THE IMPACT OF TALL FESCUE (*FESTUCA ARUNDINACEA*) ENDOPHYTE (*NEOTYPHODIUM* SPP.) ON NON-TARGET SOIL MICRO-ORGANISMS

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ABSTRACT

The impact of two strains of the tall fescue (*Festuca arundinacea*) endophyte (*Neotyphodium* spp.), (E+) on the rhizoplane and rhizosphere soil micro-organisms was examined at two sites (Lincoln: endophyte-free (E–) and the endophyte strain AR501; and Aorangi: E-, AR501 and a second strain, AR542). Rhizosphere and rhizoplane populations of bacteria and fungi, functional diversity, root fungi and soil microbial biomass carbon and nitrogen were measured. Most characteristics measured showed no differences between E+ and E– samples indicating that the presence of endophytes had no impact on non-target soil micro-organisms. At Aorangi, soil microbial biomass C and N were significantly greater ($P<0.05$) in AR542 than E1 and AR501 soils. Culturable fungal populations from both the rhizoplane and rhizosphere soil were significantly greater ($P<0.05$) in the E– than the AR501 samples at Lincoln but not at Aorangi. There were no differences in the functional diversity of rhizoplane micro-organisms due to endophyte.

Keywords: functional diversity, rhizoplane, rhizosphere, root fungi, tall fescue.

INTRODUCTION

Tall fescue (*Festuca arundinacea*) is an important cool season perennial forage plant in the humid regions of the United States and other parts of the world. Much of the tall fescue used in the USA is infected with an endophytic fungus, *Neotyphodium coenophialum*, which resides primarily in the basal stem tissue. This plant-endophyte association is one of mutual advantage to both the plant and the fungus. The endemic *N. coenophialum* endophyte reduces insect pressure through the production of alkaloids (Breen 1994) and increases tolerance to abiotic stresses such as drought (West 1994), allowing the plant to persist where endophyte-free plants do not. One of the protective alkaloids that is produced by the endemic endophyte, ergovaline, also causes fescue toxicosis in grazing cattle. In New Zealand, AgResearch Limited has sourced naturally occurring endophytes, mainly from Europe, that do not produce ergovaline and these have been introduced into existing tall fescue cultivars. These "non-toxic" endophytes produce at least one of the three loline derivatives produced by the endemic endophyte that are known to have anti-insect activity, as well as peramine, a feeding deterrent to Argentine stem weevil. One endophyte, AR542, is now marketed in the USA as Max Q™.

These endophytic fungi are not present in the roots but alkaloids they produce may be translocated to these tissues where they can adversely affect root herbivores. For instance, the presence of the endemic tall fescue endophyte adversely affects several plant parasitic nematodes (Kimmons et al. 1990; Pederson et al. 1988; West et al. 1988). However, the non-toxic endophytes, AR542 and AR501, appear to have little effect on plant feeding nematodes in the field (Timper & Bouton 2004; N.D. Bell, pers. comm.).

Ideally, a useful plant–endophyte association would provide protection against insect pests with little or no adverse effects on grazing animals and other non-target organisms. There is only a limited amount of literature on the environmental impacts of endophytes

on non-target soil organisms. In New Zealand, because tall fescue seed containing endophyte is not commercially available, field trials have been carried out to evaluate the advantages to the plant of infection with non-toxic endophytes. A study on soil and plant material from two of these tall fescue field trials was conducted to determine the environmental impacts (if any) of the presence of the endophyte on a range of soil biota. This paper reports on the effect of endophyte infection on rhizoplane and rhizosphere micro-organisms.

MATERIALS AND METHODS

In autumn 1997, field trials were established with tall fescue, cv. Advance, on a Kairanga silt loam soil at Aorangi near Palmerston North, and on a Wakanui silt loam at Lincoln in Canterbury. Both trials had four replicates of endophyte-free (E–) tall fescue and tall fescue infected with a non-toxic endophyte AR501. The Aorangi trial also contained two replicates of tall fescue infected with AR542.

