Research

Oikos 117: 310–320, 2008
doi: 10.1111/j.2007.0030-1299.15973.x,

Subject Editor: Tadashi Fukami, Accepted 5 October 2007

Balancing multiple mutualists: asymmetric interactions among plants, arbuscular mycorrhizal fungi, and fungal endophytes

Keenan M. L. Mack and Jennifer A. Rudgers

K. M. L. Mack and J. A. Rudgers (jrudgers@rice.edu), Dept of Biology, Indiana Univ., Bloomington, IN 47405, USA. JAR also at: Dept of Ecology and Evolutionary Biology, Rice Univ., Houston, TX 77005, USA.

Most organisms engage in beneficial interactions with other species; however, little is known regarding how individuals balance the competing demands of multiple mutualisms. Here we examine three-way interactions among a widespread grass, *Schedonorus phoenix*, a protective fungal endophyte aboveground, *Neotyphodium coenophialum*, and nutritional symbionts (arbuscular mycorrhizal fungi) belowground. In a greenhouse experiment, we manipulated the presence/absence of both fungi and applied a fertilizer treatment to individual plants. Endophyte presence in host plants strongly reduced mycorrhizal colonization of roots. Additionally, for plants with the endophyte, the density of endophyte hyphae was negatively correlated with mycorrhizal colonization, suggesting a novel role for endophyte abundance in the interaction between the symbionts. Endophyte presence increased plant biomass, and there was a positive correlation between endophyte hyphal density and plant biomass. The effects of mutualists were asymmetric: mycorrhizal fungi treatments had no significant impact on the endophyte and negligible effects on plant biomass. Fertilization affected all three species – increasing plant biomass and endophyte density, but diminishing mycorrhizal colonization. Mechanisms driving negative effects of endophytes on mycorrhizae may include inhibition via endophyte alkaloids, altered nutritional requirements of the host plant, and/or temporal and spatial priority effects in the interactions among plants and multiple symbionts.

Mutualisms are common in nature, and organisms frequently engage in more than one mutualism at a time (Bronstein 1994a, 1994b, Richardson et al. 2000, Stachowicz 2001, Kogel et al. 2006). For instance, plants can form mutualisms with mycorrhizal fungi, endophytic fungi, nitrogen-fixing bacteria, pollinators and seed dispersers. Yet, the vast majority of research has focused on specialized and pair-wise mutualisms neglecting the complex interactions that characterize most ecological communities (Bronstein and Barbosa 2002, Stanton 2003, Strauss and Irwin 2004, Rudgers and Clay 2005).

Theoretical models and current empirical studies suggest that the dynamics of mutualisms may not be predictable from the additive effects of pair-wise interactions alone (Stanton 2003). For example, mycorrhizal fungi can increase visitation rates by pollinating insects (Gange and Smith 2005, Wolfe et al. 2005). A recent synthesis highlighted the need for experiments that simultaneously manipulate the abundance of multiple mutualists (or antagonists) and quantify the responses of all species involved (Strauss and Irwin 2004). For mutualistic interactions in particular, most experiments have manipulated only one mutualist or functional group of mutualists, potentially overlooking interactions among species that confer different types of benefits (Stachowicz and Whitlatch 2005).

Here, we investigate a three-way interaction consisting of the widespread, invasive plant host *Schedonorus phoenix* (tall fescue), and two symbionts, the aboveground fungal endophyte, *Neotyphodium coenophialum* (hereafter, the endophyte), and belowground arbuscular mycorrhizal fungi (AMF). Endophytes can provide multiple benefits to tall fescue and other grasses. These include the primary benefit – production of anti-herbivore alkaloids (Clay 1996, Bush et al. 1997) – as well as increased drought tolerance (Malinowski et al. 2005), pathogen resistance (Gwinn and Gavin 1992, Mahmood et al. 1993), soil nutrient acquisition such as phosphorous uptake (Malinowski and Belesky 1999, Malinowski et al. 2000, Rahman and Saiga 2005), and overall plant vigor (Marks et al. 1991, Clay et al. 2005). These benefits can translate into higher plant fitness for infected plants relative to endophyte-free, including for tall fescue (Rice et al. 1990, Clay and Holah 1999, Clay et al. 2005). Arbuscular mycorrhizal fungi (AMF) are widely thought to function as mutualists, but, like fungal endophytes, may become parasitic under certain conditions (Johnson et al. 1997, Allen et al. 2003). These soil fungi associate with the roots of ~80% of angiosperms (Smith and Read 1997, Brundrett 2002). The main benefit attributed to mycorrhizal symbiosis is increased nutrient uptake from the soil, particularly of phosphorus and nitrogen (Smith and Read 1997). However, other benefits

may include enhancements of resistance to root parasites (Ingham and Molina 1991, Borowicz 2001), water uptake (Marulanda et al. 2003), photosynthetic rate (Black et al. 2000), and carbohydrate metabolism (Smith and Read 1997). Like endophytes, AMF can receive shelter and nutrients (mainly carbon) from the plant host (Smith and Read 1997, Brundrett 2002).

Although endophytic and mycorrhizal fungi receive similar benefits from the plant host, they differ not only in the primary benefits conferred, but also in their location within plants and in the timing of host colonization. These differences in spatial and temporal priority offer clues to how the two symbionts may interact. First, the endophyte is confined to aboveground tissues whereas AMF colonize roots. Therefore, these mutualists likely only interact indirectly through the shared host plant. Furthermore, although both mutualists acquire carbon from hosts, only endophytes inhabit tissues where photosynthesis occurs. Thus, the endophyte may gain first access to carbon resources, taking spatial priority over AMF. Second, *Neotyphodium* endophytes are strictly vertically transmitted from maternal plants to seeds (Clay 1990), but AMF colonize hosts horizontally, after the seed germinates and roots grow (Brundrett 2002). Thus, the potential exists for a temporal priority effect as well as a spatial one. Theory predicts that exclusively vertically transmitted symbionts should maximize host fitness because the symbiont's fitness is equivalent to that of its host (Ewald 1987, Maynard Smith and Szathmary 1995). Thus, if an association with a second symbiont, like AMF, benefits the plant, then that association might be expected to be promoted (or at least tolerated) by the vertically transmitted symbiont. However, it is possible that endophytes could redirect plant resources away from AMF, despite a potential advantage to the plant. This pattern may only be adaptive if some component of the endophyte's fitness is independent of host fitness, as would occur if higher endophyte density in host tissues increased the likelihood of vertical transfer of the endophyte to seeds (Schardl et al. 2004).

