Functional Ecology

British Ecological Society

Functional Ecology 2011, **25**, 910–920 doi: 10.1111/j.1365-2435.2011.01853.x

Competition between foliar *Neotyphodium lolii* endophytes and mycorrhizal *Glomus* spp. fungi in *Lolium perenne* depends on resource supply and host carbohydrate content

Qianhe Liu[1], Anthony J. Parsons[1], Hong Xue[1], Karl Fraser[1], Geraldine D. Ryan[2], Jonathan A. Newman[2] and Susanne Rasmussen[1,*]

[1]*AgResearch, P.B. 11008, Palmerston North, New Zealand; and* [2]*School of Environmental Sciences, University of Guelph, Guelph, Ontario N1G 2W1, Canada*

Summary

1. Cool-season grasses can be simultaneously infected by foliar fungal endophytes and colonised by mycorrhizal fungi, the integrated functions of which are strong predictors of plant fitness within grassland ecosystems. Evidence has been presented previously that infection of grass species with foliar endophytes can negatively affect mycorrhizal colonisation. Here, we tested the hypothesis that mycorrhizal colonisation in turn adversely affects *Neotyphodium* endophyte concentrations and that the competitive interaction between the two endosymbionts is affected by resource supply.

2. Specifically, we report how competition between *Glomus* (*G. mosseae* – GM, *G. intraradices* – GI) mycorrhizal fungi and *N. lolii* (common strain (CS) and AR1) foliar endophytic strains is affected by P supply and water-soluble carbohydrate (WSC) content in two *Lolium perenne* (perennial ryegrass) cultivars: a high sugar grass, AberDart, and a conventional (control) grass, Fennema.

3. The presence of *Glomus* mycorrhizae reduced the concentrations of endophytes and alkaloids in leaf blades and pseudostems. The reduction depended on P supply, ryegrass cultivar (notably WSC content) and endophyte strain. Conversely, foliar endophyte infection reduced mycorrhizal colonisation rates and concentrations in the roots of the control cultivar Fennema, although not in the high sugar cultivar, AberDart.

4. Neither GM nor *N. lolii* infection had an effect *per se* on the yield of root or blade compared with mycorrhiza-free (M-) and endophyte-free (E-) plants, respectively; though, yield of roots and blades was reduced by GI infection and at low P.

5. Competitive interactions between ecologically widespread foliar endophytes (valuable for plant protection) and mycorrhizal endosymbionts (valuable for P acquisition) as seen in this study are of critical importance especially in areas of high pest prevalence and low P availability. Our work stresses the need for elucidating the physiological/metabolic basis for such interactions between endosymbionts to understand how these processes contribute to plant performance and fitness in grassland ecosystems.

Key-words: alkaloids, arbuscular mycorrhizal fungi, foliar endophytes, *Glomus intraradices*, *Glomus mosseae*, grassland ecosystems, high sugar grass, lolitrem B, peramine, perennial ryegrass

Introduction

Grass species belonging to the family Poaceae are often hosts of systemic symbiotic fungal endophytes that reside in aerial plant parts and are vertically transmitted through the

*Correspondence author. E-mail: susanne.rasmussen@agresearch.co.nz

seeds of infected grasses (Leuchtmann 1992; Clay & Schardl 2002). In agricultural systems, associations between (i) *Lolium perenne* L. (perennial ryegrass) and *Neotyphodium lolii* (Latch, Christensen & Samuels) Glenn, Bacon & Hanlin, and (ii) *Schedonorus phoenix* (Scop.) Holub (tall fescue) and *N. coenophialum* (Morgan-Jones & Gams) Glenn, Bacon & Hanlin predominate (Christensen *et al.* 1993). Endophyte infection is proposed to benefit agricultural grasses through increased tolerance to abiotic stress (e.g. drought), enhanced growth and competitive ability, and increased resistance to herbivory because of the production of endophyte-derived alkaloids (Clay & Schardl 2002). Major alkaloids produced by the common *N. lolii* strain (CS = Lp19) in ryegrass are peramine, lolitrem B and ergovaline, while *N. coenophialum* produces peramine, ergovaline and lolines in fescue (Powell & Petroski 1992). The neurotrophic alkaloids lolitrem B and ergovaline are thought to be toxic to mammalian herbivores and are the putative causes of ryegrass staggers in sheep and fescue toxicosis in cattle, respectively, while peramine and lolines are associated with resistance to invertebrates. For use in pasture grazing systems, novel associations between naturally occurring endophyte strains and high yielding grass cultivars have been developed to retain insect-deterrent properties while reducing the detrimental impact on grazing herbivores (Fletcher & Easton 1997). For example, the *N. lolii* strain 'AR1' contains peramine only and 'AR37' produces only janthitrems, while the *N. coenophialum* strain 'AR542' produces peramine and *N*-acetyl norloline only. Although these are widely commercially marketed, few studies have examined the broader ecological implications of such associations and how resource supply, plant and fungal genotypes and competitive interactions may alter the grass–endophyte relationship and the putative benefits derived by their use in agricultural systems. A recent study by Rudgers, Fischer & Clay (2010) found that both endophytic strain and host plant cultivar play an important role in plant species diversity and plant competitive interactions in tall fescue plots.

Grasses are also able to form associations with a second type of obligate symbiotic fungi belonging to the phylum Glomeromycota (Smith & Read 1997; Schüssler, Schwarzott & Walker 2001). Arbuscular mycorrhizal fungi such as *Glomus mosseae* (GM) and *G. intraradices* (GI) colonise host roots and form specific structures (vesicular and arbuscular) as well as extensive intra- and extra-radical hyphal nets, which facilitate the uptake of mineral elements, mainly phosphorus (P), from the soil (Smith & Gianinazzi-Pearson 1988; Marschner & Dell 1994; Bucher 2007). Mycorrhizae are thought to be widely beneficial in agricultural systems because of improvements in plant nutrition status and yield, resistance to environmental stresses, improved soil aggregate formation and stability against erosion (Smith & Read 1997).

