Effect of resource supply on interactions between foliar endophytic and root mycorrhizal fungi in perennial ryegrass

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Abstract
Both foliar endophytes and root arbuscular mycorrhizal fungi can infect perennial ryegrass and improve its performance, mainly by improving herbivore resistance and phosphorus (P) uptake, respectively. Here, we report a study of how the endophytic fungus (*Neotyphodium lolii*) and two mycorrhizal fungi (*Glomus* spp.) are affected by P supply and perennial ryegrass (*Lolium perenne*) cultivars differing in water soluble carbohydrate content. Endophyte and alkaloid concentrations were reduced at high P supply and in the high sugar grass (HSG) ‘AberDart’, and these effects were modulated by the presence of mycorrhizal fungi. High P supply resulted also in a strong reduction of mycorrhizal colonisation, while the effect of the high sugar grass was subtle and modulated by endophyte infection. Our results show that the simultaneous association of perennial ryegrass with both endophytic and mycorrhizal endosymbionts can negatively affect each endosymbiont, but this depends on other factors such as resource supply and genetic background of the partners.

Keywords: ryegrass, phosphate, high sugar grass, endophyte, mycorrhizae, alkaloids

Introduction
Field grown perennial ryegrass plants are often infected with endophytic *Neotyphodium* spp. and root specific arbuscular mycorrhizal *Glomus* spp. fungi. Both endosymbionts can confer benefits to temperate grasses, mainly by improving herbivore resistance and phosphorus (P) uptake, respectively. Studies on the interactions of host plants with either one of these fungal symbionts are numerous, but studies considering tripartite interactions between endophyte and mycorrhizal fungi and host plants are rare. It has been shown that endophytic fungi can suppress mycorrhizal colonisation and fungal spore germination (Müller 2003; Omacini et al. 2006; Antunes et al. 2008). Effects of mycorrhizal colonisation on endophyte infection have been much less studied, although it has been reported that mycorrhizal infection can reduce the level of endophyte-confferred herbivore resistance (Vicari et al. 2002). Alkaloid concentrations in the grass-endophyte symbiosis vary considerably depending on season, weather and nutrient supply, and there is conflicting evidence of the effects of nutrients on alkaloid production (Malinowski & Belesky 2000; Rasmussen et al. 2007).

In a previous study we have shown that both endophyte and alkaloid concentrations were reduced in perennial ryegrass at high nitrogen (N) and in the HSG ‘AberDove’ (Rasmussen et al. 2007), demonstrating that simple changes in nutrient supply and resource (energy) related plant traits can affect the symbiotic association between ryegrass and its fungal endophytes. Here, we tested if P supply had a similar effect on endophytes and if interactions between mycorrhizal *Glomus* fungi and foliar endophytes are affected by nutrient and resource supply in a different HSG ‘AberDart’.

Methods
We used two *Lolium perenne* cultivars, a HSG ‘AberDart’ and a control ‘Fennema’ that differ inherently in water soluble carbohydrate contents. Seedlings were artificially inoculated by two *N. lolii* endophyte strains, Common Strain Lp19 (CS) or AR1; and/or by two mycorrhizal fungi, *G. mosseae* (GM) or *G. intraradices* (GI). Non-inoculated endophyte-free (EF) and/or mycorrhizal-free (MF) plants were used as controls. A total of 360 plants were grown in sand media and kept in controlled climate chambers for 12 weeks (14 h light, 20°C/ 10 h dark, 10°C). Half-strength Hoagland nutrient solution containing either 0.05 mM (low P) or 2 mM (high P) KH₂PO₄ was added weekly. During the whole growth period plants were watered every second day to “pot field capacity”. Plants were trimmed every 4 weeks to 6 cm above-ground, trimmings were oven-dried and dry weights recorded. At the final harvest leaf blades were frozen and ground in liquid nitrogen and then freeze-dried. Endophyte concentrations were estimated based on quantitative polymerase chain reaction (qPCR) of genomic DNA (gDNA) isolated from infected plant tissues (Rasmussen et al. 2007). Colonisation of roots with mycorrhizae was microscopically assessed. Peramine and water soluble carbohydrates were extracted and quantified as described previously (Rasmussen et al. 2007).

The overall experiment was a split-unit design, with
two cultivars, three levels of foliar endophytes, and three levels of mycorrhizal fungi as the whole unit treatment (2 x 3 x 3 x 10 replicates) with two levels of P supply as the sub-unit treatment (genotypes across P treatments being identical). All analyses were conducted using JMP statistical software version 7.0. Box-Cox transformation was used to homogenise the error variances for statistical analysis and report, uniformly, the untransformed means and standard error of the means. Tukey’s Honestly Significant Difference (HSD) test was used to assist in the interpretation of significant effects and interactions.

Results

In leaf blades, endophyte concentrations were significantly reduced at high P compared with low P supply (P<0.001); the reduction was stronger in the HSG significantly reduced at high P compared with low P supply. Peramine concentrations in leaf blades were significantly reduced at high P (Fig. 1c; P<0.001), in the HSG ‘AberDart’ (Fig. 1d; P<0.001), and in plants colonised with both mycorrhizal species (Fig. 1e; P<0.001); none of the interactions between above factors were significant.

Mycorrhizal colonisation rates in plants grown at high P supply were 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. At low P supply, mycorrhizal colonisation rates were affected by a three-way cultivar x mycorrhizae x endophyte interaction (P<0.05). Colonisation rates of G. mosseae were not significantly affected by endophyte infection or grass cultivar (Fig. 2). However, colonisation rates of G. intraradices were significantly reduced in endophyte infected ‘Fennema’ plants, but not in ‘AberDart’.