Prior research has shown that endophytes in grasses can reduce mycorrhizal colonization of host roots as well as spore densities in the soil (Chu-chou et al. 1992, Guo et al. 1992, Mueller 2003). Further, in one case, the presence of endophyte-infected hosts increased mycorrhizal colonization of endophyte-free conspecifics growing in the same pot (Omacini et al. 2006). However, previous research has focused exclusively on responses of AMF and (in a few cases) also the plant, but has not quantified reciprocal effects on the endophyte. For example, it remains unknown whether AMF can alter endophyte concentrations in the host plant. In addition, prior work has largely been conducted under constant environmental conditions, even though abiotic factors can shift the outcomes of species interactions along a continuum from mutualism to parasitism (Bronstein 1994a, Neuhauser and Fargione 2004). Alteration of environmental factors that affect the costs and benefits of mutualisms can aid in understanding the dynamics of multi-species interactions. For example, under high nutrient levels, mycorrhizal fungi can become parasitic (reviewed by Johnson et al. 1997, 2003). We might expect that if the endophyte or plant host can inhibit AMF colonization, then such inhibition would be more likely under high than low nutrient levels, when the benefits of AMF are diminished. Similarly, if AMF alleviate nutrient deficiencies, then plants may reduce allocation to endophyte growth and promote AMF association under low nutrient conditions.

We experimentally manipulated the presence of an endophyte, AMF, and nutrient availability to explore how plants balance symbionts that confer different functional benefits. To our knowledge, our work is the first to simultaneously examine responses of all three interacting species at high and low soil fertility. Specifically, we addressed the following questions: (1) does the endophyte or fertilizer addition reduce AMF colonization? (2) Does AMF or fertilizer affect endophyte hyphal density? (3) How does the host plant respond to interactions among the endophyte, AMF, and fertilizer?

Methods

The study system

Tall fescue *Schedonorus phoenix*, formerly *Lolium arundinaceum,* is native to Europe and Africa and was introduced to the USA during the 1800s. This species currently covers more than 22 million ha of the continental USA and is widely naturalized and sometimes invasive (Hiebert 1990, Ball et al. 1993, Raloff 2003, Fribourg and Hannaway 2007). *Neotyphodium coenophialum* is the dominant fungal endophyte in this system and the primary systemic endophyte occupying individual plants; ~75–80% of tall fescue in the USA is infected (Ball et al. 1993, Clay and Holah 1999). While fungal endophyte – grass interactions can span a continuum from mutualism to parasitism (Saikkonen et al. 1998, 2006, Schardl 2001, Schardl et al. 2004), the endophyte in tall fescue is commonly mutualistic in the USA (Ball et al. 1993, Clay and Schardl 2002, Clay et al. 2005).

We used the KY-31 cultivar of tall fescue because it has received the most attention in the literature on grass-endophyte interactions and is the most widely planted in the USA (Clay and Schardl 2002, Rudgers et al. 2004, 2005, 2007, Clay et al. 2005, Rudgers and Clay 2005, Saikkonen et al. 2006). While tall fescue is not intended to be representative of all grass-endophyte associations, it, and specifically cultivar KY-31, is currently one of the most widespread and dominant C3 grass ecotypes, making it a common member of many ecological communities. Given the sheer abundance of tall fescue across a range of ecosystems, understanding the dynamics of multiple symbioses in this species is highly ecologically relevant.

Experimental design

We conducted a greenhouse experiment in Bloomington, Indiana, USA manipulating *N. coenophialum* infection, AMF infection, and nutrient availability in a $2 \times 3 \times 2$ factorial design ($n = 50$ plants per treatment combination, 600 plants total). We used two endophyte treatments: endophyte-infected (E+) and endophyte-free (E−). We initially removed the endophyte from half of the seeds using long-term seed storage. All seeds were then propagated for

several generations in adjacent field plots that freely cross-pollinated, allowing for homogenization of the plant genetic background with respect to the endophyte treatment (Clay and Holah 1999, Clay et al. 2005). Seeds used in the experiment were several generations distant from the original storage treatment. Because tall fescue is self-incompatible, each seed should represent a unique host genotype.

We manipulated the abundance of AMF by applying three soil treatments: live soil inoculum (from a Bloomington, Indiana grassland (39°13′9″N, 86°32′29″W) formerly in cultivation for corn, but abandoned for many years, MF), commercial inoculum containing only a single, but common, AMF species (*Glomus intraradices,* MI), or sterilized soil inoculum (same source location as live inoculum, M −). Sterilization was achieved by autoclaving the field soil for 7 h at 250°C. Soil treatments were added to 2.6 l plastic pots by layering 200 ml of live (MF) or sterilized (MI, M −) soil inoculum halfway between two layers of pasteurized potting soil. Of the pots treated with sterilized field soil, half were inoculated with 1 g of commercial mycorrhizal inoculum consisting of spores and root fragments, as recommended by the supplier (Endonet Turbo, provided by Dr. T. St. John, BioNet, LCC, Marina, California, USA). The commercial inoculum was sandwiched in the center of the pot on top of the sterilized field soil layer (MI). Because a small amount was added ($<0.2\%$ of total soil volume), we did not control for the addition of commercial inoculum by adding sterilized commercial inoculum; if commercial inoculum had a fertilizing effect, this did not affect plant growth.

