{10} CFU/g DRS followed by the same letter do not differ significantly within each column.**

	Sweet corn	Ryegrass	Onion	Clover	Cauliflower	Carrot
***T. harzianum* LU673**	6.52×10^4 (4.81) a	2.30×10^5 (5.36) ab	2.24×10^4 (4.35) b	1.10×10^4 (4.04) a	1.06×10^4 (4.03) c	5.13×10^4 (4.71) a
***T. harzianum* LU151**	6.01×10^4 (4.78) a	3.53×10^5 (5.55) ab	1.85×10^4 (4.27) b	8.38×10^3 (3.92) a	2.81×10^5 (5.45) ab	4.80×10^4 (4.68) a
***T. atroviride* LU132**	1.08×10^5 (5.03) a	7.74×10^5 (5.89) a	2.58×10^4 (4.41) b	5.75×10^3 (3.76) a	9.79×10^5 (5.99) a	4.05×10^4 (4.61) a
T. virens LU556	5.05×10^3 (3.70) b	1.55×10^5 (5.19) b	7.46×10^3 (3.87) b	6.27×10^3 (3.80) a	6.43×10^4 (4.81) b	7.85×10^3 (3.90) b
T. harzianum LU672	5.82×10^3 (3.77) b	2.33×10^5 (5.37) ab	2.13×10^5 (5.33) a	1.57×10^4 (4.20) a	1.13×10^5 (5.05) b	1.21×10^4 (4.08) ab
T. crassum LU555	7.78×10^3 (3.89) b	1.44×10^5 (5.16) b	2.06×10^4 (4.31) b	1.26×10^4 (4.10) a	6.07×10^4 (4.78) b	1.40×10^4 (4.15) ab
Control*	1.25×10^4 (3.764)	4.86×10^3 (3.660)	3.42×10^3 (4.187)	1.54×10^4 (3.534)	4.57×10^3 (3.687)	5.81×10^3 (4.097)
LSD 5%			0.6981			

* Data for untreated controls not included in the analysis. Note-**LU132, LU673, LU151** selected as highly rhizosphere competent on sweet corn in chapter 2; LU556, LU555, LU672 selected as weakly rhizosphere competent on sweet corn in Chapter 2.

B2 2 **Log_{10} values and mean number of *Trichoderma* CFU/g DRS of six plant species. Mean CFU/g DRS followed by the same letter do not differ significantly within the column.**

Plant	Log_{10} CFU/g DRS	Mean CFU/g DRS	
Ryegrass	5.419	2.62×10^5	a
Cauliflower	5.018	1.04×10^5	b
Onion	4.424	2.65×10^4	c
Carrot	4.353	2.25×10^4	c
Sweet corn	4.331	2.14×10^4	c
Clover	3.969	9.31×10^3	d
LSD 5%	0.6968		

B2 3 **Log_{10} values and mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) recovered from six plant species for six different isolates. Mean CFU/g DRS followed by the same letter do not differ significantly within the column.**

Isolate	Log_{10} CFU/g DRS	Mean CFU/g DRS	
T. atroviride LU132	4.948	8.87×10^4	a
T. harzianum LU151	4.775	5.96×10^4	ab
T. harzianum LU672	4.632	4.29×10^4	bc
T. harzianum LU673	4.55	3.55×10^4	bc
T.crassum LU555	4.399	2.51×10^4	cd
T. virens LU556	4.211	1.63×10^4	d
LSD 5%	0.6968		

B.3 Rhizosphere competence result tables for experiment 2

B3 1 Mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) (log_{10} values are indicated between brackets) from selected plant species treated with six different *Trichoderma* isolates. Mean CFU/g DRS followed by the same letter do not differ significantly within each column.

