An investigation of non-target impacts of ryegrass endophytes on nematodes and soil microorganisms

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Abstract

A pot trial with two sampling times was conducted to compare the non-target effects of Nil endophyte ryegrass with three endophyte/ryegrass combinations, two selected endophytes (AR1 and AR37) and a wild-type, in the same cultivar. Shoot dry weight, soil nematode and microbial abundance and community diversity were assessed at each sample time. Plants infected with wild-type endophyte produced significantly less shoot weight than all other treatments due to slow initial growth. None of the plant-parasitic nematodes were consistently affected by any of the endophyte combinations. There was a significant overall effect of endophyte infection on some bacterial-feeding nematode families which was reflected in the community indices calculated but these were not consistent across sample times. Wild-type endophyte differed significantly from AR1 for one bacterial-feeding nematode family, and differed significantly from all other treatments in composition of *Pseudomonas* and Actinobacteria communities. Neither total fungal populations nor functional diversity were consistently different amongst treatments. While there were some differences in the microbial and nematode communities at one or other of the sampling times these did not appear to be consistent nor represent a threat to the non-target community as a whole.

Keywords: *Neotyphodium,* Denaturing Gradient Gel Electrophoresis, Enrichment Index, Structure Index, Nematode Channel Ratio

Introduction

The ability of *Neotyphodium* endophyte-infection to confer pest resistance on important forage grasses has resulted in incorporation of new strains into a range of cultivars such that they are currently the most widely distributed plant biotechnology product in New Zealand pasture land. Their impact on New Zealand's agriculture has been to allow more reliable pasture production as a result of reduced pest populations with minimal undesirable effects on livestock health (Easton & Fletcher 2007).

With a widely used biotechnology such as the new endophytes, which have been in use for a number of years, there is a potential risk of unintended non-target effects becoming widespread. While there is a large literature on the non-target effects of biotechnologies involving genetic modifications (e.g. O'Callaghan *et al.* 2005; Squire *et al.* 2003) there is a small but growing literature on non-target effects of novel plant-endophyte combinations, which do not involve genetic modifications (e.g. Bell 2005; Bultman *et al.* 2009; Matthews & Clay 2001; Popay & Jensen 2005; Sayer *et al.* 2004).

Analogous to some modified plants, endophytes produce different types of compounds in their host plant which affect pests but which can also affect livestock. For endophytes the compounds that are currently known to deter insects are largely produced in above ground tissue (Ball *et al.* 1997) and therefore affect above ground pests. There are, however, some compounds which are found in roots and are thought to be responsible for deterring root-feeding pests (Patchett *et al.* 2008) including insects (Popay & Gerard 2007) and nematodes (Elmi *et al.* 2000; Timper *et al.* 2005). Given that many compounds in roots are exuded into soil around the roots (rhizosphere) as part of normal root functioning (Bais *et al.* 2006), and that these have been shown to affect the structure and activity of microorganisms in the rhizosphere of many plant species (e.g. Costa *et al.* 2006), it is possible that endophytes could influence organisms other than the targeted pests that inhabit this important and dynamic habitat.

This study aimed, therefore, to compare non-target effects of perennial ryegrass plants infected with (i) the non-selected wild-type endophyte, producing mammalian toxic and pest resistance metabolites, with (ii) perennial ryegrass plants infected with selected endophytes (AR37 and AR1) that have different metabolic profiles which reduce or eliminate mammalian toxicity but retain pest resistance properties. Nil endophyte plants were also included as a control.

Materials and Methods

Perennial ryegrass (*Lolium perenne*) seeds (cultivar 'Grasslands Samson') with endophytes AR1, AR37, wild-type or Nil (no endophyte) were germinated on damp filter paper for 5 days at 20ºC. Seedlings were transferred into planting boxes (50 seedlings per box) containing potting mix and allowed to grow for 10

Table 1 Raw mean (log transformed data in parentheses) plant (g/ pot) and nematode (number/100 g soil) data over both sample times. Values in italics are sub-totals of the non-italic value immediately above. Note: *, ** and *** = P<0.05, 0.01 and 0.001 respectively.