The experiment began 8 July 2004. Three tall fescue seeds (*Schedonorus phoenix* var. KY-31) were placed on the soil surface of each pot approximately 2.5 cm distant. After 20 days, pots were weeded to achieve one plant per pot. Plants were grown without supplemental light at an average temperature of 21–24°C. Randomization of plants in the greenhouse was accomplished by assigning each plant to a random position using a random number table.

We altered nutrient availability by applying 5.0 g of fertilizer per liter (15-16-17 NPK ratio) to half of the pots (F) 40 days after planting. The other half (NF) were treated with an equal volume of tap water. Thereafter, fertilizer or water was applied weekly. Three weeks prior to harvest (27 September 2004) NF plants showed signs of nutrient deficiency and were treated once with a half-strength fertilizer treatment.

Plants were harvested after $\sim$103 days, beginning 18 October 2004. At harvest, there was no evidence for growth limitation imposed by the size of the pots. To harvest, roots were washed thoroughly with tap water over a 3×3 mm mesh screen for $\sim$7 min until soil was removed. At this time, 2 g (wet) root tissue samples were taken from each plant for AMF detection and stored in tissue cassettes submerged in 50:50 glycerol and water. In addition, two leaf sheath peels ($\sim$0.5 cm) were removed from each of two live tillers per plant for endophyte detection (Clark et al. 1983). Leaf peels represented a negligible amount of biomass, and equal amounts were removed from all plants.

Effectiveness of experimental treatments

At the end of the experiment, every plant was checked for endophyte presence. We also determined independently of the experiment whether the endophyte treatment had any indirect effects on seed mass or germination. For each endophyte treatment (E+ or E −), 25 batches of 10 seeds were weighed to the nearest 0.001 g. In addition, twenty seeds were germinated in each of 15 replicate closed plastic containers per treatment in a growth chamber (12 h day length, 15–24°C). Every two days, we recorded the number of germinated seeds.

We tested the infection potential of the AMF inoculum by conducting a mean infection percentage (MIP) assay using sorghum *Sorghum bicolor* as a host standard. Seeds were planted on 8 July 2004 and grown for 36 days in the same size pots as the tall fescue, with five replicate pots per AMF treatment. Root tissue samples (2 g) were stained following the procedure described in INVAM (http://invam.caf.wvu.edu/methods/mycorrhizae/staining.htm) using 0.05% trypan blue. After staining, we created microscope slides containing 9–14 rows of 3-cm long root sections. Slides were scored at 400 × magnification with a compound microscope. We estimated AMF infection per plant by determining the proportion of $\sim$60 non-overlapping "views" per slide that showed evidence of mycorrhizal colonization (hyphae, arbuscules, vesicles, or coils). Only a single root was visible in each "view;" thus, a standard length of root tissue was observed for each plant. Only mycorrhizal fungi were scored; other types of fungi identifiable as non-mycorrhizal (e.g. dark septate fungi) were not recorded.

Endophyte responses

We assessed the presence/absence of endophyte hyphae in the leaves and also quantified hyphal density in response to the experimental treatments. Two leaf-sheath peels from each of two randomly selected live tillers were collected and prepared on slides using lactophenol cotton blue stain (a total of four peels per plant, 200 peels per treatment) (Clark et al. 1983). We scored slides for *N. coenophialum* hyphal density by counting the number of hyphal intersections along a 200 μm transect placed perpendicular to the longitudinal axis of the leaf sheath cells. We used an ocular micrometer at 200 × on a compound microscope. On each slide, we counted intersections with hyphae for a randomly chosen subset of 24 non-overlapping transects and determined the density of hyphal intersections per mm.

AMF responses

Root tissue (2 g wet) was stained to score colonization, as described for the MIP experiment. Slides from 10 plants per treatment combination (out of 50 total) were evaluated because of the labor intensiveness of the scoring process. This resulted in a total of 120 plants for mycorrhizal colonization (rather than the 600 total). Despite the reduced sample size, statistical power was not compromised. Analysis yielded significant treatment effects, and the ANOVA model explained 30% of the variation in the data.

Plant responses

Root and shoot tissues were dried in a convection oven at 60°C for a minimum of 3 d then weighed. Removed root biomass was accounted for each plant by dividing the dry root mass by the wet root mass after 2 g were removed then, multiplying this number by the 2 g removed and adding it back to the dry root mass yielding final dry root biomass. The root:shoot ratio was calculated. Vegetative tillers were counted on each plant as an additional measure of fitness. Once it has become established, like many perennial grasses (Snaydon 1978, Eriksson 1992), tall fescue spreads largely through clonal growth with infrequent seedling establishment. Thus, vegetative biomass and tiller number constitute important components of fitness for this species.

Colonization by aphids

Aphids (*Rhopalosiphum padi*) naturally colonized the greenhouse during the experiment. Plants were classified into three broad infestation levels: (1) fewer than 10 aphids per plant, (2) between 10 and 100 aphids, and (3) more than 100 aphids. Many aphids were naturally parasitized by an unidentified wasp, leaving mummies (exoskeletal remains); therefore, counts included both live aphids and mummies.

Statistical analyses

Analyses were conducted using SAS ver. 9.1. For evaluating treatment effectiveness, a log linear model tested whether the endophyte treatment affected the infection status of tillers, applying a binomial distribution (Proc GENMOD). To test for differences in mean seed mass between endophyte treatments, we performed ANOVA. We used repeated measures ANOVA to test for differences between endophyte treatments in proportional seed germination across time. To test the statistical significance of the mycorrhizal treatments on sorghum, we performed ANOVA with the independent factor of mycorrhizal treatment and used post-hoc Tukey HSD (honestly significantly different) tests to compare among the three mycorrhizal treatments (M −, MI, MF).