	Sweet corn	Ryegrass	Onion	Clover	Cauliflower	Carrot
***T. harzianum* LU673**	9.95×10^4 (5.00) a	1.85×10^5 (5.27) a	2.71×10^4 (4.43) a	3.63×10^4 (4.56) a	2.98×10^4 (4.47) b	2.59×10^4 (4.41) a
***T. harzianum* LU151**	7.11×10^4 (4.85) ab	1.93×10^5 (5.29) a	6.14×10^4 (4.79) a	3.20×10^4 (4.51)	1.78×10^5 (5.25) a	2.64×10^4 (4.42) a
***T. atroviride* LU132**	1.87×10^5 (5.27) a	4.49×10^5 (5.65) a	1.57×10^4 (4.20) a	1.55×10^4 (4.19) a	1.34×10^5 (5.13) ab	3.84×10^4 (4.58) a
T. virens LU556	1.37×10^4 (4.14) bc	1.17×10^5 (5.07) a	3.52×10^4 (4.55) a	1.87×10^4 (4.27) a	7.03×10^4 (4.85) ab	1.47×10^4 (4.17) a
T. harzianum LU672	7.82×10^3 (3.89) c	1.06×10^5 (5.02) a	7.00×10^4 (4.85) a	1.19×10^4 (4.08) a	1.04×10^5 (5.02) ab	3.13×10^4 (4.50) a
T. crassum LU555	1.12×10^4 (4.05) c	8.55×10^4 (4.93) a	1.57×10^4 (4.20) a	2.83×10^4 (4.45) a	2.10×10^5 (5.32) a	2.21×10^4 (4.35) a
Control*	4.83×10^3 (3.684)	9.71×10^3 (3.987)	8.77×10^3 (3.943)	7.26×10^3 (3.861)	5.57×10^3 (3.746)	7.91×10^3 (3.898)
LSD 5%			0.7650			

* Data for untreated controls not included in the analysis. Note-**LU132, LU673, LU151** selected as highly rhizosphere competent on sweet corn in chapter 2; LU556, LU555, LU672 selected as weakly rhizosphere competent on sweet corn in Chapter 2.

B3 2 **Log_{10} values and mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) of six plant species. Mean CFU/g DRS followed by the same letter do not differ significantly within the column.**

Plant	Log_{10} CFU/g DRS	Mean CFU/g DRS	
Ryegrass	5.205	1.60×10^5	a
Cauliflower	5.006	1.01×10^5	a
Sweet corn	4.533	3.41×10^4	b
Onion	4.501	3.17×10^4	b
Carrot	4.405	2.54×10^4	b
Clover	4.343	2.20×10^4	b
LSD 5%	0.7742		

B3 3 **Log_{10} values and mean number of *Trichoderma* CFU/g dry rhizosphere soil (DRS) recovered from six plant species for six different isolates. Mean CFU/g DRS followed by the same letter do not differ significantly within the column.**

Isolate	Log_{10} CFU/g DRS	Mean CFU/g DRS	
T. harzianum LU151	4.850	7.08×10^4	a
T. atroviride LU132	4.836	6.85×10^4	a
T. harzianum LU673	4.691	4.91×10^4	a
T. harzianum LU672	4.558	3.61×10^4	a
T. crassum LU555	4.549	3.54×10^4	a
T. virens LU556	4.507	3.21×10^4	a
LSD 5%	0.7742		

B.4 Growth promotion tables

B4 1 Seedling emergence experiment 1 (out of 10)

Plant	Control	132	151	555	556	672	673
carrots	5	5	4.67	7	4	8.33	4.67
cauliflower	8.89	9	9.67	6.67	9.67	8.67	8
clover	4.77	4.67	5.33	5.33	6	5.33	5
onion	6.22	7.33	8.33	5	8	7	7.33
ryegrass	8.33	8.33	8	8.67	8.33	7.67	8.33
s. corn	5.55	7	6.67	5.67	8	6.67	7.33
LSD 5%				2.343			

B4 2 Seedling emergence experiment 2 (out of 10)

Plant	Control	132	151	555	556	672	673
carrots	1.78	2.33	1.33	4	5.33	3	2.33
cauliflower	9.11	9	8	8.67	7.67	9	6.67
clover	4.44	5	3.33	4.67	4	4.67	1.67
onion	4	4.33	4.67	5.67	5.33	4	2.33
ryegrass	7	7.33	7	8	8	6.67	6
s. corn	5.78	7.67	7.33	8.67	6.33	6.33	6.33
LSD 5%				2.181			

