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Amorpha canescens and *Andropogon gerardii* recruit comparable foliar fungal communities across the steep precipitation gradient in Kansas

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Tallgrass prairies in North America have endured substantial losses due to anthropogenic environmental change. Plant-associated phyllosphere fungi are a largely overlooked aspect of diversity in grassland systems. Phyllosphere fungi are important in plant health as pathogens, commensals, and potential mutualists. We aimed to determine how host plant species affected the community-level diversity of phyllosphere fungi across a steep precipitation gradient and two co-occurring prairie species – big bluestem (*Andropogon gerardii*, Poaceae) and leadplant (*Amorpha canescens*, Fabaceae). We sampled leaves of both plant species from five sites across a portion of the precipitation gradient in Kansas, USA, each site with a different mean annual precipitation (MAP; 615 mm – 1038 mm). Leaf disks were homogenized, and the extracted DNA was Illumina MiSeq sequenced to characterize the foliar fungal communities. We compared several richness and diversity estimates using Wilcoxon rank sum tests and multiple linear regression analyses and tested for compositional differences among the communities using permutational analysis of variance analogs (PERMANOVA). The data suggested minor differences in the fungal communities between the two hosts, whereas MAP had no effect. Similarly, linear regression analyses indicated no host or precipitation gradient effects on the fungal community composition. Both phyllosphere fungal communities and the factors affecting them in prairie systems remain poorly understood and results suggest that more research is needed to fully understand the compounding biodiversity which includes microbial communities within this system.

Key Words: phyllosphere, fungal diversity, operational taxonomic unit, prairie

INTRODUCTION

Prairie ecosystem total area has declined across the United States' Great Plains due to anthropogenic environmental change, particularly the conversion to monocrop agriculture (Lark et al. 2020). Tallgrass prairie systems have suffered the greatest losses compared to other grassland types and have been reduced to 4% of their original extent (Samson and Knopf 1994). The largest contiguous tracts of tallgrass prairie today are in Kansas, whereas other states have lost up to 99% of their tallgrass prairie cover (Samson and Knopf 1994; Eilers and Roosa 1994). These losses have resulted in declines in plant, animal, and microbial diversity, richness, and functional groups (Sala et al. 2000; Hirsh et al. 2013; Jain et al. 2014). Prairies provide crucial ecosystem services such as carbon storage, water filtration, and soil degradation mitigation (DeLuca and Zabinski 2011). They also provide forage for livestock and other animals. Dominant plants in the tallgrass prairie are grasses such as *Andropogon gerardii* Vitman (Big Bluestem), *Sorghastrum nutans* (L.) Nash (Indiangrass), *Panicum virgatum* L. (Switchgrass), and *Schizachyrium scoparium* (Michx.) Nash (Little Bluestem). Forbs contribute substantially to floristic diversity and can increase in abundance after fires or other disturbances (Fuhlendorf and Engle 2004; Weir and Scasta 2017). These diverse plant communities host hyperdiverse fungal communities reflective of the plant diversity (Peršoh 2015) and lead to compounding diversity across trophic levels. Such diverse fungal communities can occur at the scale of plant leaves (e.g., Dea et al. 2022), as well as vary with nutrient quality within plants (Borruso et al. 2021; Lekberg et al.

2021).

One example of a unique fungal community is that of the phyllosphere fungi. These fungi can either occur on the leaf surface (epiphytic fungi) or within leaf tissues (endophytic fungi), and are all collectively called phyllosphere fungi. Phyllosphere fungi include plant pathogens but may also include taxa that can reduce pathogen infection in their host plants by spatial exclusion on the leaf surface otherwise open for pathogens (Blakeman and Fokkema 1982). Epiphytic fungi can also reduce the harmful UV radiation affecting the leaf surfaces (Barrera et al. 2020; Solhaug et al. 2003) and can aid host plant water retention (Rho and Kim 2017). A single plant organ (e.g., leaves) can support a diverse fungal community and individual host plants can filter fungal communities in prairies, resulting in distinct fungal assemblages associating with different host plants (Yao et al. 2019; DeMers and May 2021; Liu et al. 2021). Therefore, fungal diversity is an exaggerated reflection of botanical diversity, and fungal endophytes represent a diverse component of the prairie community, from within an individual plant to the ecosystem level (Saikkonen, Mikola, and Helander 2015).