For many response variables in the tall fescue experiment, residuals were non-normally distributed, and normality could not be obtained through transformations. Therefore, randomization tests were used to evaluate differences among treatments (Edgington 1987, Manly 1991). Randomization tests determine p-values by comparing an observed test statistic (e.g. F-ratio from ANOVA) to a distribution of the test statistic that is expected under the null hypothesis. To create the expected distribution, the response variable values from treatments being compared are pooled, permuted, and randomly assigned to the treatments for 9999 iterations. We used randomization test equivalents of ANOVA by encompassing Proc GLM code within a SAS randomization test macro program (Cassell 2002). The models included the fixed effects of endophyte, mycorrhizae, and fertilizer treatments and all interactions.

Further tests were used to examine the relationship between symbiont densities. Unlike the endophyte, which is present prior to germination, mycorrhizal hyphal density could vary in two distinct ways: (1) differential probability of colonization or (2) differential growth after initial colonization. Therefore, first, we conducted a logistic regression to assess whether treatments affected the presence/absence of mycorrhizae. Second, we performed regression analysis on mycorrhizal colonization as a function of endophyte hyphal density; we used only the subset of plants that had the endophyte and were scored for mycorrhizae, a total of 19 plants. Assumptions of normality and homogeneity of residuals were satisfied.

Finally, we used a proportional odds model to analyze treatment effects on the ordinal, polytomous response of aphid score (i.e. 1, 2 or 3) following Stokes et al. (2000). We included total plant biomass as a covariate to control for differences in aphid abundance due to plant size.

Results

Were treatments effective?

The long-term storage treatment eliminated the endophyte in later plant generations, with no endophyte hyphae present in the E − treatment and ∼15 hyphae per mm (on average) in the E + treatment (Table 1, 2, $p < 0.0001$). There were no side effects of the endophyte treatment on mean mass per seed with standard error (SE) [E + = 0.0019 g (0.00007), E − = 0.0020 g (0.00005), $F_{1,48} = 1.81$, $p = 0.19$] or mean percentage of seeds germinated (SE) [E + = 89% (1%), E − = 86% (2%), endophyte $F_{1,28} = 1.17$, $p = 0.29$, time × endophyte $F_{3,26} = 0.64$, $p = 0.60$].

Mycorrhizal treatments significantly altered AMF colonization of both the bioassay plant (mean percentage colonization for sorghum (SE), M − = 17% (2%), MI = 41% (3%), MF = 41% (3%); $F_{2,12} = 27.66$, $p < 0.0001$)

Table 1. Means [SE] of plant (*Schedonorus phoenix*), endophyte (*Neotyphodium coenophialum*), and arbuscular mycorrhizal fungi responses to the fertilizer (fertilized = F, not fertilized = NF) and endophyte (present = E +, absent = E −) treatments. Only means for significant treatment main effects are given; significant interaction effects are displayed in the figures.

	NF	F	E +	E −
Total mass (g)	9.03 [0.23]	36.16 [0.71]	24.84 [0.99]	20.46 [0.89]
Root mass (g)	3.32 [0.09]	4.99 [0.18]	4.61 [0.16]	3.70 [0.14]
Shoot mass (g)	5.76 [0.16]	31.21 [0.57]	20.32 [0.89]	16.89 [0.80]
Root:shoot ratio	0.60 [0.01]	0.16 [0.00]	n.s.	n.s.
Tiller number	19.18 [0.74]	66.46 [1.77]	44.19 [2.34]	41.01 [2.52]
Endophyte hyphae per mm	6.74 [0.52]	8.38 [0.63]	15.05 [0.53]	0.00 [0.00]
Mycorrhizal colonization (%)	2.81 [0.47]	1.22 [0.32]	1.34 [0.33]	2.69 [0.47]

Table 2. Results from randomization tests (9999 iterations) examining the effects of E = endophyte treatment (present or absent), M = mycorrhizal treatment (sterilized soil = M −, sterilized soil + commercial inoculum = MI, sterilized soil + live field inoculum = MF), and F = fertilizer treatment (added or not) on the host plant *Schedonorus phoenix* (total biomass, root biomass, shoot biomass, root/shoot ratio, and tiller number), on the endophyte, *Neotyphodium coenophilum* (hyphal density per mm of leaf tissue, Endo), on arbuscular mycorrhizal fungi (% of roots colonized, AMF), and on aphids (score of abundance 1, 2, or 3). Significant results ($p < 0.05$) are shown in bold. For aphid score, total plant biomass was included as a covariate.

Treatment	Total	Root	Shoot	Ratio	Tiller number	Endo	AMF	Aphid score	
								Wald χ^2	p
Endophyte	**0.0000**	**0.0000**	**0.0000**	0.2723	**0.0272**	**0.0000**	**0.0098**	7.21	**0.0073**
Mycorrhizae	0.0656	0.6473	**0.0473**	0.8581	0.4780	0.8700	**0.0001**	0.17	0.9199
Fertilizer	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0062**	**0.0023**	0.67	0.4139
E × M	0.5441	0.2597	0.6991	0.6571	0.9911	0.5754	0.2589	1.55	0.4609
M × F	**0.0424**	0.1623	**0.0007**	0.8345	0.5976	0.8734	**0.0399**	0.40	0.8191
E × F	**0.0015**	**0.0442**	**0.0276**	0.5277	0.4624	**0.0064**	0.3900	0.38	0.5363
E × M × F	0.9860	0.8738	0.9982	0.8644	0.1766	0.5748	0.9317	0.88	0.6436
Plant biomass (covariate)	–	–	–	–	–	–	–	1.59	0.2074

and tall fescue (Fig. 1a, Table 2). Plants were protected from water splash, and in the M − treatment, levels of colonization were very low for tall fescue (< 1%), but there was apparently some contamination in the sorghum trial. For tall fescue, overall AMF colonization was low, 5% or less. The M − treatment was significantly lower than in the commercial inoculum (85%) or live field soil inoculum (87%) (Fig. 1a). Commercial inoculum (MI) and live field soil (MF) treatments did not significantly differ (Fig. 1a). Differences between M − and inocula treatments were stronger in the absence of fertilizer than with fertilizer addition, as indicated by a significant mycorrhizal treatment × fertilizer (M × F) interaction (Table 2, Fig. 1a).