Dual infection of host plants with both mycorrhizal and endophytic fungi occurs in both natural and agricultural ecosystems. Figure 1 depicts the anatomy of a dually infected grass plant. While studies on the interactions of host plants with either one of these fungal symbionts are numerous, studies considering tripartite interactions between endophytic and mycorrhizal fungi, and host plants, are rare. Foliar endophytic fungi can suppress mycorrhizal colonisation of roots in tall fescue (Chu-Chou *et al.* 1992; Mack & Rudgers 2008), perennial ryegrass (Müller 2003) and annual ryegrass (Omacini *et al.* 2006) but they can also enhance mycorrhizal colonisation in *Bromus setifolius* (Novas, Cabral & Godeas 2005). Effects of mycorrhizal colonisation on endophyte infection have been much less studied, although it has been reported that mycorrhizal infection reduced the level of resistance to Argentine stem weevil in *L. perenne* infected with *N. lolii* (Barker 1987) and that foliar *N. lolii* endophyte-conferred resistance in *L. perenne* against the moth *Phlogophora meticulosa* was reduced by mycorrhizal (GM) colonisation (Vicari, Hatcher & Ayres 2002).

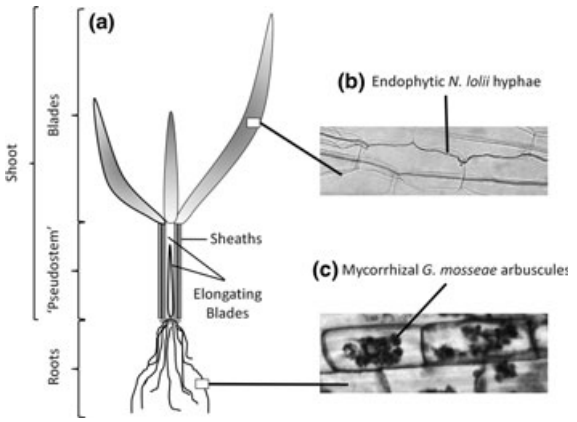

Fig. 1. Schematic diagram of the anatomy of a dually infected grass plant (a) showing endophytic *Neotyphodium* hyphae in leaf blades (b) and colonisation by mycorrhizal arbuscules in root tissues (c).

While host plant associations with both foliar endophytes and mycorrhizal fungi are generally regarded as mutualistic, recent evidence suggests that these associations represent a mutualism–parasitism continuum. In mycorrhizal associations, host growth depression at high levels of soil P availability can occur (Hall, Johnstone & Dolby 1984; Peng *et al.*1993), while the relationship between foliar endophytes and their hosts depend on host genotype, endophytic strain and environmental conditions, notably those affecting resource availability (Müller & Krauss 2005; Saikkonen *et al.* 2006; Cheplick 2007). In a previous controlled environment study, we used quantitative polymerase chain reaction (qPCR) to estimate endophyte concentrations in *N. lolii*-infected ryegrass to test whether resource supply affected the interactions between the host plant and its endophyte (Rasmussen *et al.* 2007). We found that high N supply reduced both endophyte and alkaloid concentrations by 50% and that endophyte and alkaloid concentrations in a high sugar grass (HSG) cultivar, bred for elevated water-soluble carbohydrate (WSC) content (AberDove-HSG), were also reduced to 50% compared with a control cultivar (Fennema). This clearly shows that simple changes in nutrient supply and resource (energy)-related cultivar traits can affect the symbiotic association between ryegrass and its fungal endophyte, and it is

likely that any association of plants with endosymbionts that would change the host nutrient status, e.g. mycorrhizal fungi, might also affect endophyte and alkaloid concentrations.

Here, we performed an environmental study to test the hypothesis that mycorrhizal fungi (GM, GI) and *N. lolii* strains (CS, AR1) interact antagonistically in simultaneously infected ryegrass, and that the outcomes of these interactions depend on resource supply (P) and WSC content of the *L. perenne* host.

Materials and methods

PLANT MATERIAL AND GROWTH CONDITIONS

We used two *L. perenne* cultivars, a HSG 'AberDart' (IGER, UK) and a control 'Fennema', that have been shown to differ inherently in WSC contents (Parsons *et al.* 2004). Two strains of *N. lolii* endophytes and two species of mycorrhizal *Glomus* fungi were used to examine the generality of interactions between these two types of plant fungal symbionts.

Mature plants of ryegrass cannot be artificially inoculated with *Neotyphodium* fungi, and *de novo* infection of uninfected plants requires the inoculation of the meristematic region of endophyte-free germinating grass seeds with fungal mycelia (Latch & Christensen 1985). Endophyte-free germinating seeds of the two cultivars were inoculated with one of two *N. lolii* strains differing in their alkaloid profiles: Common Strain Lp19 (CS, produces all three major alkaloids: peramine, lolitrem B and ergovaline) and AR1 (produces only peramine; Fletcher & Easton 1997). A further set of plants was mock-inoculated (endophyte free: E-). Five weeks after inoculation, endophyte presence was confirmed in epidermal sheath strips by aniline blue staining of hyphae (Latch, Potter & Tyler 1987).

Two tillers of approximately equivalent size were separated from each plant (hence clonal replicates) and individually planted into non-drainage pots containing 720 g sterilised quartz sand (particle size 0·5–1 mm). The original inocula of *G. mosseae* (Nicol. & Gerd.; GM) Gerdemann & Trappe (UK115) and *G. intraradices* (Smith & Schenck UT126; GI) were supplied by INVAM [International Culture Collection of (Vesicular) AM Fungi, USA] and propagated separately on ryegrass roots of E-plants grown in autoclaved sand for 12 weeks before use to generate large amounts of inocula for the experiment. For mycorrhizal treatments, the sand was mixed with 40 g whole-mycorrhizal-inocula of either GM or GI. Mycorrhiza-free (M-) controls were mixed with the same amount of autoclaved inocula and 30 mL of non-autoclaved inocula filtrate (passed through 10 μm nylon mesh). The surface of the pots was covered with filter paper to minimise cross-contamination.

Plants were randomly distributed and grown in controlled climate chambers (14-h light, 20 °C; 10-h dark, 10 °C, light intensity 620 μmol m^{-2} s^{-1}) for 12 weeks. Half-strength Hoagland nutrient solution containing either 0·05 mM (low P) or 2 mM (high P) KH_2PO_4 was added weekly to each pot (60 mL per pot). During the whole growth period, plants were watered every second day to 'pot field capacity'.

A total of 360 pots (see Statistical Analysis) representing 180 genotypes (plants grown at the two different P treatments were of identical genetic background) were kept under these conditions for 12 weeks. Plants were cut back every 4 weeks to approximately 6 cm above-ground, the clippings were oven-dried (65 °C, 1 week) and the dry weight (DW) recorded.

