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Mechanisms underlying the costs and benefits in grass-fungal endophyte symbioses

by

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ABSTRACT

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Nearly all plants have developed symbiotic associations with microbes above- and belowground. These symbionts often alter the ecology of their hosts by enhancing nutrient uptake, increasing stress tolerance, or providing protection from host enemies. Understanding the dynamics of symbiosis requires testing how ecological factors alter not only the fitness consequences of the symbiosis, but also the rate of symbiont transmission. Here we asked how changes in the biotic and abiotic context alter both the costs and benefits of interactions between grass hosts and symbiotic fungal endophytes and rates of symbiont transmission. First, we assessed how shade and the presence of endophyte symbiosis affected host plant growth across six grass species. Our results demonstrate a novel benefit of endophyte symbiosis via the amelioration of shade stress. Second, we examine how interactions between a fungal endophyte and its grass host change along a gradient of water availability and in the presence versus absence of soil microbes. We show that benefits of the symbiosis were strongest when water was limiting. Together, our results highlight the context dependent nature of grass endophyte symbioses.
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TABLE OF CONTENTS

1. List of tables v
2. List of figures vi
3. List of appendices vii
4. Chapter 1: Do the costs and benefits of fungal endophyte symbiosis vary with light availability? 1
5. Chapter 2: Understanding context dependency in plant microbe symbioses: the influence of abiotic and biotic contexts on host fitness and the rate of symbiont transmission 44
LIST OF TABLES

Table 1.1. List of grass and endophyte species used for the study, including information on original source population location, natural endophyte frequencies, treatment for endophyte removal, and the range of light habitats in which they occurred. 39

Table 1.2. Statistical results from ANOVA analyzing effects of endophyte treatment, shade treatment and species on total biomass, root:shoot ratio, SLA, and endophyte density. 40

Table 2.1. Statistical results from M/ANOVA examining the effects of the endophyte treatment, water treatment, and soil treatment on plant reproductive responses: days to flowering, the number of inflorescences, inflorescences per tiller, seed weight, and reproductive effort. 77

Table 2.2. Statistical results from M/ANOVA examining the effects of the endophyte treatment, water treatment, and soil treatment on plant growth responses: total biomass, belowground biomass, aboveground biomass, root:shoot ratio, and the final number of tillers. 78
LIST OF FIGURES

Figure 1.1. Differences in the frequency of endophyte symbiosis for grass species from shaded habitats vs. not restricted to shaded habitats. 35

Figure 1.2. Effects of the shade treatment on total biomass across all species (a) and effect of the shade and endophyte treatment in P. alsodes (POAL) (b). 36

Figure 1.3. Effects of the shade and endophyte treatment on the number of inflorescences in A. perennans (AGPE). 36

Figure 1.4. Effects of the shade and endophyte treatment on specific leaf area. 37

Figure 1.5. Effects of the shade treatment on root:shoot ratio across all species (a) and effects of the endophyte treatment on species grouped by habitat type (b). 38

Figure 1.6. Effects of the shade treatment on endophyte density. 38

Figure 2.1. Effects of the fungal endophyte and water availability on days to flowering in Agrostis hyemalis. 73

Figure 2.2. Effects of the fungal endophyte and water availability on plant reproductive responses. 74

Figure 2.3. Effects of the fungal endophyte and soil type (live or sterile) on total plant biomass. 75
LIST OF APPENDICES

Appendix 1. Phylogenetic trees used for examining relationships between endophyte frequency and habitat type. 41
CHAPTER 1: DO THE COSTS AND BENEFITS OF FUNGAL ENDOPHYTE SYMBIOSIS VARY WITH LIGHT AVAILABILITY?
Summary

We examined whether fungal endophytes modulated host plant responses to light availability, a potentially novel benefit of endophyte symbiosis.

To look for general patterns across host plant species, we conducted a review of the literature to evaluate whether natural frequencies of endophyte symbiosis in grass species from shaded habitats were higher than frequencies in grass species spanning more diverse habitats. Then, in a greenhouse experiment, we assessed how four levels of shade and the presence of endophyte symbioses affected host plant growth across six grass species, and we compared species restricted to shaded environments to species that occupied a broader range of light environments.

In our literature survey, endophytes were more common in grasses from shaded habitats than those that were not. In the greenhouse, we found large benefits of endophyte symbiosis for one species (*Poa alsodes*), and strong negative effects of shade for all but one species (*Festuca subverticillata*). However, only one species showed evidence of an endophyte-mediated growth response to shade. In *Agrostis perennans* symbiotic plants produced 22% fewer inflorescences than non-symbiotic plants at the lowest level of shade, but made 53% more inflorescences at the highest level of shade, showing greater benefits of symbiosis under light limitation. Some species exhibited endophyte-mediated allocation to traits in response to shade. Under high shade, symbiotic *Poa autumalis* invested in greater specific leaf area than symbiont-free plants, but this effect did not occur under high light. Furthermore, in grass species from broad habitat ranges, symbiosis increased the root:shoot ratio, whereas species from shade-restricted habitats
showed no symbiont-mediated response. Finally, shade increased endophyte density in host leaf tissues across all species tested.

Our results demonstrate a novel benefit of endophyte symbiosis via the amelioration of shade stress for one grass host and highlight the potential for symbiosis to alter the plasticity of hosts.

Keywords: mutualism, context-dependency, phylogenetically independent contrasts, *Epichloë, Neotyphodium,* tall fescue
Introduction

Nearly all plants have developed symbiotic associations with endophytic or mycorrhizal fungi (Petrini, 1986). The fossil record suggests that some of these interactions are older than 400 million years (Redecker et al., 2000), suggesting that fungal associations have played a long and important role in the evolution of life on land. These symbionts have been important in plant evolution because they alter the ecology of their hosts, often by enhancing nutrient uptake, increasing stress tolerance, or providing protection from host enemies (Clay & Schardl, 2002; Smith & Read, 1997; Hartley & Gange, 2009). Furthermore, both mycorrhizal fungi, which occur belowground, and aboveground fungal endophytes can have strong impacts on community composition (Clay & Holah, 1999; Hartnett & Wilson, 1999), succession (Janos, 1980; Rudgers et al., 2007), and nutrient cycling (Franzluebbers et al., 1999; van der Heijden et al., 2008).

Given the ecological and evolutionary importance of plant-fungal symbioses, there is great interest in understanding mechanisms through which these symbioses are maintained at high frequencies in plant populations (Rudgers et al., 2009). Isolating these mechanisms necessitates identifying the ecological factors that generate variation in the relative costs and benefits of symbiosis.

Positive, negative and neutral effects of symbionts on plant fitness are expected to arise from variation in the relative costs and benefits of the interaction under different ecological contexts (Bronstein, 1994). In some cases, identifying factors causing this variation is straightforward. For example, in plant-mycorrhizal fungi associations, plant
hosts and fungal symbionts benefit from the exchange of mineral and organic resources (Smith & Read, 1997; Hoeksema et al., 2010). Consequently, variation in nutrient availability in the soil influences both the magnitude of costs and benefits and, ultimately, the net outcome of the interaction (Johnson et al., 1997; Allen et al., 2003). Although increased access to immobilized soil nutrients has traditionally been recognized as the major benefit of mycorrhizal symbiosis, evidence suggests the costs and benefits of the interaction are influenced by factors beyond resource limitation. For example, mycorrhizal fungi can improve host resistance to soil-borne pathogens and root parasites (Ingham & Molina, 1991; Azcón-Aguilar & Barea, 1996; Borowicz, 2001), increase host water uptake (Auge, 2001; Marulanda et al., 2003), improve host tolerance to heat (Kytovitta & Ruotsalainen, 2007), and indirectly affect herbivores through changes in the host plant (Koricheva et al., 2009). Although it has been difficult to determine whether these alternative benefits arise as a result of increases in nutrient acquisition or other changes in host biology, it is possible that symbiont-induced changes in the morphology, chemistry or phenology of hosts may be just as important for host fitness as enhanced nutrient uptake. These results demonstrate the potential for microbial symbionts to provide a diverse set of benefits to host plants, and highlight the importance of measuring both changes in plant performance and symbiont-induced alterations of host phenotypes in novel environments.

In the aerial tissues of plants, endophytic and epiphytic fungal symbionts are incredibly abundant and diverse (Rodriguez et al., 2009). Here we focus on the symbiosis between grass hosts and vertically transmitted, systemic fungal endophytes because, relative to belowground symbioses, far less is known about the costs and benefits of
endophytes. Class 1 endophytes in the family Clavicipitaceae inhabit the aboveground plant tissues of their hosts, and are estimated to occur in approximately 20-30% of all grass species (Leuchtmann, 1992). Historically, endophyte symbioses have primarily been recognized for benefitting host plants through increased resistance to herbivores (Clay, 1996; Bush et al., 1997; Clay & Schardl, 2002). However, research has also shown that endophytic fungi can increase competitive ability (Clay et al., 1993), drought tolerance (Elmi & West, 1995; Malinowski & Belesky, 2000; Kannadan & Rudgers, 2008), pathogen resistance (Gwinn & Gavin, 1992; Mahmood et al., 1993), and the accumulation of nutrients (Malinowski et al., 2000; Rahman & Saiga, 2005), suggesting these symbionts may play important roles in ameliorating a wide variety of environmental stressors. The majority of this research has focused on a few species of agronomically important grasses (Saikkonen et al., 2006; Cheplick & Faeth, 2009), and far less is known about the nature of plant benefits in wild grass species. Several studies have highlighted the continuum from mutualism to parasitism in grass-endophyte interactions (Schardl et al., 2004; Saikkonen et al., 2004; Muller & Krauss, 2005), but the breadth of ecological factors that produce variation in the outcome of the interaction remains poorly characterized. A lack of evidence for symbiont-mediated host benefits could be due to the difficulty in measuring benefits that are small in magnitude (Gundel et al., 2008). Alternatively, these symbionts may provide novel benefits to the host that have yet to be characterized (Cheplick & Faeth, 2009).

In this study, we examined whether endophytes modulate plant responses to shade stress across six grass species that differed in the breadth of light habitats they inhabit. Our interest in shade stems from two observations. First, class 1 endophytic fungi
associate primarily with C3 grasses (Clay & Schardl, 2002). Evidence suggests that C3 grasses are more common in shaded habitats than their C4 counterparts (Klink & Jolly, 1989). Second, endophyte symbioses have been documented in hosts spanning a range of habitats from sunny, open fields to shaded, forest understories. These habitat types exhibit large differences in light availability which may create an important gradient along which the costs and benefits of symbiosis may vary. Despite this wide range of habitat types, there have been no experimental investigations manipulating both endophyte symbiosis and light availability. Belesky et al. (2008; 2009) examined how variation in microsites differing in shade levels influenced plant growth and nutritive values in tall fescue and found that symbiotic hosts in shaded sites had higher levels of alkaloids and phenolics than symbiotic hosts in open sites. However, sites differed in attributes other than shade, and little is known about the potential for endophyte symbioses to modulate plant growth and trait responses to shade stress alone.

