



Response of fungal endophyte communities within *Andropogon gerardii* (Big bluestem) to nutrient addition and herbivore exclusion

Monica Watson ^{a,*}, Kathryn Bushley ^b, Eric W. Seabloom ^a, Georgiana May ^{a,b}

^a Department of Ecology, Evolution & Behavior, University of Minnesota, St. Paul, MN, USA

^b Department of Plant & Microbial Biology, University of Minnesota, St. Paul, MN, USA

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ABSTRACT

Fungal endophytes may alter plant responses to the environment, but how does the environment affect the communities of fungal symbionts within plants? We examined the impact of nutrient addition and herbivore exclusion on endophyte communities of the prairie grass *Andropogon gerardii* in a full factorial field experiment. Fungi were cultured from stems, young leaves, and mature leaves, ITS sequences obtained, and endophyte incidence, community richness, and composition analyzed. Results indicate that in plots where nutrient addition and herbivore exclusion treatments had been applied separately, fungal endophyte incidence, community composition or evenness did not differ, but that greater species richness was observed in plots with nutrient addition and herbivore exclusion treatments applied in combination, compared to other treatments. Further, although fungal community composition was significantly different in stem and leaf tissues, OTU richness was greater in all endophyte communities in nutrient addition plus herbivore exclusion treatments, regardless of tissue type. Our results indicate the distinct fungal endophyte communities found in different plant tissues respond similarly to environmental factors.

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1. Introduction

What governs the diversity of microbial communities residing within a host? Plants and animals harbor a myriad of diverse microbial symbiont communities with estimates of microbial community diversity being 3–33 times more diverse than the host communities with which they associate (Arnold et al., 2000; Hawksworth, 2001; Baker, 2004; Human Microbiome Project Consortium, 2012). In addition to being incredibly diverse, endophytes, defined as asymptomatic microbial symbionts residing within plants, have a wide range of effects on their plant hosts (Saikkonen et al., 1998; Aly et al., 2011; Creamer and Baucom, 2013). Some may be latent pathogens that cause disease under stressful environmental conditions (Carroll, 1988; Slippers and Wingfield, 2007), mutualists that modulate plant responses to abiotic and biotic stress (Sieber, 2007; Rodriguez and Redman, 2008; Allan et al., 2010), or symbionts that impede or facilitate infection by plant pathogens (Adame-Álvarez et al., 2014; Busby et al., 2019). Endophytes associated with roots are often involved in both plant

nutrient uptake and in cycling of nitrogen and phosphorus (Bonfante and Genre, 2010; Lendenmann et al., 2011). Thus, our understanding of how abiotic and biotic factors affect the assembly and diversity of fungal endophyte communities (Lumibao et al., 2019; Seabloom et al., 2019) has implications for plant growth (Guo et al., 2015) and resiliency (Azad and Kaminskyj, 2016; Redman and Rodriguez, 2017) in response to human-mediated environmental challenges.

Nutrient availability affects soil microbial community and plant community structure and composition (Mitchell et al., 2002; Dybzinski and Tilman, 2007; Hautier et al., 2009; Borer et al., 2014a), but the impacts on foliar endophyte communities are less well understood. The mechanisms by which nutrient addition may directly affect endophyte communities *in planta* include increased resource availability within the host (Borer et al., 2013), changing host immune response to endosymbionts (Berger et al., 2013), and changing competitive microbial interactions within hosts (Larkin et al., 2012). Fungal endophyte communities might mirror the patterns observed in other symbiotic or soil microbial communities, where increased nutrient resources are associated with increases in faster growing taxa that apparently outcompete other species and lead to a reduction in diversity (Fontaine et al., 2003).

* Corresponding author.

E-mail address: Watso541@umn.edu (M. Watson).

Mycorrhizal communities, for example, are consistently less species diverse in high nitrogen and phosphorus soils (Egerton-Warburton et al., 2007; Camenzind et al., 2014). Of particular concern is the association between nutrient addition and increased pathogen abundance in microbial communities (Bruno et al., 2003; Mitchell et al., 2003; McKenzie and Townsend, 2007; Wiedermann et al., 2007). Nutrient addition can diminish shared benefits of mutualistic interactions, with the expectation of a lower relative abundance of mutualist versus pathogenic taxa (Johnson et al., 1997; Hoeksema and Kummel, 2003). For example, occurrence of pathogens increased with nutrient addition in several moss species compared to plants in unfertilized soils (Davey et al., 2017). Nutrient addition is often associated with reduced plant host community diversity (Mitchell et al., 2002; Dybzinski and Tilman, 2007; Hautier et al., 2009; Borer et al., 2014a), which may indirectly lead to decreased endophyte community diversity. Previous work describing the effect of nutrient addition on fungal endophyte community diversity suggests that the magnitude and direction of nutrient effects on fungal endophyte community diversity may vary across spatial and environmental scales, with results from some sites exhibiting negative correlation with nutrient addition while results for other sites do not show apparent effects of nutrient addition on diversity (Lumibao et al., 2019; Seabloom et al., 2019).

Biotic factors such as large mammal herbivory may affect fungal endophyte community diversity, either directly or through changing host plant community structure (Olff and Ritchie, 1998; Borer et al., 2009). Although herbivory provides an entry point for fungal infection, the magnitude and direction of herbivory effects are context dependent (Lumibao et al., 2019; Seabloom et al., 2019) and may be associated with either increased (Daleo et al., 2009; David et al., 2016) or decreased fungal endophyte diversity. Most previous studies on the effects of herbivory on fungal endophytes have focused on *Epichloë* spp, systemic symbionts of grasses that can produce toxic alkaloids (Leuchtmann et al., 2000; Schardl et al., 2013) and thus deter herbivores (Clay, 1988; Bastias et al., 2017; Fuchs et al., 2019). The abundance of *Epichloë* spp may increase with herbivory (Clay et al., 2005) and in *Festuca campestris* large herbivore exclusion was associated with a reduction in *Epichloë* endophyte prevalence in plants and seeds (Rudgers et al., 2016). If herbivore exclusion affects endophyte communities as it does plant communities, and reduces the abundance of a dominant taxon such as an *Epichloë* spp., greater diversity of the remaining taxa may be observed (Olff and Ritchie, 1998). However, the effect of herbivory on fungal endophyte community diversity as a whole is not well understood.

