Rhizosphere and endophytic colonisation of ryegrass and sweet corn roots by the isolate *Trichoderma atroviride* LU132 at different soil pHs

N. Cripps-Guazzone[12], E.E. Jones[1], L.M. Condron[12], K.L. McLean[23], A. Stewart[24] and H.J. Ridgway[1]

[1]*Faculty of Agriculture and Life Sciences* , [2]*Bio-Protection Research Centre, Lincoln University, Ellesmere Jct Rd, Lincoln 7647, New Zealand*
[3]*Three Miners Vineyard, 89 McPherson Rd, RD 1, Alexandra 9320, New Zealand*
[4]*Scion, Te papa Tipu Innovation Park, 49 Sala Park Street, Rotorua 3046, New Zealand*
Corresponding author: natalia.cripps-guazzone@lincoln.ac.nz

Abstract The colonisation of plant roots by biological control agents is dependent on abiotic factors, one of the most important being soil pH. The rhizosphere and endophytic colonisation of ryegrass and sweet corn roots by the biological control agent *Trichoderma atroviride* LU132 were assessed in a pot experiment with non-sterile soil at three different pHs (5.5, 6.5 and 7.5). *T. atroviride* LU132 colonised the roots of both plants regardless of the soil pH with 1.13-1.47 x 10^6 CFU/g of dry rhizosphere soil (DRS) for ryegrass and 1.36-3.50x10^5 CFU/g DRS for sweet corn. *T. atroviride* LU132 was able to colonise both plants endophytically, regardless of soil pH. However, the isolate was recovered from entire ryegrass roots but only from the upper parts of sweet corn roots. These experiments demonstrated that *T. atroviride* LU132 colonised the rhizosphere and roots within a soil pH range common to most NZ soils, which is a desirable trait for biological control agents.

Keywords *Trichoderma*, soil pH, rhizosphere competence, root colonisation, endophyte.

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 commercial uptake of *Trichoderma* products (Stewart 2010). Various biotic and abiotic soil factors 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 & Paul 2008).

Research on the effect of pH on *Trichoderma* species extracellular enzyme production, mycelial growth and root colonisation, all important to biological control capabilities, has yielded conflicting results, with no clear common trend as to the pH tolerance level of different species or

different isolates among the same species. These results are summarised in Table 1. Moreover, pH effect on *Trichoderma* species has been commonly investigated under axenic conditions and less frequently in soil, making it difficult to relate the results to field conditions. Studies in soil have also revealed contradictory results. Mondal et al. (1996) reported that acidic conditions favoured the development of *T. koningii* and *T. viride* in soil. Lo et al. (1996) reported that *T. harzianum* isolate T22 established equally high populations in turfgrass soil at pH 8.0 as in soil at pH 6.4. Furthermore, these studies have either been conducted in the absence of a plant or excluded the rhizosphere soil where the fungus interacts with the plant, the area most likely to be influenced by pH changes. The few studies addressing the effect of pH on rhizosphere colonisation of *Trichoderma* species reported that the fungus can colonise plant roots at pH levels ranging from 5 to 7 (Ahmad & Baker 1987; Jeong et al. 1997). However, the effect of soil pH on endophytic colonisation has not been reported.

Endophytic colonisation by *Trichoderma* strains has also been linked to plant beneficial traits (Vinale

Table 1 Summary of research addressing the effect of pH on different aspects of *Trichoderma* species biology, physiology and ecology.