Decreased irradiance generally reduces biomass production in grasses (Eriksen & Whitney, 1981). However, this reduction in total plant biomass may be mitigated through plastic changes in plant traits. Functionally adaptive plasticity can contribute to environmental tolerance. Ultimately, interspecific differences in plasticity may contribute to the ecological amplitude of a species and its ability to persist in novel environments (Sultan, 2000; 2004). Plant species with restricted ecological amplitudes are expected to exhibit narrow tolerance to environmental variation and smaller plastic responses (Sultan, 2000). In contrast, widely distributed species are expected to cope with broader environmental gradients and show larger plastic responses. Adaptive plastic responses to shade stress may include reducing the root:shoot ratio and increasing
specific leaf area (SLA) (Chapin et al., 1987; Sultan & Bazzaz, 1993), both of which are associated with the ability of plants to optimize light capture. The degree to which symbionts, such as endophytes, may influence a host plants’ level of plasticity remains unclear. Endophyte symbiosis may alter the plastic responses of grass hosts to shade stress, changing the net effect of the symbiosis. Here, we hypothesized that if host grasses and endophytic symbionts are adapted to high shade environments, then hosts would gain some benefit from the endophyte at high levels of shade. In addition, we hypothesized that this benefit could occur through increases in plant productivity, or changes in plant traits typically associated with adaptive plant responses to shade. Alternatively, endophytes could become more costly to host plants under shaded conditions because they acquire carbon directly from the host (Thrower & Lewis, 1973).

By examining hosts that differed in the breadth of light habitats they occupy, we evaluated whether plastic responses to increased shade and to endophyte symbioses differed between species that are restricted to shaded habitats and those that are not. Specifically, we combined a survey of the literature with experimental manipulations of light availability and endophyte presence to address the following questions:

*Are endophyte frequencies higher in shaded habitats?*

We conducted a literature review to examine whether natural frequencies of endophyte symbiosis differed between grass species from shaded habitats and those that are not restricted to shaded habitats. We predicted that if the symbiosis plays a
role in modulating plant responses to shade, endophytes would occur more frequently in host species from shaded habitats.

Do endophyte symbioses affect plant growth and plant traits in response to shade?

We assessed the effect of four levels of shade and the presence of fungal endophytes across six grass species. We expected that symbiosis would mitigate shade-induced reductions in host biomass. In addition, we compared the responses of three grass species restricted to shaded environments with three species that occupy a range of light habitats. We predicted that if endophytes can influence host phenotypic plasticity, then species from broad light habitats would show stronger, endophyte-mediated plasticity in response to shade.

Does shade alter endophyte density?

In order to examine the response of the symbiont to increased shade, we measured the density of endophyte hyphae within leaf tissues for the highest and lowest shade treatments. Endophyte density could influence both the costs and benefits of the symbiosis, and it has been hypothesized that density is linked to endophyte fitness by increasing the success of vertical transmission of the endophyte to host seeds (Mack & Rudgers, 2008). If endophyte symbiosis benefits plants in shaded habitats or endophytes benefit from shade, then endophyte density may increase with greater
shade. Alternatively, shade may limit plant carbon thereby reducing endophyte density in host tissues.

Materials and Methods

Literature Survey

Data Collection

Data on endophyte presence/absence and endophyte frequency within and among populations was collected largely from a survey conducted by Rudgers et al. (2009) and references therein. In addition, we added data from three new studies (Novas et al., 2009; Emery et al., 2010; Saha et al., 2009) that were not included in Rudgers et al. (2009) survey, and new data from our recent field surveys (Seifert, Miller & Rudgers, unpublished). Then, using data from published floras (Tutin, 1964; Gleason & Cronquist, 1991; Flora of North America Editorial Committee, 1993) we classified grass species into two groups, those that are found in habitats with high levels of shade, such as forest understories, and those that are not. In addition to endophyte presence/absence data, we calculated two metrics of endophyte frequency for the subset of symbiotic grass species: (1) the percentage of total populations that had at least one infected individual (including only the species that had at least three populations sampled) and (2) the mean percentage of plants with the endophyte per population (using only populations for which at least one plant had an endophyte).

Statistical Analysis
We compiled habitat and endophyte data for a total of 187 grass species. We used a log-linear model to test whether the endophyte status of the host grass species (symbiotic vs. not) was associated with habitat type (shaded vs. not) (N = 187 species, Proc Genmod; SAS v. 9.1.3, SAS Institute, Cary, NC, USA). We also conducted a more conservative analysis, where we excluded host grass species that were scored as "non-symbiotic" if fewer than three populations had been sampled. In addition, we tested the relationship between habitat type (shaded or not) and the two metrics of endophyte frequency: percentage of total populations (N = 101 species) or mean percentage per population (N = 82 species) (Proc GLM; SAS v. 9.1.3).

Comparisons across species risk pseudoreplication if phylogeny is ignored (Felsenstein, 1985), so we obtained phylogenetically independent contrasts for habitat type and our three measures of endophyte frequency. For each analysis, we assembled trees for host plants in Mesquite version 2.71 (Maddison & Maddison, 2009) using phylogenetic data from published trees constructed using molecular data sets (Vergara & Bughara, 2003; Catalan et al., 2004; Torrecilla et al., 2004; Blattner, 2004; Strauss et al., 2006). Because branch length information was not available, we chose the best branch length estimator for each variable following published recommendations (PDAP PDTREE; Midford et al., 2005; See Appendix). To examine the relationship between endophyte status and habitat type, we performed phylogenetic logistic regression (PlogReg.m; Ives & Garland, 2010) using MATLAB (version 5.0 MathWorks, 1996). PlogReg.m requires a phylogenetic variance-covariance matrix and a tip (variables) data file which were generated using the programs PDIST (Garland et al., 1993) and PDTREE (Midford et al., 2005), respectively. We report means and bootstrapped 95% confidence
intervals for the regression coefficient and the parameter $\alpha$ (a measure of the strength of the phylogenetic signal). To examine relationships between habitat type and our two measures of endophyte frequency, we performed regression through the origin (SAS Institute, 2004) using the phylogenetically independent trait values (i.e., standardized contrast values), and we report adjusted correlation coefficients (Garland et al., 1992). For a phylogenetic tree with some polytomous nodes, the degrees of freedom range from a minimum of the number of nodes minus one to a maximum of the number of species minus two (Midford et al., 2005). Because it remains unclear whether polytomies in our trees are hard or soft, we present the full range of $P$-values for the correlations.

Greenhouse Study

Study System

We evaluated the effects of shade and symbiosis on the growth and traits of six perennial grass species (Table 1). Natural levels of endophyte frequency from source populations of the species ranged from 41-100% (Table 1). Our field assessment of light habitats indicated that *Elymus villosus*, *Poa alsodes*, and *Festuca subverticilliata* are typically found in deep shade, whereas *Lolium arundinaceum*, *Poa autumnalis*, and *Agrostis perennans* occupy a broader range of light habitats (Table 1).

Endophyte Treatment

We collected seeds from natural source populations during summer 2006 for five native species as well as a naturalized population of the best-studied endophyte host, *Lolium arundinaceum* (tall fescue), which is non-native to the US and grown for forage and turf (Table 1). For each species we worked out the appropriate window of heat
treatment to effectively remove the endophyte (Table 1) without causing substantial reductions in germination rates (Rudgers, unpublished data). Disinfection techniques included wet treatment in a 55°C water bath or dry treatment in a 60°C drying oven, depending on the host species (Table 1). The advantage of using experimental disinfection rather than comparing naturally symbiotic and symbiont-free plants is that we can separate effects of endophyte presence and plant genotype by generating endophyte-free seeds from symbiotic plant lineages. Our recent evidence suggests that loss of the endophyte from symbiotic lineages via imperfect vertical transmission occurs commonly in nature (Afkhami & Rudgers, 2008), and our disinfection treatments were designed to mimic this process.

We began with 10 endophyte-symbiotic (E+) and 10 endophyte-disinfected (E-) genetically unique individuals of each species, grown from seed. Following heat treatment, we surface sterilized seeds and planted into 10 cm X 10 cm X 10 cm plastic pots filled with ProMix-BX (Premier Horticulture, Quakertown, PA). *A. perennans* and *F. subverticillata* required 2-4 weeks of cold stratification in 2% water agar prior to planting. Plants were grown in a common greenhouse environment for six months. Then, each individual was subdivided into four equally sized clones (2-4 tillers each). Cloning to create similarly sized individuals across treatments combined with the six months of growth in a common environment should reduce possible side-effects of the original disinfection treatment (see also Faeth & Sullivan, 2003). Clones were planted into 10 cm X 10 cm X 10 cm plastic pots filled with a 50:50 mixture of ProMix-BX (Premier Horticulture, Quakertown, PA) and QUIKRETE® Premium Play Sand (QUIKRETE® International Inc., Atlanta, GA).
Shade Treatment

Replicate clones were distributed evenly among four shade treatments. To determine the appropriate experimental gradient of light availability we collected light meter readings from natural habitats of the grasses using an AccuPAR Linear PAR Ceptometer (Decagon Devices, Inc., Pullman WA) at the Stephen F. Austin Experimental Forest, Nacogdoches, TX (5 June 2008), and a LI-COR LAI Ceptometer (LI-COR, Lincoln, NE) at Lilly-Dickey Woods Preserve, Nashville, IN (30 May 2008). Readings were taken from 10:00 to 16:00 and sampled the deepest shade (< 10 PAR) and brightest open areas (> 2000 PAR) in which the target grass species naturally occurred. Deep shade showed 99% light reduction relative to open areas. These data suggested that a 0 – 90% gradient of light reduction would mimic natural conditions.

We constructed 61 cm H x 61 cm L x 46 cm W individual shade structures from 1.27 cm diameter PVC frames. Frames were and draped with shade cloth to cover all sides. We draped frames with black knitted shade fabric (Dewitt Company, Sikeston, MO) to create 30, 60, or 90% light reduction. Control structures (0% reduction) included the PVC frame alone. We constructed a total of 40 structures, with 10 replicate structures per shade treatment.