B4 3 Shoot and root lengths (cm) for experiment 1

	Shoot length (cm)						
Plant	Control	132	151	555	556	672	673
carrots	6.33	7.25	9.33	6.92	6.67	7.58	7.08
ryegrass	21.67	20.75	22.33	21.92	18.17	20.17	18.33
LSD 5%				3.238			

	Root length (cm)

Plant	Control	132	151	555	556	672	673
onion	7.56	9.17	13.33	5.17	9.33	8.33	6.67
LSD 5%				3.489			

B4 4 Log_{10} shoot and root dry weights in experiment 1

Log_{10} shoot weight

Plant	Control	132	151	555	556	672	673
carrot	0.787	0.953	1.072	0.872	0.852	0.949	0.949
clover	0.672	0.350	0.600	0.945	0.796	0.698	0.798
LSD 5%				0.2267			

Log_{10} root weight

Plant	Control	132	151	555	556	672	673
clover	1.95	0.77	2.08	2.8	2.2	3	1.95
sweet corn	20.18	43.02	32.2	38.17	61.08	47.63	41.53
LSD 5%				9.203			

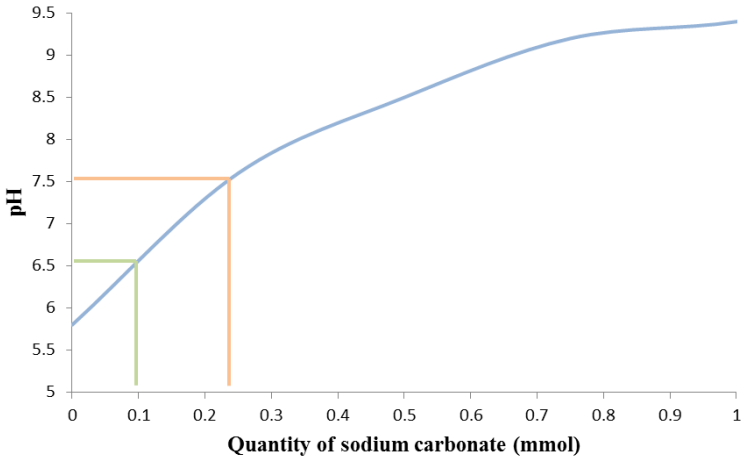
Appendix C: **Appendices for Chapter 3**

C.1 Titration curve to achieve different pH values in Wakanui silt loam soil

Ten grams of Wakanui silt loam soil at 20% GWC were placed in six 80 mL specimen containers. A total of 25 mL of different volumes of a solution of 0.05 M Na_2CO_3 (sodium carbonate) and water were added to each of the containers as in table below. Containers were shaken on a shaker for an hour and were left sitting over night before pH was measured.

Container	Water (mL)	0.05 M Na_2CO_3 (mL)	Na_2CO_3 quantity (mmol)	pH
1	25	0	0	5.8
2	20	5	0.25	7.6
3	15	10	0.5	8.5
4	10	15	0.75	9.2
5	5	20	1	9.4
6	0	25	1.25	9.8

pH values of Wakanui silt loam mixed with a solution of increasing amounts of sodium carbonate



Titration curve of Wakanui silt loam soil. To adjust the soil pH to 6.5, it was necessary to add 0.1 mmol of Na_2CO_3 for every 10 g of soil. To adjust the soil pH to 7.5, it was necessary to add 0.24 mmol of Na_2CO_3 for every 10 g of soil.

C.2 Pilot study to check the stability of Wakanui silt loam soil modified by the addition of Na2CO3

Six zip lock bags with 500 g of Wakanui silt loam soil at 20% GWC and at pH5 were prepared. Na_2CO_3 was added as a powder or as a solution to achieve three different pH levels: 5.5 (bags 1 and 2), 6.5 (bags 3 and 4) and 7.5 (bags 5 and 6). Bags were stored in an incubator at the following conditions: 16 hour day at 18°C and 8 hour night at 20°C. The pilot study was set up on the 2nd November 2011. pH was measured at four different dates.

