

Ménage à trois – are two fungi too much for ryegrass?

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Abstract

A pot experiment investigated interactions between foliar endophytic (EP, *Neotyphodium lolii*) and root arbuscular mycorrhizal (AM, *Glomus mosseae*) fungi in *Lolium perenne*. The effects of three phosphorus (P) forms (KH₂PO₄-P, phosphate rock-P (PR) and phytate-P) on EP and AM colonisation and plant growth parameters were also assessed. AM arbuscular and vesicular structures were estimated by microscopic counting and fungal concentrations were quantified by qPCR of *G. mosseae* and *N. lolii* specific genes. Significantly more AM structures were formed in roots of EP-free compared to EP-infected plants, indicating that AM development is suppressed by the presence of foliar EPs. Total AM concentrations were also generally higher in EP-free plant roots, but not significantly. EP concentrations were significantly reduced by higher plant-available P in the growth medium, but mycorrhizal infection did not significantly affect EP concentrations. Mycorrhizal inoculation increased shoot dry matter (DM) at low plant-available P in the PR treatment. Infection with the endophytic strain AR37 also significantly increased shoot DM and fungal concentrations were higher compared to CS (common or wild-type strain)- and AR1-infected plants. Insoluble phytate-P produced the same amount of biomass as soluble-P (K-Pi) did, suggesting that *L. perenne* has the ability to use organic P.

Keywords: *Neotyphodium lolii*, *Glomus mosseae*, foliar endophyte, arbuscular mycorrhizae, *Lolium perenne*, qPCR, phosphate, AR1, AR37

Introduction

Foliar endophytic (EP, *Neotyphodium* spp.) and root arbuscular mycorrhizal (AM, *Glomus* spp.) fungi are common associates of grasses. These two plant-fungal relationships are generally considered mutualistic and both offer potential benefits for the interacting host (Smith & Read 1997; Schardl *et al.* 2004; but see Saikkonen *et al.* 2006). Over the past two decades, most work on symbiotic relations in grass-endophyte systems has focused on the ecological importance of foliar EP for plant protection from herbivores and abiotic stresses. By contrast, the potential impact of foliar EP on below-ground processes and possible interactions between grass EP and AM has been largely overlooked. Although EPs occur almost exclusively in aerial plant tissues, EP effects on root-feeding herbivores (Bernard *et al.* 1997), soil-dwelling organisms (Latch 1993) and soil microbial processes (Omacini *et al.* 2004) have been reported, suggesting that EP by-products may affect the soil flora (White & Cole 1985). Previous studies demonstrated that EP infection can suppress mycorrhizal colonisation of grass roots in tall fescue (Chu-Chou *et al.* 1992; Guo *et al.* 1992), perennial ryegrass (Müller 2003) and annual ryegrass (Omacini *et al.* 2006). However, Popay and Jensen (2005) reported that under field conditions neither the proportion of root segments infected with AM nor the level of root infection differed between EP-infected and EP-free treatments. Furthermore, Novas *et al.* (2005) found that roots of EP-infected *Bromus setifolius* populations were colonised more extensively by AM fungi than those of EP-free populations. These conflicting observations have made it difficult to understand the ecological

relationship amongst the three-way interactions in EP-AM-grass system. We conducted a pot experiment with controlled levels of nutrients, AM inoculates and EP strains to test the hypothesis that endophyte and mycorrhizae have antagonistic effects on each other and to investigate endophyte, mycorrhizal and P effects on plant growth parameters.

Materials and Methods

Growth conditions

A three-way completely randomised factorial experiment was conducted with four EP treatments of *Neotyphodium lolii* (EP-, CS (common or wild type strain), AR1 and AR37 infected) × two AM (AM- and *Glomus mosseae* inoculated) × three P forms added (KH₂PO₄ (K-Pi), phosphate rock (PR) and phytate P). Each treatment was replicated nine times. *Lolium perenne* cultivar ‘Nui’ was used as a host plant.

Seeds from four seedlines infected with three EP strains (CS, AR1 and AR37) differing in their alkaloid profiles and an EP-free control, were germinated on agar-plates and cultured in root-trainers. Three-week-old seedlings were transplanted into pots (one seedling per pot) containing 600 g pre-acid washed and steam-autoclaved quartz sand and K-Pi, Sechura PR and phytic acid sodium salt were applied at a rate of 80 µg P/g sand. The AM-infected seedlings were inoculated with 20 g *Glomus mosseae* inocula. The AM-free control received the same amount of autoclaved inocula and 10 ml extracts (passed through 10 µm nylon mesh filters) from the inocula. The pots were randomly arranged in nine blocks inside a glasshouse. Plants were fertilised by adding 30 ml P-free Hoagland nutrient solution and watered to ‘pot filled capacity weight’ at 2-day intervals. The plants were cut back every 3 weeks (6 cm above ground) and harvested after 12 weeks.