	Nil	Wild type	AR1	AR37	SED
Shoot dry weight	4.84 (1.3)	4.04 (1.0)	5.06 (1.4)	5.08 (1.3)	(0.09)***
Total nematodes	3250 (8.0)	3210 (8.0)	3725 (8.2)	2638 (7.8)	(0.20)
Bacterial feeders	1505 (7.2)	1824 (7.5)	1955 (7.6)	1700 (7.4)	(0.20)
Cephalobidae	*675 (6.2)*	*1195 (6.9)*	*926 (6.8)*	*1247 (7.0)*	*(0.29)**
Rhabditidae	*404 (5.9)*	*237 (5.3)*	*633 (6.2)*	*254 (5.2)*	*(0.31)***
Fungal feeders	400 (5.8)	316 (5.5)	356 (5.8)	192 (5.1)	(0.28)
Plant feeders	341 (5.4)	279 (5.3)	396 (5.5)	209 (5.2)	(0.39)
Paratylenchus	271 (4.8)	234 (5.0)	331 (5.2)	156 (4.7)	*(0.48)*
Plant associates	131 (4.7)	102 (4.6)	149 (5.0)	47 (3.9)	(0.36)*
Omnivores	63 (4.2)	60 (4.0)	73 (4.2)	45 (4.0)	(0.40)
Nematode Channel Ratio	0.752	0.852	0.851	0.894	0.033***
Enrichment Index	67.4	45.4	64.8	44.7	8.16*
Structure Index	62.1	46.0	41.5	33.5	7.05**

weeks. A single tiller from each plant was tested for the presence of endophyte by immunoblotting (Hahn *et al.* 2003). Plants were maintained in the shade house until the pot experiment was set up.

Soil (Horotiu sandy loam) was collected from a farm near Hamilton, sieved (<4 mm) and was put into each of 64 pots (15 cm diameter) at field moist levels (660 g dry weight equivalent). Eight pots were allocated into each of the four treatments and four tillers of ryegrass with the correct endophyte status were planted into each pot. Pots were placed in a glasshouse with ambient lighting and maintained with regular overhead watering. Four replicate pots from each treatment were destructively sampled after 10 weeks growth and the remaining four replicates were sampled after a further 10 weeks. Plant shoots were all trimmed to the same height on the second batch of replicate plants after 10 weeks growth.

Nematodes were extracted from 100 g wet weight soil per pot at each of the two harvest times using the modified Whitehead & Hemming method described by Bell & Watson (2001). After extraction and fixation in 4% formalin each nematode sample was reduced to 1 ml by aspiration and centrifugation and a 100 µl subsample taken for nematode counts and identification as described in O'Callaghan *et al.* (2008). Indices of the nematode community structure (Enrichment Index, Structure Index, Nematode Channel Ratio) were calculated as outlined in Ferris *et al.* (2001) and Yeates (2003). Results were analysed by ANOVA of untransformed plant data and of natural log transformed nematode data (nematode data transformed to normalise distribution).

For plate-based microbial analyses soil was sieved (<4 mm), serially diluted and used for estimating culturable bacterial and fungal populations as well as microbial functional diversity. Total culturable bacteria and fungi were counted on tryptose soy agar (TSA) and potato dextrose agar containing the antibiotic chlortetracycline (PDAA). Inoculated plates were incubated at 25°C (bacteria) or room temperature (fungi). Functional diversity was estimated according to Zak *et al.* (1994) by inoculating soil dilutions into Biolog EcoPlates™, incubating these at 25°C and measuring the colour development in wells at 24, 48, 72 and 144 h using Bio-Rad Model 3550-UV microplate reader (Biorad Laboratories, Hercules, California) at a wavelength of 595 nm. The Shannon Diversity Index (*H*) was calculated according to Zak *et al.* (1994).

To determine whether the presence of the endophyte in ryegrass had any impact on the diversity of the soil microbial community, soil samples were collected for community DNA extraction. At each sampling, four soil minicores (5 mm diameter × 70 mm deep) were collected from each pot and immediately frozen in liquid nitrogen and stored at –80°C until DNA extraction. Total DNA was extracted from each 0.25 g subsample of soil using the PowerSoil™ DNA Isolation Kit according to the manufacturer's instructions (MO BIO Laboratories, Inc.). Taxon-specific primers were used in a nested PCR approach as described previously for alpha- and beta-proteobacteria and actinobacteria (O'Callaghan *et al.* 2008). Techniques for profiling pseudomonad populations (Evans *et al.* 2004), general

Figure 1 Principal co-ordinate analysis (PCO) of pseudomonad and actinobacterial communities in response to different endophyte/ryegrass combinations for two combined sample times. Control = Nil endophyte ryegrass. Note: Circles represent 95% confidence intervals for means of endophytic grass lines but significance of difference between plant lines must be read from Table 3.

