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Do the costs and benefits of fungal endophyte symbiosis vary with light availability?

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Summary

• Here, we examined whether fungal endophytes modulated host plant responses to light availability. First, we conducted a literature review to evaluate whether natural frequencies of endophyte symbiosis in grasses from shaded habitats were higher than frequencies in grasses occupying more diverse light environments. Then, in a glasshouse experiment, we assessed how four levels of light and the presence of endophyte symbioses affected the growth of six grass species.

• In our literature survey, endophytes were more commonly present in grasses restricted to shaded habitats than in grasses from diverse light environments.

• In the glasshouse, endophyte symbioses did not mediate plant growth in response to light availability. However, in the host grass, *Agrostis perennans*, symbiotic plants produced 53% more inflorescences than nonsymbiotic plants at the highest level of shade. In addition, under high shade, symbiotic *Poa autumnalis* invested more in specific leaf area than symbiont-free plants. Finally, shade increased the density of the endophyte in leaf tissues across all six grass species.

• Our results highlight the potential for symbiosis to alter the plasticity of host physiological traits, demonstrate a novel benefit of endophyte symbiosis under shade stress for one host species, and show a positive association between shade-restricted grass species and fungal endophytes.

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 yr (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 (Smith & Read, 1997; Clay & Schardl, 2002; 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 can influence 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 alternative benefits beyond resource limitation, including greater host resistance to soil-borne pathogens and root parasites (Azcón-Aguilar & Barea, 1996), increased host water uptake (Auge, 2001),

improved host tolerance to heat (Kytoviita & Ruotsalainen, 2007), and indirect effects on herbivores (Koricheva *et al.*, 2009). 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 under a variety of ecological contexts.

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 (class 1 endophytes, Clavicipitaceae; Rodriguez *et al.*, 2009). Relative to belowground symbioses, far less is known about the costs and benefits of endophytes. Class 1 endophytes inhabit aboveground plant tissues and are estimated to occur in *c.* 20–30% of grass species (Leuchtmann, 1992). Historically, endophyte symbioses have primarily been recognized for benefiting host plants through increased resistance to herbivores (Clay, 1996; Bush *et al.*, 1997). However, research has also shown that endophytic fungi can increase competitive ability (Clay *et al.*, 1993), drought tolerance (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 that endophytes may ameliorate the effects of a wide variety of environmental stressors. Most 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 (Saikkonen *et al.*, 2004; Schardl *et al.*, 2004; Muller & Krauss, 2005), but the breadth of ecological factors that produce variation in the outcome of the interaction remains poorly characterized.

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 typically inhabit. Our interest in shade stems from two observations. First, class 1 endophytic fungi associate primarily with C_3 grasses (Clay & Schardl, 2002), which are more common in shaded habitats than C_4 grasses (Klink & Joly, 1989). Secondly, grass–endophyte symbioses can occur across a range of habitats, from sunny, open fields to shaded, forest understories. However, to our knowledge, there have been no experimental investigations manipulating both endophyte symbiosis and light availability. Recent work on tall fescue grass (*Lolium arundinaceum*) found that symbiotic hosts in shaded microsites produced more alkaloids and phenolics than symbiotic hosts in open sites (Belesky *et al.*, 2008, 2009). 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, reduced biomass may be mitigated through plastic changes in plant traits, such as reduced root : shoot ratios or increased specific leaf areas (SLAs) (Chapin *et al.*, 1987; Sultan & Bazzaz, 1993), both of which are associated with the ability of plants to optimize light capture. Functionally adaptive plasticity can contribute to environmental tolerance, and ultimately, interspecific differences in plasticity may underlie species' ecological amplitudes and abilities to persist in novel environments (Sultan, 2000). Widely distributed species are expected to cope with broader environmental gradients and show larger plastic responses than species with restricted ecological amplitudes. The degree to which symbionts, such as endophytes, may influence a host plant's level of plasticity remains unclear. Here, we hypothesized that, if symbiota are adapted to high-shade environments, then hosts would gain some benefit from the endophyte at high levels of shade, and endophyte symbioses would be more common in hosts from shaded habitats. 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. First, we conducted a literature review to ask: Are endophyte frequencies higher in shaded habitats? Secondly, we assessed the effects of four levels of shade and the presence of fungal endophytes on the performance of six grass species to address the question: Do endophyte symbioses affect plant growth and plant traits in response to shade? Thirdly, we assessed the performance of the endophyte within leaf tissues to ask: Does shade alter endophyte density?