Greenhouse Experimental Set-up

Shade structures were assigned at random to a position on one of four greenhouse benches. Each structure was placed 0.3 m apart to minimize shading from adjacent structures. Throughout the experiment, structure positions were re-randomized every week. This process was designed to equalize exposure to any differences in light
availability that were caused by overhead obstructions in the greenhouse. To quantify differences among the treatments, we took repeated light readings over each structure, and we ensured the shade structures experienced similar exposure to ambient variation in light over the duration of the experiment.

For each of the six grass species, we paired an endophyte-symbiotic plant (E+) and an endophyte disinfected (E-) plant and randomly assigned them to a shade structure. Thus, twelve plants were located within each shade structure. We initially assigned each plant at random to a position within the structure. Plant position within the structure was then re-randomized once per week for the duration of the experiment to equalize any intra-structure positional bias. A replicate of each of the four clones per genotype per endophyte treatment was exposed to each level of shade. Each pot was supplied with a single RainBird drip emitter (Rain Bird Corporation, Tucson, AZ), and plants were watered twice daily at 09:00 and 14:00 with a 1 min drip, at 20 second intervals. The experiment began on 28 June 2008 and was harvested 20 weeks later.

Isolating the Effect of Shade

Shade not only reduces light availability, but also increases humidity and water retention in the soil. To reduce the effects of shade on water availability and isolate the influence of light availability per se, we adjusted soil moisture levels to eliminate any differences due to the shade treatment. Soil moisture readings for eight randomly selected structures (2 per shade treatment) were taken every 14 d (TDR 100 Soil Moisture Probe, 7.5 cm probes, Spectrum Technologies, Inc., Plainfield, IL). TDR probes were calibrated with measurements of gravimetric water content from a subset of trial pots ($r^2$
Using the TDR data from each shade structure, we determined average soil moisture for each level of shade, then administered supplementary watering for the treatments with higher light availability and lower soil moisture by hand-watering.

**Response Variables**

At the end of 20 weeks, we harvested all plants and measured above- and belowground biomass. Roots were washed through a 1.00 mm U.S Standard Sieve (No. 18, Soil Test Inc., Lake Bluff, IL). Mass was obtained following drying at 60°C to a constant weight, and was used to calculate the root:shoot ratio. For *A. perennans*, we recorded the number of inflorescences produced. To calculate specific leaf area (SLA), one leaf from each pot was haphazardly selected and scanned using a HP ScanJet 5590 Digital Scanner at 100 x 100 dpi. Leaf area was calculated using ImageJ Image Analysis software (Rasband, 2009). Following drying at 60°C for 48 h, leaf mass was obtained and used to calculate SLA (cm\(^2\) leaf g\(^{-1}\) leaf biomass).

We determined endophyte density in leaf tissue to estimate endophyte performance. Using a compound microscope (Leica Microsystems, Wetzlar, Germany), we examined thin sections of the inner leaf sheath stained with lacto-phenol cotton blue (Bacon & White, 1994). All species were scored at 400X excepting *L. arundinaceum*, which was viewed at 200X because sheath sections were large. Two observers independently counted the number of views with fungal hyphae present out of 30 non-overlapping slide views per plant, or until tissue was exhausted. We used the average of these independent estimates to determine mean hyphal density for each symbiotic plant.
Hyphal density can increase with nutrient availability (Mack & Rudgers, 2008), but has not always been correlated with other measures of endophyte abundance (e.g., qPCR, Spiering et al., 2005).

**Statistical Analysis**

Experimental data were analyzed with ANOVA including the fixed factors of endophyte status (E+ or E-) and shade treatment (0, 30, 60, or 90% reduction), and the random effects of plant species (six levels) and structure (nested within the shade treatment) to account for the non-independence of plants that co-occurred within each structure (ProcMixed; SAS v. 9.1.3, SAS Institute, Cary, NC, USA). We did not apply MANOVA because of the complex, mixed model analysis (Littell et al., 2002). To compare grasses differing in habitat breadth, we conducted a second analysis including the fixed factors of shade treatment, endophyte, habitat type (shaded vs. not), and the random effects of species (nested within habitat type) and structure (nested within shade treatment). Post-hoc Tukey HSD tests were used to compare treatment means. When measuring treatment effects on plant morphology and allocation patterns, it is important to differentiate between allometric and true plastic responses (Coleman et al., 1994). To correct for allometric effects, log transformed total plant biomass was used as a covariate in the analysis of root:shoot ratio, SLA, and inflorescence number. All interactions with the covariate were initially included in the model, and non-significant interactions were step-wise excluded. In our analysis of endophyte density, log-transformed aboveground biomass was used as a covariate. Analyses met assumptions of normality of residuals and homogeneity of variances following logarithmic transformation of total biomass and square-root transformation of SLA.
Results

Are endophyte frequencies higher in shaded habitats?

Endophyte symbiotic grasses were twice as likely to be occur in shady habitats than non-symbiotic grasses, with approximately 25% of symbiotic grasses restricted to shady habitats versus only 12% of non-symbiotic grasses restricted to shady habitats (Fig. 1a, Wald $\chi^2_{1,185} = 4.31$, $P = 0.0379$). This relationship remained significant after accounting for plant phylogeny ($\beta = 0.885$, 95% CI: (0.0747, 1.90), $P = 0.035$) and a phylogentic signal was not detected ($\alpha = -1.587$, 95% CI: (-3.999, -0.147), $P = 0.1095$). Although the pattern remained the same, our conservative analysis, which excluded “non-symbiotic” grasses with fewer than 3 populations sampled, was not significant (Fig. 1a, Wald $\chi^2_{1,148} = 1.54$, $P = 0.2147$). This may be due to a lack of statistical power and highlights the need for more intensive sampling of potential host grass species. For the subset of symbiotic grasses, the proportion of symbiotic populations per host species was 27% greater in hosts from shaded habitats than those that were not (Fig. 1b, $F_{1.99} = 16.65$, $P < 0.0001$), and this relationship remained significant after correcting for phylogeny using standardized independent contrasts ($r_{99\cdot 63} = 0.3991; 0.0001 < P < 0.0010$). In addition, the average frequency of endophytes within populations was 17% greater in hosts from shaded habitats than those that were not, and showed a trend toward statistical significance (Fig. 1b, $F_{1.80} = 3.35$, $P = 0.0709$). This trend became stronger when variation caused by host plant relatedness was removed ($r_{(80\cdot 50)} = 0.26; 0.0180 < P < 0.0621$).
Does endophyte symbiosis affect plant growth and plant traits in response to shade?

Despite our initial prediction that endophyte symbiosis would alter plant growth responses to shade, we found no significant endophyte x shade interactions for total plant biomass. (Fig. 2a, Table 2). Individually, both the shade and endophyte treatments significantly influenced plant growth. Shade reduced plant total biomass by 50-75% across species (Fig. 2a; Table 2), and *P. autumnalis* showed the strongest response, with high shade plants weighing on average 75% less than low shade plants ($F_{3,36} = 19.97$, $P < 0.0001$; means ± se: 0% shade = 0.617 ± 0.077, 90% shade = 0.150 ± 0.020). *F. subverticillata* was the only species that did not show a significant reduction in total biomass in response to shade ($F_{3,32} = 1.13$, $P = 0.3534$). Only one grass species showed significantly enhanced plant growth from endophyte symbiosis, resulting in a significant endophyte x species interaction (Table 2). Endophyte-symbiotic *P. alsodes* had, on average, 140% higher total biomass relative to endophyte-free plants (Fig. 2b; endophyte $F_{1,35} = 59.89$, $P < 0.0001$), regardless of the level of shade. Endophyte symbiosis did not significantly influence plant biomass for any other grass species.

Only one species flowered during the course of our experiment, and here, we detected endophyte-mediation of the plant response to shade. Presence of the endophyte in *A. perennans* altered allocation to reproduction in response to shade, as indicated by a significant endophyte x shade interaction for the number of inflorescences produced (Fig. 3; shade x endophyte $F_{3,35} = 3.09$ $P = 0.0397$). Symbiotic plants produced 22% fewer
inflorescences than non-symbiotic plants when unshaded, but made 53% more inflorescences at the highest level of shade.

Although we found no evidence for endophyte-mediated plant growth in response to shade, endophyte symbiosis did modulate changes in plant traits in response to shade. Symbiosis in _P. autumnalis_ altered specific leaf area (SLA) in response to shade, as indicated by a significant endophyte x shade interaction. Under low shade, SLA did not differ between endophyte-symbiotic and endophyte-free plants, but at high shade, SLA was 20% greater in symbiotic plants relative to symbiont free plants (Fig. 4c, shade x endophyte _F_\textsubscript{3,33} = 3.98, _P_ = 0.0159). Individually, both the endophyte and shade treatments also influenced SLA. Shade enhanced specific leaf area in four of six species, with increases ranging from 10-180% (Fig. 4a). The effect of shade differed across species, as indicated by the significant species x shade interaction for SLA (Table 2). _L. arundinaceum_ responded the strongest to shade, with a 180% increase in SLA (_F_\textsubscript{3,36} = 61.5, _P_ < 0.0001; means ± se: 0%: shade = 144.0 ± 6.26, 90% shade = 402.7 ± 18.78), while _F. subverticillata_ did not significantly respond (_F_\textsubscript{3,32} = 0.30, _P_ = 0.8328). Differences among species corresponded with differences in habitat breadth. Broad habitat grass species showed significant increases in SLA for every incremental increase in shade, while shade restricted species only altered SLA under the highest (90%) level of shade (Fig. 4d; habitat x shade _F_\textsubscript{3,380} = 8.13, _P_ < 0.0001). The symbiosis alone influenced SLA only in _P. alsodes_, with endophyte-free plants averaging 25% greater SLA than symbiotic plants, across all levels of shade (Fig. 4b), resulting in a significant species x endophyte interaction (Table 2).
The endophyte did not alter root:shoot ratios in response to shade, resulting in no significant endophyte x shade interactions. However, both the endophyte and shade altered allocation independently. As is typical of plant responses to shade, increased shade significantly decreased the root:shoot ratio in five of the six species (Fig. 5a; Table 2). *A. perennans* showed the strongest response with a 50% reduction at the highest level of shade ($F_{3,36} = 6.9, P < 0.0008$; means ± se: 0%: shade = 0.671 ± 0.060, 90% shade = 0.329 ± 0.038), while *F. subverticillata* did not respond to shade ($F_{3,32} = 0.48, P = 0.6992$). The effect of shade was consistent across habitat groups, with species from shade restricted and broad habitats responding similarly (shade x species, $F_{15,361} = 1.3, P = 0.1890$). In contrast, the influence of endophyte symbiosis on the root:shoot ratio varied among host grass species (Table 2; endophyte x species $P < 0.0001$), with species from differing habitat breadths showing different responses. The endophyte significantly increased root:shoot ratio by 26% in species from broad habitat types, while it did not significantly influence the ratio in shade restricted species (Fig. 5b; habitat type x endophyte $F_{1,400} = 15.4, P < 0.0001$).