Just as differing plant tissues can respond to environmental perturbations differently (Strauss and Agrawal, 1999; Schultz et al., 2013), so too may endophyte communities within these plant tissues. Tissue type and age of tissues affect fungal endophyte communities, with stem fungal endophyte communities usually less diverse and less abundant compared to those in foliar tissues (Verma et al., 2007; Mishra et al., 2012) and fungal endophyte communities of older plant tissues more diverse and abundant than communities in younger plant tissues (Sieber, 2007; Jin et al., 2013). Nutrient sequestration in response to nutrient addition may differ across tissues (Miao and Sklar, 1997) and herbivory can alter resource allocation to different plant tissues (Babst et al., 2005, 2008). Consequently, we might expect that perturbations of nutrients or herbivory levels may affect fungal endophyte communities of stem and leaf tissues differently, but to our knowledge there are no other studies investigating the effect nutrient addition and herbivory has on fungal communities occurring in different plant tissues.

Here, we examined the effects of nutrient addition and herbivore exclusion on endophyte communities in young leaves, mature

leaves, and stems of the grass host, *Andropogon gerardii* (Big Blue-stem), a prominent native grass throughout the Great Plains prairies. This culture-dependent study utilized replicated experimental plots manipulating nutrients and herbivore exclusion (NutNet; www.nutnet.org, Borer et al., 2014b), and dovetails with a culture independent study at sites across the Great Plains, including the site examined here (Seabloom et al., 2019). The culture-based approach provides an additional measure of fungal diversity, often identifying taxa not observed in culture-independent methods (Peršoh, 2015; Dissanayake et al., 2018), and provides an independent assessment of the abundance of endophytic fungal taxa within tissues (David et al., 2019). We asked the following questions:

- 1) How does nutrient addition and herbivore exclusion affect culturable fungal endophyte community structure, diversity and abundance? We expected lower fungal endophyte community diversity and greater pathogen abundance in nutrient addition treatments and higher fungal endophyte community diversity in herbivore exclusion treatments.
- 2) How do fungal endophyte communities of stem, young leaf, and mature leaf tissues differ in community structure, diversity, and abundance?
- 3) Do fungal communities within stem, young leaf, and mature leaf host tissues respond differently to nutrient addition and herbivore exclusion treatments?

2. Methods

2.1. Study site

We sampled *Andropogon gerardii* plants at experimental plots established in 2007 within an abandoned agricultural field (NutNet; www.nutnet.org, Borer et al., 2014b) at the Cedar Creek Ecosystem Science Reserve (<https://www.cedarcreek.umn.edu/>), a University of Minnesota biological station (45.4020° N, 93.1994° W; Mean Annual Precipitation = 750 mm). The plant community is composed of native and introduced grasses, forbs, and legumes. The experiment uses a complete randomized block design with the four treatments applied to plots within blocks: Control, nutrient addition (NPK), herbivore exclusion fencing (Fence), or combined nutrient addition and herbivore exclusion fencing (NPK + Fence). In Nutrient addition plots, 10 g N m⁻²yr⁻¹ as time-release urea, 10 g P m⁻²yr⁻¹ as triple super phosphate, 10 g K m⁻²yr⁻¹ as potassium sulfate have been added annually since 2007. In addition, 100 g/m² of a micronutrient mix (6% Ca, 3% Mg, 12% S, 0.1% B, 1% Cu, 17% Fe, 2.5% Mn, 0.05% Mo, and 1% Zn) was applied once at the establishment of the study site in 2007. Fenced plots were surrounded with 2 m tall wire mesh fences exclude large mammals such as deer and rabbits, but not burrowing herbivores. All treatments have been applied annually from 2007 up to the time of this study in 2014. Treatments and experimental plot set-up are described in detail in Borer et al. (2014b).

2.2. Tissue sampling and endophyte culturing

In August 2014 when plant biomass was greatest, four *A. gerardii* plants were identified in each replicate treatment plot (Control, Fence, NPK, NPK + Fence), in each of the four blocks, for a total of 64 plants. Two of these four plants were the same as those sampled for NextGen Sequencing (NGS) as reported previously (Grantham et al., 2019; Lumibao et al., 2019; Seabloom et al., 2019), and two additional plants were sampled per plot to increase sample size. Three tissue types from each plant were sampled: mature leaves (lowest

fully-expanded leaf that showed no signs of disease or senescence), young leaves (most recent fully emerged leaf), and stem tissue. A total of 192 tissue samples were collected (64 plants $\times$ 3 tissue types). Samples were kept on ice until processing within 48 h after collection. All samples were surface sterilized by rinsing in water, washed in 75% ethanol for 1 min, washed in 50% commercial bleach for 1 min, washed in 75% ethanol for 1 min, then rinsed in sterile distilled water for 1 min (Arnold et al., 2001). The efficacy of sterilization was evaluated by pressing sterilized and unsterilized plant tissues to 1/2 CMA plates for a few minutes, and then monitoring these plates for fungal growth. Plates in which sterilized plant tissues were pressed did not harbor fungal growth. Leaf tissues were cut into 20 4 mm $\times$ 4 mm sections and stems were cut into 4 mm lengths using flame-sterilized scissors and these sections were individually plated on 2% malt extract agar (MEA) in 1.5 ml Eppendorf tubes (slants), then incubated at room temperature. In total, 3840 slants were made (20 sections per sample $\times$ 192 tissue samples).