Did the endophyte or fertilizer reduce mycorrhizal colonization?

Endophyte presence significantly reduced AMF colonization of tall fescue plants, by 50% (Fig. 1b, Table 1, 2). Endophyte effects did not depend on the fertilizer or mycorrhizal treatment (Table 2). Fertilizer also significantly reduced AMF colonization (Fig. 1c, Table 1, 2), and the statistical model explained 30% of the variation in the data, despite the reduced sample sizes used to limit the labor intensiveness of the scoring process ($F_{11,108} = 4.27$, $p < 0.0001$, $r^2 = 0.30$). For plants with AMF, overall levels of colonization were relatively low (range 1.5%–20%), but comparable to studies on related grasses (Guo et al. 1992, Mueller 2003, Omacini et al. 2006), where means ranged from 3% colonization (E+ *S. phoenix*, Guo et al. 1992) to 45% (E− *Lolium perenne*, Mueller 2003). Low overall colonization was not due to a lack of infection potential, as revealed by the sorghum assay.

The strong negative effect of endophyte presence on mycorrhizal colonization suggested that there might be a negative correlation between the densities of these symbionts. There are two successional phases in this process (1) whether or not roots were colonized by AMF and (2) given initial colonization, the percentage of roots with AMF. First, endophyte density had no effect on the probability of roots being colonized by AMF, although fertilizer addition did reduce the odds of colonization (logistic regression, Endophyte density Wald $\chi^2 = 2.74$, $p = 0.10$; Fertilizer Wald $\chi^2 = 6.27$, $p = 0.01$; Endophyte density × Fertilizer Wald $\chi^2 = 0.23$, $p = 0.63$). Second, given that roots were initially colonized, mycorrhizal colonization was negatively correlated with endophyte density (Fig. 2). A quadratic model provided a better fit to the correlation ($r^2 = 0.83$) than a linear model ($r^2 = 0.58$), suggesting a saturating effect. Further, the negative relationship was strongest when the four fertilized plants with mycorrhizal colonization were excluded from the analysis ($r^2 = 0.83$ vs $r^2 = 0.49$).

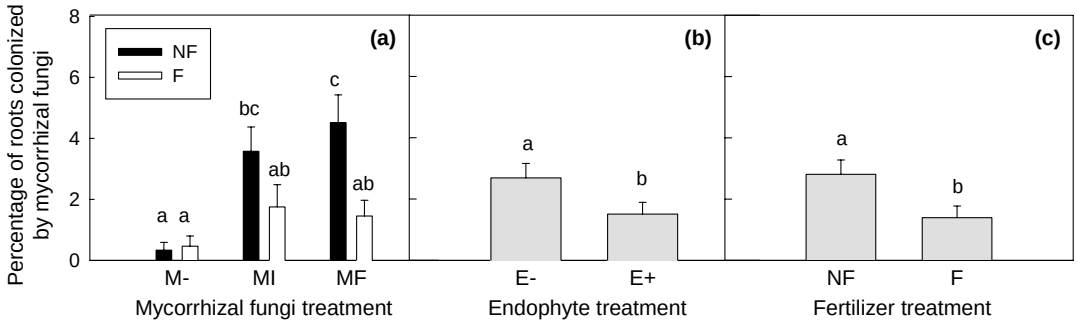

Fig. 1. Effects of (a) soil treatment (sterilized soil = M −, sterilized soil + commercial inoculum = MI, sterilized soil + live field inoculum = MF), (b) the *Neotyphodium coenophialum* endophyte (present = E+, absent = E −), and (c) fertilizer (fertilized = F, not fertilized = NF) on the percentage of non-overlapping root views colonized by mycorrhizal fungi in tall fescue grass *Schedonorus phoenix*. Bars are means + 1 SE. Significant differences as determined by randomization tests are indicated by different letters.

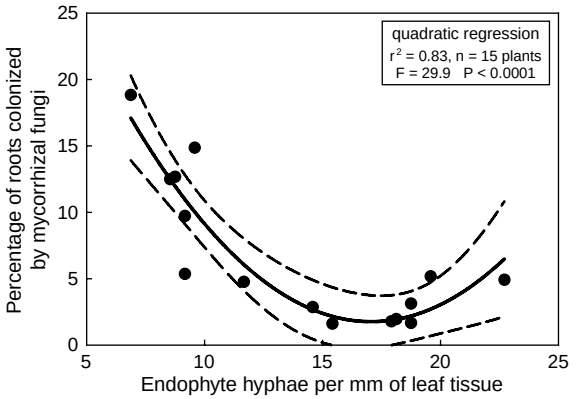

Fig. 2. Relationship between *Neotyphodium coenophialum* hyphal density per mm leaf tissue and arbuscular mycorrhizal colonization (% of non-overlapping root views with evidence of colonization). Each dot represents an individual plant. Unfertilized plants are shown with filled dots; fertilized plants (n = 4 plants with mycorrhizal colonization) are not shown. Dashed lines show 95% CI around the quadratic function.

Did AMF or fertilizer affect endophyte hyphal density?

Mycorrhizal treatments did not affect endophyte density (Table 1). However, the application of fertilizer significantly increased endophyte density, by 24% (Table 1, 2). All E − plants in the analysis had endophyte densities of zero regardless of fertilizer addition, thus, there was a significant interaction between the endophyte and fertilizer treatments (Table 2).

How did tall fescue respond to interactions among the endophyte, AMF, and fertilizer?

