Asexual Epichloë Endophytes Do Not Consistently Alter Arbuscular Mycorrhizal Fungi Colonization in Three Grasses

Author(s): Eric Kalosa-Kenyon, Lindsey C. Slaughter, , Jennifer A. Rudgers, and Rebecca L. McCulley

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Eric Kalosa-Kenyon

Department of Applied Mathematics, Brown University, 182 George St., Providence, Rhode Island 02912

Lindsey C. Slaughter[1]

Department of Plant and Soil Science, Texas Tech University, Lubbock 79409

Department of Plant and Soil Sciences, University of Kentucky, 1100 South Limestone, Lexington 40546

Jennifer A. Rudgers

Department of Biology, University of New Mexico, Albuquerque 87131

AND

Rebecca L. McCulley

Department of Plant and Soil Sciences, University of Kentucky, 1100 South Limestone, Lexington 40546

ABSTRACT.—Plants commonly host multiple microbial symbionts that regulate productivity and other ecosystem processes, yet multi-symbiont interactions within hosts are rarely examined. We evaluated how the presence of aboveground *Epichloë* fungal endophytes (E+, symbiotic, and E−, endophyte experimentally removed) altered belowground colonization by arbuscular mycorrhizal fungi (AMF) in three grass species in a common environment. We sampled from E+ and E− populations of woodland bluegrass (*Poa sylvestris* A. Gray), grove bluegrass (*Poa alsodes* A. Gray), and tall fescue (*Schedonorus arundinaceus* Schreb.) in long-term experimental plots in woodlands near Nashville, Indiana. Endophyte symbiosis aboveground increased AMF colonization of roots in both *Poa* species, although this effect was only significant for hyphal colonization in *P. sylvestris*. Endophyte symbiosis did not significantly alter AMF colonization in *S. arundinaceus*, in contrast to prior findings for this species. Our results illustrate the effects of *Epichloë* sp. on AMF cosymbionts are not easily generalizable across plant-endophyte symbiota, even those that co- occur in the same ecosystem.

INTRODUCTION

Many grasses (Family *Poaceae*) host a variety of fungal symbionts, including aboveground leaf-dwelling endophytes (White Jr., 1987) and belowground root-colonizing arbuscular mycorrhizal fungi (AMF; Smith and Read, 2008). However, multiple symbionts within plants are rarely examined in concert. The effects of multiple mutualists can produce a range of net results on plant hosts, from synergistic to antagonistic, depending both on the relative costs of mutualism and on the degree of functional overlap in benefits imparted to hosts (Afkhami *et al.*, 2014). For example asexual endophytic species of the genus *Epichloë* can defend hosts against herbivory (Clay and Schardl, 2002), whereas AMF can increase host water and nutrient uptake (Augé, 2001; Smith and Read, 2008). The environmental context, such as degree of herbivory or resource limitation, may influence the interaction of these

[1] Corresponding author present address: Department of Plant and Soil Science, Texas Tech University, Lubbock, Texas 79409; Telephone: (806) 834-1345; Fax: (806) 742-0775; E-mail: lindsey. slaughter@ttu.edu

two fungal functional types, as well as their combined effects on the shared host plant. Given current knowledge it is difficult to predict whether and when asexual *Epichloë* endophytes affect belowground AMF colonization. Because *Epichloë* endophytes and AMF intimately interact with plant and soil resources, and both are frequently mutualistic, how these two symbionts interact within a shared host may hold considerable ecological implications.