SAMPLE PREPARATION

After 12 weeks, plants were cut at soil level, the total shoot fresh weight determined, separated into leaf blades and pseudostems, immediately frozen, ground in liquid nitrogen and subsequently freeze-dried. Roots were washed under tap water to remove sand and subsequently rinsed with deionised water. Fresh weight of the roots was determined after removing excess water by pressing the roots in paper towels. A fraction of the roots was frozen immediately, ground in liquid nitrogen and subsequently freeze-dried. The other root fraction was kept on ice until AM colonisation rates were determined.

ENDOPHYTE AND MYCORRHIZAL CONCENTRATIONS

Endophyte and mycorrhizal concentrations were estimated based on qPCR of genomic DNA (*g*DNA) isolated from infected blades, pseudostems and roots as described (Rasmussen *et al.* 2007). Primers suitable for qPCR were designed for fragments of a *N. lolii*-specific chitinase A (*NlChiA*; forward 5′-aagtccaggctcgaattgtg-3′; reverse 5′-ttgaggtagcggttgttcttc-3′), a GM-specific phosphate transporter (*GmPT*; DQ074452; forward 5′-acgtgaagtcgatgaaccag-3′; reverse 5′-catgacaccgcagtaccaac-3′) and a GI-specific alkaline phosphatase (*GiAP*; AB114298; forward 5′-gggatgctggttaatgatacc-3′; reverse 5′-tggatctccgtgagtgctc-3′). The PCR protocol was as described (Rasmussen *et al.* 2007).

MYCORRHIZAL COLONISATION

Colonisation of roots with AM fungi was microscopically assessed. Fresh roots were treated with KOH and stained using trypan blue. After destaining in glycerol–water, randomly selected root segments (10 per sample) were mounted on slides and mycorrhizal structures (arbuscules, vesicles) were counted (100× magnification) and expressed as percentage of roots colonised as described (McGonigle *et al.* 1990).

ALKALOID CONCENTRATIONS

Peramine was extracted as described (Rasmussen *et al.* 2007) and quantified using a linear ion trap mass spectrometer (Thermo LTQ) coupled to a Thermo Finnigan Surveyor HPLC system (Thermo Fisher Scientific, San Jose, CA, USA). Data were acquired in positive electrospray mode and mass spectra collected as described (Koulman *et al.* 2007).

Lolitrem B was analysed using an adaptation of the method of Gallagher, Hawkes & Stewart (1985) as described (Hunt *et al.* 2005)and estimated by comparison of peak areas to those obtained with authentic lolitrem B (obtained from Dr. Chris Miles, AgResearch NZ).

WATER-EXTRACTABLE PHOSPHATE AND CARBOHYDRATE CONCENTRATIONS

Water-extractable phosphate (P*i*) was determined in blades and roots of plants grown at low P supply only, as colonisation rates were greatly reduced at high P and we were interested here in comparing colonised with mycorrhiza-free plants. P*i* was extracted by shaking 40 mg freeze-dried and powdered plant material (blades and roots separately) in 1·5 mL deionised Millipore water for 1 h at room temperature. Extracts were centrifuged (12 000 *g*, 20 min), and P*i* con-

centrations were determined in the supernatant using Malachite Green Phosphate Assay kit (POMG-25H; BioAssay Systems, Hayward, CA, USA) following the manufacturer's instructions.

Low molecular weight (LMW) WSCs (mostly glucose, fructose and sucrose) and high molecular weight (HMW) WSCs (mainly fructans) were extracted and quantified in blades and roots as described previously (Hunt *et al.* 2005; Rasmussen *et al.* 2007).

STATISTICAL ANALYSIS

The overall experiment was a split-unit (also called a split-plot) design, with the two cultivars ('Fennema' and 'AberDart'), three levels of foliar endophytes (E-, CS, AR1) and three levels of AM fungi (M-, GM, GI) as the whole unit treatments (representing $2 \times 3 \times 3 \times 10$ reps $=$ 180 individual genotypes) with two levels of P supply (low P, high P) as the subunit treatment (and so 360 pots; genotypes across P treatments being identical). For response variables that were measured on more than one tissue type (blades, pseudostems, roots), these tissue-specific responses were analysed separately. For some response variables (AM colonisation and concentrations; root and blade P*i* concentrations), our analyses were restricted to low P supply. Colonisation rates in plants grown at high P supply were very low (means ± SE: low P = 9·09 ± 0·92; high P = 0·41 ± 0·14); we therefore statistically analysed only the data sets from plants grown at low P.

In these cases, the analysis was simply a 3-way full factorial ANOVA (cultivar × AM × endophyte).

As it is known that copy numbers and nuclei per cell can vary between mycorrhizal species and strains (Corradi *et al.* 2007), results for GM and GI concentrations cannot be directly compared. We therefore analysed the effects on the concentrations of the two different mycorrhizal species separately.

All analyses were conducted using JMP statistical software version 7.0.We used Box–Cox transformation to homogenise the error variances and report, uniformly, the untransformed means and standard error of the means as a measure of data dispersion. We used Tukey's honestly significant difference (HSD) test on the Box–Cox-transformed least-squared means to assist in the interpretation of significant effects and interactions. In the results and figures, different letters denote means that are significantly different according to Tukey's HSD test.

Results

For ease of comprehension, the text and figures focus on the statistically significant main effects and interactions ($P \leq 0{\cdot}05$). In all cases, where error degrees of freedom deviate from those expected based on the experimental design, the difference was because of missing value data points (see Table 1 for *F* and *P* values). Table 2 shows means ± SE for significant effects that have not been presented graphically.

ENDOPHYTE CONCENTRATIONS IN PLANT TISSUES

Endophyte concentrations were approximately 6-fold higher in pseudostems compared with blades (200 vs. 1200 *NlChiA* copies ng^{-1} *g*DNA). In blades, endophyte concentrations were negatively affected by mycorrhizal colonisation (Fig. 2a) and high P supply. The reduction at high P was stronger in AberDart-HSG compared with Fennema (cultivar × P supply interaction; Fig. 2b).

In pseudostems, endophyte concentrations were significantly reduced by high P supply and in AberDart-HSG compared with Fennema; the interaction between cultivar × P supply was significant (Fig. 2d). The three-way interaction between P supply × mycorrhiza × endophyte was also significant and resulted in a reduction of endophyte concentrations at high P supply in GM compared with GI-colonised plants, but only if infected with the endophytic strain AR1 (Fig. 2c).