Samples were taken from each of the 10 plots at Aorangi on 11 March 2002 and from the eight plots at Lincoln on 8 April 2002. Samples consisted of soil cores (100 mm x 100 mm) and the intact plants associated with each core. Tillers from each soil core, with roots attached, were tested for the presence of endophyte by staining with aniline blue followed by microscopic examination. Only those with confirmed endophyte infection were used as E+ samples while those with no detectable endophyte from endophyte-free plots were used as E– samples.

Microbiological analyses

Microbiological analyses were done on both root washings (rhizoplane) and sieved (<4.0 mm) field-moist soil (rhizosphere). For root washings, roots were carefully separated from attached soil, rinsed in tap water to further remove adhered soil material and a weighed quantity (approximately 0.43 g from Aorangi samples and 0.6 g from Lincoln samples) was placed in a bottle containing 100 ml of sterile distilled water (SDW) and 10 g of glass beads. Glass beads were used as a gentle abrasive to remove material from the root surface. The bottle was then shaken for 20 min on a wrist-action shaker after which 10-fold serial dilutions were made and aliquots (0.1 ml) plated on to duplicate plates of each agar medium. Total bacteria were counted on $^1/_2$ strength tryptose soy agar (TSA), fluorescent pseudomonads were counted on $^1/_2$ strength Kings medium B (KB) (King et al. 1954) with antibiotics (Sands & Rovira 1970) and fungi were counted on potato dextrose agar containing the antibiotic chlortetracycline (1 mg/100 ml) (PDAA). On KB plates, fluorescent as well as non-fluorescent colonies were counted. Chitinolytic bacteria were estimated on 1/10 TSA containing 1% chitin (Sarathchandra et al. 1996). Gram positive bacteria were counted on the medium described by Stapleton & DeVay (1982) and gram negative bacteria were counted on 1/10 TSA supplemented with crystal violet (10 mg/litre). From the counts, bacteria and fungi populations per gram of root material were calculated.

Soil samples (5 g dry weight equivalent) were weighed into bottles containing 100 ml SDW and treated as for the root washings. Aliquots (0.1 ml) of the serial dilutions were plated on to TSA, KB and PDAA, as well as Caprylate Thallous Agar (Star et al. 1976) for enumerating *Serratia* species. Gram-negative and gram-positive bacteria were not enumerated in rhizosphere samples. Bacterial plates were incubated at 25ºC while PDAA plates were incubated at room temperature (20-22ºC).

Root fungi

The different types of fungi colonising tall fescue roots were determined on PDAA, water agar and on a *Pythium* selective medium (Ali Shtayeh et al. 1986). Random lengths of tall fescue roots were washed well in tap water to remove soil, surface sterilised for 3 min in dilute sodium hypochlorite solution (0.4% available Cl), and washed four times in SDW. Ten root pieces (2-5 mm length) were cut from each plant, with five being placed on PDAA and the other five on water agar containing chlortetracycline (1 mg/100 ml). The plates were incubated at 20ºC until fungal growth occurred. Fungi growing out of roots were transferred to hay agar (0.2% by weight chopped hay/litre)

for identification. A further 10 non-sterilised root pieces were placed on *Pythium* medium and incubated at 20°C until fungal growth occurred.

Soil microbial biomass

Microbial biomass C was measured by a modified fumigation-extraction method of Vance et al. (1987). Triplicate samples of soil (5 g dry wt equivalent) were fumigated with chloroform; these and corresponding non-fumigated samples were then extracted with 0.5 M K_2SO_4 (1:5 soil solution ratio) for 2 h on an end-over-end shaker at 40 rpm and 20ºC. Suspended samples were then centrifuged for 5 min at 750 × g and filtered through Whatman 42 filter paper. Amounts of C in these extracts were determined as described by Wardle & Ghani (1995). Microbial biomass C values presented in this paper have not been corrected with a *kc* factor, which accounts for the proportion of microbial biomass C not extracted into K_2SO_4. Using the same soil extracts, microbial biomass N was estimated according to Brookes et al. (1985).