Climate factors like precipitation can affect fungal community assembly and alter fungal diversity and community composition on plants and in soils (House and Bever 2018; Lagueux et al. 2020; Rudgers et al. 2021; Dea et al. 2022). For example, using Bray-Curtis dissimilarity matrices, DeMers and May (2021) documented a potential fungal community-level endophyte gradient along a precipitation gradient in Minnesota prairies. Data on the phyllosphere fungal diversity of tallgrass prairie plants are lacking and studies that focus on how these fungal communities differ among similar host species are few (LeBlanc et al. 2014). For example, LeBlanc et al. (2014) found rhizosphere fungal diversity to increase with plant diversity and fungal community structure to differ between species in the Fabaceae and Poaceae. *Andropogon gerardii* is a dominant, almost ubiquitous grass (Poaceae) in the tallgrass prairie ecosystem that depends on its symbiosis with mycorrhizal fungi (Anderson, Hetrick, and Wilson 1994; Hartnett and Wilson 1999). *Andropogon gerardii* can provide almost 100% of the canopy cover in some Kansas prairies (Hulbert 1986) and can account for up to 24% of the aboveground biomass (Owensby et al. 1993). However, the phyllosphere fungi that associate with this important dominant plant remain to be explored. *Amorpha canescens* Pursh (leadplant) coexists with *A. gerardii* and is a common semi-perennial legume (Fabaceae) in tallgrass prairies with N-fixing symbionts in its root nodules. This symbiosis with N-fixing rhizobia results in higher N content in *A. canescens* tissues compared to non-leguminous plants in the tallgrass prairie ecosystem (Adams et al. 2016). Further, *A. canescens* is widely distributed within the tallgrass prairie region and can occur at a high density relative to co-occurring forbs (Towne and Knapp 1996). We use these two important, but functionally distinct, prairie species as examples of hosts for fungal communities.

Worldwide, nitrogen content in leaves of N-fixing plants, such as those in the Fabaceae, is greater than leaf nitrogen content of non-N-fixing plants, such as grasses (Adams et al. 2016; Averill et al. 2019). Substrate nitrogen content may affect fungal growth rate, and some evidence suggests a correlation between foliar fungal communities and foliar nitrogen content in northeastern Kansas (Jumpponen and Jones 2010). Similarly, fungal communities respond to available nitrogen; atmospheric nitrogen deposition can increase fungal community diversity and functional groups in prairie soil and on deciduous tree leaves (Borruso et al. 2021; Lekberg et al. 2021). Because *A. canescens* is an N-fixing species, and *A. gerardii* is a grass, they would differ in N content and their C:N ratios in our areas of study and thus, in quality as a resource for fungal communities (Thomas and Asakawa 1993).

We assessed the effects of two common tallgrass prairie host species (*A. gerardii* and *A. canescens*) that likely differ in foliar tissue quality as habitat for foliar fungal communities along with effects of MAP gradient on phyllosphere fungi. We sampled leaves and analyzed MiSeq sequence data to characterize the phyllosphere fungal communities. We

Table 1. Characteristics of climate (MAP = mean annual precipitation, MAT = mean annual temperature), management, soil, and location of each site. Sites include Konza Prairie Biological Station (KNZ), Hays Agricultural Research Center (HAR), Rockefeller Prairie (JEF), Welda Prairie (WEL), and a private prairie in Saline County (SAL).

Site	Location	MAP (mm)	MAT (°C)	Management	Soil Type	County
WEL	38° 51', -99°23’	1038	13.4	patch-burn grazing, haying	Kenoma-Olpe (8780)	Anderson
JEF	39° 06', -99° 37'	982	12.6	haying	Pawnee (7501)	Jefferson
KNZ	38° 54', -97° 59'	864	12.8	prescribed burns	Reading (7174)	Riley
SAL	39° 03', -95° 12'	742	12.7	grazing	Wells-Edalgo (3495)	Saline
HAR	38° 11', -95° 16'	615	12.3	grazing	Harney (2612)	Ellis

hypothesized that our host species would present unique environments and would support different but overlapping fungal assemblages. Adding to host-based differences, we hypothesized that the steep precipitation gradient in Kansas would further modify habitat suitability to fungal communities. We expected that fungal communities on the mesic end of the precipitation gradient and those on *A. canescens* would be the most diverse.

METHODS

Field methods: Our study was conducted along a west to east precipitation gradient across the state of Kansas. Study sites were located at Hays Agricultural Research Center (HAR), a private prairie in Saline County (SAL), Konza Prairie Biological Station (KNZ), Rockefeller Prairie (JEF), and Welda Prairie (WEL). All sites were predominantly tallgrass prairie with management practices ranging from grazing and burning to haying, and along a precipitation that ranged from 615 to 1,038 mm annually (Table 1). Mean annual temperatures ranged from 12.3-13.4 °C among sites. The dominant flora at the sites was characteristic of typical tallgrass prairie with grasses such as *A. gerardii*, *S. nutans*, *S. scoparium*, and various forb species such as *A. canescens*, *Solidago* L. spp. (goldenrods), and *Helianthus* L. spp. (sunflowers). We chose the two focal host plants for this study because of functional differences and because of their widespread distribution and frequent occurrence in tallgrass prairies throughout the Great Plains (Great Plains Flora Association 1986).