2.3. DNA extraction and ITS sequencing

Once fungi emerged, isolates were subcultured on 2% MEA plates and DNA was extracted from small tissue samples. The ca. 700 bp region of the rDNA locus was PCR amplified with ITS1F (SSU) and LR3 reverse (LSU) primers (Gardes and Bruns 1993; Hopple and Vilgalys 1994) using REDextract'n'Amp Tissue PCR kit protocol (Thermocycler parameters: 94 °C for 3 min, 35 cycles of 94 °C for 1 min $\rightarrow$ 45 °C for 1 min $\rightarrow$ 72 °C 1 min, final extension of 72 °C for 10 min, 4 °C hold; Sigma-Aldrich, St. Louis, MO, USA). Successful amplification was confirmed via gel electrophoresis, DNA products were purified using ExoSap-IT Product Cleanup Reagent protocol (Thermo Fisher Scientific, Waltham, MA, USA) and single strand ITS1 sequences were obtained using the ITS1F primer (GeneWhiz; South Plainfield, NJ, USA). DNA sequences with multiple overlapping chromatogram peaks (Geneious v. 5.5.6) were removed from further analyses, and ITS1F primer sequences were trimmed from the remaining sequences in Geneious (Mac Version 5.5.6).

2.4. Fungal endophyte community statistical analyses

To estimate fungal endophyte incidence within plant tissues, we calculated isolation frequency as the percentage of slants (each with one plant tissue segment) in which a fungal colony grew for each tissue type (young leaf, mature leaf, stem). To evaluate the effects of the nutrient addition (NPK) and herbivore exclusion (Fence) treatments and spatial location on endophyte incidence within different plants and tissues, we used a linear mixed-effects (lme) model for differences in endophyte incidence in tissues and treatments with Plant, Plot, and Block as nested random effects. The incidence, a proportion, was log transformed and the following model evaluated: $\log(\text{incidence}) \sim \text{Tissue} \times \text{Treatment} + 1|\text{Plant}/\text{Plot}/\text{Block}$ using the R package lme4 (Bates et al., 2015). In presenting results, effect sizes were back transformed.

Sequences obtained from each culture were used to determine Operational Taxonomic Units (OTUs) using USEARCH (Edgar, 2010) and clustered in QIIME v1.8.0 (Caporaso et al., 2010) with 97% sequence similarity against the UNITE fungal ITS1 database (v8; accessed April 15, 2019; Kõljalg et al., 2013). The resulting OTU table was exported and all subsequent analyses were performed in R version 3.6.3 (R core team, 2019). To evaluate the association between nutrient addition and pathogen abundance, we used FUNGuild to assign trophic modes to OTUs for which genus-level taxonomic assignments could be made (Nguyen et al., 2016). For our analysis, we accepted all “Probable” and “Highly Probable”

trophic mode assignments generated in FUNGuild and grouped all “Possible” or unknown trophic mode assignments as “unassigned”. We grouped as “unidentified” those sequences that could not be identified to the genus level and thus trophic mode could not be assigned. We used Fisher's exact test (fisher.test() function in R) to evaluate the independence of frequencies of sequences assigned to different trophic modes and their occurrence in different nutrient addition and herbivore exclusion treatments.

To determine the effects of nutrient addition and herbivore exclusion on fungal endophyte communities (Q1), we pooled OTUs from the leaf and stem samples together by individual plants, giving a total of 64 sampled fungal communities (4 plants within each treatment plot, 4 treatments, 4 blocks). We estimated community beta diversity in order to compare the differences in community structure as unweighted Bray-Curtis distances (Bray and Curtis, 1957). We determined differences in community composition associated with treatments (Control, NPK, Fence, NPK + Fence) and spatial location (Plot within Block) using Permutational Multivariate ANOVA (PERMANOVA) which was implemented in the adonis function in the vegan R package (Oksanen et al., 2019). Bray-Curtis distances between individual plants were visualized on ordination plots using principal coordinate analysis (PCoA) in the phyloseq R package (McMurdie and Holmes, 2013).

To determine the taxa of fungi contributing to differences in fungal endophyte community composition due to treatment, we counted OTUs (ITS1 sequences >97% similar) that were uniquely found in plots of each treatment. We then evaluated differences in the relative frequencies of unique OTUs that were assigned to the two most common Classes, Dothideomycetes and Sordariomycetes, using Fisher's exact test of independence with pairwise comparisons of treatments (pairwiseNominalIndependence(fisher = TRUE), rcompanion package version 2.1.7 (Mangiafico, 2017)). We next estimated alpha diversity as OTU richness (observed number of OTUs) using the estimate_richness function in the phyloseq R package (McMurdie and Holmes, 2013). We calculated Pielou's evenness (Pielou, 1966), a measure of skewedness in the distribution of OTUs abundances within samples, using the evenness function in the vegan R package. OTU richness and Pielou's evenness were calculated for each fungal community per plant and used in linear mixed-effects (lme) models to evaluate differences in diversity or evenness between the treatments, with Block and Plot as nested random effects (Diversity or Evenness $\sim$ Treatment + (1|Block/Plot) using the lme4 R package (Bates et al., 2015). To understand how rarely observed taxa might affect our results, we used the same lme models (Diversity or Evenness $\sim$ Treatment + (1|Block/Plot) and evaluated OTU richness and Pielou's evenness for datasets with singletons (OTUs observed only once) excluded. To determine if spatial scale at the level of Plot contributed significantly to responses, Plot was dropped from each model and an ANOVA was used to evaluate differences between the reduced and complete models. In addition, to make a direct comparison of alpha diversity metrics of our results using culture-dependent approaches, and those of Seabloom et al. (2019) using NGS approaches, we calculated Effective Number of Species/Probability of Intra- or Interspecific Encounter (ENS_{PIE}), an estimate of species richness based on the probability of interspecific encounter (Chase and Knight, 2013) of mature leaf tissue endophyte communities, using the diversity() function with the “inv” method in the vegan R package (Oksanen et al., 2019). We then used the lme model described above with ENS_{PIE} as the Diversity response variable.