Studies focusing on a single mutualist, either asexual *Epichloë* endophytes or AMF, often identify significant mutualist outcomes on community and ecosystem properties. For example asexual *Epichloë* symbiosis with tall fescue (*S. arundinaceus* Schreb.) can increase fescue's competitive ability and abundance over time and reduce overall plant diversity (Clay and Holah, 1999; Iqbal *et al.*, 2013), while AMF symbioses play key roles in increasing soil aggregate stability over time and improving water holding capacity and long-term nutrient availability (Duchicela *et al.*, 2013). Interestingly, some observed effects of these mutualists are similar and suggest the potential for synergistic interactions. For example soils that support *Epichloë*-symbiotic *S. arundinaceus* tend to accumulate more soil organic carbon (SOC) than endophyte-free (Franzluebbers *et al.*, 1999; Franzluebbers and Stuedemann, 2005; Iqbal *et al.*, 2012). AMF can also increase SOC (Wilson *et al.*, 2009), including through the production of recalcitrant glycoproteins. Little is known about the effects of *Epichloë* symbiosis on soil C or soil nutrients in other grass species, but the effect in tall fescue could plausibly be influenced by *Epichloë*-associated impacts on AMF. The potentially synergistic ecosystem consequences of concurrent *Epichloë* and AMF symbiosis, such as the combined effect on soil C stocks, therefore warrant closer examination.

Prior work also suggests mutualist interactions vary across host grass species. In agronomic grasses such as tall fescue and ryegrass, studies to date have reported *Epichloë* symbiosis reduces host root AMF colonization, AMF propagules in soil, or abundance of a soil AMF lipid biomarker (Antunes *et al.*, 2008; Buyer *et al.*, 2011; Chu-Chou *et al.*, 1992; Guo *et al.*, 1992; Mack and Rudgers, 2008; Omacini *et al.*, 2006). This may be because the aboveground endophyte and belowground AMF compete for plant C (sensu Liu *et al.*, 2011). However, studies evaluating native nonagronomic grasses have shown the opposite effect: *Epichloë* symbiosis was associated with higher root AMF colonization in *Bromus setifolius* (Novas *et al.*, 2005; Novas *et al.*, 2011), *Poa bonariensis* (Novas *et al.*, 2009), and *Elymus hystrix* (Larimer *et al.*, 2012), although some of these studies were not experimental. A confounding issue in understanding host species influence on these multiple mutualist interactions is that to date no one has compared multiple host species in a common environment.

In order to assess the importance of host species in determining tripartite *Epichloë*—grass—AMF interactions and the potential soil C consequences, we quantified AMF root colonization in three grass species harboring asexual *Epichloë* endophytes in a common environment. We hypothesized: (1) endophyte symbiosis in nonnative agronomic *S. arundinaceus* would reduce AMF colonization rates, while (2) endophyte symbiosis in two native grass species, *Poa sylvestris* and *P. alsodes*, would increase AMF colonization rates, and (3) these effects would subsequently impact soil C, such as C reduction in soils under *Epichloë*-symbiotic *S. arundinaceus* and *Epichloë*-associated C increase in the two *Poa* species.

<center>METHODS</center>

<center>STUDY SITES</center>

We used an existing long-term plant demography study in Lilly-Dickey Woods (LDW) at the Indiana University Research and Teaching Preserve, located near Nashville, Indiana (39°14′29″N, 086°13′7″W). This study included eight different grass species that can host

asexual *Epichloë* endophytes, each represented in replicated E+ and E− stands. Of these the present study focused on six plots of woodland bluegrass *(P. sylvestris* A. Gray) (four E+ plots, two E− plots), 10 plots of *S. arundinaceous* (five E+ plots, five E− plots), and 10 plots of grove bluegrass (*P. alsodes* A. Gray) (five E+ plots, five E− plots), with 20 individuals planted in each plot in 2007 (for details *see* Chung *et al.,* 2015; Crawford *et al.,* 2010).

PLANT AND SOIL COLLECTION

We randomly selected three individuals from each of the 26 total plots on 8 June 2013. Of the 78 potential samples, only 71 were available for collection due to plant mortality in some treatment plots since the time of establishment. For each of the three individuals selected from each treatment plot, we collected one root sample, one soil sample, and one leaf sheath. Roots were harvested by exposing a small portion of the lateral root system, cutting off a section, and storing it in a Histosette I biopsy cassette with 0.9 mm square openings (Ted Pella, Inc.). Three 10 cm deep, 1.5 cm diameter soil samples were taken in close proximity to each plant sample and composited per plot. Leaf sheaths were collected by peeling one leaf blade away from the stem and storing it in a plastic bag with a moistened paper towel. Samples were stored in a cooler during transit to the University of Kentucky in Lexington, Kentucky, where soil samples were stored at −80°C, leaf sheaths at −20°C, and roots at 4°C.