ENDOPHYTE ALKALOID CONCENTRATIONS

In blades, peramine concentrations were significantly reduced in mycorrhizal plants (Fig. 3a), at high P supply (Fig. 3b), in AberDart (Fig. 3c) and in CS compared with AR1-infected plants (Fig. 3d). None of the interactions were significant.

In pseudostems, peramine concentrations were significantly reduced at high P supply (Fig. 3f). The interactions between cultivar × mycorrhiza and cultivar × endophytic strain, on the concentration of peramine, were also significant. Peramine concentrations were reduced in mycorrhizal compared with M- plants, but only in Fennema (Fig. 3e); they were also reduced in CS-infected plants, but only in Fennema (Fig. 3g).

Lolitrem B is produced by the CS endophyte only; its concentration was significantly reduced in blades of mycorrhizal plants, but only in Fennema (cultivar × mycorrhiza interaction: Table 2).

We performed an ANCOVA with endophyte concentration (NlChiA copies) as the covariate to examine the effects of endophyte concentration on alkaloids (Table 3). In both blades and pseudostems, peramine and lolitrem B concentration was positively correlated with NlChiA copy number and this effect was highly significant. In blades, there was a significant NlChiA × cultivar interaction for both alkaloids, whereby the slope (i.e. alkaloid accumulation per unit endophyte) was greater in the AberDart cultivar. There was also a two-way NlChiA × P and NlChiA × mycorrhiza interaction in blade tissues for peramine whereby high P supply had a higher slope than low P and both GI and GM plants had higher slopes than mycorrhiza-free plants. There was also a NlChiA × endophyte interaction for peramine in pseudostems whereby the slope was greater in the CS treatment.

MYCORRHIZAL COLONISATION RATES AND CONCENTRATIONS IN PLANT ROOTS

Mycorrhizal colonisation rates were assessed from microscopic counts of vesicular and arbuscular mycorrhizal structures in roots of colonised plants and are expressed as per cent of root colonisation. Mycorrhizal structures of GM were mainly of the arbuscular type, whereas GI structures were mainly vesicular (means ± SE: Arbuscules GM = 1·94 ±

Table 1. Summary of ANOVA results

Source	NlChiA	Peramine	Lolitrem	DW	HMW	LMW	P*i*
Blades							
CV		***		***	***		
		$F_{1,113} = 31{\cdot}82$		$F_{1,162} = 23{\cdot}71$	$F_{1,165} = 45{\cdot}39$		
P	***	***		***		***	
	$F_{1,93} = 84{\cdot}44$	$F_{1,99} = 30{\cdot}57$		$F_{1,157} = 367{\cdot}9$		$F_{1,153} = 26{\cdot}05$	
AM	**	**	*	***			
	$F_{2,111} = 5{\cdot}04$	$F_{2,113} = 5{\cdot}66$	$F_{2,61} = 3{\cdot}80$	$F_{2,162} = 13{\cdot}61$			
EP		*					
		$F_{1,113} = 4{\cdot}19$					
CV × P	*			**		**	
	$F_{1,93} = 11{\cdot}34$			$F_{1,157} = 10{\cdot}59$		$F_{1,153} = 9{\cdot}77$	
CV × AM			*	*			
			$F_{2,63} = 4{\cdot}60$	$F_{2,162} = 4{\cdot}38$			
P × AM				***			
				$F_{2,157} = 11{\cdot}60$			

Source	NlChiA	Peramine	Lolitrem	FW(shoots)	HMW	LMW	P*i*
Pseudostems							
CV	**	***					
	$F_{1,109} = 12{\cdot}40$	$F_{1,113} = 13{\cdot}37$					
P	***	***		***			
	$F_{1,94} = 147{\cdot}00$	$F_{1,95} = 47{\cdot}97$		$F_{1,156} = 438{\cdot}6$			
AM				***			
				$F_{2,166} = 13{\cdot}97$			
EP		***					
		$F_{1,113} = 12{\cdot}63$					
CV × P	*						
	$F_{1,94} = 4{\cdot}21$						
CV × AM		*					
		$F_{2,113} = 3{\cdot}74$					
CV × EP		*					
		$F_{1,113} = 4{\cdot}13$					
P × AM				**			
				$F_{2,156} = 7{\cdot}22$			
P × AM × EP	*			*			
	$F_{2,94} = 3{\cdot}53$			$F_{4,156} = 2{\cdot}44$			

Source	AM colonisation	GmPT	GiAP	FW	HMW	LMW	P*i*
Roots							
CV	**	**		**	***		
	$F_{1,106} = 181{\cdot}68$	$F_{1,53} = 8{\cdot}80$		$F_{1,168} = 10{\cdot}18$	$F_{1,166} = 29{\cdot}94$		
P				***	***	***	
				$F_{1,149} = 296$	$F_{1,152} = 63{\cdot}19$	$F_{1,149} = 25{\cdot}17$	
AM	**						***
	$F_{2,106} = 39{\cdot}43$						$F_{2,152} = 17{\cdot}10$
EP	*		**	*			
	$F_{2,106} = 5{\cdot}31$		$F_{2,53} = 5{\cdot}77$	$F_{2,168} = 3{\cdot}28$			
CV × P					*		
					$F_{1,152} = 4{\cdot}37$		
CV × EP	*		**				**
	$F_{2,106} = 4{\cdot}6$		$F_{2,53} = 6{\cdot}03$				$F_{2,151} = 3{\cdot}64$
P × AM				**	***		
				$F_{2,149} = 6{\cdot}92$	$F_{2,152} = 14{\cdot}34$		
CV × AM × EP							

Only significant main effects and interactions are shown.
*$P < 0{\cdot}05$; **$P < 0{\cdot}01$; *** $P < 0{\cdot}001$; blank fields: $P \geq 0{\cdot}05$; grey fields: not analysed.
CV, cultivar; P, phosphorus; AM, arbuscular mycorrhiza; EP, endophyte; GIAP, GI-specific alkaline phosphatase; GmPT, GM-specific phosphate transporter.