**Does shade alter endophyte density?**

Endophyte density was 10-85% greater across species in the 90% shade treatment relative to ambient light (0% shade). Despite this range of increases, we detected no significant interaction between grass species and the shade treatment (Table 2), and on average across all six grass species, the highest shade level increased endophyte hyphal density by 41% relative to no shade (Figure 6). *P. alsodes* and *L. arundinaceum* expressed the strongest responses to shade with 85% and 86% increases in the endophyte density, respectively.
Discussion

Relatively few controlled experiments examining the benefits or costs of endophyte symbioses have been conducted with native grass species (Saikkonen et al., 2006; Cheplick & Faeth, 2009), and to our knowledge, this is the first study to experimentally investigate whether endophyte symbioses can alter plant responses to light availability. Despite a high frequency of endophyte symbioses in grasses occupying shady habitats, endophyte symbioses do not appear to mediate plant growth in response to light alone, at least across the range of host grasses we tested. This perceived lack of benefit does not rule out the possibility that variation in levels of shade may play an important role in determining the costs and benefits of endophyte interactions. Recent studies demonstrate endophyte costs and benefits may vary with water availability (Morse et al., 2002; Kannadan & Rudgers, 2008) and herbivore presence (Clay et al., 2005). Compared to plants growing in shade, those in full sunlight typically have greater structural defenses against herbivores (Roberts & Paul, 2006), and are often more prone to drought due to stronger winds, higher temperatures, and lower air humidity (Larcher, 1975). Our experiment decoupled the effect of light from other microclimate (and biotic) variation by controlling water availability, and pests were minimal in the greenhouse. In the future, it would be useful to explore these factors in combination as they are often correlated in natural settings.

Although we found no endophyte-mediated changes in plant biomass production in response to shade, we showed for the first time that plant reproductive fitness can be increased by endophyte symbiosis when light is limiting. The endophyte and shade interacted to alter reproductive allocation in *A. perennans*, with symbiotic plants at high
levels of shade producing 53% more inflorescences than endophyte-free plants. If high levels of shade reduce the long-term survival of the perennial host, this symbiont-mediated shift towards investing more in reproduction than growth could increase host fitness. This would also be an adaptive strategy for the endophyte if vertical transmission rates to seeds are high. However, for both host and symbiont, an assessment of effects throughout host ontogeny would be required to fully characterize benefits and costs (Rudgers et al., in press Am Nat).

Independently, both shade and the endophyte influenced biomass accumulation in some species. As predicted, shade reduced biomass in five of the six species examined. Most strikingly, in *P. alsodes*, loss of the endophyte reduced biomass by 55%, highlighting the importance of the symbiont for host growth in this species (see also Kannadan & Rudgers, 2008). In fact, the magnitude of the effect of endophyte symbiosis was comparable to the magnitude of the shade effect in *P. alsodes*, for which the 90% shade treatment resulted in a 47% reduction in biomass.

The ability of plants to adjust allocation in an adaptive manner is important to their ecological success (Hutchings et al., 2000). Phenotypically plastic allocation patterns can determine a plant’s ability to capture resources (Poorter et al., 1990), produce offspring (Sultan, 2000), and compete with neighbors (Tilman, 1988). As predicted, increased shading reduced the root:shoot ratio in five of the six grass species, confirming results from previous studies (Hunt, 1973; Olff et al., 1990). Given that water and nutrients were not limited, this reduction in root:shoot ratio could be explained by plants optimizing biomass allocation for light harvesting.
Only a few other studies have examined endophyte-mediated changes in biomass partitioning, and ours is the first to examine a suite of native host species. Results have been variable, with some studies documenting decreases in root:shoot ratios (Lewis et al., 1996; Cheplick, 2007; Lehtonen et al., 2005;), and others finding the opposite (or no) effects (Hesse et al., 2003; Kannadan & Rudgers, 2008). Our study suggests that species' habitat breadths may help to explain these prior idiosyncrasies, because our shade-restricted and unrestricted host species responded differently to the symbiosis. In species from shade restricted habitats, symbiosis had no effect on the root:shoot ratio. However, in species from broad light habitats, symbiosis increased the root:shoot ratio, with symbiotic plants investing relatively more resources belowground. This provides some evidence for a potential long-term cost of the endophyte symbiosis for these species in shaded habitats, as greater partitioning to belowground biomass could limit the host’s ability to capture light. However, over the course of our four month experiment, the reduction in root:shoot ratio did not result in differences in total biomass between symbiotic and symbiont-free hosts at high levels of shade. Although potentially costly in shaded habitats, this symbiont-mediated increase in root:shoot ratio could be beneficial in habitats where nutrients or water are limiting.

In addition to changing the proportion of biomass allocated to above- and belowground structures, plastic changes in the morphology of plant tissues can play an important role in adaptive plasticity. Increases in SLA in response to shade can improve the ability of plants to capture light by increasing leaf surface area relative to the amount of energy invested in the production of plant tissue (Evans & Poorter, 2001). In one species tested (P. autumnalis), symbiotic plants showed greater plasticity in SLA in
response to shade than did symbiont-free plants. This symbiont-mediated change in host phenotype could benefit host survival or reproduction in deeply shaded environments and may ultimately allow symbiotic hosts to persist in a broader range of habitats. In contrast, for *P. alsodes*, endophyte symbiosis reduced SLA regardless of the shade level. This effect of symbiosis could be costly in shaded habitats; however, symbiotic plants were significantly larger across all shade levels, suggesting that the decrease in SLA had little effect on plant growth, and that endophyte-mediated changes in host growth are likely occurring through alternative mechanisms. Finally, as predicted, greater shading generally increased SLA, and the response of species restricted to shaded habitats differed from those that occupied diverse habitat types. Broad habitat species were more sensitive to increased shade than restricted species, which could contribute to plants' ability to occupy a wider range of habitat types. This result also suggests that species differing in habitat breadth may differ in their ability to perceive or respond to the environmental cues that trigger plastic responses to shade.

Given the fundamental importance of examining the responses of both partners for understanding the context-dependency of symbioses, surprisingly few studies have examined how variation in abiotic factors influence both host and symbiont growth (Rasmussen *et al.*, 2007; Mack & Rudgers, 2008). To our knowledge, our study is the first to quantify the response of endophytes to changes in levels of shade, and although there was no interaction between the endophyte and shade in affecting plant growth, shade had a consistently positive influence on endophyte density. Prior studies suggest that host and endophyte genotype (Rasmussen *et al.*, 2007), abiotic factors such as nitrogen level (Rasmussen *et al.*, 2007; Mack & Rudgers, 2008), and biotic interactions
with mycorrhizal fungi (Mack & Rudgers, 2008) may influence fungal concentration in plant tissues. Much remains to be elucidated regarding the mechanisms that regulate endophyte growth and fitness, but several hypotheses have been proposed. Changes in density could occur through a dilution effect (Lane et al., 1997), which can occur if an environmental factor stimulates growth of the grass host more than growth of the fungus. In the present study, we are not able to rule out this explanation as we did not measure plant or endophyte growth rates, and our shade treatment significantly altered the aboveground biomass of grass hosts. However, inclusion of aboveground biomass as a covariate in the statistical model did not eliminate the significant effect of shade on endophyte density (Table 2), suggesting this dilution effect may not be strong.

Alternatively, changes in host metabolic profiles in response to the environmental context could also play a role in altering endophyte density (Rasmussen et al., 2008).

Although little is known about mechanisms controlling hyphal growth within hosts and direct evidence of host control is lacking, it is likely that variation in hyphal density can have diverse effects on host ecology. Endophytes rely on hosts for carbon and use host nitrogen for alkaloid synthesis; thus, changes in hyphal abundance could directly alter the costs and benefits of these resource exchanges. Changes in density could also have strong indirect effects by altering biotic interactions. Several studies have reported positive correlations between endophyte abundance and alkaloid concentrations in host tissue (Spiering et al., 2005; Rasmussen et al., 2007), and alkaloid levels in hosts can be positively related to greater herbivore resistance (Clay & Schardl, 2002; Schardl et al., 2007). Given that shade-grown plants may be more vulnerable to herbivores and pathogens due to lower amounts of structural defenses (Roberts & Paul, 2008).
2006), increased alkaloid concentrations associated with higher hyphal densities could be advantageous for hosts growing in shady habitats and contribute to the persistence of higher symbiont frequencies in shade restricted species. Alternatively, endophyte density could be an important component of endophyte fitness that may be independent of host fitness. This could occur if increases in hyphal density increase rates of vertical transmission resulting in higher symbiont fitness at no cost to the host. Data on the ecological factors that influence rates of vertical transmission are lacking, but high frequencies of endophyte symbioses in grasses restricted to shady habitats could ultimately reflect changes in transmission rates and be unrelated to host fitness (see Gundel et al., 2008). Future studies examining how both shade and herbivory, in combination, alter host fitness and rates of symbiont transmission should prove fruitful for understanding mechanisms of endophyte persistence.

Understanding the breadth of factors that generate variation in the costs and benefits of interactions between plants and their microbial symbionts is of fundamental importance to elucidating the mechanisms of symbiont persistence. In this study we found that endophyte symbioses can alter plant reproduction as well as the plasticity of plant traits associated with light capture in response to shade, thereby enhancing understanding of the role of light in the net outcome of the symbiosis. In addition, by examining these interactions across a suite of host species, we found that hosts differing in ecological breadth also differed in their response to the endophyte. This study highlights the importance of examining symbioses across multiple host species and in novel environments for understanding the factors that alter costs and benefits of symbioses and may ultimately influence the persistence of symbioses in host populations.
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**Hunt R, Burnett JA. 1973.** Effects of light intensity and external potassium level on


FIG. 1.1. Differences in the proportion of grass species from shaded habitats for endophyte symbiotic grass species versus endophyte-free hosts (a). The full data set included "non-symbiotic" species where only one population had been sampled, whereas the conservative set was limited to "non-symbiotic" species that had at least three populations sampled. Differences in the frequency of endophyte symbiosis for grass species from shaded habitats vs. not shaded habitats (b) showing both the percentage of populations with the endophyte per grass species and mean endophyte frequency per grass population. Bars show means + SE, and sample sizes (number of species) are indicated on each bar.
FIG. 1.2. Effects of the shade treatment on total biomass across all species (a) and effect of the shade and endophyte treatment in *P. alsodes* (POAL) (b). Bars show means + SE.

(A) All Species

Shade: $P < 0.0001$

Endophyte: $P < 0.0001$

(B) POAL

Shade (% Light Reduction)

FIG. 1.3. Effects of the shade and endophyte treatment on the number of inflorescences in *A. perennans* (AGPE). Bars show means + SE.