LEAF ENDOPHYTE DETECTION

To test for endophyte presence, we used the leaf sheath epidermal peel methods outlined in Clark *et al.* (1983). We peeled a thin layer from the inside of each individual leaf sheath and stained them with cotton (aniline) blue dye (2:1 1% aniline blue solution: 85% lactic acid) on microscope slides. We determined positive or negative endophyte status via microscopy at 100x magnification. Individuals were considered endophyte-free if we detected no hyphae within 5 min of microscopic inspection. Endophyte presence within approximately 80% of total samples reflected treatment conditions. Four individuals sampled from E+ plots were found to be instead E−, and 11 individuals from E− plots were found to be E+. These were kept in their original treatment group for statistical analyses, although we note moving individuals into another endophyte class based on leaf endophyte detection did not affect statistical results.

ARBUSCULAR MMYCORRHIZAL FUNGI QUANTIFICATION

We assessed mycorrhizal colonization of plant roots via microscopy modified from McGonigle *et al.* (1990) to increase magnification and the number of root subsamples per slide and to reduce the number of intersects counted. We cleared root samples in 10% KOH, neutralized the roots in 10% HCl, rinsed in deionized (DI) water, and stained fungal hyphae within roots using 0.05% trypan blue. We destained the roots in a 1:1 glycerol:DI water solution. To examine the roots, we arranged ten 1 cm long subsamples per individual into two columns on a microscope slide. Slides were air-dried for 24 h, cover slides secured using PVLG (INVAM), and dried at 55C for 3 d. To determine mycorrhizal colonization, we visually transected the slides at 400x magnification and recorded either the presence or absence of stained AMF structures (counting only aseptate hyphae, vesicles, or arbuscules) for each view. AMF colonization (%) was calculated as the number of fungal presences divided by the total views and multiplied by 100.

TABLE 1.—Rate of total AMF colonization (including arbuscule, vesicle, and hypha presence; %), detected in roots of three grass species, and associated soil C concentration (%). Values are means (±1 SE). Within species for each parameter, values sharing no common letter (a, b) indicate statistically significant differences (α = 0.05)

	Grass Species		
	S. arundinaceus	*P. alsodes*	*P. sylvestris*
Total AMF (%)	66 (3)[a]	53 (4)[b]	57 (3)[b]
Soil C (%)	2.27 (0.06)[b]	2.77 (0.15)[a]	2.87 (0.16)[a]

SOIL C ANALYSIS

To determine whether endophyte-mediated effects on AMF colonization altered ecosystem C stocks, we also measured SOC in composited soil samples from each study plot. Total C (%) was determined via combustion on a Flash Elemental Analyzer 1112 (Thermo Fisher Scientific Inc., Waltham, Massachusetts), after testing with 1 N HCL to confirm absence of inorganic C. We did not conduct other soil nutrient analyses on soils from the plots. Based on prior analysis of 30 homogenized soil samples to 30 cm depth collected from beneath each of eight naturally occurring grass species in May 2011, average soil nutrients at LDW (ppm ± SE) were 2.9 NO_3-N ± 0.6, 13.6 P ± 1.8, 102.6 K ± 7.2, 979.1 Ca ± 167.5, 122.9 Mg ± 16.7, and 17.0 S ± 1.1. The pH was 5.4 (Rudgers *et al.*, unpubl. data).