High molecular weight water soluble carbohydrates (HMW WSC) were significantly higher in leaf blades of the HSG ‘AberDart’ compared with the control cultivar ‘Fennema’ (Fig. 3a; P<0.001). Even though the interaction between P supply and mycorrhizal inoculation was significant (P<0.05), Tukey’s HSD test was unable to differentiate between the different treatment combinations (Fig. 3b). Low molecular weight water soluble carbohydrates (LMW WSC) were not different between the two cultivars, but the observed increase of LMW WSC levels at high P compared with low P was significant only in ‘Fennema’ (Fig. 3c; P<0.001). HMW WSCs in roots were increased at high P compared with low P supply in both cultivars, and this effect was stronger in ‘Fennema’, resulting in significantly higher levels of HMW WSCs in roots of ‘Fennema’ compared with the HSG ‘AberDart’ at high P (Fig. 3d; P<0.001). P supply also interacted with mycorrhizal inoculation (P<0.01), but the interaction was too subtle for Tukey’s HSD test to distinguish between treatment combinations (Fig. 3e). LMW WSC concentrations in roots were higher at high P compared to low P supply (Fig. 3f; P<0.001); none of the other main effects or interactions were significant.
Discussion
Effects of P supply on foliar endophytes and mycorrhizal fungi
In the present study high P supply had a strong negative effect on foliar endophyte and alkaloid concentrations in infected leaf blades of perennial ryegrass plants. This effect of high P supply, in reducing endophyte and alkaloids concentrations, is similar to what has been seen for high N supply in our previous study. There we hypothesised that high resource supply might stimulate plant shoot growth more than endophyte growth, thereby 'diluting' endophyte and alkaloid concentrations (Rasmussen et al. 2007). High P supply also strongly depressed mycorrhizal colonisation, consistent with previous studies on other plants (Smith & Read 1997), and it has been shown that signalling molecules such as strigolactones, which are exuded by P starved plants only, mediate this mycorrhizal response (Yoneyama et al. 2007).

Effects of carbohydrate content on foliar endophytes and mycorrhizal fungi
High sugar grasses (HSG) have been bred for accumulation of high fructan levels in leaf blades, and here we show that ‘AberDart’ did accumulate higher levels of fructans compared to ‘Fennema’, consistent with previous studies (Parsons et al. 2004). Endophyte and alkaloid concentrations were reduced in the HSG ‘AberDart’ compared with the control cultivar ‘Fennema’, also consistent with a previous study using the HSG ‘AberDove’ (Rasmussen et al. 2007; 2008a). However, we also note here that the negative effect of the HSG ‘AberDart’ on endophyte concentrations was modulated by P supply and mycorrhizal inoculation; endophyte concentrations were different between the two cultivars only at high P supply, clearly showing that the interaction between high sugar grasses and endophytes can be affected by other factors such as nutrient supply or the presence of mycorrhizal fungi.

In roots, water soluble carbohydrate (WSC) concentrations were the same in the two cultivars at low P supply. At high P supply, high molecular weight WSCs were lower in the HSG ‘AberDart’ compared with ‘Fennema’, possibly indicating a difference in sugar translocation in HSG compared with other ryegrass cultivars. Earlier we reported that endophyte infection increased WSC concentrations in the control sugar grass ‘Fennema’, but not in the HSG ‘AberDart’ (Rasmussen et al. 2007; 2008a). We did not see an effect of endophyte infection on WSC levels in the present study, but concentrations were much higher compared with our previous study (approx. 350 and
Interactions between mycorrhizal fungi and foliar endophytes

In this study, we hypothesised that foliar endophyte and root mycorrhizal fungi interact with each other if simultaneously present in perennial ryegrass plants, and that the interactions between them are affected by resource supply. Our results show that the effects of mycorrhizal inoculation on endophyte and peramine concentrations interacted with P supply, cultivar, and endophytic strain. In leaf blades, endophyte concentrations were reduced by mycorrhizal inoculation, but only at high P supply and in CS infected plants, but not at low P supply or in AR1 infected plants. This indicates that studies on the effects of mycorrhizal colonisation need to control for factors like mycorrhizal species, resource supply, genetic background of plants and even foliar endophytic strains, if the outcomes of mycorrhizal infection are to be compared or generalised across a range of ecological studies.

The effect of endophyte infection on mycorrhizal colonisation was subtle and also depended on other factors; specifically mycorrhizal colonisation in *G. intraradices* inoculated ‘Fennema’ plants. A reduction in mycorrhizal colonisation has previously been reported for several endophyte-infected *Lolium* species (Müller 2003; Omacini *et al.* 2006) and it has been suggested that endophytic alkaloids directly transferred to the roots or via leachates from endophyte-infected grass litter might inhibit mycorrhizal fungi (Antunes *et al.* 2008). We show here that mycorrhizal colonisation was reduced in both CS (produces peramine, lolitrem B, and ergovaline) and AR1 (does not produce lolitrem B or ergovaline) *N. lolii* infected ‘Fennema’. This makes it unlikely that the antagonistic effects on mycorrhizal fungi are caused by the endophytic alkaloids lolitrem B and ergovaline, but our results do not rule out that peramine negatively affected mycorrhizal colonisation. However, we have demonstrated in previous metabolomics studies of the *N. lolii* – perennial ryegrass association (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 the negative effects of foliar-based endophytic fungi on the root-based mycorrhizal fungi seen in this and other studies.

Our results show that the simultaneous association of temperate grasses with both foliar and root symbiotic fungi can result in the reduction of each endosymbiont, but this depends on other factors such as resource supply or grass cultivar. 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 from 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.

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