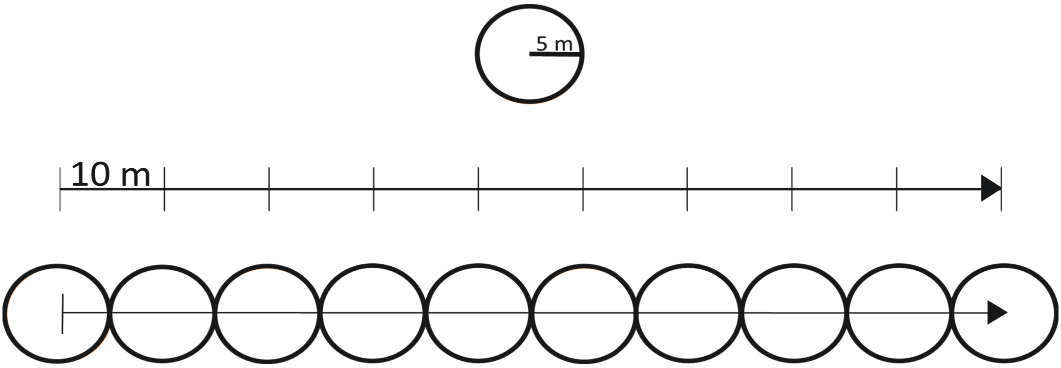

Figure 1. A schematic example of a transect used to gather host plant clippings. The center of each circle is a sampling start point 10 m from the next, each with a 5 m search radius for either *Andropogon gerardii* or *Amorpha canescens*, whichever was found first. Five specimens of each species were collected along each transect.

We laid 100 m transects starting at a random point at each site. Transects followed elevation isolines and represented the trajectory with the least variation in elevation from the starting point. A sample collection point (n = 10 per transect) was located every five meters along the transect to avoid overlapping the 5 m search radius of each point (Fig. 1). Plants from which clippings were taken were the individuals of each species closest to the sampling point, and within the 5 m radius of each point along the transect. A total of 10 plant individuals were sampled along each transect (five individuals for each of the two focal species). Only leaves without any clear pathogen infection were sampled to avoid skewing fungal community characteristics. For *A. canescens*, we sampled distal stems with new foliage, and for *A. gerardii*, we cut two to four mature blades at the base of each plant, excluding leaves on the flowering stalk. *Amorpha canescens* was absent from the SAL site, potentially because of selective grazing by cattle. We included the five *A. gerardii* leaf samples from this site in our analyses. Leaf samples were sealed into plastic bags and transported on ice to the laboratory within 24 hours (h) and stored in a -20 °C freezer for at least 24 h before sample processing.

Laboratory methods: A total of 16, 2 mm (diam.) discs were excised with a sterile biopsy punch from each individual plant (total surface area sampled = 0.5 cm^2 per plant). To remove unattached fungal spores and hyphae, discs were removed from the punch using a sterile dissecting needle and transferred to a 1.5 mL Eppendorf tube filled with 1,000 µL of 0.1% Triton-X. The discs were then shaken vigorously by hand for 30 s. After shaking, the solution was removed with a micropipette and the discs were rinsed by shaking them three times in sterile, deionized water for 30 s. The rinsed discs were transferred to a PowerSoil homogenization tube (2 mL) with PowerSoil beads (1.4 mm bead size, Qiagen, Hilden, Germany), two 2.4 mm zirconium beads, and 500 µL of dilution buffer (Plant Direct Phire Kit, Thermo Scientific) to homogenize the samples. The homogenization tubes with discs and beads were kept on ice and stored at -20 °C until all samples had been processed. The leaf disks were homogenized in a Savant Fastprep FP120 (MP Biomedicals, Irvine, CA) at speed six. Because Poaceae leaf tissues tend to be tougher than those of Fabaceae, we conducted a pilot study on extra host material to determine the optimal homogenization for each host. Based on that pilot study, the *A. canescens* discs were homogenized for 30 s, whereas the *A. gerardii* discs were homogenized for 60 s, with the addition of 200 µL of 0.15 mm garnet beads.

To choose the optimal dilution for PCR-amplification with the Phire Plant Direct Kits, we 10-fold diluted the leaf extracts (10^{-3} - 10^{0}) in sterile molecular grade RNA- and DNA-free water and compared the Internal Transcribed Spacer (ITS2) metabarcode amplification success with the forward primer fITS7 (Ihrmark et al. 2012) and the reverse primer ITS4 (White et al. 1990). The 10^{-2} dilution produced PCR-amplicons consistently and was chosen for library preparation. The ITS2 amplicons were generated with fITS7-ITS4 primers in 30 cycles with primer pads that permitted sample-specific indexing and MiSeq adapter addition in five PCR cycles. The MiSeq libraries were prepared at the Integrated Genomics Facility at Kansas State University. The raw sequence data are available at the sequence read archive under BioProject number PRJNA934065.