Both endophyte presence and fertilizer addition significantly increased tall fescue biomass (Fig. 3a–c, Table 1, 2). E+ plants had higher total (21% more), root (25%), and shoot (20%) biomass as well as higher mean tiller numbers (8%) than E − (Table 1). Fertilization increased total biomass by 403% in the absence of the endophyte and by

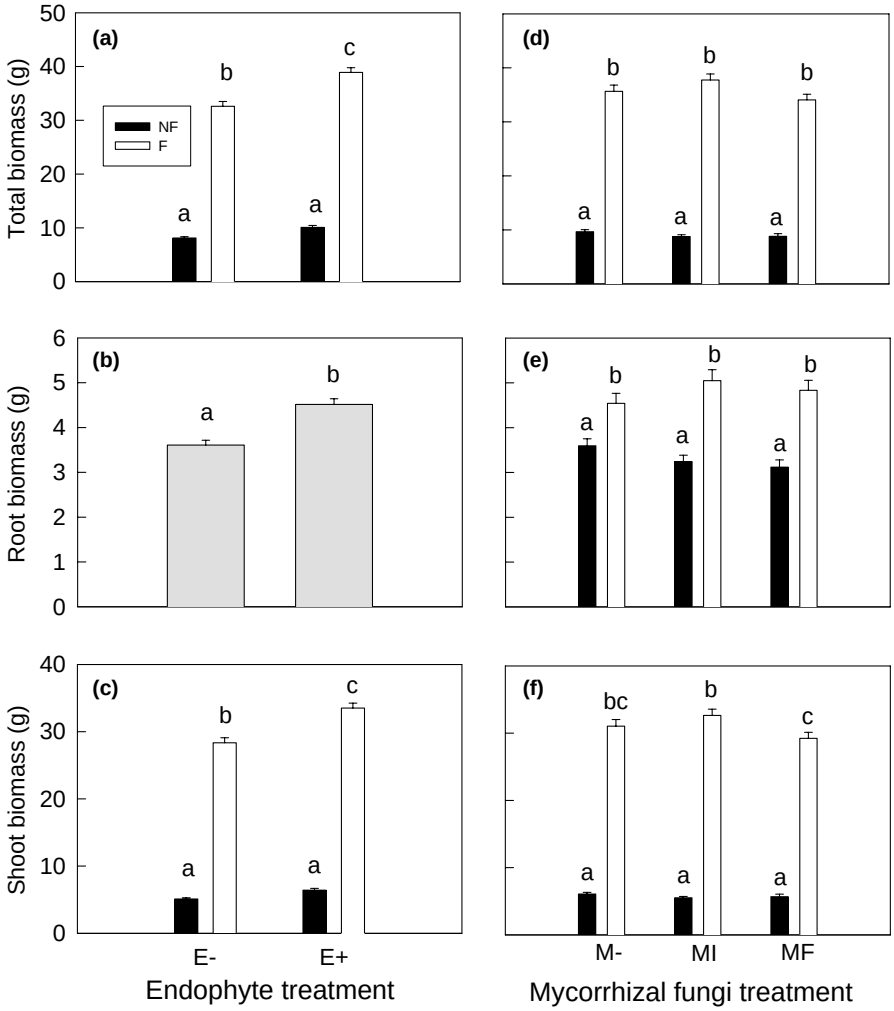

Fig. 3. The effects of the *Neotyphodium coenophialum* endophyte (present = E+, absent = E −) and soil treatment (sterilized soil = M −, sterilized soil + commercial inoculum = MI, sterilized soil + live field inoculum = MF) on the host plant, *Schedonorus phoenix*, including total biomass (a) and (d), root biomass (b) and (e), and shoot biomass (c) and (f) measured in grams. Bars are means + 1 SE. When symbiont treatments significantly interacted with fertilization, results were split by fertilizer treatment (white bars, fertilized = F, black bars, not fertilized = NF). Significance, as determined by randomization tests, is indicated by different letters.

386% (significantly less) in the presence of the endophyte ($E \times F$, $p < 0.01$, Table 2). Only fertilizer application reduced the root:shoot ratio (Table 1).

Because of the strong effect of endophyte presence on plant biomass, we also examined the relationship with endophyte hyphal density by replacing endophyte presence (category) with endophyte hyphal density (continuous, no zeros) in randomization ANCOVA models ($n = 270$ plants). This relationship depended on fertilizer addition (Endophyte density $\times$ Fertilizer, $p = 0.0012$). For fertilized plants, there was a significant positive correlation between tall fescue biomass and endophyte density, whereas unfertilized plants showed no relationship (Fig. 4). There was substantial variation in endophyte density in both the fertilized (variance = 75.2) and unfertilized (variance = 50.8) treatments; detection of the correlation only under fertilized conditions did not result from unequal variances between treatments.

AMF treatments had less pronounced effects on tall fescue than either the endophyte or fertilizer (Fig. 3d–f). The only detectable AMF effect was a reduction in mean shoot biomass for live field soil inoculated plants (MF) compared to commercial inoculum (MI) or inoculum-free (M −) treatments (Fig. 3f). This effect was only observed under the addition of fertilizer (Mycorrhizae $\times$ Fertilizer interaction, Table 2) and could indicate the presence of soil pathogens or other root parasites in the field soil, rather than a cost of association with AMF per se.

Finally, aphid infestation was reduced by 26% in the presence of the endophyte (mean (SE) aphid score E+ = 1.66 ± 0.04, E− = 2.23 ± 0.05; Table 2), but was unaffected by all other treatments. These results contrast with prior work suggesting that mycorrhizae weaken insect deterrence by endophyte-infected *Lolium perenne* (Vicari et al. 2002); however, aphid scores in our study provide a fairly coarse measure of treatment effects on herbivores.