STATISTICAL ANALYSIS

This study was designed to examine whether endophyte symbiosis significantly influenced root AMF colonization or soil C for three grass species. With plot included as a random factor nested within endophyte status, we used a mixed model to evaluate significant (α = 0.05) main effects of grass species and endophyte treatment and the interactive effects of species × endophyte treatment on measured parameters via PROC MIXED in SAS (9.3 SAS Institute Inc., Cary, North Carolina, U.S.A.). Within significant main or interactive effects of species and endophyte treatment, significant differences between means for each grass species were determined using differences in least squares means (LSMEANS, /pdiff) in SAS at (α = 0.05). We also explored relationships among measured parameters within each grass species using linear regression analyses (PROC REG) in SAS.

RESULTS

ARBUSCULAR MYCORRHIZAL FUNGI

We detected no significant differences in AMF arbuscule, vesicle, or hyphal colonization percentages due to main or interactive effects of endophyte treatment, with one exception (Table 2). AMF hyphal prevalence in *P. sylvestris* roots was significantly (16%) higher in E+ samples than in E− samples (effect size = 1.3, Fig. 1). A similar, though nonsignificant, trend was observed in *P. alsodes*, with 8% higher AMF hyphal colonization in E+ than E− plants (Fig. 1, Table 2).

Overall, *S. arundinaceus* exhibited significantly higher total AMF colonization rates (%) than the two *Poa* species, yet no significant difference was found between E+ and E− samples for *S. arundinaceus* (Tables 1, 2). Total AMF colonization (aggregated across arbuscules,

TABLE 2.—ANOVA results assessing the main and interactive effects of grass species (*S. arundinaceus, P. alsodes, P. sylvestris*), and asexual *Epichloë* endophyte symbiosis (E+, E− treatment) on measured parameters. Statistically significant effects ($\alpha = 0.05$) are denoted in bold type

	Effect								
	Species			Endophyte			Species × Endophyte		
Response	df	F-value	P-value	df	F-value	P-value	df	F-value	P-value
Total AMF (%)	2, 45	3.98	**0.0256**	1, 20	0.97	0.361	2, 45	1.54	0.225
Arbuscules (%)	2, 45	1.74	0.1869	1, 20	0.03	0.8686	2, 45	0.29	0.7508
Vesicles (%)	2, 45	0.95	0.3937	1, 20	2.44	0.134	2, 45	0.62	0.5433
Hyphae (%)	2, 45	5.32	**0.0084**	1, 20	4.09	0.0568	2, 45	3.4	**0.042**
Soil C (%)	2, 45	3.47	**0.0396**	1, 20	2.55	0.126	2, 45	1.36	0.2667

vesicles, and hyphae) also did not significantly differ between endophyte treatments in the two *Poa* species (Table 2), despite E+ samples hosting approximately 11% and 9% higher AMF colonization rates on average than E− samples in *P. alsodes* and *P. sylvestris*, respectively (not shown).

<div align="center">SOIL C</div>

Soil C concentration (%) also did not significantly differ based on endophyte status (Table 2), yet plots containing *Poa* species exhibited significantly higher soil C than *S. arundinaceus* plots (Tables 1, 2). We observed no correlations between soil C and total AMF
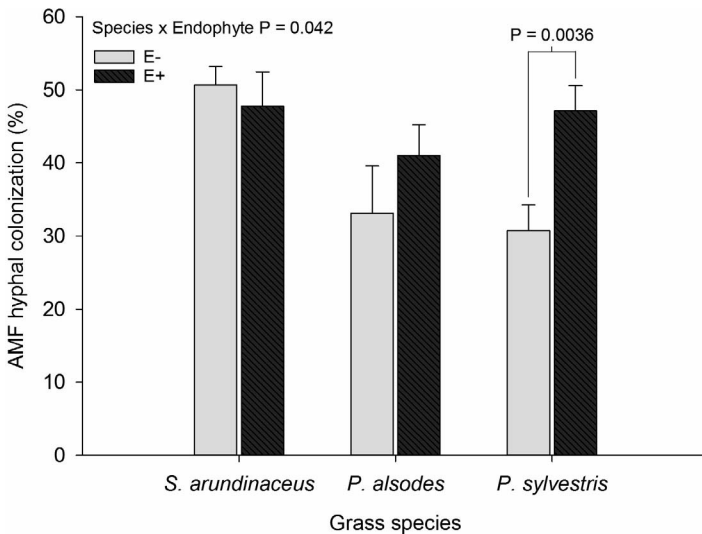