Materials and Methods

Literature survey

Data collection Data on endophyte presence/absence and endophyte frequency within and among populations were 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; Saha *et al.*, 2009; Emery *et al.*, 2010) and data from our recent field surveys (E. K. Seifert, T. E. X. Miller and J. A. 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. For the subset of symbiotic hosts, we calculated two metrics of endophyte frequency: the percentage of total populations that had at least one infected individual (including only the species that had at least three populations sampled) and 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 used a log-linear model to test whether the endophyte status of the host grass species (symbiotic vs not symbiotic) was associated with habitat type (shaded vs not shaded) (n = 187 species; Proc Genmod; SAS v. 9.1.3; SAS Institute, Cary, NC, USA). We also conducted a more conservative analysis, which excluded host grass species that were scored as 'nonsymbiotic' if fewer than three populations were sampled. In addition, we tested the relationship between habitat type (shaded or not shaded) and the two metrics of endophyte frequency, percentage of total populations (n = 101 species) and mean percentage per population, using a general linear model (n = 82 species) (Proc GLM; SAS v. 9.1.3). Comparisons across species risk pseudoreplication if phylogeny is ignored (Felsenstein, 1985). Thus, we obtained phylogenetically independent contrasts for habitat type and our three measures of endophyte frequency (see Supporting Information Notes S1 and Fig. S1). To examine the relationship between endophyte status and habitat type, we performed phylogenetic logistic regression (PlogReg.m; Ives & Garland, 2010) and report means and bootstrapped 95% confidence intervals (CIs) for the regression coefficient and the parameter α (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 report adjusted correlation coefficients with the full range of P-values accounting for changes in the degrees of freedom associated with polytomous nodes (Garland *et al.*, 1992; Midford *et al.*, 2005).

Glasshouse study

Study system We evaluated the effects of shade and symbiosis on the growth and traits of six perennial grass species (Table 1). Our field assessment of light habitats indicated that *Elymus villosus* Muhl. ex Willd, *Poa alsodes* A. Gray, and *Festuca subverticilliata* (Pers.) Alexeev occurred in shade, whereas *Lolium arundinaceum* (Schreb.) S. J. Darbyshire, *Poa autumnalis* Muhl. ex Elliot, and *Agrostis perennans* (Walter) Tuck. occupied a broader range of light habitats (Table 1).

Endophyte treatment We collected seeds from natural populations during summer 2006 for five native species as well as a naturalized population of *L. arundinaceum* (tall fescue), which is nonnative to the USA and grown for forage and turf (Table 1). Endophytes in these populations appear to be primarily vertically transmitted, as stromata formation has not been observed on plants in the source populations, in experimental field plots (100 plants per species), or in our glasshouse. For each species, we worked out the appropriate window of heat treatment (wet treatment in a 55°C water bath or dry treatment in a 60°C drying oven) to effectively remove the endophyte (Table 1) without causing substantial reductions in germination rates. By using experimental removal of the endophyte rather than comparing naturally symbiotic and symbiont-free plants, we can

Table 1 List of plant and endophyte species used in 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