Soil bags	Method	pH on the 18th Nov.	pH on the 29th Nov.	pH on the 12th Dec.	pH on the 19th Dec.
1	Powder	5.5	5.5	5.5	5.5
2	Solution	5.5	5.6	5.4	5.3
3	Powder	7.2	6.9	6.7	6.5
4	Solution	7	6.7	6.5	6.5
5	Powder	7.7	7.5	7.2	7.5
6	Solution	7.6	7.5	7	7.2

pH of Wakanui silt loam soil, measured at several time points during incubation, after the addition of Na2CO3 either as a powder or as a solution.

C.3 Soil nutrient analysis of Wakanui silt loam samples corrected to three pHs. Analysis conducted by Hills Laboratories.

	pH soil experiment 1		
pH at start measured in Lincoln University	**5.5**	**6.5**	**7.5**
pH (pH Units) at start measured by Hill	5.7	7	8.3
Olsen Phosphorus (mg/L)	23	23	20
Potassium (me/100g)	0.48	0.46	0.44
Calcium (me/100g)	4.6	4.5	4.0
Magnesium (me/100g)	0.67	0.63	0.47
Sodium (me/100g)	0.14	2.12	4.56
CEC (me/100g)	11	11	10
Total Base Saturation (%)	53	71	96
Volume Weight (g/mL)	1.03	1.10	1.16
Available Nitrogen (15cm Depth) (kg/ha)	70	68	81
Anaerobically Mineralisable N (µg/g)	45	41	47

C.4 Soil pH analysis of bulk soils collected from each treatment pot at the end of pH experiments 1 and 2. Values are a mean of all pots for each treatment.

		Bulk soil pH at the end of experiments	
Plant	Soil pH at start	experiment 1	experiment 2
Ryegrass	5.5	5.5	5.5
Ryegrass	6.5	5.5	5.6
Ryegrass	7.5	7.2	6.9
Sweet corn	5.5	7	6.7
Sweet corn	6.5	7.7	7.5
Sweet corn	7.5	7.6	7.5

C.5 Soil nutrient analysis of Wakanui silt loam samples corrected to three available nitrogen contents. Ananlysis conducted by Hills Laboratories.

	Experiment 1		
Desired nitrogen level (kg N/ha)	**75**	**150**	**300**
pH (pH Units)	5.7	5.6	5.4
Olsen Phosphorus (mg/L)	23	23	23
Potassium (me/100g)	0.43	0.48	0.48
Calcium (me/100g)	4.7	4.4	4.6
Magnesium (me/100g)	0.63	0.60	0.62
Sodium (me/100g)	0.11	0.12	0.12
CEC (me/100g)	11	10	11
Total Base Saturation (%)	51	56	55
Volume Weight (g/mL)	0.98	1.14	1.04
Available Nitrogen (15cm Depth) (kg/ha)	65	50	65
Anaerobically Mineralisable N (µg/g)	44	29	44

Appendix D: **Appendices for Chapter 5**

D.1 Soil nutrient analysis of Wakanui silt loam for Chapter 5. Ananlysis conducted by Hills Laboratories.

Ananlysis	Level
pH (pH Units)	6.2
Olsen Phosphorus (mg/L)	20
Potassium (me/100g)	0.33
Calcium (me/100g)	7.4
Magnesium (me/100g)	0.78
Sodium (me/100g)	0.08
CEC (me/100g)	13
Total Base Saturation (%)	68
Volume Weight (g/mL)	1.11
Available Nitrogen (15cm Depth) (kg/ha)	70
Anaerobically Mineralisable N (µg/g)	42

D.2 Arbuscular mycorrhizal fungi PERMANOVA analysis

Main soil analysis: AMF	**df**	**SS**	**MS**	**Pseudo-F**	**P(perm)**
Soil type	2	18958	9478.9	3.3382	0.0001
Residual	58	164690	2839.5		
Total	60	183650			