Functional diversity

Functional diversity of rhizoplane micro-organisms was determined by inoculating root washings (prepared as previously described) into Microlog Ecoplates™ (Biolog Inc., Hayward, California). Each plate was inoculated with 100 µl of 10^{-4} dilution from three rhizoplane samples (each plate contains three identical sets of substrate wells). Plates were incubated at 25ºC and the absorbance measured using a Bio-Rad Model 3550-UV microplate reader (Biorad Laboratories, Hercules, California) at a wavelength of 595 nm. Readings were made after 1, 2, 3 and 6 days. Each plate was shaken for 2 sec prior to reading. The average well colour development (AWCD) was calculated according to Garland (1996). The Shannon Diversity Index (*H*) was calculated using data from 31 wells according to the equation:

$$H = -\sum_{i=1}^{31} p_i \ln p_i$$

where p_i is the proportion of microbial activity (colour intensity in Biolog plates) on a given substrate (Zak et al. 1994).

Statistical analysis was done using analysis of variance. The microbial numbers were log transformed ($\log_{10}$) prior to analysis.

RESULTS AND DISCUSSION

The rationale for the present study was to detect any effects of endophyte infection on rhizoplane and rhizosphere organisms. The rhizoplane is the root surface and the rhizosphere is the region immediately surrounding the roots. In most pastoral soils, all of the topsoil can be regarded as the rhizosphere because of the extensive root mass. There were no significant differences in the microbial populations recovered from either the rhizoplane or from rhizosphere soil of tall fescue plants from E+ or E- samples from the Aorangi site (Tables 1 & 2). However, at the Lincoln site, there were significantly higher ($P<0.05$) populations of culturable fungi in both the rhizoplane and rhizosphere samples of E– samples compared with AR501 samples (Tables 1 & 2). The other groups of micro-organisms tested showed no significant treatment differences in rhizoplane or rhizosphere samples from the Lincoln site. *Serratia* spp. were detected in all the soil samples from the Lincoln site but at the Aorangi site, *Serratia* were present in only very few samples.

TABLE 1: Populations (log_{10} per g of root) of rhizoplane micro-organisms recovered from endophyte infected (AR501 and AR542) and endophyte free (E–) tall fescue plants from the two sampling sites.

	Aorangi				Lincoln		
	AR501	AR542	E-	SEM[1]	AR501	E-	SEM[1]
Culturable bacteria	8.26	8.00	7.76	0.31	8.37	8.15	0.15
Culturable fungi	5.65	5.62	5.13	0.43	6.42	7.00*	0.14
Pseudomonas	6.55	5.80	5.86	0.43	6.80	6.65	0.22
Fluorescent *Pseudomonas*	5.15	4.12	5.17	0.72	5.48	6.04	0.43
Chitinolytic bacteria	6.46	6.90	6.03	0.29	4.99	4.31	0.70
Gram +ve bacteria	6.55	6.46	6.24	0.24	6.32	6.48	0.17
Gram –ve bacteria	5.86	5.67	5.82	0.33	6.23	6.00	0.19

[1]Average Standard Error of the mean; *differences significant at P<0.05.

TABLE 2: Populations (log_{10} per g of soil) of rhizosphere soil micro-organisms recovered from endophyte infected (AR501 and AR542) and endophyte free (E-) tall fescue plants from the two sampling sites.

	Aorangi				Lincoln		
	AR501	AR542	E–	SEM[1]	AR501	E -	SEM[1]
Culturable bacteria	7.07	7.16	7.01	0.10	7.35	7.76	0.18
Culturable fungi	5.46	5.50	5.64	0.11	5.83	6.19*	0.10
Pseudomonas	5.60	5.41	5.38	0.12	6.49	6.57	0.15
Fluorescent *Pseudomonas*	4.57	4.24	4.48	0.43	5.17	4.73	0.35
Chitinolytic bacteria	6.01	6.32	5.94	0.29	4.21	4.39	0.31
Serratia spp.	n.d.	n.d.	n.d.		3.93	4.32	0.36

[1]Average Standard Error of the mean; *differences significant at P<0.05; n.d. not detected in the majority of samples

There were no differences in the microbial biomass C and N or the extractable organic C and N (the latter values obtained from non-fumigated soil extracts) in soils from the Lincoln site (Fig. 1). The microbial biomass C and N were significantly (P<0.05) greater in AR542 soil compared with AR501 and E– soils from the Aorangi site. The reasons for these differences are not clear but they indicate that presence of the endophyte has no adverse effects on the soil microbial biomass.