AGPE

Endophyte x Shade: $P = 0.0397$
FIG. 1.4. Effects of the shade treatment on specific leaf area (SLA) across all species (a), effects of the shade and the endophyte treatment on SLA in P. alsodes (POAL) (b) and P. autumnalis (POAU) (c), and effects of the shade treatment on all species grouped by habitat type (d). Bars show means + SE.
FIG. 1.5. Effects of the shade treatment on root:shoot ratio across all species (a) and effects of the endophyte treatment on species grouped by habitat type (b). Bars show means + SE.

(a) All Species

(b) Habitat Type

FIG. 1.6. Effects of the shade treatment on endophyte density across all species. Bars show means + SE.
Table 1.1 List of plant and endophyte species used for this study, including information on original source population location, natural endophyte frequencies (Rudgers et al., 2009), treatment for endophyte removal, and the range of light habitats in which they occurred.

<table>
<thead>
<tr>
<th>Grass Species</th>
<th>Code</th>
<th>Endophyte species</th>
<th>Source population</th>
<th>Endophyte frequency</th>
<th>Disinfection treatment</th>
<th>Light Habitat % PAR Reduction (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrostis perennans</td>
<td>AGPE</td>
<td><em>Epichloë amarillans</em></td>
<td>Lilly-Dickey Woods Preserve, Nashville, IN</td>
<td>88 - 100%</td>
<td>Water bath 7.5-8 min</td>
<td>10 - 98% (77%)</td>
</tr>
<tr>
<td>Lolium arundinaceum</td>
<td>LOAR</td>
<td><em>Neotyphodium coenophialum</em></td>
<td>Lilly-Dickey Woods Preserve, Nashville, IN</td>
<td>98 - 100%</td>
<td>Water bath 10-11min</td>
<td>13 - 96% (79%)</td>
</tr>
<tr>
<td>Poa autumnalis</td>
<td>POAU</td>
<td><em>Neotyphodium sp.</em></td>
<td>Stephen F. Austin Experimental Forest, Nacogdoches, TX</td>
<td>83 - 100%</td>
<td>Drying oven 12 days</td>
<td>16 - 94% (77%)</td>
</tr>
<tr>
<td>Elymus villosus</td>
<td>ELVI</td>
<td><em>Epichloë sp.</em></td>
<td>Griffy Lake, Bloomington, IN</td>
<td>41 - 81%</td>
<td>Drying oven 6 days</td>
<td>75 - 97% (94%)</td>
</tr>
<tr>
<td>Festuca subverticillata</td>
<td>FESU</td>
<td><em>Neotyphodium sp.</em></td>
<td>Lilly-Dickey Woods Preserve, Nashville, IN</td>
<td>83 - 100%</td>
<td>Water bath 11-12min or Drying oven 7 days</td>
<td>95 - 99% (97%)</td>
</tr>
<tr>
<td>Poa alsodes</td>
<td>POAL</td>
<td><em>Neotyphodium sp.</em></td>
<td>Lilly-Dickey Woods Preserve, Nashville, IN</td>
<td>88 - 100%</td>
<td>Drying oven 7 days</td>
<td>83 - 97% (92%)</td>
</tr>
</tbody>
</table>
Table 1.2: Statistical results from ANOVA analyzing effects of endophyte treatment, shade treatment and species on total biomass, root:shoot ratio, SLA, and endophyte density. Log transformed total biomass was included as a covariate in the analysis of root:shoot ratio and SLA. Log transformed above-ground biomass was included as a covariate in the analysis of endophyte density. P-values < 0.05 are shown in bold face.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Biomass</th>
<th>Root:Shoot Ratio</th>
<th>Specific Leaf Area</th>
<th>Endophyte Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endophyte</td>
<td>1,369</td>
<td>10.3</td>
<td>0.0015</td>
<td>1,361</td>
</tr>
<tr>
<td>Shade</td>
<td>3,36</td>
<td>51.3</td>
<td>&lt;0.0001</td>
<td>3,36</td>
</tr>
<tr>
<td>Endophyte x Shade</td>
<td>3,369</td>
<td>1.00</td>
<td>0.3974</td>
<td>3,361</td>
</tr>
<tr>
<td>Species</td>
<td>5,369</td>
<td>130.2</td>
<td>&lt;0.0001</td>
<td>5,361</td>
</tr>
<tr>
<td>Species x Endophyte</td>
<td>5,369</td>
<td>7.49</td>
<td>&lt;0.0001</td>
<td>5,361</td>
</tr>
<tr>
<td>Species x Shade</td>
<td>15,369</td>
<td>1.16</td>
<td>0.3037</td>
<td>15,361</td>
</tr>
<tr>
<td>Species x Endophyte x Shade</td>
<td>15,369</td>
<td>0.70</td>
<td>0.7844</td>
<td>15,361</td>
</tr>
<tr>
<td>Log transformed biomass</td>
<td>1,361</td>
<td>1.3</td>
<td>0.2524</td>
<td>1,349</td>
</tr>
<tr>
<td>Biomass x species</td>
<td>5,361</td>
<td>2.51</td>
<td>0.0299</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 1. Phylogenetic trees used for examining relationships between endophyte frequency and habitat type.

A. Phylogenetic tree used for examining the relationship between mean endophyte frequency per population and habitat type (shaded or not). For the calculation of independent contrasts, the best branch length estimator for the mean frequency per population was Nee’s arbitrary branch length, in which the depths of the nodes equal the log of the number of extant (tip) species descendent in the tree (Midford et al. 2005) For habitat type the best branch length estimator was all branch lengths = 1.
B. Phylogenetic tree used for examining the relationship between percentage of populations with the endophyte and habitat type (shaded or not). For the calculation of independent contrasts, the best branch length estimator for the percentage of populations was Nee’s arbitrary branch length. For habitat type the best branch length estimator was all branch lengths = 1.
C. Tree used for examining the relationship between the endophyte status of potential grass hosts and habitat type (shaded or not). The best branch length estimator for both variables was Nee's arbitrary branch length (Midford et al. 2005).
CHAPTER 2: UNDERSTANDING CONTEXT-DEPENDENCY IN PLANT-MICROBE SYMBIOSIS: THE INFLUENCE OF ABIOTIC AND BIOTIC CONTEXTS ON HOST FITNESS AND THE RATE OF SYMBIONT TRANSMISSION
Abstract

Understanding the dynamics of a hereditary symbiosis requires testing how ecological factors alter not only the fitness consequences of the symbiosis, but also the rate of symbiont transmission to the next generation. The relative importance of these two mechanisms remains unresolved because studies have not simultaneously examined how the ecological context of the symbiosis influences both costs/benefits and the rate of vertical transmission. Fungal endophytes in grasses have provided particularly tractable systems for investigating the ecological and evolutionary dynamics of hereditary symbiosis. Here we examine interactions between a fungal endophyte, \textit{Epichloë amarillans}, and its grass host, \textit{Agrostis hyemalis}, under altered abiotic and biotic contexts: a gradient of water availability and in the presence versus absence of soil microbes. We show that benefits of the symbiosis were strongest when water was limiting. Symbiotic plants at the lowest watering level produced $\sim 40\%$ more inflorescences and greater seed mass than non-symbiotic plants, while at the highest watering level, symbiotic and non-symbiotic plants did not significantly differ in reproductive fitness. Benefits appear to accrue by allowing hosts to escape from drought, a response that has not been previously reported to be endophyte-mediated. Symbiotic plants at the lowest watering level flowered nine days earlier than non-symbiotic plants. Interestingly, our results suggest the symbiosis may be costly in the presence of soil microbes, as on live soil, the biomass of symbiotic plants was lower than the biomass of symbiont-free plants. We detected no effect of either the biotic or abiotic context on the rate of symbiont vertical transmission, suggesting that the context-dependent benefits of the symbiosis are the more important driver of variation in symbiont frequency in this system.
Introduction

Nearly all plants form symbiotic relationships with microbes (Petrini, 1986; Fitter and Moyenson, 1996; Saikkonen et al., 1998). These symbionts can alter the ecology of their hosts, often by enhancing nutrient uptake, increasing tolerance to stress, or providing protection from host enemies (Smith and Read, 1997; Clay and Schardl, 2002; Hartley and Gange, 2009). They can also affect community composition (Clay and Holah, 1999; Hartnett and Wilson, 1999), succession (Janos, 1980; Rudgers et al., 2007), and nutrient cycling (Franzluebbers et al., 1999; van der Heijden et al., 2008). Given their potential for strong ecological impacts, not only on host plants but also on the surrounding community and ecosystem, there is great interest in understanding the factors that influence the persistence of these symbioses.

Theory suggests that the persistence of symbionts in host populations is dependent upon the net effect of the symbiont on host fitness and the mode and rate of symbiont transmission (Gundel et al., 2008). Thus, understanding the dynamics of the symbiosis at the population level requires testing how ecological factors alter not only the fitness consequences of the symbiosis, but also the rate of symbiont transmission.

Positive, negative and neutral effects of symbionts on plant fitness are expected to arise from variation in the relative costs and benefits of the interaction under different ecological contexts. Variation in the mode and rate of symbiont transmission is also common, with some symbionts transferred horizontally through contagious spread and others transferred vertically from parent to offspring (Bright et al., 2010).

Fungal endophytes in grasses have provided particularly tractable systems for investigating the ecological and evolutionary dynamics of symbiosis. Fungal endophytes
occur commonly, can be easily manipulated, and are not obligate for the plant; thus allowing for comparisons of symbiotic and symbiont-free hosts. Within the grass family, Poaceae, approximately 20-30% of species host systemic class 1 endophytic fungi in the fungal family Clavicipitaceae (Leuchtmann, 1992). These symbionts are often vertically transmitted through seeds of the host plant and can increase host growth and reproduction by enhancing host resistance to both biotic and abiotic stress (Cheplick and Faeth, 2009).