Grass species	Code	Endophyte species	Source population	Endophyte frequency (%)	Disinfection treatment	Light habitat % PAR reduction (mean)
Agrostis perennans	AGPE	*Epichloë amarillans*	Lilly-Dickey Woods Preserve, Nashville, IN	88–100	Water bath 7.5–8 min	10–98 (77)
Lolium arundinaceum	LOAR	*Neotyphodium coenophialum*	Lilly-Dickey Woods Preserve, Nashville, IN	98–100	Water bath 10–11 min	13–96 (79)
Poa autumnalis	POAU	*Neotyphodium* sp.	Stephen F. Austin Experimental Forest, Nacogdoches, TX	83–100	Drying oven 12 d	16–94 (77)
Elymus villosus	ELVI	*Epichloë* sp.	Griffy Lake, Bloomington, IN	41–81	Drying oven 6 d	75–97 (94)
Festuca subverticillata	FESU	*Neotyphodium* sp.	Lilly-Dickey Woods Preserve, Nashville, IN	83–100	Water bath 11–12 min or drying oven 7 d	95–99 (97)
Poa alsodes	POAL	*Neotyphodium* sp.	Lilly-Dickey Woods Preserve, Nashville, IN	88–100	Drying oven 7 d	83–97 (92)

PAR, photosynthetically active radiation.

separate the effects of endophyte presence and plant genotype. 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 20 genetically unique individuals of each species (10 endophyte-symbiotic (E+) and 10 endophyte-disinfected (E−)), grown from seed. Following heat treatment, we surface-sterilized seeds and planted into 10 × 10 × 10 cm plastic pots filled with ProMix-BX (Premier Horticulture, Quakertown, PA, USA). Plants were grown in a common glasshouse environment for 6 months. Then, each individual was subdivided into four equally sized clones (two to four tillers each). Cloning to create similarly sized individuals across treatments following 6 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 × 10 × 10 cm plastic pots filled with a 50 : 50 mixture of ProMix-BX (Premier Horticulture) and QUIKRETE® Premium Play Sand (QUIKRETE® International Inc., Atlanta, GA, USA).

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, USA) at the Stephen F. Austin Experimental Forest, Nacogdoches, TX (5 June 2008), and a Li-Cor LAl Ceptometer (Li-Cor, Lincoln, NE, USA) at Lilly-Dickey Woods Preserve, Nashville, IN (30 May 2008). Readings were taken from 10:00 to 16:00 h and sampled the deepest shade (< 10 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)) and brightest open areas (> 2000 μmol m^{-2} s^{-1} PAR) in which the target grass species 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 40 (10 replicate structures per shade treatment) individual shade structures (height 61 cm × length 61 cm × width 46 cm) from 1.27-cm-diameter PVC frames. Frames were draped with black knitted shade fabric (Dewitt Company, Sikeston, MO, USA) to create 30, 60, or 90% light reduction. Control structures (0% reduction) included the PVC frame alone.

Glasshouse experimental set-up Shade structures were assigned at random to a position on one of four glasshouse benches. To ensure that structures experienced similar exposure to ambient variation in light over the duration of the experiment, we took repeated light readings over each structure and re-randomized structure positions every week.

For each of the six grass species, we randomly paired an endophyte-symbiotic plant (E+) and an endophyte disinfected (E−) plant and randomly assigned each pair to a shade structure. Plant position within the structure was re-randomized once per week for the duration of the experiment to equalize any intra-structure positional bias. For each plant genotype (10 E+ and 10 E−), one replicate clone was exposed to each level of shade. Every pot was supplied with a single RainBird drip emitter (Rain Bird Corporation, Tucson, AZ, USA), and plants were watered twice daily at 09:00 and 14:00 h with a 1-min drip, at 20-s intervals. The experiment began on 28 June 2008 and was harvested after 20 wk.

Isolating the effect of shade Shade not only reduces light availability, but also increases humidity and water retention in the soil. To isolate the influence of light availability *per se*, we adjusted soil moisture levels to eliminate any differences caused by the shade treatment. Soil moisture readings for eight randomly selected structures (two per shade treatment) were taken every 14 d (TDR 100 Soil Moisture Probe, 7.5 cm probes; Spectrum Technologies, Inc., Plainfield, IL, USA). TDR probes were calibrated with measurements of gravimetric water content from a subset of trial pots (r^2 = 0.83, $F_{1,21}$ = 104.9, P < 0.0001, n = 22 pots). Using the TDR data, we determined average soil moisture for each level of shade, and then watered plants by hand to homogenize soil moisture across the four shade treatments.