The data set initially consisted of 3,848,465 raw fungal sequences, which were processed using the mothur pipeline (v. 1.44.1, Schloss et al. 2009) as per the MiSeq standard operation protocol (Kozich et al. 2013) where possible. Sequences were extracted from paired-end .fastq files, contiged and any sequences with ambiguous bases, sequences with more than 1base pair (bp) mismatch with primer, or homopolymers longer than 9 bp were omitted. This resulted in a total of 3,007,181 sequences. The sequences were truncated to the length equal to the shortest high-quality read (237 bp excluding primers). The > 99% similar sequences were pre-clustered (Huse et al. 2008), checked for potential chimeras using UCHIME algorithm (Edgar et al. 2011) and putative chimeras were removed. The remaining sequences were assigned to the

Table 2. Multiple linear regression model statistics for fungal Operational Taxonomic Unit (OTU) community diversity, richness evenness, compositional estimates, and percentage of plant pathogen guild predicted by plant host species (Host) and mean annual precipitation centered around the mean (MAP) main effects and their interaction (Host x MAP) with *Amorpha canescens* as reference (0) compared to *Andropogon gerardii* (1). Statistically significant models and predictors (P<0.05) are bold-faced. Parameter estimate significances are denoted as ‘ns’ for not significant, ‘(*)’ for 0.05 ≤ P < 0.10, ‘*’ for 0.01 ≤ P < 0.05, ‘**’ for 0.001 ≤ P < 0.01, and ‘***’ for P < 0.001.

Response	Model	Predictor	Estimate ± SE	t-value
Richness (S_{Obs})	**$F_{3,38}$ = 4.13*, R^2_{adj} = 0.186**	**Intercept**	**105.00±15.90**	**6.60*** **
	AIC = 475.30	Host	–33.02±20.74	-1.59^{ns}
		MAP	**$2.8x10^{-1} \pm 1.1x10^{-1}$**	**2.67***
		Host × MAP	$-2.4x10^{-1} \pm 1.4x10^{-1}$	–1.79(*)
Diversity (H')	$F_{3,38}$ = 0.45^{ns}, R^2_{adj} = –0.042	**Intercept**	**$3.19 \pm 2.1x10^{-1}$**	**15.27*** **
	AIC=111.49	Host	$1.3x10^{-2} \pm 2.7x10^{-1}$	0.05^{ns}
		MAP	$1.5x10^{-3} \pm 1.3x10^{-3}$	1.14^{ns}
		Host × MAP	$-1.5x10^{-3} \pm 1.8x10^{-3}$	-0.86^{ns}
Evenness (E_H)	$F_{3,38}$ = 1.70^{ns}, R^2_{adj} = 0.049	**Intercept**	**$7.6x10^{-1} \pm 3.2x10^{-2}$**	**24.01*** **
	AIC = –46.66	Host	$5.5x10^{-2} \pm 4.2x10^{-2}$	1.34^{ns}
		MAP	$-2.3x10^{-4} \pm 2.1x10^{-4}$	-1.08^{ns}
		Host × MAP	$4.8x10^{-5} \pm 2.7x10^{-4}$	0.18^{ns}
PCoA Axis 1	$F_{3,38}$ = 0.59^{ns}, R^2_{adj} = –0.031	**Intercept**	**$-6.6x10^{-2} \pm 7.0x10^{-2}$**	**–0.94*** **
	AIC = 19.04	Host	$1.2x10^{-1} \pm 9.1x10^{-2}$	1.29^{ns}
		MAP	$-8.5x10^{-6} \pm 4.6x10^{-4}$	-0.02^{ns}
		Host × MAP	$9.6x10^{-5} \pm 6.0x10^{-4}$	0.16^{ns}
PCoA Axis 2	$F_{3,38}$ = 0.09^{ns}, R^2_{adj} = –0.071	Intercept	$-7.8x10^{-3} \pm 5.1x10^{-2}$	-0.15^{ns}
	AIC = –6.44	Host	$8.2x10^{-3} \pm 6.7x10^{-2}$	0.12^{ns}
		MAP	$9.9x10^{-5} \pm 3.4x10^{-4}$	0.29^{ns}
		Host × MAP	$-2.2x10^{-4} \pm 4.4x10^{-4}$	-0.49^{ns}
PCoA Axis 3	**$F_{3,38}$ = 3.33*, R^2_{adj} = 0.146**	Intercept	$3.4x10^{-2} \pm 3.7x10^{-2}$	0.92^{ns}
	AIC = –33.66	Host	$-3.3x10^{-2} \pm 4.9x10^{-2}$	-0.68^{ns}
		MAP	**$-7.3x10^{-4} \pm 2.5x10^{-4}$**	**–2.97** **
		Host × MAP	**$9.5x10^{-4} \pm 3.2x10^{-4}$**	**2.97** **
% Pathogens	$F_{3,38}$ = 2.78(*), R^2_{adj} = 0.115	**Intercept**	**$3.31 \pm 6.7x10^{-1}$**	**4.93*** **
	AIC = 209.53	Host	$-7.6x10^{-1} \pm 8.8x10^{-1}$	-0.86^{ns}
		MAP	$6.7x10^{-3} \pm 4.5x10^{-3}$	1.50^{ns}
		Host × MAP	**$-1.5x10^{-2} \pm 5.8x10^{-3}$**	**–2.58***

Operational Taxonomic Units (OTUs) at 97 % similarity and clustered using VSEARCH (Rognes et al. 2016). Rare OTUs (fewer than ten) and those that were detected in the negative controls were removed from further analyses. Remaining OTUs were assigned to taxa using the Naïve Bayesian Classifier (Wang et al. 2007) and International Nucleotide Sequence Database – reference database (UNITE) (Abarenkov et al. 2021). Non-target OTUs which did not match the UNITE dataset or were assigned to groups outside of the Kingdom Fungi were removed.