Species tested (number of isolates)	pH	Media	Key results	Reference
Trichoderma aureoviride (1), *T. harzianum* (2) and *T. viride* (2)	2 - 9	Yeast and soil extract	Maximal mycelial growth rate observed at pH 4. Optimal pH for six different extracellular enzymes' activity varies from 5 to 7.	Kredics et al. 2004
T. asperellum (4)	4.5 - 8.5	Potato dextrose agar (PDA)	Mycelial growth for the four strains highest between 4.5 and 6.5, and also possible at 8.5	Begoude et al. 2007
T. asperellum (3), *T. aureoviride* (4), *T. harzianum* (3), *T. reesei* (1), *T. longibrachiatum* (10), *T. virens* (1) and *T. viride* (3)	4.5 - 7.5	Liquid Czapeks-dox	Biomass production varied among species as well as within the same species. 64% of isolates produced the highest biomass at pH 7.5. None produced highest biomass at pH 4.5.	Kolli et al. 2012
T. atroviride (1)	3 - 7	Low nutrient agar	pH did not affect the development of conidia	Schubert et al. 2010
T. atroviride LU132 (1)	3.5 - 8.5	PDA	Production of conidia was higher at pH 6.5 and possible at all pH levels tested. Conidia germination was highest at 7.5 and lowest at 5.5.	Daryaei et al. 2016
T. harzianum (2)	5 - 7	Unsterilized Nunn sandy loam soil	Both isolates colonised entire root depth of cucumber and radish plants regardless of pH level.	Ahmad & Baker 1987
T. harzianum (1)	5 - 7	Soil	Higher rhizosphere competence in soils at pH 5 & 6 compared to pH 7.	Jeong et al. 1997

et al. 2008). Recent research showed *T. atroviride* isolate LU132 is rhizosphere-competent on a range of plant species, including ryegrass and sweet corn (Cripps-Guazzone 2014). *Trichoderma atroviride* LU132 can reduce infection of onion by *Sclerotium cepivorum* and is the active agent in the biocontrol product Tenet® (Agrimm Technologies, Lincoln, New Zealand), with rhizosphere competence linked to its biocontrol efficacy (McLean et al. 2005). This isolate also increased ryegrass growth both in the presence and absence of the soilborne pathogen *Rhizoctonia solani* (Kandula et al. 2015). Rhizosphere competence is important in both growth promotion and biocontrol. However, the effect of soil pH on rhizosphere competence of this strain has not been investigated.

The objectives of this study were to investigate the influence of pH on the rhizosphere competence of *T. atroviride* LU132 and to ascertain if the effect was consistent in two plant species, ryegrass and sweet corn. Additionally, the effect of soil pH on endophytic colonisation of both ryegrass and sweet corn was assessed.

MATERIALS AND METHODS

The ability of the isolate *T. atroviride* LU132 to colonise ryegrass and sweet corn roots was assessed in non-sterile soil at three pH levels: 5.5, 6.5 and 7.5. These pH levels were selected as they are representative of the pH range of soils in New Zealand. *T. atroviride* LU132 was chosen after showing rhizosphere competence on both plants in previous experiments (Cripps-Guazzone 2014). This isolate was stored in 25% glycerol solution at -80°C and sub-cultured onto potato dextrose agar (PDA) plates incubated at 18-23°C under constant blue light for 8 days to stimulate conidiation. Spore suspensions were produced by flooding the plates with sterile water and rubbing with a sterile glass rod to release the conidia from the mycelium. Spore suspensions were filtered through two layers of Miracloth™ (Calbiochem, EMD Biosciences Inc., La Jolla, California, USA) to remove hyphal fragments and the concentration adjusted to $1x10^8$ conidia/ml based on haemocytometer counts. Conidia were applied to sweet corn (*Zea mays*, variety Chieftain, Corson Grain Ltd, Gisborne, New Zealand) and ryegrass seeds (*Lolium perenne*, variety Impac, Agriseeds, New Zealand) by mixing a spore suspension of *T. atroviride* LU132 with a 2% methyl cellulose sticker solution (MC, viscosity 400 cP, Sigma Aldrich Chemie GmbH, Steinheim, Germany). Seeds were surface sterilized in a 1% v/v sodium hypochlorite (Advance liquid bleach, 5g/100 ml sodium hypochlorite) and 5% ethanol prior to applying the spore suspension. On average each seed was coated with 10^6 spores. *T. atroviride* LU132 coated seeds and control seeds (coated with water and MC solution only) were sown in 500 ml plastic pots filled with Wakanui silt loam soil (cropping soil) at 20% (w/w) soil moisture content at pH 5.5 or amended to pH 6.5 or 7.5 (10 seeds per pot). The soils were prepared one week prior to the set-up of the experiment. The quantity of water needed to achieve 20% (w/w) soil moisture content (SMC) was added to each of three bags (7.5 kg of cropping soil per bag). To determine the SMC, the gravimetric water content (GWC) was calculated using the following formula: $GWC = (FS_g - DS_g) / DS_g * 100$. FS is the weight in grams of a fresh soil sample and DS is the weight in grams of a dry soil sample after drying at 105°C for 24 h. Sodium carbonate (Na_2CO_3) was added to the soil of two bags to increase the pH of the soil to 6.5 and 7.5, respectively. Moisture content was adjusted if necessary, and soil moisture content and pH were measured before being used in the study. Five pots per treatment were set up. Each pot was placed in a sealed plastic bag to maintain soil moisture content and then placed in an incubator with the following layout: five randomized blocks divided into two sub-blocks (one per plant species) with six pots each (three treatments and their control) and grown in 16:8 h light:dark 18:20°C. Light intensity was measured with a quantum radiometer (Li 188B, Li-Cor Biosciences, Inc., Lincoln, Nebraska, USA) and averaged 124 μmol photons /m^2/s (top half