To determine differences in the endophyte communities living within different plant tissues (young leaves, mature leaves, and stems), (Q2), we pooled OTUs by tissue type for all plants within each plot, for a total of 48 fungal communities (3 tissues, 4 treatments, 4 blocks). We did not have sufficient culture representation to evaluate differences in communities within tissues of individual

plants. As above, PERMANOVA was performed on unweighted Bray-Curtis distances between fungal endophyte communities associated with different tissues (young leaf, mature leaf, stem), treatments (Control, NPK, Fence, NPK + Fence), or with spatial location (Block) using the vegan R package (Oksanen et al., 2019). Because of the low frequency occurrence of many Classes, we performed two pairwise comparisons using Fisher's exact test of independence (pairwiseNominalIndependence (fisher = TRUE), rcompanion package version 2.1.7; Mangiafico, 2017). First, we evaluated whether the relative frequencies of OTUs assigned to the two most common Classes, Dothideomycetes and Sordariomycetes, were independent of tissue type and second, we performed a separate analysis to evaluate whether the relative frequency of Ustilaginomycete OTUs versus all other OTUs was independent of tissue type.

We used a linear mixed-effects model (lme4 R package) for observed OTUs and Pielou's evenness as described above for treatment effects, except that we only evaluated the spatial variable at the level of Block because samples were pooled by tissue for all plants within a plot. (Diversity or Evenness ~ Treatment*Tissue + (1|Block)). Both the PERMANOVA of Bray-Curtis distances and lme models of observed OTUs and Pielou's evenness included an interaction term between tissue and treatment to determine if fungal endophyte communities within young leaf, mature leaf, and stem host tissues responded differently to nutrient addition and herbivore exclusion (Q3).

3. Results

From a total of 64 plants and 192 tissue samples, we plated a total of 3840 tissue sections onto agar mini-slants from which a total of 1344 demonstrated fungal growth, giving an overall isolation frequency of 35%. From these 1344 cultures, after discarding sequences of low quality and those indicating multiple fungal sequences, we obtained 875 ITS1 sequences (Table S1). Clustering in QIIME resulted in 437 OTUs at the 97% sequence similarity level of which, 314 were singletons (observed once). The relatively high number of singletons across all treatments and tissues suggests that these communities may be structured with a few abundant taxa and many, more rare endophytes. While this pattern may also be due to under sampling the fungal endophyte communities, we obtained useable sequence for 65% of the cultures that grew (Table S1). Of the 437 OTUs above, most were assigned to Ascomycota (87%), and the remainder were assigned to Basidiomycota (9%), or were unidentified Fungi (4%). The most common Ascomycota Classes were Dothideomycetes and Sordariomycetes, as in most studies of foliar endophytes. In stems, the Ustilaginomycetes were also commonly observed (Fig. 1).

Q1: How does nutrient addition and herbivore exclusion affect culturable fungal endophyte communities?

The results of PERMANOVA analysis on Bray-Curtis distances indicated that community composition did not significantly differ due to NPK or Fence treatments applied individually or in combination (Fig. S1), but that fungal endophyte community composition did differ significantly at the Plot ($p = 0.001$) and Block ($p = 0.001$) spatial scales (Table 1; Fig. S2). We found eight "core" OTUs shared across all treatments, and at least 20% of OTUs in any treatment were shared with at least one other treatment (Fig. S3). The 8 core OTUs were assigned to the genera *Alternaria*, *Anthracycystis*, *Didymella*, *Phoma*, *Pyrenophora*, with two OTUs assigned to *Epicoccum*.

A significantly greater number of OTUs were observed in plots with the NPK + Fence treatment compared to the Control treatment ($p < 0.001$, Table 2, Fig. 2) and while more OTUs were observed in the single factor NPK and Fence treatments than in Control plots, these were not significantly different ($p > 0.05$, Table 2, Fig. 2). When singletons were excluded from the dataset, treatment was not a

significant term, a result suggesting that rare taxa contribute importantly to community diversity. To compare our results with those of Seabloom et al. (2019), we calculated log ENS_{PIE} for alpha diversity in mature leaves only. As with Seabloom et al. (2019), lme results for our culture-dependent data show no significant effect of treatment on ENS_{PIE} . However, the sign of effect size of the NPK + Fence treatment was positive for our results while negative for the previously published results (Seabloom et al., 2019, Table S2).

To understand which taxa were contributing to the greater richness of NPK + Fence communities, we then examined the identity of OTUs that were only found in the NPK + Fence treatment plots, compared to the OTUs found other treatments. Of the OTUs unique to the NPK + Fence treatment, the relative frequency of unique Sordariomycete OTUs was greater than that of the unique Dothideomycete OTUs in NPK + Fence compared to the Control treatment ($p_{Fisheradj} = 0.04$), and NPK treatment ($p_{Fisheradj} = 0.004$) communities, but not the Fence treatment ($p_{Fisheradj} = 0.35$) (Table S3).

The results of the lme analyses showed that Pielou's evenness (Table 2, Fig. S4) and endophyte incidence (Table 2, Fig. S5) were not significantly different across treatments. When singletons were excluded from the dataset, treatment effect was still not significant for Pielou's evenness. The ANOVA of complete (Plot nested in Block) and reduced (Block only) lme models indicated Plot was a significant term for observed number of OTUs ($p = 0.001$) and endophyte incidence ($p = 0.003$), but was not for evenness. Thus, while overall incidence of endophytes and their diversity varied at the spatial scale of Plot, that diversity was fairly uniformly distributed.

We used FUNGuild to assign trophic modes to the taxa identified at the genus level (Nguyen et al., 2016) and asked whether the relative abundance of apparent pathogens differed among treatments. Of the 875 cultures for which sequences were obtained, 6.6% were not identified at the genus level and could not be assigned trophic modes and 24.5% were identified to the genus-level but were not assigned a trophic mode using our criteria. For those sequences that could be assigned trophic modes, taxa were related to those previously described as belonging to the pathotroph-saprotroph trophic mode (36.7%), pathotrophs (23.8%), saprotrophs (6.7%), pathotroph-symbiotrophs (1.0%), pathotroph-saprotroph-symbiotrophs (0.5%), saprotroph-symbiotrophs (0.1%), and symbiotrophs (0.1%). Surprisingly, although all isolates were cultured from asymptomatic, living plant tissues, the majority of assigned trophic modes included the term pathotroph or saprotroph, and trophic modes that included the term symbiotroph were assigned to less than 2% of the taxa. Although the frequency of OTUs assigned to different trophic modes was not independent of treatment (fisher.test(), simulated $p < 0.001$, Table S4), we did not obtain evidence that the frequency of taxa assigned to any functional group that included pathotrophs, was greater than the frequency of those assigned to other symbiotroph modes in nutrient addition plots. Importantly, our results suggest that many endophyte taxa of *A. gerardii* are multi-trophic.