After removing poor quality, chimeric, and rare sequences, the final dataset included 1,426,547 sequences representing 886 OTUs (Coverage: 0.999 ± 0.002). We iteratively (100 iterations) estimated fungal richness and diversity for each sample using mothur (v. 1.44.1, Schloss et al. 2009). To minimize biases resulting from differences in sequencing depths among the libraries, we rarefied the sequence data to 8,500 sequences per sample as recommended in Gihring, Green, and Schadt (2012). To estimate richness and diversity, we estimated observed OTU richness (S_{Obs}), Shannon Diversity (H'), and Shannon Evenness (E_H).

Statistical analyses: We used R (R Core Team 2022) and RStudio (version 2022.7.1.554, RStudio Team 2022) for all statistical analyses and graphical data representations. Some experiment groups violated assumptions of normality (Shapiro Wilks tests) or homoscedasticity (Bartlett's test), so we chose to use a nonparametric test. To determine if one of the host species harbored greater fungal richness, diversity, or evenness, we used the Wilcoxon rank sum test to compare the fungal observed species richness (S_{Obs}), Shannon Diversity (H'), and Shannon Evenness (E_H) between plant host species within each site. To detect any relationships between fungal richness (S_{Obs}), diversity (H'), or evenness (E_H), and mean annual precipitation (MAP) or the two plant hosts, we used multiple linear regressions. Each model included the linear term "MAP" centered around the mean (861.11 mm/yr) and the categorical term "Host" as well as their interaction. We visually evaluated residuals to ensure they did not blatantly violate assumptions of linear regression analyses and performed outlier analyses. We identified outliers as those more than two standard deviations from the mean in each site-species combination and proceeded with linear regressions with and without outliers to evaluate the effects of potential outliers. These regressions yielded comparable results suggesting that the outliers minimally impacted our conclusions. As a result, we present here models with potential outliers retained. To further infer the ecology of the OTUs, we used the function "funguild_assign()" in R package "FunGuildR" (Nguyen et al. 2016). We used these data to test if the proportion of assigned plant pathogens differed between plant hosts and across the precipitation gradient. We calculated the percentage of sequences assigned to the guild "Plant Pathogen" and used Wilcoxon rank sum test and multiple linear regressions similar to those for richness and diversity (Host × MAP).

For community compositional analyses, we rarefied our community abundance data to 10,500 sequences per sample using the function "rarefy_even_depth()" in "phyloseq"(McMurdie and Holmes 2013). This resulted in the removal of three low-yielding samples (one *A. gerardii* sample from site JEF and two *A. canescens* samples from HAR) which were excluded from compositional analyses. We calculated the Bray-Curtis distance matrix and visualized it with Principal Coordinates Analysis (PCoA) using the "ordinate()" function in "phyloseq" (McMurdie and Holmes 2013). To test for compositional differences in fungal communities associated with the two host plants (main effect term nested within site) and between the sites along the MAP gradient, we used a permutational analysis of variance (PERMANOVA) with 10,000 permutations with function "adonis()" in the R package "vegan" (Oksanen et al. 2020). To test for any linear relationships between MAP and ordination space, we used multiple linear regression models with the first three PCoA axes as the response. These models included the linear term "MAP" centered around the mean (861.11 mm/yr) and the categorical term "Host" as well as their interaction, similar to the analyses for fungal richness and diversity.

Finally, to identify OTUs disproportionally more abundant in either of the two host species or in the various sites, we used function "multiplatt()" in R package "indicspecies" (v. 1.7.12, De Caceres and Legendre 2009) to run indicator taxon analysis (method = IndVal.g) and niche preference analysis (method=r.g). Both analyses compared either sites or plant hosts in three distinct data subsets: 1) the top 100 most abundant OTUs present in sites in which both hosts were sampled (SAL was excluded); 2) the top 100 most abundant OTUs in the two terminal sites (HAR and WEL); and 3) all OTUs assigned to the guild "Plant Pathogen" (64 OTUs total) present in sites in which both hosts were sampled (SAL was excluded). To correct for multiple testing, we used function "p.adjust()" and corrected acquired p-values for false discovery rate (FDR) in program R.