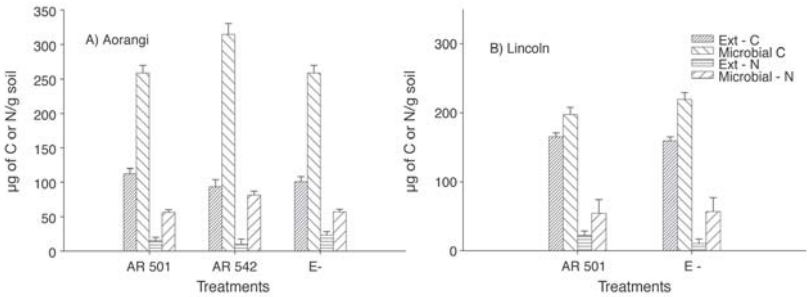

FIGURE 1: Extractable and microbial C and N in soils from (a) Aorangi and (b) Lincoln field sites containing tall fescue with (AR501 and AR542) and without (E–) endophyte. Bars represent SEM.

Table 3 lists the genera and species of root surface fungi isolated from root pieces of E+ and E– plants from both sites. A total of 155 and 131 fungal isolates were obtained from Aorangi and Lincoln sites, respectively. There were 30 species of fungi from 26 genera. Because the individual species were so few, statistical analyses could not be done on the data. No treatment effects could be detected. However, the numbers of *Trichoderma viride* and *Verticillium* spp. were greater in Lincoln samples compared to Aorangi samples. *Pythium* species were not detected from any root samples.

TABLE 3: Species of fungi isolated from root pieces of tall fescue plants with (endophyte strains AR501, and AR542) and without (E–) endophyte from Aorangi and Lincoln field sites.

	Aorangi			Lincoln	
Fungal species identified	AR501	AR542	E–	AR501	E–
Acremonium spp.		+	+	+	+
Arthrobotyrs spp.				+	
Chaetomium globosum	+++				
Chaetomium spp.	+	+	+		
Cladosporium cladosporioides					+
Cylindrocarpon spp.	+	+		+	+
Codinea fertilis	+	+			
Dactylaria spp.		+			
Dictyosporium elegans					+
Fusarium crookwellense			+	+	
Fusarium culmorum	+	+			
Fusarium oxysporum	+	+	++	+	+
Gliocladium roseum	++		+		
Humicola grisea				+	
Monocillium spp.	+				
Mortierella spp.	+		+		+
Mucor spp.	+	+	+	+	
Mariannaea elegans	+			++	
Myrothecium cinctum			+		+
Paecilomyces spp.	+		+	+	++
Periconia spp.		+	+		
Penicillium spp.	++		+	++	
Phialophora spp.			+		
Pithomyces chartarum	+	++			
Pyricularia spp.		++		+	+
Rhizoctonia spp.	+		+		
Sporomia spp.	+	+	+		+
Sterile fungi	++	+	++	+	
Trichoderma hamatum				+	
Trichoderma spp.	++	++	++		+
Trichoderma viride			+	+++	+++
Unidentified	+	+			+
Verticillium spp.	+		+	+++	+++

+ = 1-2 isolates; ++ = 2-4 isolates; +++ = >5 isolates

The microbial functional diversity (estimated using Microlog Ecoplates™) in the rhizoplane was also unaffected by the presence of either of the two endophyte strains (Fig. 2). This is an interesting observation since any adverse or inhibitory effects of toxic alkaloids present in root tissue or root exudates would be expected to affect the

root surface micro-organisms at the community level leading to differences in diversity patterns. Average well colour development (AWCD) was also not affected by the endophytes (data not shown). While AWCD summarises the information about total microbial activity in the plate, the Shannon Diversity Index provides information about the diversity of microbes in samples (as they utilise different carbon sources, Harch et al. 1997).