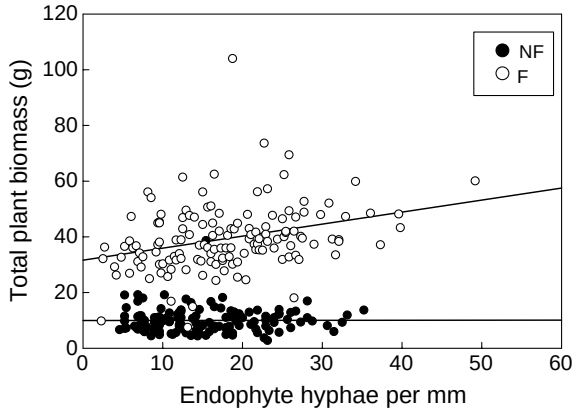

Fig. 4. Relationship between *Neotyphodium coenophialum* hyphal density per mm leaf tissue and total biomass of tall fescue grass *Schedonorus phoenix*. Each dot represents an individual plant. Fertilized plants (open dots) showed a significant positive slope (Spearman rank correlation, $r = 0.35$, $p < 0.0001$, $n = 139$) while unfertilized plants (filled dots) showed no significant slope ($r = 0.03$, $p = 0.72$, $n = 131$). Randomization test: Endophyte density $p = 0.0032$; Endophyte density $\times$ mycorrhizal treatment, $p = 0.79$; Endophyte density $\times$ mycorrhizal treatment $\times$ fertilizer, $p = 0.67$.

Discussion

Systemic fungal endophytes associate with ~20–30% of all grasses (Leuchtmann 1992), and AMF infect ~80% of angiosperms (Brundrett 2002), making these three-way plant-fungal interactions ecologically common ones. To our knowledge, our work is the first to quantify the responses of all three organisms (the plant host, arbuscular mycorrhizal fungi, and the endophyte) under varied resource availability. In general, we found strong asymmetry in responses. Endophyte infection had a large negative effect on mycorrhizal colonization (50% reduction), supporting previous documentation of AMF inhibition by endophytes (Chu-chou et al. 1992, Guo et al. 1992, Mueller 2003; but see Novas et al. 2005). The endophyte also increased plant growth (Rice et al. 1990, Clay and Holah 1999, Clay et al. 2005, Omacini et al. 2006) and deterred aphids. In contrast, mycorrhizal fungi did not affect the endophyte or strongly influence plant biomass or aphids; this was the first test for reciprocal effects of AMF on endophytes. Although we did not observe benefits of AMF for plant growth and biomass, it is possible that AMF affected tall fescue more through changes in plant nutrient status than through changes in plant growth. Future work assessing effects of AMF and endophyte treatments on nutrient levels in host tissues would be informative.

Altering nutrient availability yielded greater insight into the dynamics of both symbionts. Fertilizer additions had strong effects on both fungi, but in opposite directions. Fertilizer increased endophyte hyphal density (by 24%) and plant growth (by ~400%), suggesting an overall promotion of the endophyte-plant mutualism and demonstrating the ability of the endophyte to respond plastically to nutrient availability. This result contrasts with a recent study which found that high nitrogen supply lowered endophyte concentrations in perennial ryegrass (Rasmussen et al. 2007). In contrast, fertilizer decreased the odds of colonization by AMF and the percentage of roots colonized, following predictions from prior studies (Chambers et al. 1980, Azcon et al. 1982, Hepper 1983, Bååth and Spokes 1989, Titus and Leps 2000, Blanke et al. 2005). Finally, although endophyte-infected plants had greater overall biomass, plant biomass was less responsive to increases in fertilizer when plants were endophyte-infected. This pattern may reflect a resource shunt to the endophyte as nutrient availability to the host plant increases. Consistent with the hypothesis of a resource shunt, we found a positive correlation between plant biomass and endophyte density only in plants that were fertilized. However, it remains unclear whether increased endophyte density produces larger plants, or if larger plants can simply support higher endophyte densities. Herbivory could additionally interact with this relationship. Also to be resolved is whether variation in endophyte hyphal density reflects genetic variation in the fungus, the plant, or both (Christensen and Latch 1991, Xu et al. 1994).

Endophyte density was negatively correlated with mycorrhizal colonization. To our knowledge this is the first attempt to describe the *quantitative* relationship between these two symbionts. Several possible mechanisms could produce this pattern. First, endophyte hyphal concentration can explain >30% of the variation in endophyte alkaloid

concentrations (Spiering et al. 2005, see also Rasmussen et al. 2007) and thus could directly reduce mycorrhizae through chemical inhibition. Despite their localization in above-ground tissues, endophytes can increase defensive chemicals in plant roots (Ball et al. 1997, Malinowski et al. 1998), negatively affect belowground herbivores (Humphries et al. 2001) and plant pathogens (Gwinn and Gavin 1992), and alter soil chemistry (Franzluebbers and Hill 2005, Franzluebbers and Stuedemann 2005) and soil respiration (Van Hecke et al. 2005). Thus, the ecological impacts of endophytes are not confined above-ground.

Second, endophyte density could alter the nutritional requirements of the host plant, thereby indirectly affecting mycorrhizae. For example, endophyte infection has been shown to increase plant uptake and storage of phosphorus (Azevedo and Welty 1995, Malinowski et al. 1998, 2000, 2005, Malinowski and Belesky 1999, Rahman and Saiga 2005), which could minimize the benefits of association with AMF (Chambers et al. 1980, Azcon et al. 1982, Hepper 1983, Bååth and Spokes 1989, Titus and Leps 2000, Blanke et al. 2005). In addition, one study on tall fescue has reported longer root hair lengths and shorter root diameters in endophyte infected compared to endophyte-free plants (Malinowski et al. 1999). This result suggests the endophyte can increase root surface area for nutrient absorption, which may thereby reduce the need for AMF. Cool season grasses, such as tall fescue, often have low mycotrophy, and may depend more than other plant species on intrinsic plant traits (or even endophytes) for nutrient acquisition (Wilson and Hartnett 1998).