FIG. 1.—Effects of grass species and asexual *Epichloë* endophyte symbiosis (E+, E− treatment) on percentage of AMF hyphae detected in grass roots. Only significant P-values for differences between endophyte treatments are shown
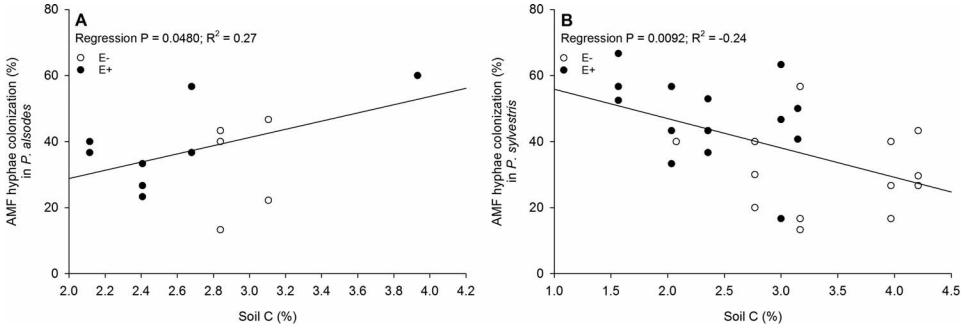
FIG. 2.—Linear regression between root AMF hyphae colonization and soil C in **(A)** *P. alsodes*, and **(B)** *P. sylvestris*

colonization for any of the three grass species. However, soil C correlated positively with AMF hyphae in *P. alsodes* and negatively in *P. sylvestris* samples (Figs. 2 A,B).

DISCUSSION

Our study demonstrates effects of asexual *Epichloë* fungal endophytes on belowground AMF and soil C vary across grass host species. While this finding is consistent with our general expectations, our specific results often countered our hypotheses and contrasted with existing work on the subject.

Based on prior work demonstrating *E. coenophiala* symbiosis negatively impacts AMF in this host species (Chu-Chou *et al.,* 1992; Guo *et al.,* 1992; Mack and Rudgers, 2008), we expected E+ *S. arundinaceus* would exhibit reduced AMF colonization compared to E−, yet this was not supported by our results (Table 2). In fact E− plants tended to have lower hyphal colonization than E+ (Fig. 1), but the difference was not significant. Several differences exist between our work and prior studies, which might account for this discrepancy. Our study occurred in a relatively undisturbed forest site, on 6 y old field-grown plant material, whereas previous studies were conducted over one growing season (Guo *et al.,* 1992; Mack and Rudgers, 2008) or used soils from 3 to 4 y old grassland plots (Chu-Chou *et al.,* 1992). It is likely the natural soil or commercial inoculum (Mack and Rudgers, 2008), single-species grassland soil isolates (Guo *et al.,* 1992), or managed grassland soils (Chu-Chou *et al.,* 1992) used in the prior studies harbored different AMF communities compared to the indigenous community of our forest site (Moora *et al.,* 2014), which included a diverse plant understory underlain by deciduous tree roots. It is also likely light and soil nutrient availability differed across these various experiments, which may have influenced the nature of these tripartite interactions (Davitt *et al.*, 2010, Mack and Rudgers, 2008). We show here that endophyte symbiosis does not always inhibit AMF colonization in *S. arundinaceous*, and interactions between AMF and asexual *Epichloë* endophytes within this agronomic grass are likely environment-dependent.