Table 2. Means ± SE for all significant effects that have not been presented graphically

	Fennema	AberDart	
Lolitrem B (blades) (μg g DM^{-1})			
M-	3·43 (0·61)^{a}	2·74 (0·37)^{ab}	
GM	1·51 (0·08)^{b}	2·35 (0·46)^{ab}	
GI	1·52 (0·22)^{b}	3·00 (0·73)^{a}	
GM concentration (copies *GmPT* ng^{-1} gDNA)	665 (101)	1074 (149)	
Blade yield (g DM)			
M-	0·45 (0·03)^{b}	0·56 (0·03)^{a}	
GM	0·44 (0·02)^{b}	0·53 (0·02)^{a}	
GI	0·40 (0·02)^{b}	0·41 (0·03)^{b}	
Low P	0·33 (0·01)^{c}	0·35 (0·02)^{c}	
High P	0·53 (0·01)^{b}	0·65 (0·02)^{a}	
	Fennema	*AberDart*	
Root yield (g FM)	7·82 (0·24)	6·84 (0·21)	
	E-	CS	AR1
	7·04 (0·26)†	7·1 (0·29)†	7·85 (0·29)†
Extractable phosphate (μg g^{-1})	M-	GM	GI
	242·4 (4·7)^{b}	290·6 (11·6)^{a}	300·1 (9·2)^{a}
	Fennema	AberDart	
E-	286·6 (15·2)^{ab}	278·0 (11·3)^{ab}	
CS	246·6 (6·76)^{b}	306·3 (17·6)^{a}	
AR1	270·3 (14·6)^{ab}	279·2 (11·7)^{ab}	

Different letters denote means that are significantly different.
†Too weak to be separated by a Tukey test.
CS, common strain; GmPT, GM-specific phosphate transporter.

0·32, GI = 0·39 ± 0·14; Vesicles GM = 0·65 ± 0·1, GI = 6·44 ± 0·94).

Mycorrhizal colonisation rates were significantly reduced in endophyte-infected plants, but only in Fennema (endophyte × cultivar interaction; Fig. 4a). They were also lower in GM compared with GI-colonised plants (means ± SE: GM = 4·97% colonisation ± 0·44, GI = 13·20% colonisation ± 1·10).

Mycorrhizal concentrations were assessed by qPCR and are expressed as the number of copies of genes specific for each mycorrhizal species (*GmPT*, *GiAP*) per total (plant + fungal) root *g*DNA. As pointed out previously, colonisation rates were very low at high P supply; we therefore only report mycorrhizal concentrations in roots of plants grown at low P supply.

Concentrations of GI (expressed as copies of *GiAP* ng^{-1} gDNA) were decreased in endophyte-infected compared with E- plants but this was observed only in Fennema (cultivar × endophyte interaction; Fig. 4b). Concentrations of GM (expressed as copies of *GmPT* ng^{-1} gDNA) were lower in Fennema compared with AberDart-HSG (Table 2); none of the other main effects or interactions were significant.

PLANT GROWTH

Blade yield (g DM) was analysed from the sum of two cuttings prior to the final harvest. A significant interaction of P supply × mycorrhiza (Fig. 5a) resulted in reduced blade yield in GI compared with M- and GM-colonised plants at low P; at high P, both mycorrhizal species reduced blade yield significantly compared with M- plants. The two-way interactions cultivar × mycorrhiza and cultivar × P supply (Table 2) were

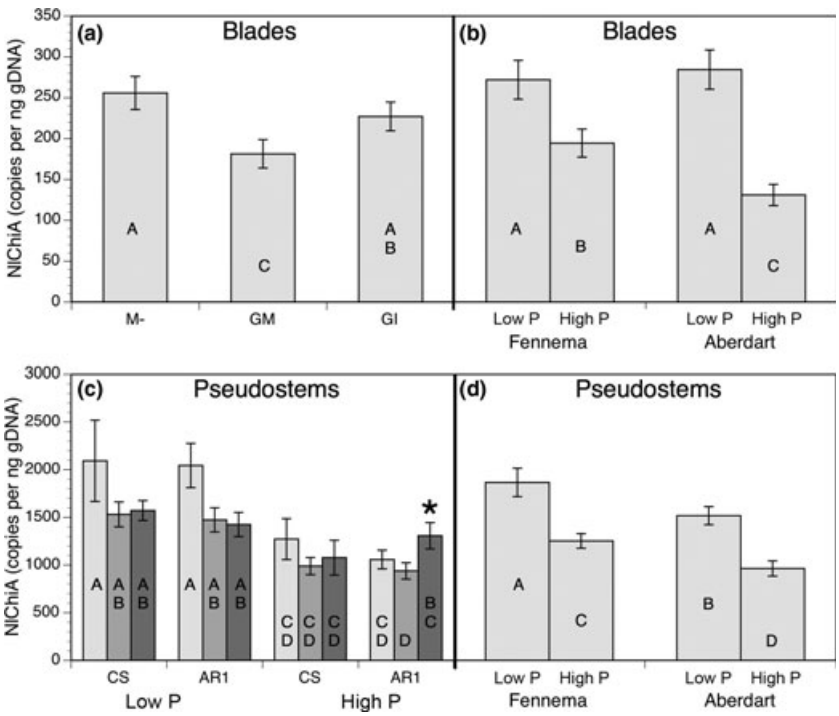

Fig. 2. Top panel shows the effects of (a) mycorrhiza and (b) the cultivar × P interaction on endophyte concentrations in blades. Bottom panel shows the effects of the interactions (c) between P × mycorrhiza × endophytic strain (light grey denotes 'M-', medium grey denotes 'GM' and dark grey denotes 'GI') and (d) between cultivar × P on endophyte concentrations in pseudostems (means ± SE). Different letters denote means that are significantly different.

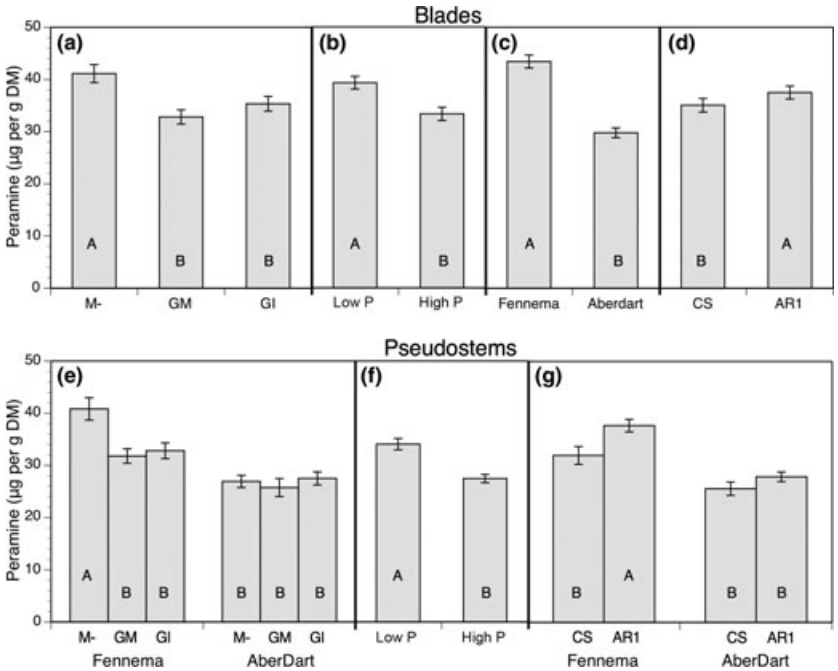

Fig. 3. Top panel shows the effects of (a) mycorrhiza, (b) P supply, (c) cultivar and (d) endophytic strain on peramine levels in blades. Bottom panel shows the effects of (e) the cultivar × mycorrhiza interaction, (f) P supply and (g) the cultivar × endophyte interaction on peramine levels in pseudostems (means ± SE). Different letters denote means that are significantly different.