Q2: How do fungal endophyte communities of stem, young leaf, and mature leaf tissues differ?

Endophyte community composition differed across stems, young leaf, and mature leaf tissues. Results of the PERMANOVA of Bray-Curtis distances of communities pooled by tissue type within plots showed a significant effect of plant tissue on endophyte community composition ($p = 0.005$, Table 1). Block also had a significant effect. Results of the principal coordinates analysis of Bray-Curtis distances suggests that endophyte communities within stem tissues were more similar to each other and represent a subset of communities found in young leaf or mature leaf tissues (Fig. 3).

The OTUs assigned to Sordariomycetes occurred more frequently than did Dothideomycete OTUs in stem tissues, compared to mature

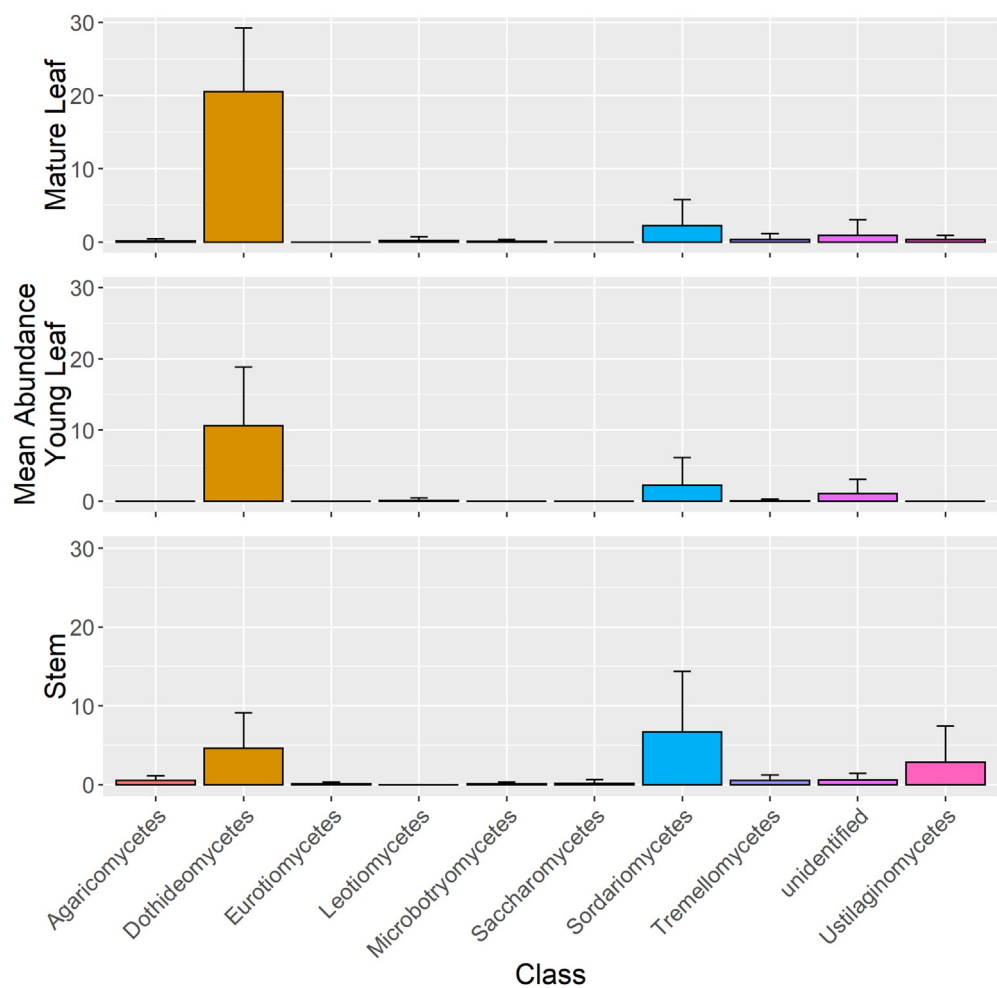


Fig. 1. Mean abundance of Classes of fungal endophytes cultured from *Andropogon gerardii* stem, young leaf, and mature leaf tissue samples and pooled by tissue type within plots. The most commonly isolated fungi identified in both young and mature leaf tissues belonged to the ascomycete Class Dothideomycetes, while Sordariomycetes were the most commonly isolated Class from stem tissues. Error bars represent one standard deviation. Pairwise Fisher's exact test of independence of frequency of Dothideomycete and Sordariomycete OTUs in different tissue types demonstrated that Sordariomycete OTUs were overrepresented compared to Dothideomycetes in stem tissue compared to mature leaf ($p < 0.001$) and young leaf ($p < 0.001$) tissues.

Table 1
Results of PERMANOVA analyses of Bray-Curtis distances to evaluate treatment (NPK + Fence, NPK, Fence, Control) effects (model: Bray-Curtis Distance ~ Treatment + Block/Plot) with communities pooled by individual plants. To evaluate tissue (young and mature leaves, stems) effects and treatment interaction effects on Bray-Curtis distances (model: Bray-Curtis Distance ~ Treatment*Tissue + Block), communities were pooled by tissue type within plots.