Response variables After 20 wk, we harvested all plants. Roots were washed through a 1.00-mm US Standard Sieve (No. 18; Soil Test Inc., Lake Bluff, IL, USA). Above- and belowground biomass was obtained following drying at 60°C to a constant weight. For *A. perennans*, the only species to flower, we additionally recorded the number of inflorescences produced. To calculate SLA, we haphazardly selected one fully expanded nonsenescent leaf from each pot, scanned it at 100 × 100 dpi (HP ScanJet 5590 Digital Scanner) and calculated leaf area with IMAGEJ Image Analysis software (Rasband, 1997–2009). 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. 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). 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 ×400 except *L. arundinaceum*, which was viewed at ×200 because sheath

sections were large. Two observers independently counted the number of views with fungal hyphae out of 30 nonoverlapping slide views per plant, or until tissue was exhausted. We used the average of these independent estimates to determine mean hyphal density (hyphal views per mm^2 of plant tissue examined).

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 nonindependence of plants that co-occurred within each structure (Proc Mixed; SAS v. 9.1.3, SAS Institute). We did not apply MANOVA because of the complex, mixed model structure (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 shaded), and the random effects of species (nested within habitat type) and structure (nested within shade treatment). For this analysis we standardized SLA to a mean of 0 and a standard deviation of 1 to facilitate comparisons of the effect size across species that varied greatly in SLA. Use of the standardized data did not qualitatively change the results. For all analyses, *post hoc* Tukey HSD tests were used to compare treatment means. When assessing 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 nonsignificant interactions were step-wise excluded. In our analysis of endophyte density, log-transformed aboveground biomass was used as an allometric 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?

Approximately 25% of symbiotic grasses were restricted to shady habitats vs only 12% of nonsymbiotic grasses (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 phylogenetic 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 'nonsymbiotic' grasses with fewer than three populations sampled, was not significant (Fig. 1a; Wald $\chi^2_{1,148} = 1.54$, $P = 0.2147$) because of a lack of statistical power. This conservative analysis 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 shade-restricted hosts than in hosts from diverse light environments (Fig. 1b; $F_{1,99} = 16.65$, $P < 0.0001$), and this relationship remained significant after correcting for phylogeny ($r_{(99-63)} = 0.3991$, $0.0001 < P < 0.0010$). In addition, the average frequency of endophytes within populations was 17% greater in hosts that were from shaded habitats than in 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 ($r_{(80-50)} = 0.26$, $0.0180 < P < 0.0621$).

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

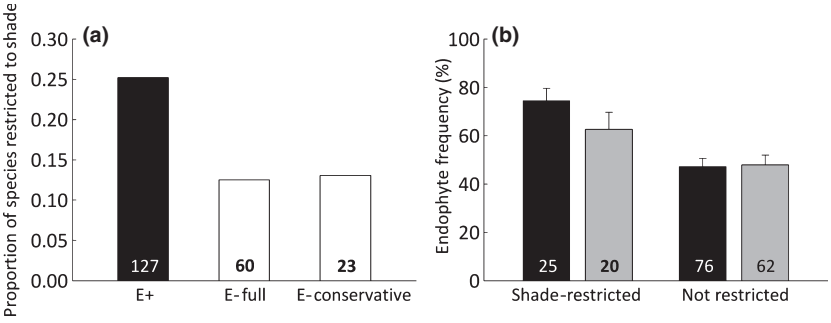

Fig. 1 (a) Differences in the proportion of grass species restricted to shaded habitats for endophyte symbiotic grass species vs endophyte-free hosts. The full data set included 'nonsymbiotic' species where only one population had been sampled, whereas the conservative set was limited to 'nonsymbiotic' species that had at least three populations sampled. (b) Differences in the frequency of endophyte symbiosis for grass species restricted to shaded habitats vs those not restricted to shaded habitats, showing both the percentage of populations with the endophyte per grass species (black columns) and mean endophyte frequency per grass population (gray columns). Bars show mean + SE, and sample sizes (number of species) are indicated on each bar.