Harvest and analysis of plant material

Plants were separated into shoots and roots, frozen in liquid nitrogen, freeze dried, weighed (for DM determination) and ground. Sand was removed from the roots under tap water. AM colonisation was determined microscopically in Trypan blue stained roots as described by McGonigle *et al.* (1990). EP concentrations in shoots and AM concentrations in roots were estimated by quantitative real-time PCR (qPCR) of genomic DNA. Primers suitable for qPCR were designed for fragments of an endophyte specific gene (non-ribosomal peptide synthetase; NRPS-1) and a *G. mosseae* mycorrhizal specific gene (phosphate transporter; Accession No. DQ074452). The cloning and transformation procedures for purifying plasmid DNA to standardise qPCR analyses were as described in Rasmussen *et al.* (2006). The data were statistically analysed using GenStat v.9. We report the means of main effects as a measure of data dispersion. Significant differences between means were established by LSDs at P<0.05.

Results and Discussion

Effects of P availability and EP infection on AM colonisation

Higher levels of plant-available P significantly limited the colonisation and development of AM fungi on *L. perenne* roots. Vesicular and arbuscular structures were very low in both K-Pi

Table 1 Treatment effects on plant growth parameters. Data represent the mean and standard error (in bracket) of main effects. Different letters following the mean value denote significant differences between treatments at $P < 0.05$.

Factor	----AM----		-----P form-----			-----EP-----			
Treatment	AM-	AM+	KH ₂ PO ₄	PR	Phytate	EP-	CS	AR1	AR37
Shoot DW (g)	0.62 x (0.05)	0.79 y (0.07)	4.06 A (0.20)	0.71 B (0.06)	4.04 A (0.17)	2.96 b (0.12)	2.72 c (0.16)	2.84 bc (0.14)	3.22 a (0.16)
Root DW (g)	0.54 x (0.05)	0.52 x (0.05)	2.38 A (0.23)	0.53 B (0.05)	2.52 A (0.26)	1.86 ab (0.19)	1.74 ab (0.19)	1.68 b (0.15)	1.96 a (0.19)
Shoot : root ratio	1.21 x (0.14)	1.58 y (0.16)	1.93 A (0.30)	1.40 B (0.15)	1.77 A (0.19)	1.73 a (0.22)	1.71 a (0.25)	1.66 a (0.17)	1.71 a (0.17)
Tiller No.	9.00 x (0.87)	10.78 x (1.04)	32.14 A (2.08)	9.89 B (0.96)	31.48 A (1.99)	24.07 c (1.57)	22.0 bc (2.09)	24.42 b (1.48)	27.48 a (1.57)

and phytate treatments (less than 0.5%) compared to 6-17% in the PR treatment. Effects of EP infection on AM colonisation were therefore only assessed in the PR treatment.

Significantly fewer AM structures were formed on roots from EP-infected plants compared to roots of EP-free plants (Fig. 1A). Total AM concentrations quantified by qPCR were also higher in roots from EP-free plants (Fig. 1B), but the difference was not significant due to large variations in the sample set. There were no significant interactions between endophyte strains and AM infection rates. These results suggest that AM formation and development is suppressed by the presence of foliar endophytes and confirms previous studies on grass-endophyte associations (Chu-Chou *et al.* 1992; Guo *et al.* 1992; Müller 2003; Omacini *et al.* 2006; but see Novas 2005).

It has been suggested that alkaloids produced by the foliar EP may be responsible for the suppressive effect of leaf endophytes on AM fungi (Guo *et al.* 1992). Nutrient competition between the above- and below-ground fungi may also play a role (Müller 2003). Another explanation may be a systemic induction of resistance mechanisms of the host plant by EP; these resistance mechanisms could reduce the formation of intracellular structures necessary for the nutrition of the biotrophic mycorrhizal fungus

(Müller 2003). However, the exact mechanisms for different AM colonisation rates between EP-infected and EP-free plants have yet to be identified.