Pair wise test: Soil	**t**	**P(perm)**
Rhizosphere, Bulk	2.1323	0.0001
Rhizosphere, Set-up	1.4711	0.0013
Bulk, Set-up	1.5134	0.002

Rhizosphere soil analysis: AMF	df	SS	MS	Pseudo-F	P(perm)
Isolate	2	15295	7647.3	2.4411	0.0002
Days	3	6433	2144.3	0.68448	0.9684
Isolate.Days	6	14214	2369	0.7562	0.9704
Residual	22	68921	3132.8		
Total	33	104830			

Pair wise test: Isolate	t	P(perm)
Control, LU132	1.6724	0.0007
Control, LU556	1.8021	0.0003
LU 132, LU 556	1.1733	0.1452

D.3 Bacteria PERMANOVA analysis

Main soil analysis: Bacteria	df	SS	MS	Pseudo-F	P(perm)
Soil type	2	13262	6631.1	2.6983	0.0001
Residual	56	137620	2457.5		
Total	58	150880			

Pair wise test: Soil	t	P(perm)
Rhizosphere, Bulk	2.0467	0.0001
Rhizosphere, Set-up	1.1274	0.0927
Bulk, Set-up	1.1966	0.0853

Rhizosphere soil analysis: Bacteria	df	SS	MS	Pseudo-F	P(perm)
Isolate	2	7054.8	3527.4	1.4175	0.0142
Days	3	6545	2181.7	0.87669	0.8188
Isolate.Days	6	10781	1796.9	0.72207	0.9997
Residual	20	49771	2488.5		
Total	31	74282			

Pair wise test: Isolate	t	P(perm)
Control, LU132	1.3027	0.0047
Control, LU556	1.2093	0.0666
LU132, LU556	1.0754	0.2259

D.4 Fungi PERMANOVA analysis

Main soil type: Fungi	df	SS	MS	Pseudo-F	P(perm)
Soil type	2	13903	6951.4	2.7278	0.0001
Residual	58	147800	2548.4		
Total	60	161710			

Pair wise test: Soil	t	P(perm)
Rhizosphere, Bulk	1.8607	0.0001
Rhizosphere, Set-up	1.4074	0.0009
Bulk, Set-up	1.4762	0.0086

Rhizosphere soil analysis: Fungi	df	SS	MS	Pseudo-F	P(perm)
Isolate	2	8994.2	4497.1	1.5846	0.007
Days	3	5020.6	1673.5	0.58969	0.9997
Isolate.Days	6	12144	2024.1	0.7132	0.999
Residual	22	62436	2838		
Total	33	88488			

Pair wise test: Isolate	t	P(perm)
Control, LU132	1.4017	0.0038
Control, LU556	1.1722	0.0919
LU132, LU556	1.1972	0.0856

E.1 Soil nutrient analysis of Wakanui silt loam for Chapter 6. Ananlysis conducted by Hills Laboratories.

Ananlysis	Level
pH (pH Units)	5.9
Olsen Phosphorus (mg/L)	19
Potassium (me/100g)	0.30
Calcium (me/100g)	6.6
Magnesium (me/100g)	0.72
Sodium (me/100g)	0.09
CEC (me/100g)	12
Total Base Saturation (%)	66
Volume Weight (g/mL)	1.14
Available Nitrogen (15cm Depth) (kg/ha)	87
Anaerobically Mineralisable N (µg/g)	51

E.2 Endophytic colonisation table for bottom root plant segements untreated and treated with *T. atroviride* LU132 and *T.virens* LU556

		Root segment position															
Isolate	**Plant**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Control	1	x	x	x	x	x	x	x	x	x		x	x	x	x		x
	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	3	x	x		x	x	x		x	x	x	x	x	x	x	x	x
	4	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	6	x	x	x	x	x	x	x	x	x	x	x					
LU132	1	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	3	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	4	x	x	x	x	x	x	x	x	x	x	x	x	x			
	5	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	6	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	
LU556	1	x	x	x	x	x	x	x	x	x	x	x	x	x			
	2	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
	3	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	4	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x
	5	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	6	x	x	x	x	x	x	x	x	x	x	x	x				