Table 2 Statistical results from ANOVA analyzing effects of endophyte treatment, shade treatment and species on total biomass, root : shoot ratio, specific leaf area (SLA), and endophyte density

	Total biomass			Root : shoot ratio			SLA			Endophyte density		
Effect	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Endophyte	1, 369	10.3	**0.0015**	1, 361	4.6	**0.0327**	1, 349	3.2	0.0756			
Shade	3, 36	51.3	**< 0.0001**	3, 36	11.4	**< 0.0001**	3, 36	40.3	**< 0.0001**	1, 18	7.2	**0.0151**
Endophyte × shade	3, 369	1.00	0.3974	3, 361	0.2	0.8314	3, 349	0.6	0.6010			
Species	5, 369	130.2	**< 0.0001**	5, 361	5.1	**0.0001**	5, 349	42.0	**< 0.0001**	5, 71	6.6	**< 0.0001**
Species × endophyte	5, 369	7.49	**< 0.0001**	5, 361	5.3	**< 0.0001**	5, 349	2.3	**0.0488**			
Species × shade	15, 369	1.16	0.3037	15, 361	1.3	0.1890	15, 349	5.3	**< 0.0001**	5, 71	0.5	0.7990
Species × endophyte × shade	15, 369	0.70	0.7844	15, 361	0.5	0.9139	15, 349	1.1	0.3354			
Log-transformed biomass				1, 361	1.3	0.2524	1, 349	15.1	**0.0001**	1, 71	0.2	0.6834
Biomass × species				5, 361	2.51	**0.0299**						

Log-transformed total biomass was included as a covariate in the analysis of root : shoot ratio and SLA. Log-transformed aboveground biomass was included as a covariate in the analysis of endophyte density. *P*-values < 0.05 are shown in bold.

significant endophyte × shade interactions for total plant biomass (Table 2). Individually, both the shade and endophyte treatments significantly influenced plant growth. Shade reduced plant total biomass by 50–75% across species, and *Poa 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; mean ± SE: 0% shade = 0.617 *g* ± 0.077 *g*; 90% shade = 0.150 *g* ± 0.020 *g*). *Festuca 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 × species interaction (Table 2). Endophyte-symbiotic *P. alsodes* had, on average, 140% higher total biomass relative to endophyte-free plants (Fig. 2a; 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 × shade interaction for the number of inflorescences (Fig. 2b; shade × endophyte: $F_{3,35}$ = 3.09, P = 0.0397). Under low shade, the number of inflorescences produced did not differ between endophyte-symbiotic and endophyte-free plants, but at high shade, symbiotic plants produced 53% more inflorescences than symbiont-free plants.

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. In *P. autumnalis*, symbiosis altered SLA in response to shade, as indicated by a significant endophyte × 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 (Fig. 3c; shade × endophyte: $F_{3,33}$ = 3.98, P = 0.0159).

Individually, both the endophyte and shade treatments also influenced SLA. Shade enhanced SLA in four of six species, with increases ranging from 10 to 180% (Fig. 3a). The

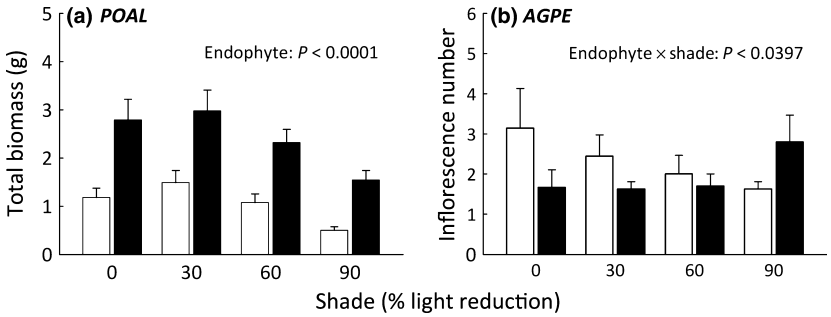

Fig. 2 Effects of the shade and endophyte treatment on total biomass in *Poa alsodes* (POAL) (a) and the number of inflorescences in *Agrostis perennans* (AGPE) (b). Bars show mean + SE. Endophyte treatment: open columns, endophyte-disinfected (E–); closed columns, endophyte-symbiotic (E+).