It remains unclear what factors contribute to the context-dependency and long-term persistence of grass-endophyte symbioses. Across host species, there exists substantial variation in the frequency of endophyte symbiosis, both within and among populations (Rudgers et al., 2009), suggesting that endophytes may not be universally beneficial and/or may have low rates of transmission across generations. Experimental evidence generally supports the hypothesis that the outcome of grass endophyte symbioses spans a continuum from parasitism to mutualism, with outcomes dependent upon the biotic and abiotic context (Cheplick et al., 1989; Schardl et al., 2004; Saikkonen et al., 2004; Muller and Krauss, 2005). For example, in tall fescue grass (*Lolium arundinaceum*) the endophyte symbiosis can enhance herbivore resistance (Rudgers and Clay, 2007), competitive ability (Clay et al., 1993) and drought tolerance (Elmi and West, 1995) but can also reduce host biomass under nutrient poor conditions (Cheplick et al., 1989). These context-dependent benefits may ultimately underlie the observed variation in endophyte frequency or persistence. For instance, one study has shown that increased herbivore pressure can drive increases in endophyte frequency (Clay et al., 2005). However, observational evidence suggests that imperfect vertical transmission of symbionts is also common (Afkhami and Rudgers, 2008), and could provide an
alternative explanation for variation in symbiont frequency. The relative importance of these two mechanisms remains unresolved because studies have not simultaneously examined both the costs/benefits of the symbiosis and the rates of vertical transmission under altered abiotic or biotic contexts. This is an important step for understanding the degree to which intrinsic dynamics vs. factors extrinsic to the symbiosis influence symbiont frequencies in natural populations.

Historically, endophyte symbioses have primarily been recognized for benefitting host plants through increased resistance to herbivores (Clay, 1996; Bush et al., 1997; Clay and Schardl, 2002). However, research has also shown that endophytic fungi can increase host competitive ability (Clay et al., 1993), drought tolerance (Elmi and West, 1995; Malinowski and Belesky, 2000; Kannadan and Rudgers, 2008), pathogen resistance (Gwinn and Gavin, 1992; Mahmood et al., 1993), and the accumulation of nutrients (Malinowski et al., 2000; Rahman and Saiga, 2005), suggesting these symbionts may play important roles in ameliorating a wide variety of environmental stressors. These alternative pathways of benefits have received far less attention than herbivory in the current literature.

Endophyte mediated benefits to hosts under water stress have been well documented in several agronomically important forage and turf grass species from the genera *Festuca* and *Lolium* (reviewed by (Bacon, 1993; Malinowski and Belesky, 2000; Clay and Schardl, 2002; Muller and Krauss, 2005; Saikkonen et al., 2006), and more recently in two native grass species (Morse et al., 2002; Kannadan and Rudgers, 2008). Surveys of native grasses have also documented higher frequencies of symbiosis in drier habitats (Lewis et al., 1997; Leyronas and Raynal, 2001; Novas et al., 2007; Saona et al.,
suggesting the potential for a widespread function of endophytes in mediating plant responses to water stress.

Local and seasonal availability of soil moisture is a critical factor determining the distribution and abundance of plant species (Cornwell and Grubb, 2003). Limited water availability can have strong, negative impacts on plant productivity, and plants have evolved adaptations in numerous physiological, developmental, and life history traits to cope with this stress (Geber and Dawson, 1990; Ackerly et al., 2000). These traits have historically been grouped into strategies that enable plants to avoid, tolerate, or escape drought, although it has been recognized that these strategies are not mutually exclusive (Levitt, 1980; Ludlow, 1989). When subjected to slowly developing water shortages (days to months) some plants can optimize their long-term resource gain through acclimation responses that allow them to avoid or tolerate drought. Avoidance mechanisms allow plants to maintain tissue water potential as high as possible by minimizing water loss or maximizing water uptake. Endophyte-mediated drought avoidance mechanisms have been documented in several species, including changes in the timing and rate of stomatal closure (Elmi and West, 1995; Buck and Elbersen, 1997; Malinowski et al., 1997), increases in root dry matter (Latch et al., 1985; De Battista et al., 1990; Malinowski et al., 1997), and greater storage of water in tillers (Elberson and West, 1996; Buck et al., 1997) of symbiotic plants relative to symbiont-free. Endophyte-mediated changes in root traits associated with enhanced water uptake, such as increased root hair length, have also been reported (Malinowski et al., 1999). Tolerance mechanisms allow plant tissues to withstand negative water deficits through changes in physiological and biochemical properties. Endophyte-mediated changes in several
drought tolerance mechanisms have also been documented, including the translocation of assimilates to leaves (Richardson et al., 1992), osmotic adjustment (reviewed in Malinowski and Belesky, 2000), and changes in cell wall elasticity (White et al., 1992). As an alternative to acclimation responses, plants can escape drought stress. Escape strategies rely on successful reproduction before the most intense period of drought, allowing plants to maximize fitness. This can be accomplished by increasing growth rates, flowering early, and allocating more resources to reproduction to maximize resource use while water is available. Historically, most attention has been placed on avoidance and tolerance, and, to our knowledge, no investigations have examined the potential for endophyte-mediated escape.

In addition to changes in the abiotic context, the costs and benefits of grass-endophyte interactions may also vary with biotic factors other than herbivory. In this study, we additionally examined the potential role of soil microbes in altering the costs and benefits of the symbiosis. Some prior studies suggested antagonism between foliar endophytes and soil communities, including decreased soil microbial biomass (Jenkins et al., 2006), suppressed plant pathogenic nematodes (Kimmons et al., 1990; Elmi et al., 2000), and reduced mycorrhizal fungi colonization and spore abundance in the soil (Chu-Chou et al., 1992; Guo et al., 1992; Mueller, 2003; Omacini et al., 2006; Mack and Rudgers, 2008). Endophyte density has also been negatively correlated with rates of mycorrhizal colonization of roots, and it has been hypothesized that variation in endophyte density could influence rates of vertical transmission (Mack and Rudgers, 2008). While endophytes can clearly have strong impacts on belowground communities
and processes, little is known about how microbes belowground influence the costs and benefits of these aboveground symbioses.

Here we examine interactions between a fungal endophyte, *Epichloë amarillans*, and its grass host, *Agrostis hyemalis*, under altered abiotic and biotic contexts: a gradient of water availability and in the presence versus absence of soil microbes. Specifically, we address the following questions:

1. Do the costs or benefits of endophyte symbiosis vary with changes in water availability and/or the presence of the soil microbial community?

2. Do these ecological factors influence the rate of vertical transmission of the endophyte?

To our knowledge, this is the first study to evaluate the context-dependency of these two, alternative pathways (costs/benefits, symbiont transmission) that can influence symbiont frequency and persistence.

**Methods**

**Study Organisms**

*Agrostis hyemalis* is a weak perennial or facultatively annual C₃ grass distributed throughout eastern North America and frequently occurring in pastures, along roadbanks and ditches, and in open woods. It is widely distributed throughout the state of Texas, flowering in late-April to early May. *A. hyemalis* hosts the endophyte *E. amarillans* (Craven, 2001). In our Texas field sites, mean endophyte frequency (% symbiotic plants/population) was 84% (range 50-96%; 8 populations), and all populations surveyed were symbiotic (Rudgers et al., 2009). During summer 2006, we collected seeds from
approximately 100 individuals in a natural population at the Stephen F. Austin Experimental Forest (31°29'52"N, 94°46'46"W). Endophyte frequency in the source population was 96%. The endophyte appears to be primarily vertically transmitted to seeds, as stromata formation has rarely been observed in the field (< 1% of plants across all of our sampled populations).

*Endophyte Disinfection Treatment*

By using experimental removal of the endophyte rather than comparing naturally symbiotic and symbiont-free plants, we can separate the effects of endophyte presence and plant genotype. To remove the endophyte, we heat treated a subset of randomly chosen seeds in a water bath at 62°C for 6 minutes. To minimize the potential side effects of the endophyte removal treatment, the treated seeds were grown in the greenhouse until they flowered and set seed; this second generation of seeds was then used to plant the experimental treatments. Our disinfection treatment was designed to mimic the natural process of endophyte loss from symbiotic lineages, which can occur through imperfect vertical transmission (Afkhami and Rudgers, 2009).

Prior to establishment of the experiment, we made several leaf peels from 30 plants per treatment, stained the endophyte in the leaves with rose bengal, and examined tissue under a microscope at 200x (Leica Microsystems, Wetzlar, Germany) to check the effectiveness of the treatment (Belanger, 1996). In addition, to assess if the treatment had any effects on seed germination for the seeds used in our experiment, 20 seeds from each treatment were planted in 10 replicate petri dishes in a growth chamber (12 h day length, 15-24°C), and we recorded the proportion of seeds that germinated.
**Soil Community Treatment**

We manipulated the biotic component of the soil community via sterilization of live field-collected soil (sterilized vs. control). We collected soil from Stephen F. Austin Experimental Forest on 16 Jun 2008 at a site near the source *A. hyemalis* population. Soil was taken from the top c. 15 cm of the soil horizon to match the rooting zone of *A. hyemalis*, sieved to 4 mm (U.S Standard Sieve No. 5, Soil Test Inc., Lake Bluff, IL) and stored at 4 °C until establishment of the experiment. Soil was sterilized twice in an autoclave at 121 °C for 1.5 hours. Control soil remained untreated. Our soil inoculum constituted of ~3% of the total soil volume; therefore, any differences between the live and sterile treatments were not likely driven by nutrient differences caused by sterilization. At the end of the experiment, root tissue samples (~1 g) were stained to assess colonization by arbuscular mycorrhizal fungi, following the procedure described by INVAM (http://invam.caf.wvu.edu/methods/mycorrhizae/staining.htm) using 0.05% trypan blue. After staining, we mounted roots onto slides and examined them under a microscope at 400X (Leica Microsystems, Wetzlar, Germany).

**Greenhouse Experiment**

On 15 July 2008, symbiotic and symbiont-free seeds that were one generation removed from the endophyte-disinfection treatment were planted in plastic seedling trays (4.1 x 4.1 cm cells) filled with an autoclave-sterilized 50:50 mixture of screened and washed sand (QUIKRETE® International Inc., Atlanta, GA) and Metromix 200 (SunGro Horticulture Inc, Corvalis, OR). Each of 96 E+ and 96 E- seedlings were randomly assigned to a soil (live or sterile) and watering treatment (20ml, 40ml, 60ml, 80ml per
day), for a total of 16 treatment combinations (12 replicates per endophyte x water x soil combination, 192 individual plants). On 25 Aug 2008, seedlings were transplanted into 10 cm X 10 cm X 10 cm deep plastic pots filled with 600ml of the sterile 50:50 sand and Metromix, and 20 ml of live or sterile field soil. To reduce splash contamination, the 20 ml of soil used as inoculum was sandwiched in the middle of the pot between layers of the sterile soil mixture. We arranged pots in a randomized order in the greenhouse, and watered them with 40 ml tap water twice per day for 1 week prior to establishment of the watering treatment. Throughout the experiment, greenhouse temperature was maintained at c. 24°C with no supplemental light.