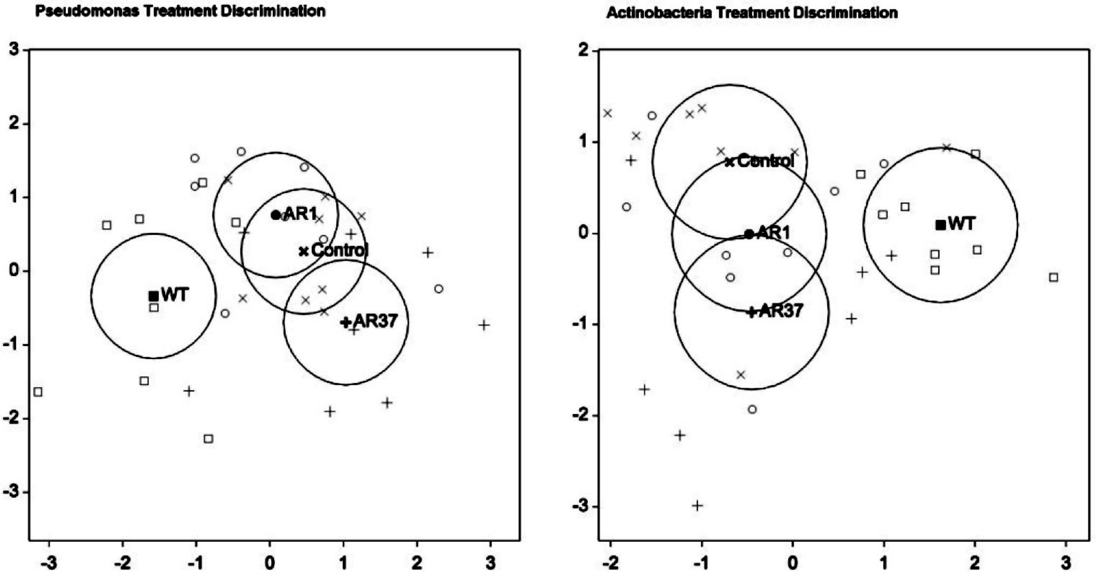

Table 2 Culturable bacteria, fungi (log_{10} colony forming units) and Shannon functional diversity index of soils from pots growing endophytic or Nil endophyte plants at two sampling times. Note: * = P<0.05.

	Culturable bacteria		Culturable fungi		Functional diversity	
	10 weeks	20 weeks	10 weeks	20 weeks	10 weeks	20 weeks
Control	7.11	7.18	4.40	5.29	1.364	1.424
WT	7.19	7.31	4.55	5.27	1.385	1.447
AR1	7.33	7.03	4.97	5.17	1.381	1.390
AR37	7.26	7.28	5.05	5.26	1.373	1.433
SEM[1]	0.088	0.111	0.146*	0.043	0.0150	0.0136*

[1]SEM = Standard error of the mean

fungi (Hoshino & Matsumoto 2007) and arbuscular mycorrhizal fungi (Liang *et al.* 2008) have been reported previously. Following PCR amplification of 16S rDNA, PCR products were separated by Denaturing Gradient Gel Electrophoresis (DGGE) and acid silver staining was used to visualise the bands on gels (O'Callaghan *et al.* 2008).

DGGE gels were scanned and intensity and position of bands were determined using the Diversity Database software (Bio-Rad). Discriminant analysis was carried out using GenStat. The 95% confidence regions around the means for each plant line (depicted as circles in Fig. 1) were produced by the GenStat DISCRIMINATE procedure. The significances of the treatment differences (using combined data from both sampling dates) were accessed using a Hotelling T2-test (O'Callaghan *et al.* 2008).

Results and Discussion

Wild type endophyte-infected plants produced significantly less shoot matter than all the other treatments for the combined sample times (Table 1) which was due to poor initial growth up to the first sampling (treatment P<0.01, data not shown) but not for the second sampling (treatment × sampling date interaction, P<0.05). There was no significant difference in abundance of total bacterial feeding nematodes

Table 3 Hotelling T^2 probabilities of the level of difference between the structures of microbial communities associated with each plant line under the null hypothesis that all plant lines will support the same soil microbial community. Values of less than 0.0500 indicate a significant difference in microbial community structure.

Taxonomic group	Endophyte	Control	AR1	AR37
Actinobacteria	Control	–		
	AR1	0.5356	–	
	AR37	0.1399	0.5182	–
	WT	0.0100	0.0259	0.0164
Pseudomonas	Control	–		
	AR1	0.8629	–	
	AR37	0.4831	0.1106	–
	WT	0.0290	0.0475	0.0045
Betaproteobacteria	Control	–		
	AR1	0.1208	–	
	AR37	0.0615	0.0937	–
	WT	0.0256	0.0333	0.4334
Alphaproteobacteria	Control	–		
	AR1	0.0250	–	
	AR37	0.1368	0.3593	–
	WT	0.1757	0.7073	0.6884
General fungi	Control	–		
	AR1	0.0421	–	
	AR37	0.1013	0.1923	–
	WT	0.6269	0.0799	0.1159
Arbuscular mychorrizal fungi	Control	–		
	AR1	0.0118	–	
	AR37	0.1151	0.0021	–
	WT	0.1123	0.0258	0.0067

WT= wild type

amongst the treatments, but there was a difference for the Cephalobidae and Rhabditidae bacterial feeding families (Table 1). Cephalobidae nematodes were significantly more abundant in soil growing wild-type or AR37 than Nil endophyte plants for combined sample dates, with this effect being most pronounced at the second sampling (treatment × sampling date interaction P=0.05). Conversely, Rhabditidae nematodes were more abundant beneath Nil endophyte and AR1 than wild-type or AR37 plants, an effect that was greater at the first (treatment $P<0.05$, data not shown) than the second sampling.