Community pooled by: Dependent variable:	Individual Plants		Tissue Type in Plot	
	Bray-Curtis Distance		Bray-Curtis Distance	
	R ²	p-value	R ²	p-value
Plot	0.19	0.001*	NA	
Block	0.07	0.001*	0.11	0.0001*
Treatment	0.05	0.082	0.07	0.086
Tissue	NA		0.06	0.005*
Treatment*Tissue	NA		0.1	0.961

leaf tissues ($p_{\text{Fisher_adj.}} < 0.001$) and young leaf tissues ($p_{\text{Fisher_adj.}} < 0.001$), both of which harbored more Dothideomycete than Sordariomycete OTUs (Fig. 1, Table S5). Ustilaginomycetes were almost

exclusively detected in stem tissue and the frequency of Ustilaginomycete OTUs was greater than other Classes in stems compared to mature ($p < 0.001$) or young leaves ($p < 0.001$) (Table S5). The results of lme model analyses indicated no significant association of tissue and observed number of OTUs (Table 2, Fig. S6) or evenness (Table 2, Fig. S7). Endophyte incidence was significantly different between tissue types, with young leaf tissue harboring significantly fewer endophytes ($p < 0.01$, Fig. 4, Table 2). Q3: Do fungal communities within stem, young leaf, and mature leaf host tissues respond differently to nutrient addition and herbivore exclusion treatments? In the PERMANOVA analysis, the interaction term for Tissue*Treatment did not significantly contribute to differences of fungal community composition (Bray-Curtis distances; Table 1). In the lme model analyses, the Tissue*Treatment effect did not significantly contribute to endophyte incidence, observed number of OTUs, or evenness (Table 2). These results suggest that while endophyte community composition differs across different tissues, these communities respond similarly to the treatments. Specifically, greater observed OTUs were found in communities in the NPK + Fence treatment than in communities of other treatments regardless of tissue type ($p < 0.01$, Fig. 5, Table 2).

Table 2

Results of the linear mixed-effects (lme) models for fixed effects (treatments and tissues) fitted to the observed OTUs and Pielou's evenness of fungal endophyte communities in two analyses, first when communities were pooled by individual plants and second, by tissue type within plots. The third lme model for fixed effects (treatments and tissues) was fitted to the incidence of fungal endophytes (% of slants per sample that grew cultures) were pooled by individual tissue samples.

Community pooled by: Dependent variable:	Individual plants				Tissue type in plots				Tissue sample	
	Observed OTUs		Pielou's Evenness		Observed OTUs		Pielou's Evenness		Incidence	
	Effect Size	Std. Error	Effect Size	Std. Error	Effect Size	Std. Error	Effect Size	Std. Error	Effect Size	Std. Error
Fence Treatment	3.12	2.737	−0.055	0.050	3.750	5.322	−0.045	0.060	0.77	−0.46
NPK Treatment	3.562	2.737	0.003	0.048	9.250	5.322	.050	0.060	1.02	−0.46
NPK + Fence Treatment	9.437***	2.737	0.023	0.048	16.250**	5.322	−0.059	0.066	1.18	−0.46
Young Tissue					−4.000	4.316	0.045	0.066	−0.79**	−0.44
Stem Tissue					−1.750	4.316	0.031	0.066	−0.44	−0.44
Fence*Young Tissue					−6.500	6.104	0.177	0.084	0.19	−0.55
NPK*Young Tissue					−3.000	6.104	0.029	0.084	0.4	−0.55
NPK + Fence*Young Tissue					−6.250	6.104	0.014	0.084	1.24	−0.55
Fence*Stem Tissue					−4.250	6.104	−0.089	0.084	−0.22	−0.55
NPK*Stem Tissue					−11.250	6.104	0.067	0.084	−0.66	−0.55
NPK + Fence*Stem Tissue					−5.500	0.084	−0.075	0.084	0.56	−0.55
Constant	6.438 ***	1.935	0.937***	0.034	10.250 ***	3.763	0.917 ***	0.047	−0.8 ***	−0.35

Note: *p < 0.05; **p < 0.01; ***p < 0.001.

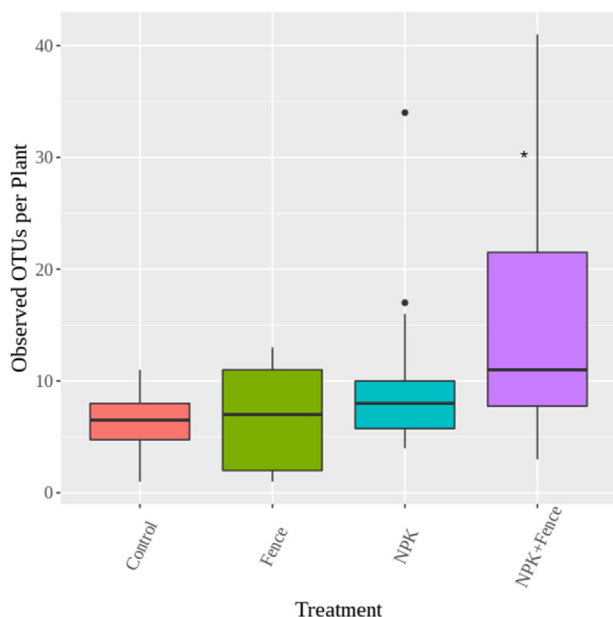


Fig. 2. Observed number of fungal OTUs per individual *Andropogon gerardii* plant under each treatment, displayed as boxplots of median and interquartile range, with outliers indicated as points. Results of the linear mixed-effects (lme) model indicated a significantly greater number of observed OTUs in communities in the NPK + Fence treatment than in the control treatment ($p < 0.01$). Communities in the NPK + Fence communities also demonstrated greater variation in observed OTUs.

4. Discussion

We investigated the effects of nutrient addition and herbivore exclusion on fungal endophyte community diversity in different tissues of *Andropogon gerardii*. Three striking patterns emerged. First, fungal endophyte communities showed greater richness (observed OTUs) in the combined nutrient addition and herbivore exclusion treatment than in plots of single factor or control treatments. Overall, fungal endophyte community composition did not significantly differ by treatment, although a greater relative frequency of Sordariomycetes contributed to increased richness observed in the combined treatment. Second, stem, young leaf, and mature leaf tissues harbored compositionally dissimilar fungal endophyte communities. Sordariomycetes and Ustilaginomycetes

were relatively more abundant in communities of stem tissues, while Dothideomycetes dominated fungal endophyte communities of young leaf and mature leaf tissues. Incidence of fungi also differed significantly by tissue type with fewer endophyte colonies recovered from young leaves from the other tissues. Third, because we did not see significant interaction effects of tissue and treatment on either community composition or richness, we conclude that endophyte communities in different plant tissues responded similarly to treatments, with greater OTU richness in combined nutrient addition and herbivore exclusion plots observed across all tissues. To our knowledge, this is the first study investigating the effects of different environmental factors on fungal endophyte communities in different plant tissues.