**Watering Treatment**

We imposed the water manipulation on 1 September 2008. Using automatic emitters (Rain Bird, San Diego, CA), we watered plants with tap water twice per day with 10ml, 20ml, 30ml, or 40ml. Using a soil moisture probe (TDR 100 Soil Moisture Probe, 7.5 cm probes, Spectrum Technologies, Inc., Plainfield, IL), we measured soil moisture 1 week after the establishment of the water treatment and again just prior to harvesting to check the effectiveness of our treatment. TDR probes were calibrated with measurements of gravimetric water content from a subset of trial pots ($r^2 = 0.81, F_{1,19} = 75.9, P < 0.0001, n = 20$ pots).

**Response Variables**

To assess treatment effects on plant growth, we counted tillers five times throughout the experiment. Here, we report final tiller numbers, as results were consistent through time. At the end of 10 weeks (beginning 15 November 2008), we harvested all
plants and measured above- and belowground biomass. Roots were washed through a
1.00 mm U.S Standard Sieve (No. 18, Soil Test Inc., Lake Bluff, IL). Above- and below-
ground mass was obtained following drying at 60°C to a constant mass and used to
calculate the root:shoot ratio (root mass (g) x shoot mass$^{-1}$ (g)). To track changes in plant
phenology, we recorded the number of days to flowering (i.e., the date that flowers
opened on the first inflorescence produced). To quantify plant reproduction, we collected
all inflorescences individually. *A. hyemalis* exhibits dual dispersal modes, such that
some seeds are dispersed locally, falling off the mature inflorescence while it is still
attached to the plant, while others are dispersed when the inflorescence breaks loose from
the parent and rolls in the wind (Rabinowitz and Rapp, 1979). In order to obtain accurate
measures of seed production, we removed individual inflorescences from plants just prior
to the release of seeds. In addition, for the highest and lowest watering treatments, we
manually removed seeds from all inflorescences per plant and weighed them to obtain
total seed mass; we limited this assessment to the treatment extremes due to the labor
intensiveness of seed removal. To assess treatment effects on reproductive allocation,
we calculated inflorescences per tiller (inflorescence number x total tiller number$^{-1}$) and
reproductive effort (seed weight x total biomass$^{-1}$).

**Endophyte Transmission**

To assess treatment effects on the viability of seeds produced, a randomly
selected subset of 20 seeds from each symbiotic and symbiont-free plant from the highest
and lowest watering treatments were planted in sealed petri dishes filled with wet sterile
sand then placed in a growth chamber (12 h day length, 15-24 °C). After 4 weeks,
representing the typical germination window, we recorded the number of seeds that
germinated. For the subset of symbiotic plants, we assessed the rate of vertical transmission by scoring endophyte presence/absence for a minimum of ten seedlings per parent plant using rose bengal stain (Belanger, 1996). We then calculated the proportion of seeds that germinated as an additional measure of reproductive fitness of the plant, and the proportion of symbiotic seedlings as a measure of the rate of vertical transmission and therefore, the fitness of the endophyte.

Statistical Analysis

We constructed two MANOVA models (SAS Institute 2004). The first model tested for treatment effects on plant growth by combining the responses of total biomass, aboveground biomass, belowground biomass, final tiller number, and the root:shoot ratio. The second model tested for treatment effects on plant reproduction by combining the responses of days until flowering, inflorescence number, total seed mass, inflorescences per tiller, and reproductive effort. For this model, our MANOVA was restricted to the highest and lowest watering levels, because seed mass was not measured for the intermediate watering levels. All statistical models included the fixed factors of endophyte treatment (E+ or E-), water treatment (four levels), and soil type (live or sterile). If MANOVA detected significant treatment effects, we decomposed the effects using individual ANOVA. For the plant reproductive traits, for which we had data at all four water levels, we present results from the full model, as results did not qualitatively differ from the model restricted to the highest and lowest water level. Post-hoc Tukey HSD tests were used to compare treatment means. At the lowest watering level, we also calculated Pearson correlation coefficients to examine the relationships between days to flowering, the number of inflorescences produced, and seed mass. For examining
treatment effects on the rate of vertical transmission, we performed ANOVA with the fixed factors of water availability and soil type. All analyses met assumptions of normality of residuals and homogeneity of variances following log transformation of aboveground biomass, total biomass and seed mass, and square-root transformation of inflorescence number.

Results

**Treatment Effectiveness**

**Endophyte treatment**

The heat treatment to remove the endophyte had no effect on the proportion of seeds that germinated in the second generation ($F_{1,19}=0.66$, $P=0.4287$; means ± se: E+ = 0.785 ± 0.039, E- = 0.822 ± 0.033). Symbiont frequency in the seedlings used to establish the experiment was 0% in the E- treatment and 100% in the E+ treatment ($n=30$ plants per treatment).

**Water treatment**

One week after the establishment of the water treatment, the lowest water level represented a 53% reduction in soil volumetric water content relative to the highest water level, and all four levels of the treatment differed significantly from each other ($F_{3,188} = 47.35$, $P < 0.0001$; Means ± se: 20ml = 12.4 ± 0.55; 40ml = 15.64 ± 0.68; 60ml = 19.41 ± 0.99; 80ml = 26.08 ± 1.22). After ten weeks, the percentage reduction in soil volumetric water content between the highest and lowest watering level increased to 85%. All treatment levels remained significantly different from each other ($F_{3,188} =$
118.17, \( P < 0.0001 \); Means ± se: 20ml = 2.29 ± 0.13; 40ml = 4.61 ± 0.24; 60ml = 9.06 ± 0.47; 80ml = 15.72 ± 0.99). Unexpectedly, the endophyte treatment also affected volumetric water content, with on average 11% higher soil water content in pots with endophyte-symbiotic plants relative to the endophyte-free treatment \( (F_{1,19} = 5.23, P < 0.0233; \text{Means ± se: } E^+ = 13.82 ± 0.75 E^- = 12.48 ± 0.60) \).

**Soil treatment**

Examination of 30 slides from the live soil treatment revealed no arbuscular mycorrhizal fungi colonization, suggesting our live soil treatment was not effective in manipulating this component of the biotic soil community. Other soil microorganisms were likely present in the live soil, but were not directly assayed.

**Plant Reproduction Responses**

The endophyte symbiosis increased several plant reproduction responses at low water availability, as indicated by a significant interaction between the endophyte and water treatments (Table 1). When water was limiting, the symbiosis altered plant phenology. Endophyte symbiotic plants began flowering 9 days earlier than non-symbiotic plants at low water availability, but did not differ from non-symbiotic plants at high water availability (Fig. 1, Table 1). The pattern was similar for the number of inflorescences produced. At the lowest water level, days to flowering was negatively correlated with the number of inflorescences produced \( (r = -0.33, P = 0.0224, n = 48) \). Symbiotic plants at the lowest watering level produced 42% more inflorescences than non-symbiotic plants, but the endophyte treatments did not significantly differ in the number of inflorescences produced at high water availability (Fig. 2a, Table 1). These
differences in inflorescence number also corresponded with differences in the total seed mass produced by the plants ($r = 0.62, P < 0.0001, n = 48$). Symbiotic plants produced 43% more seed mass than non-symbiotic plants at low water availability, but did not differ from non-symbiotic plants at high water availability (Fig. 2b, Table 1).

In addition to modulating total reproductive output, the endophyte symbiosis and water availability also interacted to alter plant allocation towards reproduction. Symbiont presence increased the number of inflorescences per tiller by 23% at low water availability, but at high water availability, symbiotic and symbiont-free plants did not significantly differ (Fig. 2c, Table 1). Reproductive effort showed a similar pattern. Symbiotic plants invested 35% more in seed mass/vegetative mass than non-symbiotic plants at low water availability, but did not differ from symbiont-free plants at high water availability (Fig. 2d, Table 1).

**Plant Growth Responses**

In contrast to its effects on plant reproduction, endophyte symbiosis did not modulate plant growth responses to water availability. The endophyte did interact with the soil treatment to alter total plant biomass. Specifically, non-symbiotic plants on live soil had 17% greater biomass than symbiotic plants on live soil, whereas symbiotic and symbiont-free plants performed equally well on sterile soil (Fig. 3, Table 2). In general, decreased water availability caused strong reductions in plant growth, reducing biomass by 68%, reducing tiller number by 56%, and increasing root:shoot ratio by 30% (Table 2), demonstrating that our treatments were effective in generating abiotic stress.

**Endophyte Transmission**
On average across all treatments, seed germination was $75\% \pm 1.3$ s.e. Neither the endophyte treatment, water availability, nor the soil treatment influenced the proportion of progeny seeds that germinated (endophyte $F_{1,95} = 0.43 \ p = 0.5154$; water availability $F_{1,95} = 0.41 \ p = 0.5227$; soil treatment $F_{1,95} = 0.10 \ p = 0.7497$). In addition, neither the soil nor water treatment influenced the rate of endophyte transmission to seedlings (water availability $F_{1,47} = 0.61 \ p = 0.4388$; soil treatment $F_{1,47} = 0.11 \ p = 0.7393$). However, transmission to seedlings was imperfect, as the mean symbiont frequency in seedlings was $90\%$ (Mean ± se: $0.90 \pm 0.02$) whereas the parental endophyte frequency was $100\%$.

**Discussion**

To our knowledge, this is the first study to simultaneously investigate how changes to the ecological context of a symbiosis alter both the costs and benefits of the interaction and the rate of symbiont vertical transmission. Specifically, we showed that fitness benefits of the symbiosis between the native grass *Agrostis hyemalis* and the fungal endophyte *Epichloë amarillans* were strongest when water was limiting. Symbiotic plants at the lowest watering level produced ~40% more inflorescences and greater seed mass than non-symbiotic plants, while at the highest watering level, symbiotic and non-symbiotic plants did not significantly differ in reproductive fitness. In addition, we found no differences in germination rates of the seeds produced by symbiotic and symbiont-free plants, suggesting that the increase in reproductive output did not decrease seed viability. Because symbiont transmission was not influenced by water availability in our study, variation in endophyte frequency in natural populations more likely reflects the relative performance of symbiotic and symbiont-free hosts in different environmental contexts.
Altogether, our results show that changes in the abiotic context influence the costs and benefits of the symbiosis, enhancing understanding of the role of endophytes in ameliorating stress in native grasses and providing insight into the future implications of long-term changes to the environment. In other systems, changes in the costs and benefits of symbiosis in response to abiotic stress have been linked to symbiont loss in local populations. Perhaps most notably, in the symbiosis between reef-building corals and zooxanthellae, increased light and temperature can lead to coral bleaching, in which the algal symbionts are expelled from the host (Abrego et al., 2008). This process can have strong negative consequences for biodiversity and ecosystem functioning (Hughes et al. 2003; Baker et al., 2008), and highlights the importance of understanding how environmental changes influence the dynamics of symbioses. We hypothesize that changes in soil moisture levels, particularly those accompanying climate change, could ultimately impact endophyte persistence in native populations, and we predict increased frequencies of the endophyte in *Agrostis hyemalis* in drier environments.