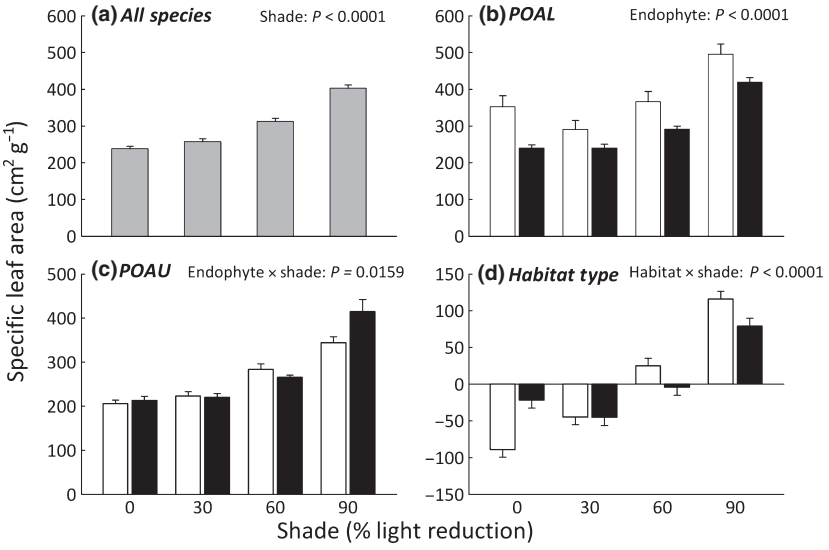

Fig. 3 Effects of the shade treatment on specific leaf area (SLA) across all species (a), effects of the shade and the endophyte treatment on SLA in *Poa alsodes* (POAL) (b) and in *Poa autumnalis* (POAU) (c), and effects of the shade treatment on SLA (standardized to a mean of 0 and standard deviation of 1) for species grouped by habitat type (d). Bars show mean + SE. Endophyte treatment (b, c): open columns, endophyte-disinfected (E–); closed columns, endophyte-symbiotic (E+). Habitat type (d): open columns, broad habitat; closed columns, shade restricted.

effect of shade differed across species, as indicated by a significant species × shade interaction for SLA (Table 2). *Lolium arundinaceum* responded the most strongly to shade, with a 180% increase in SLA ($F_{3,36}$ = 61.5, P < 0.0001; mean ± SE: 0% shade = 144.0 ± 6.26; 90% shade = 402.7 ± 18.78), while *F. subverticillata* did not significantly respond ($F_{3,32}$ = 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 significantly altered SLA when challenged with the highest (90%) level of shade (Fig. 3d; habitat × shade: $F_{3,389}$ = 8.63, P < 0.0001). Endophyte symbiosis influenced SLA only in *P. alsodes*, with endophyte-free plants averaging 25% greater SLA than symbiotic plants across all levels of shade (Fig. 3b), resulting in a significant species × endophyte interaction (Table 2).

The endophyte did not alter root : shoot ratios in response to shade, resulting in no significant endophyte × shade interactions. However, the endophyte and shade altered plant 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. 4a; Table 2). *Agrostis perennans* showed the strongest response with a 50% reduction at the highest level of shade ($F_{3,36}$ = 6.9, P < 0.0008; mean ± SE: 0% shade = 0.671 ± 0.060; 90% shade = 0.329 ± 0.038), while *F. subverticillata* did not respond ($F_{3,32}$ = 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 × species: $F_{15,361}$ = 1.3, P = 0.1890). By contrast, the influence of endophyte symbiosis on the root : shoot ratio varied among host grass species and host habitat breadths (Table 2; endophyte × species, P < 0.0001). The presence of the endophyte significantly increased the root : shoot ratio by 26% in grass species from broad habitat types, but did not significantly influence the ratio in shade-restricted species (Fig. 4b; habitat type × endophyte: $F_{1,394}$ = 16.7, $P \leq$ 0.0001).