We found greater OTU richness in fungal endophyte communities in plants from the combined treatment plots of nutrient addition and herbivore exclusion than in plants from single factor or control treatment plots. The effects of nutrient addition and herbivore exclusion on OTU richness may have been additive as we show that both single factor treatments were associated with small, although non-significant, positive effects on the number of OTUs of culturable fungi. The increased richness in the nutrient addition plots was an unexpected result because previous empirical results for plant, endophyte, and microbial soil communities have found lower species diversity in response to nutrient addition (Allison et al., 2007; Ruppel et al., 2007; Camenzind et al., 2014; Leff et al., 2015; Seabloom et al., 2019), consistent with resource competition theory (Tilman, 1977). Interestingly, the greater richness of communities in NPK treated plots is, in part, accounted for by an increase in Sordariomycete taxa, a result also observed in soil fungal communities with nutrient addition (Mueller et al., 2015). This increase, in combination with the tendency for culture-dependent studies to detect more Sordariomycetes (Al-Sadi et al., 2015; Tian et al., 2019) may explain why we observed greater fungal endophyte community diversity in NPK + Fence treated mature leaf tissue whereas the culture-independent study of Seabloom et al. (2019) did not.

While the combined nutrient addition and herbivore exclusion treatment was associated with greater OTU richness, the taxonomic and functional community composition did not differ greatly across treatments. Most of the variation in community composition was due to spatial structure at the Plot (5m) and Block (100m) scale, both of which also significantly affected alpha diversity. Our results are concordant with those of many studies of endophytic fungi (Zimmerman and Vitousek, 2012; Higgins et al., 2014; Whitaker

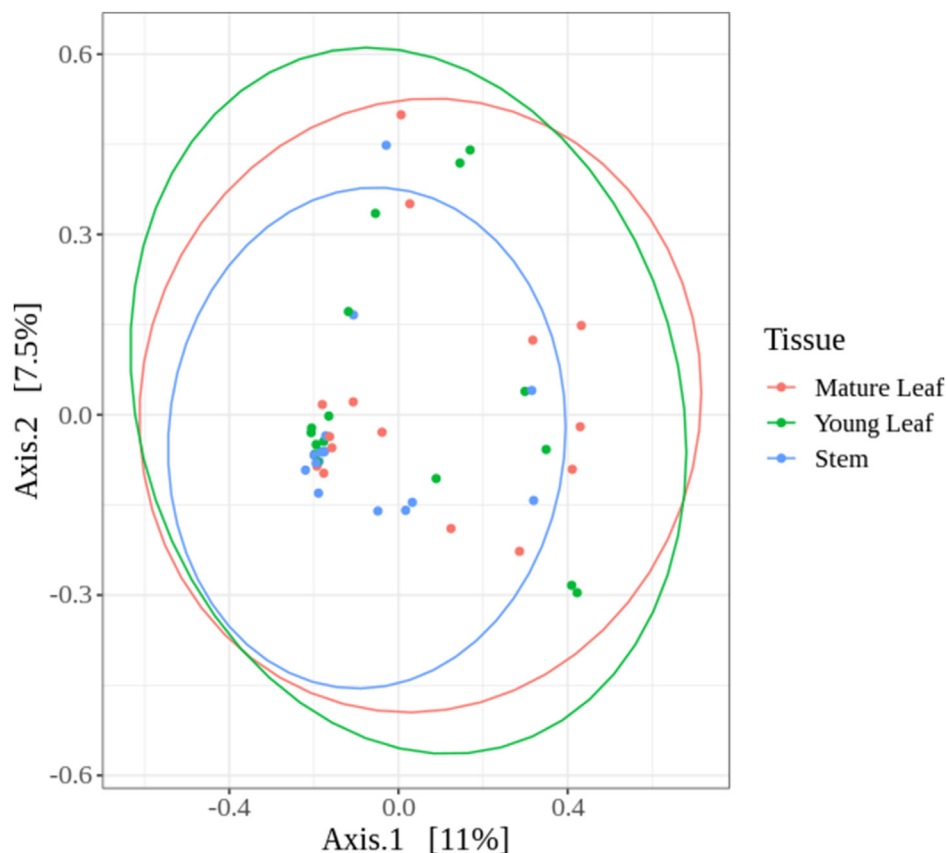


Fig. 3. Principle coordinate analysis of Bray-Curtis distance between fungal endophyte communities within *Andropogon gerardii* in mature leaf, young leaf, and stem tissues. Each point represents the fungal endophyte community pooled by tissue type in each replicate plot. Results of PERMANOVA analysis showed significant effects of tissue type on Bray-Curtis distances ($p = 0.005$).

et al., 2018; Lumibao et al., 2019; Seabloom et al., 2019), with spatial structure across geographic scales contributing significantly to fungal endophyte community composition and diversity. Together, these results suggest that unmeasured environmental heterogeneity contributes to the maintenance of diversity in fungal endophyte communities.

We had expected, but did not observe, that nutrient addition would be associated with increased representation of pathogenic taxa, as has been observed in plant and animal microbial communities (Bruno et al., 2003; Mitchell et al., 2003; McKenzie and Townsend, 2007; Wiedermann et al., 2007; Davey et al., 2017). Perhaps more surprisingly, only 2% of isolates were assigned as symbiotroph trophic modes (FUNGuild; Nguyen et al., 2016) despite the fact that all samples were taken from healthy plant tissues. This may be due to a variety of reasons, one being that culturing methods cannot detect obligate symbionts. For example, *Puccinia* spp., common rust pathogens of grasses including *A. gerardii*, are not present in our samples but did increase in response to nutrient addition in studies in which it was visually surveyed (Mitchell et al., 2003; Barnes et al., 2005). In light of the remarkable ecological plasticity exhibited by fungi (Wrzosek et al., 2017), the considerable variation in trophic modes maintained within fungal species (Slippers and Wingfield, 2007; Arnold et al., 2009; May 2016; Busby et al., 2019), and the dearth of ecological information for many fungal species, assignment of trophic modes to fungal taxa based on barcode sequences alone remains challenging.