of the incubator) and 136 µmol photons/m^2/s (bottom half). The experiment was duplicated.

Rhizosphere competence assessment

Rhizosphere competence was assessed 21 days after sowing for ryegrass and 7 days after sowing for sweet corn. The harvest times for the two plant species differed due to their relative growth rates, with the harvest times selected based on results of a previous experiment, regarding the age of the plants which supported sufficient rhizosphere soil for the analysis. At harvest, 10 seedlings per treatment (two seedlings per sub-block) were randomly chosen, gently shaken to remove any loosely adhering soil, cut from the crown, and added to a 50 ml capacity plastic tube (one seedling per tube). The seedlings were individually weighed and kept at 4°C overnight until processed. A fresh soil sample was collected from each pot and dried in an oven at 105°C for 24h to calculate the GWC of each sample. The following day, 10 ml of a 0.5% v/v Triton X 100 (BDH Chemicals Ltd., England) solution was added to each plastic tube. Tubes were shaken for 30 min (94 rpm) in an orbital shaker (Ratek EOM5, Ratek Instruments PTY Ltd, Boronia, Australia) to suspend propagules. Suspensions were serially diluted to 10^{-1} and 10^{-2} before 200 µl aliquots of the initial suspensions and each dilution were plated onto each of three *Trichoderma*-selective medium (TSM) plates (McLean et al. 2005), which were incubated at 20˚C in the dark. After plating the suspensions, seedlings roots were removed from the tubes, washed to remove adhering soil and weighed again. These weights were subtracted from the initial weights at harvest which allowed the amount of fresh rhizosphere soil present in each tube to be determined. The dry weight of rhizosphere soil was then calculated by using the GWC of each pot. The total number of *Trichoderma* colony-forming units (CFU) was counted 10 to 13 days after incubation to calculate the number of *Trichoderma* CFU/g of dry rhizosphere soil (DRS).

Endophytic colonisation

At harvest, a qualitative endophytic assessment was carried out on 10 seedlings per treatment (2 per sub-block, randomly chosen). Roots and shoots were washed under tap water before their surfaces were sterilised. Sterilisation was performed according to plant species and was done as follows: sweet corn plant material was immersed in a 1% v/v sodium hypochlorite for 4 min followed by two 2 min rinses in sterile water. The same technique was used for ryegrass. However, the sodium hypochlorite bath was 1.5 min long followed by two 2 min rinses in sterile water. The entire shoots and roots were subsequently cut into 2 cm segments with a sterile scalpel blade in a laminar flow unit and plated on TSM. For ryegrass, all shoot pieces from each replicate plant were plated onto one Petri dish. All root samples were plated onto one or two Petri plates depending on the root length. For sweet corn, all shoot pieces from each replicate plant were plated onto one Petri dish and all root pieces onto another. After incubation in the dark at 20°C for 5 to 8 days the presence or absence of colonies growing from the shoot and root pieces were recorded for all the plates. Plates where ≥1 *Trichoderma* colony was present in either shoot or root pieces were recorded as positive for endophytic colonisation. As background *Trichoderma* levels were low, it was assumed that *Trichoderma* colonies isolated from plant tissues were those of *T. atroviride* LU132. However, no identification other than morphology was used to assess endophytic colonisation.