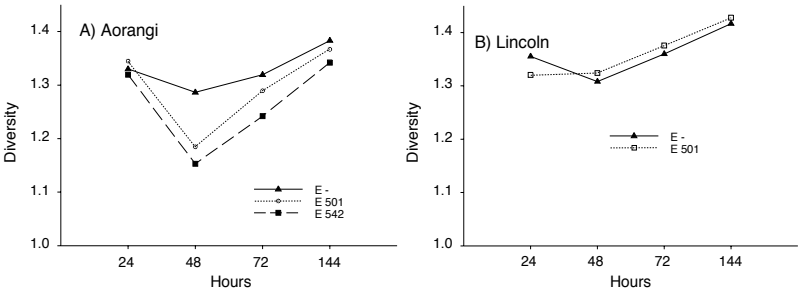

FIGURE 2: Functional diversity (Shannon Diversity Index) patterns of rhizoplane micro-organisms after 24, 48, 72 and 144 hours of incubation on Microlog Ecoplates from (a) Aorangi and (b) Lincoln field sites containing tall fescue with (AR501 and AR542) and without (E–) endophyte.

Humphries et al. (2001) found that when earthworms (*Eiseinia fetida)* were fed solely on roots of E+ tall fescue they lost weight and reproduced less. However, in the same study, there was increased weight gain of earthworms fed solely on E+ leaves. Mycorrhizal propagule densities and sporulation and root colonisation by *Glomus* species were adversely affected by the presence of the endophyte (Chu-Chou et al. 1992; Guo et al. 1992). Toxic alkaloids in leaf litter could also have negative impacts on soil microbes and other fauna leading to decreased litter breakdown and organic matter mineralisation. Microbial breakdown of plant residues containing endemic endophyte was found to be slower compared to E- residues leading to accumulation of organic matter in soil (Franzluebbers et al. 1999). In the present study, apart from very few instances, the presence of endophyte had no significant effects on the characteristics measured. Even those characteristics which showed differences between E+ and E– treatments (for example, greater populations of fungi in E– samples compared to AR 501 in Lincoln samples) the differences observed may well be due to factors other than the presence of the endophyte.

The microbial fauna of pasture soils are usually found to be dominated by bacteria. In the present study, although bacteria were still dominant, relatively high populations of fungi were detected, possibly due to low soil moisture content of 25% (g water/100 g dry soil) at the time of sampling.

In conclusion, the composition and the activities of the rhizosphere soil and rhizoplane micro-organisms were not adversely affected by the presence of non-toxic endophytes in tall fescue either directly through the translocation of alkaloids into the roots or indirectly through the natural incorporation of leaf litter into soil.

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REFERENCES

AgResearch Limited 2003: Variety: 'AR542'. Application no. 1999/198. *Plant Var. J. 16*: 59-60.

Ali-Shtayeh, M.S.; Len, L-H.C.; Dick, M.W. 1986: An improved method and medium for quantitative estimations of populations of *Pythium* species from soil. *Trans. Brit. Mycol. Soc. 86*: 39-47.

Breen, J.P. 1994: *Acremonium* endophyte interactions with enhanced plant resistance to insects. *Ann. Rev. Entomol. 39*: 401-423.

Brookes, P.C.; Landmann, A.; Pruden, G; Jenkinson, D.S. 1985: Chloroform fumigation and the release of soil nitrogen: A rapid direct extraction method to measure microbial biomass nitrogen in soil. *Soil Biol. Biochem. 17*: 837-842.

Chu-Chou, M.; Guoa, B.; An, Z-Q.; Hendrix, J. W.; Ferrissa, R.S.; Siegela, M.R.; Dougherty, C.T.; Burrus, P.B. 1992: Suppression of mycorrhizal fungi in fescue by the *Acremonium coenophialum* endophyte. *Soil Biol. Biochem 24*: 633-637.