Fungal endophyte communities in stems showed compositional differences from those in young leaf and mature leaf tissues, a result

concordant with those of other studies in plant hosts (Herrera et al., 2010; Wearn et al., 2012). Differences in the composition between tissue types were attributable to greater relative abundance of Sordariomycetes and Ustilaginomycetes in stem endophyte communities, the latter not usually one of the most abundant Classes in foliar endophyte communities (Arnold, 2007; Peršoh et al., 2010; Seabloom et al., 2019). There was less variation in community composition among stem communities in *A. gerardii*, as in other plant species (Verma et al., 2007; Gazis and Chaverri, 2010; Mishra et al., 2012). Comparing results for young and mature leaf tissues, the younger leaves demonstrated lower endophyte incidence that likely contributed to the lower OTU diversity in this tissue, which is in agreement with other observations in which leaves accumulate fungal endophytes with age (Ercolani, 1991; Arnold and Herre, 2003; Arnold et al., 2003). Putting our richness and composition results together, fungal endophyte communities were richer in NPK + Fence treated plots across the three *Andropogon gerardii* tissue types we sampled, suggesting that these communities exhibit functional redundancy, with different fungal endophyte taxa responding similarly to environmental effects (Gosling et al., 2016; Louca et al., 2016).

5. Conclusions

Our results suggest that fungal endophyte diversity is maintained by both deterministic factors such as nutrient addition, herbivory, and plant tissue type and by stochastic factors such as dispersal across a spatially heterogeneous environment. We show that while richness of OTUs increased in all tissues and plots in the

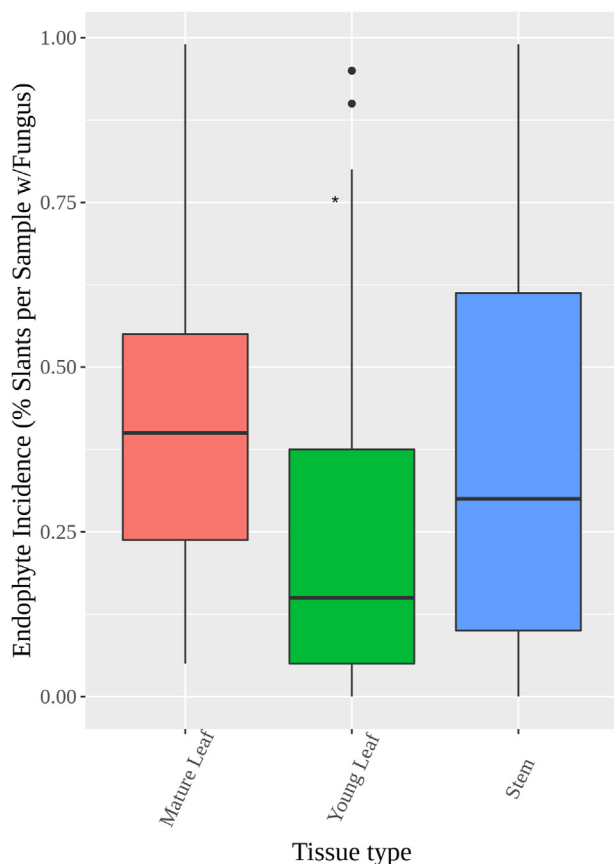


Fig. 4. Incidence of fungal endophytes in *Andropogon gerardii* mature leaf tissue, young leaf tissue, and stem tissue samples. Median and interquartile ranges are shown with outliers indicated as points. Incidence of endophytes was calculated as the percentage of slants in which a fungal colony grew per sample of an individual tissue. On average, fungal endophyte incidence was significantly lower in young leaf tissues than in the mature leaf and stem tissues ($p < 0.01$) and was highest in mature leaf tissues.

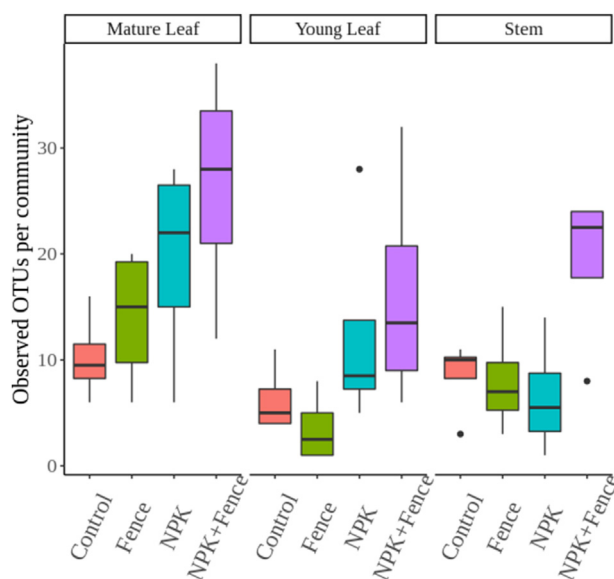


Fig. 5. Observed OTUs in fungal endophyte communities of *Andropogon gerardii* in different tissue types (mature leaf, young leaf, and stem) across treatments. Communities from NPK + Fence treatment plots had significantly more observed OTUs compared to other treatments regardless of the tissue type from which they were isolated. While there were some differences in the observed OTUs in different tissue types across treatments, results of the linear mixed-effects model for observed OTU showed no significant Tissue*Treatment interaction effects.

NPK + Fence treatments, fungal endophyte communities vary compositionally across different plant tissues and spatial location, suggesting functional redundancy in these communities. Our work underscores the power of manipulative field experiments to evaluate ecological patterns in microbial communities and contributes to our ability to predict the effect that anthropogenic changes may have on microbial endosymbiont communities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funeco.2021.101043>.

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