RESULTS

After quality control and removal of rare sequences, a total of 1,426,547 high quality sequences and 886 OTUs remained. Sequence yields ranged from 3,817 to 65,371 per sample with a mean yield of 31,701 ± 15,375 (SD). The fungal communities represented primarily Ascomycota (70.4% sequences and 68.3% OTUs) and Basidiomycota (28.2% sequences and 24.8% OTUs) with the remaining data (<1% sequences and ≤ 3% OTUs) representing Mortierellomycota, Chytridiomycota, Glomeromycota, Mucoromycota, Rozellomycota (following Tedersoo et al. 2018) and unclassified Fungi. Relative abundances of fungal orders can be found in Appendix Figure 1. A large majority of OTUs (78.0% sequences and 74.6% OTUs) were assigned to a genus. Among those with a genus-level assignment (373 genera total), the most abundant were *Darksidea* (3 OTUs, 4.57 % sequences), *Sporidiobolus* (2 OTUs, 4.46% sequences), and *Neoascochyta* (2 OTUs, 3.38% sequences). The additional ten most abundant genera were mainly fungi commonly observed in phyllosphere samples – including *Alternaria*, *Aureobasidium*, *Fusarium*, *Epicoccum*, *Magnaporthiopsis*, *Marasmiellus*, *Naganishia*, *Phaeosphaeria*, *Talaromyces*, and *Tilletiopsis*.

Our analyses provided no evidence for differences in fungal richness, diversity, or evenness between the two plant hosts within any of the sites (Wilcoxon: S_{Obs}: W < 6, P > 0.222; H': W < 18, P > 0.310; E_H: W < 17, P > 0.111). Multiple linear regression models explained a small proportion of the variation in fungal richness (Table 2). Fungal richness increased with MAP in *A. canescens* and there was some limited evidence that *A. canescens* harbored greater richness than *A. gerardii* as MAP increased (Table 2, Fig. 2a). However, there was no evidence for a host plant main effect showing that at the mean precipitation, fungal richness did not differ between the two plant hosts.

In contrast to richness, models poorly explained variation in fungal diversity, evenness, and first or second PCoA axis scores (Table 2, Fig. 3). Similarly, there was no evidence for an effect of plant host, MAP, or their interaction on fungal diversity, evenness, or first or second PCoA axes (Table 2, Fig. 2b-e).

Although there was no evidence for the host or MAP effects on the first two PCoA axes, models predicted that the third PCoA axis explained some variation (Table 2). There was strong evidence that with increasing MAP, the third PCoA axis scores decreased in *A. canescens* plants (Fig. 2f). Although third PCoA scores were not significantly different between the host plants at the mean precipitation, there was evidence for interaction between the hosts and MAP: *A. canescens* PCoA axis scores decreased whereas *A. gerardii* did not respond strongly.

Models explained a small proportion of the variation in percentage of fungi assigned to the guild plant pathogen in our samples (Table 2; Fig. 2g). There was evidence for interaction between plant host and MAP – with *A. gerardii* harboring lower percentage of plant pathogens with increasing MAP whereas the percentage did not change in *A. canescens* (Table 2, Fig. 2g). This was further shown in site-by-site comparisons of plant hosts: we found evidence of greater pathogen percent in *A. canescens* than *A. gerardii* in the terminal site WEL, while in sites with lower MAP, we found no