Franzluebbers, A.J.; Nazih, N.; Stuedemann, J.A.; Fuhrmann, J.J.; Schomberg, H.H.; Hartel, P.G. 1999: Soil carbon and nitrogen pools under low- and high-endophyte-infected tall fescue. *Soil Sci. Soc. Amer. J. 63*: 1687-1694.

Garland, J.L. 1996: Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. *Soil Biol. Biochem 28:* 213-221.

Guo, B.Z.; Hendrix, J.W.; An, Z-Q.; Ferriss, R.S. 1992: Role of *Acremonium* endophyte of fescue on inhibition of colonisation and reproduction of mycorrhizal fungi. *Mycologia 84*: 882-885.

Harch, B.D; Correll, R.L.; Meech, W.; Kirkby, C.A.; Pankhurst, C.E. 1997: Using the Gini coefficient with BIOLOG substrate utilisation data to provide an alternative quantitative measure for comparing bacterial soil communities. *J. Microbiol. Methods 30*: 91 - 101.

Humphries, S.S.; Gwinn, K.D.; Stewart, A.J. 2001: Effects of endophyte status of tall fescue tissues on the earthworm (*Eisenia fetida*). *Environ. Toxicol. Chem. 20*: 1346-1350.

Kimmons, C.A.; Gwinn, K.D.; Bernard, E.C. 1990: Nematode reproduction on endophyte infected and endophyte-free tall fescue. *Plant Disease 74*: 757-761.

King, E.O.; Ward, M.K.; Raney, D.E. 1954: Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med. 44*: 301-307.

Pederson, J.F.; Rodriguez-Kabana, R.; Shelby, R.A. 1988: Ryegrass cultivars and endophyte in tall fescue affect nematodes in grass and succeeding soybean. *Agron. J. 80*: 811-814.

Sands, D.C.; Rovira, A.D. 1970: Isolation of fluorescent pseudomonads with a selective medium. *Appl. Micobiol. 20*: 513-514.

Sarathchandra, S.U.; Watson, R.N.; Cox, N.R.; di Menna, M.E.; Brown, J.A.; Burch, G.; Neville, F.J. 1996: Effects of chitin amendment of soil on microorganisms, nematodes, and growth of white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.). *Biol. Fert. Soil. 22*: 221-226.

Stapleton, J.J.; DeVay, J.E. 1982: Effect of soil solarization on populations of selected soilborne microorganisms and growth of deciduous fruit tree seedlings. *Phytopath. 72*: 323-326.

Starr, M.P.; Grimont, P.A.; Grimont, F.; Starr, P.B. 1976: Caprylate-thallous agar medium for selectively isolating *Serratia* and its utility in the clinical laboratory. *J. Clin. Microbiol. 4*: 270-276.

Timper, P.; Bouton, J.H. 2004: Effect of endophyte status and tall fescue cultivar on reproduction of lesion and stubby-root nematodes. *Proceedings 5th International Symposium on Neotyphodium/Grass Interactions*: (in press).

Vance, E.D.; Brookes, P.C.; Jenkinson, D.S. 1987: An extraction method for measuring soil microbial biomass C. *Soil Biol. Biochem. 19*: 703-707.

Wardle, D.A.; Ghani, A. 1995: Why is the strength of relationships between pairs of methods for estimating soil microbial biomass often so variable? *Soil Biol. Biochem. 27*: 821-828.

West, C. P. 1994: Physiology and drought tolerance of endophyte-infected grasses. *In*: Bacon, C.W.; White, J.F. *ed*. Biotechnology of Endophytic Fungi of grasses. CRC Press, Boca Raton. Pp. 87-99.

West, C.P.; Izekor, E.; Oosterhuis, D.M.; Robbins, R.T. 1988: The effect of *Acremonium coenophialum* on the growth and nematode infestation of tall fescue. *Plant Soil 112*: 3-6.

Zak, J.C.; Willig, M.R.; Moorhead, D.L.; Wildman, H.G. 1994: Functional diversity of microbial communities: A quantitative approach. *Soil Biol. Biochem. 26*: 1101-1108.