Statistical analyses

Rhizosphere *Trichoderma* CFU counts were $\log_{10}$ transformed prior to being analysed by analysis of variance (ANOVA). *Trichoderma* CFUs in the rhizosphere of control seedlings were near zero for both plant species, and therefore omitted from the analysis and summarized as means only. Data ($\log_{10}$) for sweet corn seeds inoculated with *T. atroviride* LU132 were more variable than

Table 2 Experiment 1: Log_{10} CFU/g DRS for ryegrass and sweet corn grown in soil at three different pH levels (5.5, 6.5 and 7.5) from seeds either MC-only coated (control) or coated with *Trichoderma atroviride* LU132. Log_{10} values followed by the same letter in a column are not significantly different.

	Ryegrass log_{10}		Sweet corn log_{10}	
pH	Control	LU132	Control	LU132
5.5	1.73*	5.86 a	2.78*	4.62 a
6.5	1.73*	5.77 a	2.84*	4.86 a
7.5	2.09*	6.09 a	1.88*	5.27 a
LSD (5%)	-	0.346	-	0.902

* Data for untreated controls not included in the analysis; MC = methyl cellulose sticker solution

Table 3 Experiment 2: Log_{10} CFU/g DRS for ryegrass and sweet corn grown in soil at three different pH levels (5.5, 6.5 and 7.5) from seeds either MC-only coated (control) or coated with *Trichoderma atroviride* LU132. Log_{10} values followed by the same letter in a column are not significantly different.

	Ryegrass log_{10}		Sweet corn log_{10}	
pH	Control	LU132	Control	LU132
5.5	2.03*	6.05 a	2.03*	5.13 a
6.5	2.03*	6.17 a	2.09*	5.42 a
7.5	2.03*	6.09 a	2.03*	5.54 a
LSD (5%)	-	0.382	-	1.101

* Data for untreated controls not included in the analysis; MC = methyl cellulose sticker solution

ryegrass and violated the ANOVA assumption of homogeneity of variance. Therefore for each experiment, two randomized block ANOVAs were carried out, one for each plant species. ANOVA analyses were performed in GenStat 15th edition (VSN International, Hertfordshire, UK) with treatment means compared using Fishers unprotected LSD at $P = 0.05$.

RESULTS

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. *Trichoderma* CFUs for control treatments and for both plant species were low (10^1 – 10^2 CFU/g DRS) in both experiments and were not included in the analysis. In both Experiment 1 and 2, *Trichoderma* CFUs in the rhizosphere of ryegrass and sweet corn plants did not differ between soils at different pH levels (Tables 2 and 3).

Endophytic assessment

In Experiment 1, *Trichoderma* colonies were isolated from shoots and roots of *T. atroviride* LU132 inoculated ryegrass and sweet corn plants regardless of the soil pH level (Table 4). For those plants, a higher number of ryegrass roots compared to shoots appeared to have hosted *Trichoderma* colonies regardless of pH level. For inoculated sweet corn plants this number was similar. Regardless of the pH level, a higher percentage of inoculated ryegrass roots compared to inoculated

Table 4 Experiment 1: Isolation of *Trichoderma* from shoot and root pieces (2 cm) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or coated with MC-only (control) 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	
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)
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)

MC = methyl cellulose sticker solution

Table 5 Experiment 2: Isolation of *Trichoderma* from shoot and root pieces (2 cm) from sweet corn and ryegrass plants treated with *Trichoderma atroviride* LU132 or coated with MC-only (control) 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	
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)
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)

sweet corn roots appeared to be colonised. Twenty percent of the ryegrass control plants contained *Trichoderma* colonies in their roots at pH 5.5.