Due to their high fecundity and short generation times Rhabditidae nematodes are considered good indicators of any environmental resource enrichment which leads to increased bacterial growth (Ferris *et al.* 2001). In contrast, the Cephalobidae nematodes are almost ubiquitous in soil, have relatively slow population growth and the ability to withstand environmental stress and so are poorer indicators of bacterial populations. The Enrichment Index is designed to reflect this and, as for Rhabditidae, this was significantly greater with Nil endophyte and AR1 than wild-type or AR37 plants for combined samplings (Table 1). This suggests that there were greater resources available for bacteria to utilise beneath Nil endophyte and AR1 plants with the most obvious source being root exudates.

The Structure Index incorporates disturbance-sensitive nematode taxa and is a measure of environmental stability (greater values) or disturbance (lower values). For the pots sampled here, Nil endophyte plants supported a nematode community indicative of greater stability than any of the endophyte-infected plants, most notably at the second sampling. Nil endophyte plants also supported a nematode community more dominated by fungal-feeders than the endophyte plants as indicated by a lower Nematode Channel Ratio. Again this was most obvious at the second sampling (treatment × sampling date interaction, $P<0.001$).

Most plate-count microbial characteristics estimated were not consistently affected by the presence or absence of endophyte (Table 2). In the 10 week sampling, fungal populations were greater ($P<0.05$) in soil beneath AR37 and AR1 compared to Nil endophyte and Wild type plants. In the 20 week sampling this difference no longer existed. Work with tall fescue endophytes (Sayer *et al.* 2004), showed culturable fungi from one field site was greater in Nil endophyte plots compared to others. In the 20 week sample, functional diversity was lower in AR1 soils compared to others. Overall, the presence or absence of different types of endophytes or no endophytes had very little impact on non-target soil microorganisms.

Molecular profiles of the microbial communities found in soil in which Nil endophyte or various lines of endophytic grasses had been grown were consistent between replicates, allowing robust statistical analysis of treatment effects. In general, the structure of the actinobacterial, betaproteobacterial and pseudomonad communities associated with the wild-type endophyte line differed significantly from the structure of these groups on Nil-, ARI- and AR37- plant lines, which were all quite similar to each other (Fig. 1, Table 3).

The communities of alphaproteobacteria and, as for plate-count results, general fungi showed significant differences between AR1 and Nil endophyte plants (Table 3).

When primers specific to arbuscular mycorrhizal fungi (AMF) were used, more differences were detected between treatments (Table 3). AMF communities associated with AR1, AR37 and wild-type plants were significantly different to each other in structure, but only AR1 plants had significantly different communities to the Nil-endophyte communities.

Few studies have examined the microbial communities associated with plants harbouring endophytes which range in the profile of metabolites they produce. This study provides a "snap-shot" of microbial communities associated with endophytic and non-endophytic ryegrass in an experiment conducted in only one soil type with only two sampling dates. Results from this preliminary analysis suggest that some microbial taxa examined (e.g. general soil fungi) were largely unaffected by the presence of endophyte in ryegrass. In contrast, communities of AMF, which form very close associations with plant roots varied widely between the various endophyte-ryegrass combinations; each combination supported a significantly different AMF community, although only the AR1 ryegrass combination differed significantly from the Nil endophyte ryegrass.

Considered together, results suggest that the presence or absence of selected endophytes in ryegrass is not likely to affect the nematode or microbial community function in the short term. A study examining responses of prokaryotic communities in rhizosphere soils to tall fescue endophyte infection (Jenkins *et al.* 2006) found that some groups of microorganisms were suppressed in the presence of endophyte. The authors suggested that long-term suppression of these microbial communities may play a role in enhanced soil carbon sequestration associated with endophytic tall fescue (Franzluebbers 2006) but further work is needed to test this hypothesis.

From this and other studies it would appear that many selected endophytes in pasture grasses pose little threat to non-target beneficial organisms in soil. Larger-scale field studies are now needed to confirm results from pot and small-scale, short-term field trials so that what appears to be a high degree of environmental safety of endophytes can be confirmed.

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