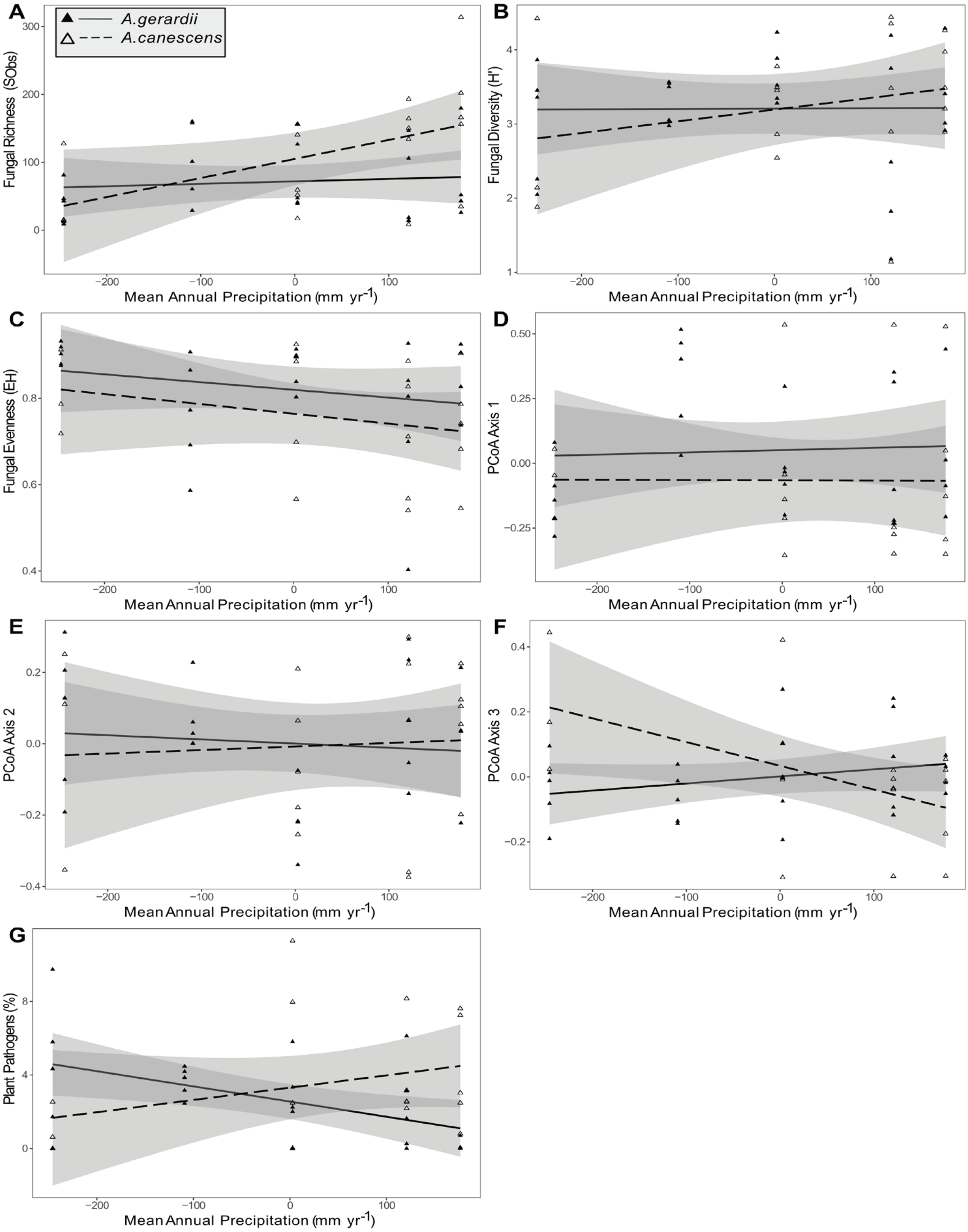

Figure 2. Fungal Operational Taxonomic Unit (OTU) responses to mean annual precipitation (MAP) centered around the mean in *Amorpha canescens* (dashed line and open symbols) and *Andropogon gerardii* (solid line and filled symbols). Models predicting observed species richness (S_{Obs}) (A), Shannon diversity (H') (B), evenness (E_H) (C), PCoA Axis 1 scores (D), PCoA Axis 2 scores (E), PCoA Axis 3 (F), and percent of plant pathogen guild (G). The shaded areas represent 95% confidence intervals around the model predictions.

evidence for differences between the hosts (Wilcoxon: HAR: W = 11.5, P > = 0.294; JEF: W = 7, P = 0.310; KNZ: W = 10.5, P = 0.753; WEL: W = 0, P = 0.019).

Our PERMANOVA provided no evidence for distinct fungal communities between the two host species ($F_{4,36} = 0.88$, $R^2 = 0.099$, P = 0.764) or among the sites (PERMANOVA: $F_{3,36} = 0.98$, $R^2 = 0.083$, P = 0.500) – a proxy for precipitation as each site differed in MAP. Similarly, analyses of dispersion aiming to evaluate the fungal community heterogeneity among samples provided no evidence for differences in dispersion between the two host species ($F_{1,36} = 0.04$, P = 0.854) or between sites ($F_{3,36} = 0.737$, P = 0.551).

Indicator taxon and niche preference analyses identified few or no indicator OTUs (P<0.05) between plant hosts or among the sites when we included the 100 most abundant OTUs in sites where both hosts were sampled (SAL was excluded), the 100 most abundant OTUs in terminal sites (HAR and WEL), or OTUs assigned to the guild "Plant Pathogen" (see Appendix Fig. 2 for guild proportions). No indicator OTUs in these analyses remained significant after false detection rate (FDR) correction for multiple testing (Appendix Table 1). Among the indicators that were significant before the FDR correction were an unidentified member of the genus *Coniothyrium* as an indicator for WEL site; for *A. canescens*, an unidentified member of the order Pleosporales as an indicator for WEL site and *A. gerardii*; and, a member of the genus *Curvularia* as an indicator for *A. canescens*. Our analyses identified far more indicator OTUs for *A. canescens* than for *A. gerardii* (~5 times more, Appendix Table 1).

DISCUSSION

We aimed to evaluate how foliar fungal communities associated with two host species representing two functional groups (a grass and a legume) would respond to MAP along the steep precipitation gradient in Kansas. Previous research has highlighted that edaphic