Does shade alter endophyte density?

Across the six grass species, endophyte density was 10–86% greater in the 90% shade treatment relative to ambient light (0% shade). *Poa alsodes* and *L. arundinaceum* expressed the strongest responses to shade, with 85 and 86% increases in

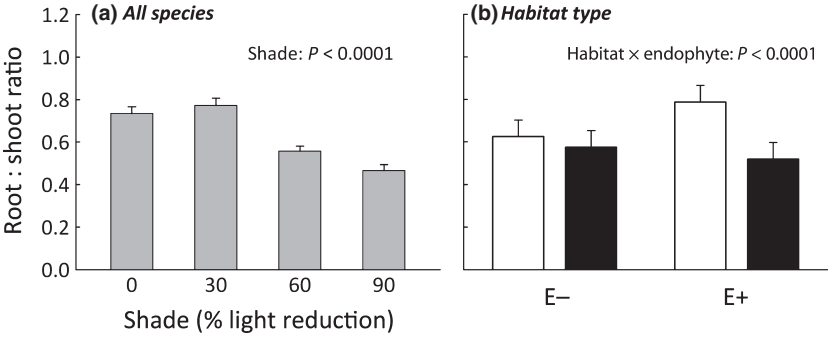

Fig. 4 Effects of the shade treatment on root : shoot ratio across all species (a) and effects of the endophyte treatment on root : shoot ratio for species grouped by habitat type (b; open columns, broad habitat; closed columns, shade restricted). Bars show mean + SE. E–, endophyte-disinfected; E+, endophyte-symbiotic.

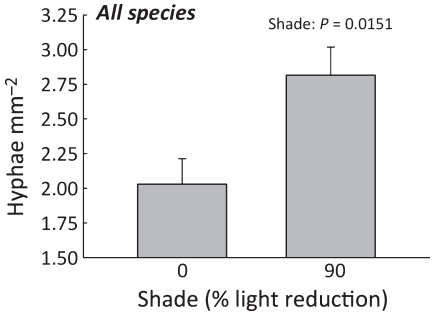

Fig. 5 Effects of the shade treatment on endophyte density across all grass species. Bars show mean + SE.

endophyte density, respectively. Despite this range of increases, we detected no significant interaction between grass species identity and the shade treatment (Table 2). Averaged across all six grass species, the highest shade level increased endophyte hyphal density by 39% relative to no shade (Fig. 5).

Discussion

To our knowledge, this is the first study to experimentally investigate whether endophyte symbioses can alter plant responses to light availability. Despite the significantly higher 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 species we tested. Compared with 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 as a result of 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 glasshouse. In the future, it would be useful to explore these factors in combination as they are often correlated in natural settings, and endophytes are known to mediate both drought stress and herbivory (e.g. Clay *et al.*, 2005; Kannadan & Rudgers, 2008).

Although the endophyte did not alter plant biomass production in response to shade for any species, in the only host that reproduced during our short-term glasshouse experiment, endophyte symbiosis increased plant reproductive fitness when light was limiting. Under high shade, symbiotic *A. perennans* produced 53% more inflorescences than endophyte-free plants, but endophyte presence had no effect under high light. If shading reduces the long-term survival of this perennial grass, this symbiont-mediated shift toward reproductive investment 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 symbiont effects throughout host ontogeny would be required to fully characterize the fitness consequences of symbiosis, as there could be trade-offs between reproduction and survival (Rudgers *et al.*, 2010). In addition, the generality of this result remains unclear as only a single species flowered during the experiment.

Independently, both shade and endophyte symbiosis influenced biomass accumulation in some grass species. As predicted, shade reduced biomass (up to 75%) 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.