Relatively few controlled experiments examining the benefits or costs of endophyte symbioses have been conducted with native grass species (Saikkonen et al., 2006; Cheplick and Faeth, 2009). To date, our work represents one of three published studies to show that endophyte symbiosis can confer benefits to native grasses under water stress (Morse et al., 2002; Kanadaan and Rudgers, 2008). Prior studies in grass-endophyte systems have provided support for endophyte-mediated benefits to plants under water stress through enhancements of both drought avoidance and drought tolerance mechanisms (see references in introduction). However, our study demonstrates that benefits may also accrue by allowing hosts to escape from drought. Symbiotic
plants at the lowest watering level flowered 9 days earlier than non-symbiotic plants, and this plastic change in flowering time was correlated with an increase in the number of inflorescences plants produced. Given that the severity of our watering treatment increased through time, this symbiont-mediated shift in phenology could have benefited hosts by allowing them to maximize resource utilization prior to the most severe level of stress, and is consistent with other studies that have demonstrated the adaptive significance of early flowering for escaping drought (Sherrard and Maherali, 2006; Franks et al., 2007). It is also in agreement with results from previous studies in the related species Agrostis tenuis. Bradshaw (1959a) reported evidence for population differentiation in flowering time, with lowland populations from warmer habitats flowering earlier than highland populations, when plants were grown in a common environment. McNeill and Antonovics (1968) found a similar result, with plants from a contaminated mine site flowering earlier than plants from a nearby pasture. In their study, early flowering was also correlated with warmer, drier soils. Both studies attributed these changes to genetic differentiation between populations, although it seems plausible that some changes could have been mediated by the presence of an endophyte, particularly because Bradshaw (1959b) reported variation in the frequency of choke disease (caused by sexual reproduction and of an endophyte) in the populations of A. tenuis he surveyed.

In our study, in addition to flowering early, symbiotic plants at the lowest water level invested more resources in reproduction than vegetative growth than did non-symbiotic plants. Because A. hyemalis is a perennial, increased allocation to reproduction in one year could potentially decrease reproductive output in future growing seasons.
However, if severe reductions in water availability reduce plant survival, a greater investment in current reproduction could maximize lifetime plant fitness. Our field data also suggest that *A. hyemalis* is likely not a long-lived perennial in the habitat where we collected seeds, as 12.5% of plants (out of 200) in field plots established near the source population were annuals and 71% of plants were biennials. Future work extending experiments through multiple years would help to elucidate longer-term effects of endophytes on plant survival.

In addition to potentially allowing plants to escape drought, symbiont-mediated changes to plant phenotypes under water stress could have interesting evolutionary implications. Specifically, a shift in the flowering phenology of symbiotic plants could increase assortative mating among symbiotic plants, ultimately leading to genetic divergence between symbiotic and symbiont-free subpopulations of hosts. Additionally, symbiont-mediated benefits to grass hosts at low soil moisture levels could lead to habitat specialization of symbiotic lineages, and further promote the reproductive isolation of symbiotic and symbiont-free hosts through spatial habitat segregation. Combined, these processes could ultimately lead to symbiont-induced speciation (Thompson, 1987).

Manipulation of the soil community suggested a potential cost of the symbiosis. In the presence of soil microbes, symbiont-free hosts accumulated more biomass than symbiotic hosts. These increases in biomass did not result in increases in reproductive fitness, but could alter reproductive output over the lifetime of a perennial host, or have consequences for long-term plant survival. Prior research has similarly demonstrated that endophytes can be costly under extreme resource limitation, such as the absence of soil nutrients (Cheplick et al., 1989; 2007). Although our soil treatment had no effect on
mycorrhizal fungi (we found no root colonization in any treatment), other components of
the soil microbial community could underlie the positive effects of live soil for
endophyte-free plants. For example, plant-growth promoting bacteria or rhizospheric
nitrogen-fixers (Bergman et al., 2009; Lugtenberg and Kamilova, 2009) could benefit
endophyte-free plants, and like other soil bacteria, may show no effect on endophyte-
symbiotic plants if the endophyte generally suppresses bacteria populations
(Franzluebbers et al., 1999; Jenkins et al., 2006).

Finally, our work also has potential implications for improvements to turfgrass
production. The genus *Agrostis* includes 150-200 species, a few of which are widely
planted for turf, particularly creeping bentgrass (*A. stolonifera*). Bentgrass turfs are
sensitive to summer heat and drought, and crop improvement efforts have aimed to
increase drought tolerance for enhanced performance (e.g., Xu and Huang, 2000). To our
knowledge, endophytes have not yet been investigated as a potential mechanism to
improve climate tolerance in this group. However, artificial inoculations of endophytes
into novel plant lineages can be achieved (Latch and Christensen, 1985; Tintjer and
Rudgers, 2006), and thereby may benefit turf improvement efforts. Although benefits in
our study appeared to primarily occur through changes in phenology that allowed
symbiotic plants to escape drought and may not be relevant to mowed, non-reproducing
turf systems, the symbiosis also influenced soil moisture levels, with symbiotic plants
retaining higher soil moisture than symbiont-free plants across all water levels. This
suggests that endophyte symbiosis may also improve drought avoidance by altering rates
of transpiration or water use efficiency, and reduce the need for intensive water additions;
both of which could improve turf performance during summer heat and drought.
Understanding how changes in abiotic and biotic factors alter the costs and benefits of symbiosis and the rate of symbiont transmission is important for gaining insight into the mechanisms of symbiont persistence. Our results highlight the complexity of plant-microbe symbiosis. Benefits to hosts were stronger when water was limiting, but the symbiosis may be costly in the presence of soil microbes. Given their ecological, evolutionary, and economic importance, elucidating the breadth of factors that influence symbiont persistence will be critical for understanding the impacts of anthropogenic changes to the environment.

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Fig. 2.1. Effects of the fungal endophyte and water availability on days to flowering in *Agrostis hyemalis*. Bars show means ± SE. Dark bars (E+) are plants grown from symbiotic seeds collected from a population in the field. Light bars (E-) are plants grown from experimentally disinfected seeds from the same population. Significant differences between the endophyte treatments within each water level are noted on top of bars.
Fig. 2.2. Effects of the fungal endophyte and water availability on (a) the number of inflorescences produced, (b) seed mass, (c) inflorescences per tiller, and (d) reproductive effort measured as the ratio of seed mass to total plant biomass. Bars show means + SE. Significant differences between the endophyte treatments within each water level are noted on top of bars.
Fig. 2.3. Effects of the fungal endophyte and soil type (live or sterile) on total plant biomass. Bars show means + SE. Different letters on top of bars denote means that significantly differ.
Table 2.1. Statistical results from MANOVA examining the effects of the endophyte treatment, water treatment, and soil treatment on plant reproductive responses: days to flowering, the number of inflorescences, inflorescences per tiller, seed weight, and reproductive effort. P-values < 0.05 are shown in bold face. MANOVA was restricted to the highest and lowest watering levels, because seed mass was not measured for the intermediate watering levels. ANOVA (full) results presented for days to flowering, inflorescences, and inflorescences per tiller are from the full data set (N = 192 plants). ANOVA (subset) results for seed mass and reproductive effort include only the highest and lowest watering treatment (N = 96 plants).

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<th>Effect</th>
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<th>F</th>
<th>P</th>
<th>ANOVA (full) d.f.</th>
<th>F</th>
<th>P</th>
<th>Days to Flowering</th>
<th>Inflorescences</th>
<th>Inflorescences per Tiller</th>
<th>ANOVA (subset) d.f.</th>
<th>F</th>
<th>P</th>
<th>Seed Mass</th>
<th>Reproductive Effort</th>
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<td>0.421</td>
<td>0.14</td>
<td>0.935</td>
<td>0.30</td>
<td>0.828</td>
<td>0.00</td>
<td>0.959</td>
<td>1.02</td>
<td>0.316</td>
</tr>
<tr>
<td>Endo x Water x Soil</td>
<td>5, 84</td>
<td>0.39</td>
<td>0.852</td>
<td>3</td>
<td>0.57</td>
<td>0.636</td>
<td>0.32</td>
<td>0.821</td>
<td>0.15</td>
<td>0.933</td>
<td>0.03</td>
<td>0.859</td>
<td>0.64</td>
<td>0.427</td>
</tr>
</tbody>
</table>
Table 2.2  Statistical results from MANOVA examining the effects of the endophyte treatment, water treatment, and soil treatment on plant growth responses: total biomass, belowground biomass, aboveground biomass, root:shoot ratio, and final tiller number. P-values < 0.05 are shown in bold face.

<table>
<thead>
<tr>
<th>Effect</th>
<th>MANOVA</th>
<th>ANOVA</th>
<th>Total Biomass</th>
<th>Belowground Biomass</th>
<th>Aboveground Biomass</th>
<th>Root:Shoot ratio</th>
<th>Tiller Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.f.</td>
<td>F</td>
<td>P</td>
<td>d.f.</td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Endophyte</td>
<td>5,170</td>
<td>1.67</td>
<td>0.145</td>
<td>1.10</td>
<td>0.149</td>
<td>1.64</td>
<td>0.202</td>
</tr>
<tr>
<td>Water</td>
<td>15, 516</td>
<td>17.5</td>
<td><strong>&lt;0.001</strong></td>
<td>164.8</td>
<td><strong>&lt;0.001</strong></td>
<td>40.1</td>
<td><strong>&lt;0.001</strong></td>
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<tr>
<td>Soil Type</td>
<td>5, 170</td>
<td>1.21</td>
<td>0.305</td>
<td>3.98</td>
<td><strong>0.048</strong></td>
<td>3.27</td>
<td>0.072</td>
</tr>
<tr>
<td>Endo x Water</td>
<td>15, 516</td>
<td>1.28</td>
<td>0.213</td>
<td>1.75</td>
<td>0.158</td>
<td>0.83</td>
<td>0.478</td>
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<tr>
<td>Endo x Soil Type</td>
<td>5, 170</td>
<td>2.10</td>
<td>0.068</td>
<td>7.99</td>
<td><strong>0.005</strong></td>
<td>1.62</td>
<td>0.205</td>
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<tr>
<td>Water x Soil Type</td>
<td>15, 516</td>
<td>1.21</td>
<td>0.265</td>
<td>0.80</td>
<td>0.498</td>
<td>1.24</td>
<td>0.296</td>
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<td>15, 516</td>
<td>0.58</td>
<td>0.893</td>
<td>0.88</td>
<td>0.452</td>
<td>0.51</td>
<td>0.677</td>
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