Comparable to the results of Experiment 1, in Experiment 2 *Trichoderma* colonies were isolated from shoots and roots of both inoculated plant species regardless of the soil pH level (Table 5). *Trichoderma* colonies were isolated at a higher percentage from inoculated ryegrass shoots and roots compared to inoculated sweet corn shoots and roots. Shoot and root colonisation by *Trichoderma* species were lower for inoculated sweet corn plants compared to the first experiment. Across both experiments, the extent of shoot and root colonisations differed according to plant species. Endophytic *Trichoderma* colonies were isolated from the entire length of ryegrass shoots and roots. However, in sweet corn endophytic *Trichoderma* colonies were isolated from the base of the shoot and the first 2 cm of the root at the

crown level. A large proportion of untreated roots and some shoots of ryegrass plants had colonies of *Fusarium* species growing endophytically. Colonies of both *Fusarium* and *Trichoderma* species grew from *T. atroviride* LU132 treated ryegrass and sweet corn in both Experiment 1 and 2.

DISCUSSION

This study is one of the first to investigate the influence of soil pH on endophytic colonisation by a *Trichoderma* strain. The results confirmed *T. atroviride* LU132 as an endophyte, with the high levels of endophytic colonisation unaffected by soil pH within the range (pH 5.5 to 7.5) tested.

Although *T. atroviride* LU132 was recovered as an endophyte from both plant species, *T. atroviride* LU132 had different colonisation patterns on sweet corn and ryegrass shoots and roots. Ryegrass roots and shoots were extensively colonised endophytically, whereas in sweet corn colonisation occurred at the base of the shoot, the top part of the root and only found sporadically along the root length. The difference in the age of the plants, ryegrass being harvested after 21 days and sweet corn after 7 days, may partly account for these differences with the additional growing time allowing increased endophytic colonisation by *T. atroviride* LU132.

T. atroviride LU132 was rhizosphere competent on sweet corn and ryegrass regardless of soil pH within the range tested. Many *in vitro* studies have shown that *Trichoderma* strains can grow at a range of pHs, but rhizosphere competence in soils with different pH has seldom been addressed (Kolli et al. 2012; Begoude et al. 2007; Daryaei et al. 2016, Mondal et al. 1996). The study by Ahmad and Baker (1987) is one of the only papers of those reviewed that addressed rhizosphere competence in soil at different pHs. They found that *T. harzianum* T-95 could colonise the entire roots of cucumber and radish at pH levels from 5 to 7. They found that with increasing pH, larger populations were isolated from roots at greater soil depths. In the current study, rhizosphere competence was not measured at different root depths, but population size obtained for the entire roots of sweet corn and ryegrass were similar to those obtained for cucumber and radish. Therefore, this study agrees with previous research that shows *Trichoderma* species can 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. The current experiment assessed rhizosphere colonisation and endophytic colonisation at one point in time only and it is therefore not representative of all the fluctuations that might occur in the field. However, the soil pH for both plant species varied between 5.5 and 7.5, which is within the range expected for *Trichoderma* to grow. It also offers an explanation for pH changes not affecting *T. atroviride* LU132's colonisation of sweet corn and ryegrass. This study confirms 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 in the conditions tested. However, in field conditions, other abiotic factors such as temperature and soil moisture content might interact with pH and potentially influence rhizosphere colonisation patterns. Further studies are needed to determine the potential of those interactions to affect *T. viride* LU132 colonisation capabilities in field conditions. Nevertheless, the ability to colonise the rhizosphere and internal parts of ryegrass plants within the range of pHs found for most New Zealand agricultural soils is a major asset of the biological control agent *T. atroviride* LU132.

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