Phenotypically plastic allocation patterns can influence a plant's ability to capture resources (Poorter *et al.*, 1990), produce offspring (Sultan, 2000), and compete with neighbors (Tilman, 1988). As predicted, greater shading generally increased SLA and reduced the root : shoot ratio. For SLA, grass species from broad habitats were more sensitive to increasing levels of shade than shade-restricted species. Few other studies have examined class 1 endophyte-mediated changes in plant biomass allocation, and ours is the first to examine a suite of native host species. Previous results have been variable, with some studies documenting decreases in root : shoot ratios (Lewis *et al.*, 1996; Lehtonen *et al.*, 2005; Cheplick, 2007), 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 previous 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, perhaps indicative of a relatively 'fixed' allocation strategy. However, in species from broad light habitats, the presence of symbiosis increased the root : shoot ratio, with symbiotic plants investing relatively more resources belowground. This result does not support the hypothesis that endophyte symbiosis mediates plant response to shade stress, but does highlight the ability of symbiosis to alter the plasticity of some host species. Symbiosis has the potential to alter host plasticity in two ways. First, the presence vs absence of the symbiont may alter host phenotype, allowing the host to adjust phenotype through the gain or loss of symbiosis. Secondly, symbiont presence may increase the degree of plasticity of the host response to altered ecological conditions. This effect occurred in *P. autumnalis,* for which symbiotic plants showed greater plasticity in SLA in response to shade than did symbiont-free plants. Neither of these types of host plasticity led to endophyte-mediated increases in plant

growth in our experiment, and it remains unclear whether such plastic responses could alter other aspects of host demography, such as survival or reproduction.

Given the fundamental importance of examining the responses of both partners for understanding the context dependence of symbioses, surprisingly few studies have examined how variation in biotic or abiotic environments influences the performance of the endophyte (Rasmussen *et al.*, 2007; Mack & Rudgers, 2008). Previous studies suggest that host and endophyte genotype (Rasmussen *et al.*, 2007), abiotic factors such as nitrogen concentration (Rasmussen *et al.*, 2007; Mack & Rudgers, 2008), and biotic interactions with mycorrhizal fungi (Mack & Rudgers, 2008) may influence fungal concentration in plant tissues. Here we showed that shade had a consistently positive influence on endophyte density across the six host species. 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 host aboveground biomass. 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 that 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). Variation in hyphal density could additionally alter the costs and benefits of host–symbiont resource exchanges (e.g. carbon and nitrogen) or indirectly alter biotic interactions (e.g. endophyte abundance has been positively correlated with antiherbivore alkaloid concentrations (Spiering *et al.*, 2005; Rasmussen *et al.*, 2007)).

The consistent effect of shade on endophyte density across the six grass species suggests alternative explanations for the higher frequency of symbiosis in shade-restricted host species. First, given that shade-grown plants may be more vulnerable to herbivores and pathogens because of reduced structural defenses (Roberts & Paul, 2006), increased alkaloid concentrations associated with higher hyphal densities could be advantageous for hosts growing in shady habitats and thereby contribute to the persistence of higher symbiont frequencies in shade-restricted species. Our glasshouse experiments, conducted in the absence of herbivores, were not designed to test this mechanism. Secondly, endophyte density could constitute an important component of endophyte fitness that may be independent of host fitness if increases in hyphal density increase rates of vertical transmission of the endophyte to seeds. With the exception of conditions of high heat and humidity, which kill the endophyte, 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).

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 a strong association between endophyte symbiosis and plant species restricted to shaded habitats. However, our glasshouse experiment detected few endophyte-mediated effects under shade, and no enhancement of host plant growth. Notable exceptions included the findings that endophyte symbioses increased plant reproduction in the one species that flowered and altered the plasticity of plant traits associated with light capture in response to shade in another species. 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 that, ultimately, influence the persistence of symbioses in host populations.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Phylogenetic trees used for examining relationships between endophyte frequency and habitat type.

Notes S1 Methods for phylogenetically independent contrasts.

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