Table 3. Summary of ANCOVA results

	Blades		Pseudostems	
	Peramine	Lolitrem B	Peramine	Lolitrem B
NlChiA	***	***	***	***
	$F_{1,54}$ = 85·32	$F_{1,54}$ = 14·02	$F_{1,54}$ = 30·51	$F_{1,54}$ = 23·36
NlChiA slope	0·049	0·009	0·006	0·002
NlChiA*CV	*	*		
	$F_{1,54}$ = 4·04	$F_{1,54}$ = 7·32		
Aberdart slope	0·059	0·011		
Fennema slope	0·039	0·007		
NlChiA*P	*			
	$F_{1,54}$ = 4·61			
High P slope	0·059			
Low P slope	0·039			
NlChiA*AM	*			
	$F_{2,54}$ = 3·98			
MF slope	0·031			
GI slope	0·058			
GM slope	0·058			
NlChiA*EP			*	
			$F_{1,54}$ = 4·99	
CS slope			0·007	
AR1 slope			0·004	

*P < 0·05; ***P < 0·001; blank fields: $P \geq 0{\cdot}05$; grey fields: not analysed. Slope units: µg per g DM/copies per ng gDNA.
N1ChiA, *N. lolii* specific chitinase A gene = endophyte concentration; CV, cultivar; P, phosphorus; AM, arbuscular mycorrhiza; EP, endophyte; CS, common strain.

also significant. Blade yield was reduced by GI colonisation, but only in AberDart-HSG. It was increased at high P supply, and this effect was stronger in AberDart-HSG compared with Fennema.

For total shoot (pseudostems and blades) yield (g FM), the three-way interaction P supply × mycorrhiza × endophyte was significant, resulting in reduced shoot yield (i) in GI-colonised plants compared with M- plants at high P, but only if infected with the endophytic CS strain and (ii) in GI-colonised compared with GM-colonised plants at low P supply, but only in E- and AR1-infected plants (Fig. 5c).

Root yield (g FM) was increased in Fennema and in endophyte-infected plants (Table 2). Mycorrhizal infection

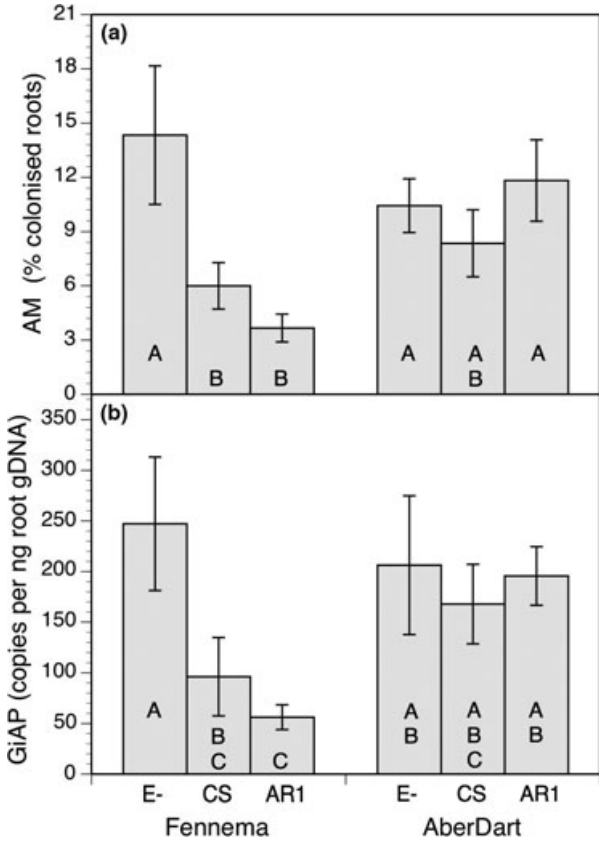

Fig. 4. The effects of the cultivar × endophyte interaction on (a) mycorrhizal colonisation and (b) on GI concentration in *L. perenne* roots grown at low P supply (means ± SE). Different letters denote means that are significantly different.

interacted with P supply (Fig. 5b), resulting in decreased root yield in GI-colonised compared with GM-colonised plants, but only at low P.

CARBOHYDRATE AND EXTRACTABLE PHOSPHATE CONCENTRATIONS IN PLANT TISSUES

In blades, high molecular weight WSC concentrations were higher in AberDart-HSG compared with Fennema (Fig. 6e), confirming that AberDart-HSG is a cultivar with the capacity to accumulate higher levels of sugars in the grazed plant component compared with conventional cultivars like Fennema. In roots, high molecular weight WSCs were higher in Fennema compared with AberDart. They were also higher at high P; this effect was strongest in GI-colonised plants (P supply × mycorrhiza interaction; Fig. 6b) and in Fennema (cultivar × P supply interaction: Fig. 6a).

Blade low molecular weight WSCs were significantly higher at high P supply, but only in Fennema (cultivar × P supply interaction; Fig. 6d). Low molecular weight WSCs in roots were also higher at high P supply (Fig. 6c). None of the other main effects or interactions were significant.

Extractable phosphate was significantly increased in roots of mycorrhizal plants compared with M- plants (Table 2). There was also a weakly significant cultivar by endophyte interaction. The interaction occurs only because Fennema infected with the CS endophyte has lower P_i than AberDart infected with the CS endophyte, but the rest of the endophyte-cultivar combinations were intermediate and not different from each other (Table 2). None of the other interactions were significant.