Because effects of *Epichloë* symbiosis on AMF have differed in prior studies on agronomic versus native plants, we expected the native species *P. sylvestris* and *P. alsodes* would exhibit higher AMF colonization in E+ than in E− grasses, which was only partially supported by our results. Aboveground endophyte symbiosis increased total AMF colonization of roots in both grasses, yet these differences were not statistically significant (Table 2). Observed AMF hyphal colonization was significantly higher in E+ *P. sylvestris* than in E−, but a similar trend

was not statistically significant in *P. alsodes* (Fig. 1). This partially complements findings by Novas *et al.* (2009) that naturally-occurring asexual *Epichloë* sp. symbiosis stimulated AMF colonization in the native grass *Poa bonariensis* compared to naturally nonsymbiotic plants amongst wild populations. A recent study of two *Poa* species with differing asexual *Epichloë* prevalence found *P. leptocoma*, which had naturally high leaf endophyte prevalence, exhibited significantly higher AMF colonization rates than nonsymbiotic *P. reflexa* (Kazenel *et al.,* 2015), illustrating how AMF symbiosis can vary within co- existing North American subalpine meadow grasses. Our current results add new experimental field results to the existing, albeit small, literature on nonagronomic grasses, by suggesting that asexual *Epichloë* species may stimulate AMF colonization in C_3 grasses in their native habitat but to varying degrees across grass or *Epichloë* species. Although our study focused on endophyte presence and absence effects on AMF colonization, an important question in future studies where strong effects of *Epichloë* symbiosis on AMF have been observed should be to investigate how *Epichloë* colonization density influences AMF (sensu Mack and Rudgers, 2008) across different host-endophyte symbiota, which may help delineate these relationships. Even so our work helps to overcome the confounding issue in prior studies of these multiple mutualist interactions, which was to date, no one had compared multiple species of host-endophyte symbiota in the same environment.

Because prior work demonstrated *Epichloë*-associated increases in soil C and reductions in AMF for *S. arundinaceus*, we expected grass species-specific differences in *Epichloë*—AMF relationships would produce synergistic effects on soil C stocks. We found grass species-specific interactions between AMF communities and soil C concentrations but not in the direction we expected, indicating the potential complexity of environment-plant-microbe interactions and subsequent ecological effects. We observed no significant endophyte effect and no significant correlations between AMF total colonization and soil C within *S. arundinaceus*, yet we found a positive relationship between root AMF hyphae and soil C in *P. alsodes* (Fig. 2A), which has been a common observation in other studies (Wilson *et al.,* 2009). The negative relationship between AMF hyphae and soil C in *P. sylvestris* (Fig. 2B) may indicate that increased root AMF hyphae instead stimulated C decomposition as in Cheng *et al.* (2012). Because *Epichloë* presence significantly stimulated root AMF hyphae in *P. sylvestris* (Fig. 1), and increased hyphal colonization rates were associated with lower soil C (Fig. 2B), our data suggest *Epichloë* symbiosis could amplify a negative association between AMF and soil C for this host species. We used experiments in a common field environment to demonstrate that *Epichloë* effects on AMF and soil C vary in symbiotum-specific ways. The driving factors and tripartite interactions creating this diversity of effects remains to be further explored, such as through direct experimental manipulation of AMF and abiotic cofactors.

To our knowledge we are the first to experimentally quantify the effect of aboveground asexual *Epichloë* associations on belowground AMF colonization across multiple grass species within a common field environment. Our results suggest relationships between aboveground asexual *Epichloë* fungal endophytes and belowground AMF in cohabitating grass hosts are not universally antagonistic or mutualistic, nor do they produce consistent outcomes for ecosystem processes. Rather, the tripartite interactions and effects on ecosystem parameters, such as soil C, depend on the grass-*Epichloë* species involved and are likely environmentally sensitive. These findings also highlight the need for further research to explore potential host-specific drivers of these interactions, such as indigenous microbial community effects, land use, and resource availability (*e.g.*, Ahlholm *et al.*, 2002; Johnson *et al.,* 2015; Vályi *et al.,* 2015). In addition our results represent a single temporal examination of *Epichloë*-grass-AMF

relationships. Future studies should consider how both interannual and intra-annual variation in the above host- or environmentally specific drivers such as resource availability influence these interactions. Such investigations will be necessary to determine or predict long-term ecosystem implications of these tripartite symbioses.

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