Third, host plants may face a tradeoff in resource allocation between symbionts. Both endophytes (Thrower and Lewis 1973) and AMF (Brundrett 2002) acquire carbon from plant photosynthesis. Because the endophyte inhabits shoots, it may gain spatial priority, leaving fewer carbon reserves for AMF, and thereby decreasing mycorrhizal colonization. For example, aboveground fungal pathogens can alter assimilate flow in plants away from roots, toward shoots (Whipps and Levis 1981). Also, a recent study on the endophyte *Epichloë glyceriae* showed that carbon was more mobile in endophyte-infected than endophyte-disinfected plants (Pan and Clay 2004). The degree to which tradeoffs exist will depend on the total carbon allocated to each symbiont. Carbon costs for mycorrhizae have been estimated at 4–20% of total plant carbon (Snellgrove et al. 1982, Koch and Johnson 1984, Douds et al. 1988, Peng et al. 1993, Fitter et al. 1998). Similar estimates are not yet available for endophytes. *Neotyphodium* endophytes only occupy intercellular spaces and do not invade cells (Hinton and Bacon 1985). They are thought to rely on free glucose in the intercellular spaces. However, the amount used has only been quantified in defined media, not *in planta* (Kulkarni and Nielsen 1986, Pope and Hill 1991, Naffaa et al. 1998). Endophyte produced loline alkaloids can reach up to 2% of total plant dry weight (Craven et al. 2001), but the relative carbon drain of endophytic vs mycorrhizal fungi is not yet clear. Finally, both symbionts can, under certain conditions, enhance photosynthesis, potentially increasing total carbon reserves (Peng et al. 1993, Richardson et al. 1993, Marks and Clay 1996, Wright et al. 1998, Black et al. 2000, Newman et al. 2003). Increased photosynthesis is not consistently observed for either symbiont, however (Belesky et al. 1987, Spiering et al. 2006), and can vary with plant carbon availability (Gavito and Olsson 2003), temperature (Marks and Clay 1996), water availability (Morse et al. 2002), genotype (Richardson et al. 1993), and nutrient status (Newman et al. 2003), supporting a complex role for abiotic factors in the three-way interaction.

Fourth, endophytes have temporal priority relative to AMF: endophytes are present in seeds (vertical transmission) prior to germination, while AMF are transmitted horizontally. Like spatial priority, temporal priority could generate the observed asymmetric and negative relationship between endophyte and AMF densities. In this study, we lack the ability to link transmission mode to the asymmetry of response, but future work could compare the effects of horizontal endophytes (Arnold et al. 2003, Schardl et al. 2004) vs vertical endophytes for AMF. Although caution should be used when inferring causation, in our study it is likely that endophyte density caused the decline in AMF colonization because AMF inoculation treatments had no effect on endophyte density or presence/absence. In this study, we lacked the ability to examine whether nutrient addition altered the endophyte-AMF correlation because too few fertilized plants were colonized by mycorrhizal fungi. But, given the increase in endophyte density caused by fertilizer addition, impacts may be even stronger under high nutrient availability. Future research could help distinguish among these four hypothesized mechanisms by manipulating endophyte density, mycorrhizae density and plant biomass independently. In addition, while we focused on density and colonization of the fungal symbionts as measures of success, the link between the concentration of symbionts in host plants and symbiont fitness also requires further resolution (Allen 2001, Schardl et al. 2004).

Both antagonisms and synergisms among symbionts have been reported in other multispecies symbioses. For instance, in legumes, AMF can colonize nodules formed by nitrogen-fixing bacteria, and colonized nodules do not fix nitrogen (Scheublin and van der Heijden 2006). It is possible that AMF directly inhibit nitrogen fixation. In contrast, ectomycorrhizal symbiosis can promote nodulation by the nitrogen-fixing bacteria, *Bradyrhizobium* (Andre et al. 2005), and *Frankia* can promote or have neutral effects on mycorrhizal colonization (Sempavalan et al. 1995). Overall, few experiments have manipulated more than one symbiont for any multispecies system (Chalk et al. 2006); drawing general conclusions about the outcomes of these interactions awaits further study.

The negative effects of endophytes on mycorrhizal fungi, shown here and elsewhere also raise important questions about the community level consequences of multispecies symbioses (Muller and Krauss 2005). For example, endophyte-infected plots of tall fescue had reduced plant diversity, fewer trees, and reduced decomposition rates compared to endophyte-free plots (Clay and Holah 1999, Lemons et al. 2005, Rudgers and Clay 2005, Rudgers et al. 2007). Whether some of these community-wide effects are driven by changes belowground in the abundance or composition of mycorrhizal fungi is unclear. Recent work by Omacini et al. (2006) showed that mycorrhizal colonization of uninfected *Lolium multiflorum* plants was

higher when grown in association with endophyte-infected conspecifics than when grown with endophyte-free conspecifics. Such benefits could extend to heterospecific plants, thereby mitigating some of the negative effects of endophytes on other plant species. Finally, these prior studies have largely examined grasses in non-native habitats. It could be informative to examine interactions where the hosts, AMF, and endophytes are native (Saikkonen et al. 2006).

Our study revealed strongly asymmetric interactions among *N. coenophialum*, arbuscular mycorrhizal fungi, and their shared host plant, tall fescue. Further, nutrient additions contributed to the imbalance between the symbionts – benefiting the host plant and endophyte, but reducing mycorrhizal colonization. The observed asymmetry could result from several life history traits of the species involved, including both spatial and temporal priority effects among the symbionts. By examining other multispecies interactions involving mutualists that differ in their functional benefits, general predictions about which mutualist is likely to be the dominant player may be possible.

Acknowledgements – We thank Jim Bever, Keith Clay and both of their lab groups for helpful discussion and comments on the manuscript, and the IU greenhouse staff, Brette Thompson, Lindsey Miller, Elizabeth Porter-Middleton and Allison Bennett for their help with experimental implementation. Summer Nijjer, Ken Whitney and Kelly Lyons also improved the manuscript. Work was supported by the National Science Foundation DBI-0200485 to J.A.R.

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