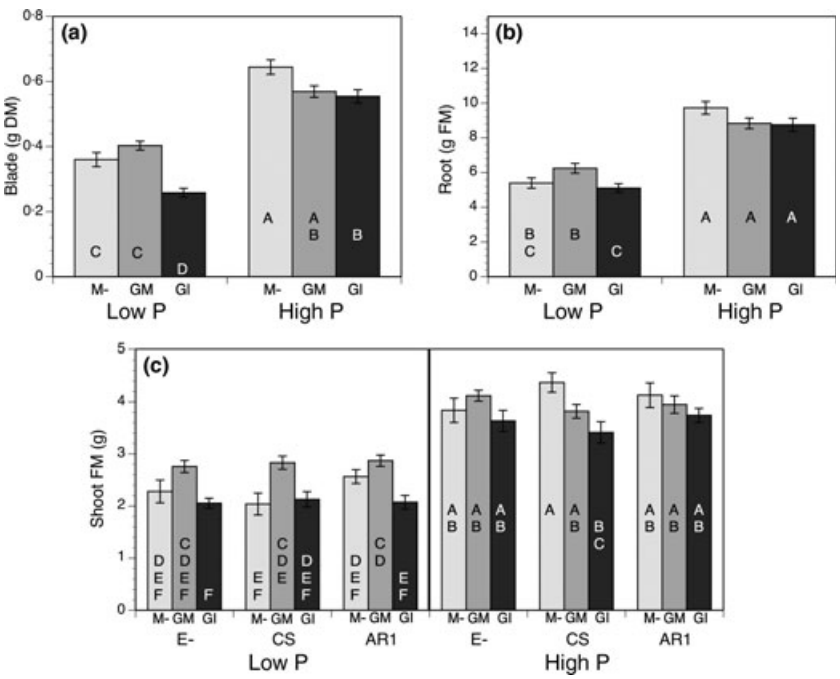

Fig. 5. The effects of the P supply × mycorrhiza interactions on yield of (a) blades and (b) roots (means ± SE). The effect of the three-way interaction (P supply × endophyte × mycorrhizal) on shoot yield (c). Different letters denote means that are significantly different.

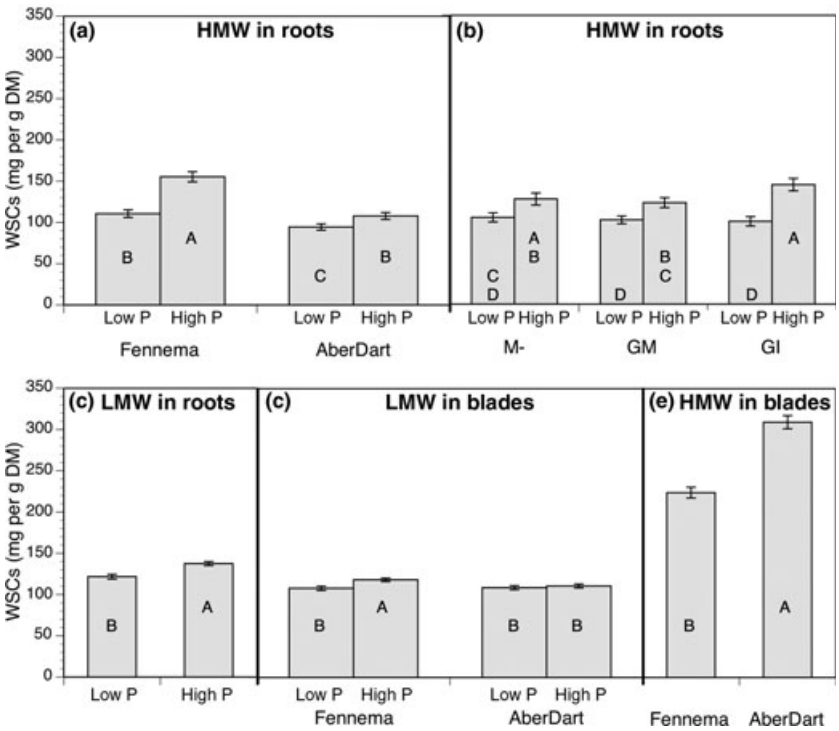

Fig. 6. Top panel shows the effects of (a) a P × cultivar interaction and (b) a P × AM interaction on HMW water-soluble carbohydrates (WSCs) in roots. Bottom panel shows the effect of (c) P on LMW WSCs in roots,(d) a P × cultivar interaction on LMW WSCs in blades and (e)the effect of cultivar on HMW WSCs in blades (means ± SE). Different letters denote means that are significantly different.

Discussion

COMPETITIVE INTERACTIONS BETWEEN MYCORRHIZAL FUNGI AND FOLIAR ENDOPHYTES

Here, we show that colonisation by mycorrhizal fungi reduced the concentrations of foliar endophytes and of the anti-herbivore associated alkaloids peramine and lolitrem B. Very few studies on the effects of mycorrhizal colonisation on the ryegrass–endophyte association have been reported. Barker (1987) and Vicari, Hatcher & Ayres (2002) showed that insect resistance conferred to ryegrass by *N. lolii* was reduced in *G. fasciculatum*- and GM-colonised plants, but neither author reported endophyte or alkaloid concentrations and it is not clear if the reduced insect resistance was because of decreased endophyte and/or alkaloid concentrations. It is also possible, but at this stage speculative, that the endophyte responses are a result of mycorrhiza-induced systemic plant responses, which have been shown to increase resistance against bacterial and fungal pathogens in roots and shoots of mycorrhizal plants (Volpin *et al.* 1994; Cordier *et al.* 1998; Liu *et al.* 2007a). We show here that alkaloid production per unit endophyte is increased in mycorrhizal plants relative to mycorrhiza-free plants. This suggests that the observed decrease in alkaloids in mycorrhizal plants is not because of an inhibition of alkaloid production *per se*, but is more likely an effect of reduced endophyte.