Effects of P availability and AM infection on EP concentrations

Using qPCR we compared EP concentrations in shoots of K-Pi treated (high plant-available P) with PR treated plants (low plant-available P). Similar to the effect of high available P levels on mycorrhizal colonisation rates described above, we found that K-Pi treatment significantly reduced EP concentrations in shoots compared to PR treatment (Fig. 2A). Interestingly, a previous study reported that high nitrogen availability also reduced EP concentrations (Rasmussen *et al.* 2006). These results suggest that the interactions between plants and EP fungi depend strongly on available soil nutrients (Müller 2003). Possible mechanisms for these nutrient effects might be: (i) nutrients like N or P could stimulate the growth of the host plant 'more' than it stimulates the growth of the fungus, thereby causing an overall 'dilution effect'; or (ii) elevated N/P could affect the EP infection rate in newly developing tillers, thereby resulting in a relatively high number of uninfected tillers (Rasmussen *et al.* 2006).

Figure 1 Effects of endophyte strains on (A) the development of arbuscular/vesicular mycorrhizal structures (microscopically counted) and (B) total mycorrhizal concentration (qPCR) in *L. perenne* roots grown at low P availability (PR treatment). Different letters denote means that are significantly different at $P < 0.05$.

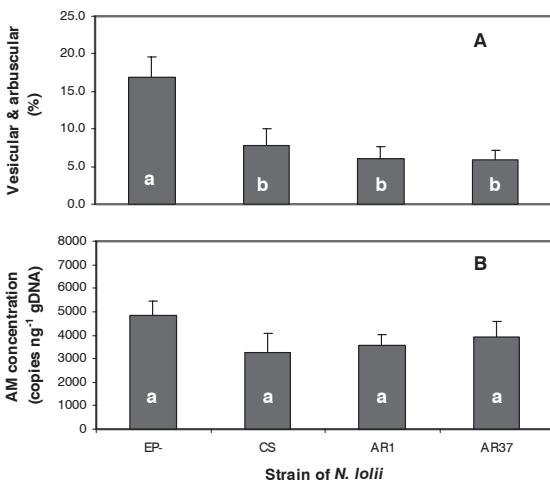
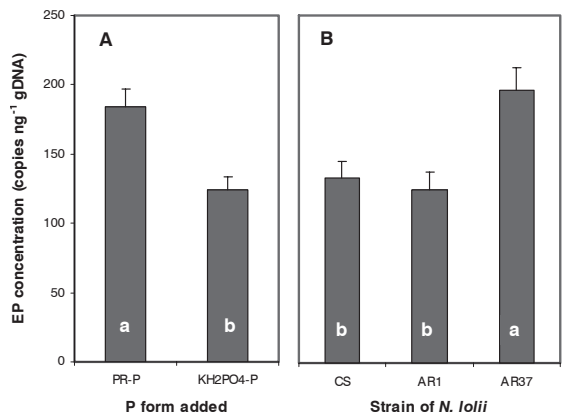


Figure 2 Effects of (A) P treatment and (B) endophytic strain on endophyte concentrations (qPCR) in *L. perenne* shoots. Plotted are the means of main effects. Different letters denote means that are significantly different at $P < 0.05$.



In the present study, we also found significantly higher EP concentrations in plants infected with AR37 compared to CS- and AR1- infected plants (Fig. 2B). This difference could be due to the genetic variation between the strains, but it might also be due to differences between the four seedlines used in this experiment. In the present study, we did not find any significant difference in EP concentration between AM-free and AM-infected treatments, mainly due to the large variation amongst the replicates.

Effects of fungi and P availability on plant growth parameters

Significant effects of mycorrhizal inoculation on plant growth were only observed in the PR treatment (low plant-available P). Compared to AM-free controls, AM inoculation resulted in significantly higher shoot DM and shoot: root ratios in this treatment (Table 1). The beneficial effects of mycorrhizal infection on plant growth are well known and are probably due to the ability of mycorrhizal fungi to exploit P forms in soil that are usually unavailable to plants (Smith & Read 1997; Wanlander 2000).

P deficiency due to low P bioavailability in the PR treatment (Liu *et al.* 2004) resulted in significantly reduced plant shoot and root DM (Table 1). Interestingly, treatment with phytate-P resulted in the same amounts of plant DM as K-Pi treatment (Table 1). Phytate, typically accounting for 30–40% of total organic phosphorus in soils, is quite insoluble and plants have a limited ability to obtain their P requirements from phytate in both agars and sand-vermiculite media (Richardson *et al.* 2001) or soil environments (Adams & Pate 1992) in a range of species. In contrast to above studies, our results suggest that *L. perenne* may have the ability to use this organic form of P.

The presented experiment also resulted in significantly higher DM and tiller production of plants infected with the endophytic strain AR37 (Table 1). The improved plant growth was associated with significantly higher EP concentrations in grass shoots quantified by qPCR (Fig. 2B). However, the observed effects of AR37 on plant growth parameters in this study could be due to differences in the seedlines as individual genotypes have been described previously to affect ryegrass-endophyte interactions (Easton *et al.* 2002).

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