Conversely, the presence of endophyte reduced mycorrhizal colonisation by 70% and decreased GI concentrations by 60% but did not affect GM concentrations. This suggests that the antagonistic effect of endophyte on mycorrhizal fungi may be species specific, with GI being much more sensitive to the presence of endophyte than GM. The presence of endophyte reduced mycorrhizal colonisation and GI concentrations in the Fennema cultivar, which has higher concentrations of endophytes and alkaloids compared with AberDart, and so arguably, the greater concentration may have a greater adverse effect on mycorrhizal infection. A reduction in mycorrhizal colonisation has previously been reported for several endophyte-infected *Lolium* species (Chu-Chou *et al.* 1992; Müller 2003; Omacini *et al.* 2006), but only one study reported endophyte hyphal density (Mack & Rudgers 2008) and none quantified alkaloid concentrations. It has been suggested that endophyte alkaloids transferred via leachates from endophyte-infected grass litter (Antunes *et al.* 2008) or directly to the roots (Chu-Chou *et al.* 1992) might inhibit mycorrhizal fungi. However, the latter explanation is unlikely as translocation of endophyte-derived alkaloids to root material has only been shown to occur for lolines that are not produced in the *N. lolii – L. perenne* association. We have previously analysed different sample sets from the *N. lolii–L. perenne* association for alkaloids in roots, but did not detect any, even when using highly sensitive mass spectrometry (unpublished results). However, we have demonstrated in previous metabolomics of the *N. lolii–L. perenne* association (Cao *et al.* 2008; Rasmussen *et al.* 2008a,b) that a wide range of other non-alkaloid metabolites are affected in endophyte-infected tissues, which could also be involved in transferring the negative effects of foliar-based endophytic fungi to the root-based mycorrhizal fungi seen in this and other studies. On the other hand, we cannot rule out alkaloids as a mechanism for reducing mycorrhizal concentration and colonisation in endophyte-infected plants because in this

study, alkaloid production per unit endophyte increased in both GI and GM relative to mycorrhiza-free plants.

EFFECTS OF P SUPPLY ON FOLIAR ENDOPHYTES, MYCORRHIZAL FUNGI AND PLANT GROWTH

In the present study, high P supply strongly depressed mycorrhizal colonisation, consistent with previous studies on mycorrhizal plants (Menge *et al*. 1978; Jasper, Robson & Abbott 1979). Our study also showed a strong negative effect of high P supply on foliar endophyte and alkaloid concentrations, confirming results of a preliminary study performed in our laboratory (Liu *et al*. 2007b). We are not aware of any other studies on the effects of P on endophyte or alkaloid abundance in *L. perenne*. This effect of high resource supply in reducing endophyte and alkaloid concentrations in ryegrass is similar to what has been seen previously for a different resource, namely N, where high N supply also reduced endophyte and alkaloid concentrations (Stewart 1986; Hunt *et al*. 2005; Rasmussen *et al*. 2007). It is uncertain if high resource supply results in the stimulation of plant shoot growth more than of endophyte growth, thereby 'diluting' endophyte and alkaloid concentrations, although this would be consistent with our present results. Here, we show that alkaloid production per unit endophyte was significantly higher in high P supply relative to low P, suggesting that P has a stimulatory effect on alkaloid production; this further supports the idea that alkaloid reduction under high P supply is attributed to a dilution effect.

Effects of mycorrhizal colonisation on plant growth depended on the mycorrhizal species tested and on P supply. At low P supply, GM increased total shoot yield, but had no effect on blade or root yield, whereas GI reduced yield of all tissues. At high P, blade and shoot yield was also reduced in GI-colonised plants, whereas GM did not affect plant growth. This might indicate that GI, which also showed a much higher level of colonisation rates and had less arbuscular structures than GM, has a more 'parasitic' effect on plants, whereas GM is more mutualistic.

EFFECTS OF CARBOHYDRATE CONTENT ON FOLIAR ENDOPHYTES AND MYCORRHIZAL FUNGI

Endophyte and alkaloid concentrations were reduced in AberDart-HSG compared with the control grass Fennema, consistent with a previous report in a different high sugar cultivar, AberDove, also compared with Fennema (Rasmussen *et al*. 2007) possibly indicating that this effect is a general feature of high sugar content or other features specifically associated with high sugar cultivars. High molecular weight WSCs (in ryegrass mainly fructans; Pavis *et al*. 2001) were, as expected, higher in the blades of AberDart-HSG, but lower in the roots of this cultivar, compared with Fennema, indicating a difference in sugar translocation in high sugar compared with control cultivars. The higher fructan levels in blades of AberDart-HSG were associated with reduced endophyte concentration, whereas the higher fructan levels in roots of Fennema were associated with reduced mycorrhizal colonisation. Polymeric fructans are the major reserve carbohydrates of ryegrass; they are stored in vacuoles and are not accessible to fungal endosymbionts, and increased fructan accumulation could mean reduced levels of available apoplastic hexoses or other low molecular weight sugars causing reduced fungal growth. In the case of mycorrhiza, it has been shown that hexoses are taken up from the apoplast (Solaiman & Saito 1997; Pfeffer *et al*. 1999). It is possible that foliar endophytes, which exclusively reside in the apoplastic spaces of their host plants, also depend on sugars present in the apoplast, but conclusive studies on this question are still missing. As both mycorrhizal and endophytic fungi are dependent on the host plant as a source of carbohydrate, the concentration and form of plant carbohydrates may be an important factor in determining fungal growth within the host plant.

Our results show that the simultaneous association of temperate grasses with both foliar and root symbiotic fungi results in the reduction in each endosymbiont. As this could have both negative (e.g. from reduced endophytic anti-herbivorous alkaloids and/or mycorrhizal P supply) and positive effects (e.g. from reduced nutrient requirements of the endosymbionts on the host plant), tripartite associations may shift the balance of mutualism–parasitism between a host plant and its symbionts, and the degree of this shift depends on a range of other factors, like nutrient supply and genetic background of host plants, mycorrhizal and endophytic fungi. Further work should be conducted to examine the broader ecological implications of dual infection of host plants by endophytes and mycorrhizal fungi. Interactions that can shift symbiotic partnerships along the mutualism–parasitism scale may alter host plant competitive ability and plant fitness that are important determinants in shaping grassland ecosystems. From an applied perspective, the purported benefits to agriculture of either mycorrhizal fungi or endophytic fungi alone may not be additive when simultaneously infected, and interactions between dually occurring symbiotic species must be considered.

Acknowledgements

This work has been partially funded by the NZ Foundation of Research, Science and Technology (contracts AGRX0501, C10X0203 and PROJ 10333-ECOS-AGR) and was conducted at AgResearch Grasslands, New Zealand. Dr. J.A. Newman was supported by grants from the Canadian Natural Sciences and Engineering Research Council (NSERC) and the Ontario Ministry of Agriculture, Food and Rural Affairs. We thank Dr. G.A. Lane (AgResearch) for providing us with a mass spectrometry method for the analysis of peramine.

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Received 14 October 2010; accepted 3 March 2011
Handling Editor: Ken Thompson