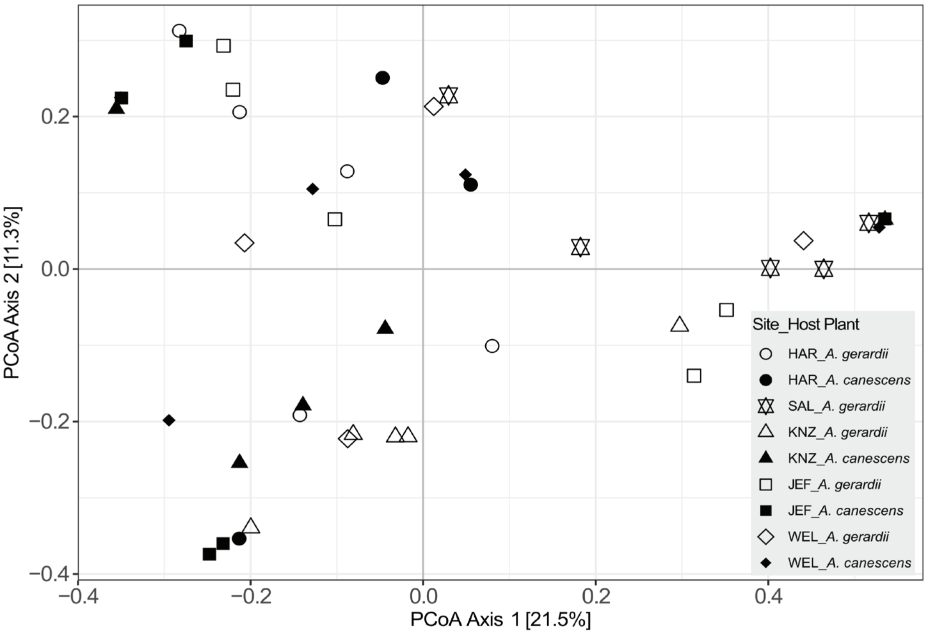

Figure 3. Principal Coordinates Analyses (PCoA) of fungal Operational Taxonomic Units (OTUs) in *Amorpha canescens* (open symbols) and *Andropogon gerardii* (filled symbols). Shapes indicate sites listed from least to most mean annual precipitation (circles = HAR, double triangle = SAL, triangle = KNZ, square = JEF, and diamond = WEL).

(Glynou et al. 2016; Rudgers et al. 2021) and climatic (Dea et al. 2022; Oita et al. 2021) variables may control the assembly of host-associated fungal communities. Similarly, host-associated communities may correlate with host communities (Dea et al. 2022) or may differ among the host species (U'Ren et al. 2012; Kembel et al. 2014). Foliar fungal communities can be particularly sensitive to climatic drivers and buffered against edaphic factors (Bowman and Arnold 2021; Oita et al. 2021) as the foliar communities do not directly interact with the soil matrix and are thus more susceptible to temporal and diurnal oscillations in the environment. Our data provided little support for responses to either host species or MAP. This is despite the two hosts representing distinct functional types and the relatively steep MAP gradient that ranged from 615mm to 1,038mm per year. Our conclusions disagree with others who have found that climatic factors strongly influence the phyllosphere fungal communities and their assembly. These studies differed from ours in several ways, however. For example, Carroll and Carroll (1978) compared fungal communities on gymnosperms at both high (dry) and low (wet) elevation sites; Zimmerman and Vitousek (2012) compared communities across a strong hydrologic and elevational gradient in Hawaii on Mauna Loa Volcano; U'Ren et al. (2012) compared fungal communities along a precipitation gradient, but at continental scale; Oita et al. (2021) compared communities at a landscape scale, but this was in the tropics and also spanned an elevation gradient; and finally, Dea et al. (2022) studied leaf fungal communities of prairies in Kansas, but their work spanned the entire precipitation gradient in Kansas sampling whole plant communities rather than a single target plant.

A lack of distinction between the foliar communities of the two hosts was surprising, particularly because differences have even been reported between co-occurring *Dalea* L. (prairie clover) species in Minnesota prairies (DeMers and May 2021). Equally surprising was the lack of any relationship between fungal diversity and MAP, because others (e.g., DeMers and May 2021; Dea et al. 2022) have documented strong differences based on sampling locations and their position along the MAP gradient in the Midwest. Despite the lack of differences in fungal communities between our plant hosts or among sampling sites, fungi represent a significant source of biodiversity, and affect ecosystem function (Perreault and Laforest-Lapointe 2022). It is of note that our sampling of 720 small leaf disks from 45 plant individuals (20 from *A. canescens* and 25 from *A. gerardii*) included 886 molecular OTUs thus emphasizing the hyperdiverse communities that phyllospheres host (see Arnold et al. 2000; Arnold et al. 2007; Jumpponen and Jones 2010).

Our data, overall, agree with earlier high throughput sequencing analyses that highlight foliar fungal communities dominated by ascomycetes (see Jumpponen and Jones 2009; Zimmerman and Vitousek 2012; Oita et al. 2021; Dea et al. 2022). Approximately 70% of our sequence data were assigned to ascomycetes, whereas the next dominant taxon - Phylum Basidiomycota – represented less than 30% of the acquired data. Among the OTUs represented by the greatest sequence counts were common foliar inhabitants, e.g., Pleosporalean genera *Alternaria* and *Epicoccum*, Dothidealean genus *Aureobasidium*, Eurotialean genus *Talaromyces*, and the basidiomycetous yeast *Tilletiopsis*, representing potential plant pathogens and saprobes. The common OTUs also included some surprising taxa. Among these was the genus *Darksidea* to which three OTUs were assigned. Fungi in genus *Darksidea* are Pleosporalean root-colonizing endophytes that have been frequently reported in European (Knapp, Pintye, and Kovács 2012; 2015) and North American grasslands (Romero-Jimenez et al. 2022). We lack a sound explanation for this observation, although it is possible that the fungi occupying belowground tissues may colonize their hosts systematically or adhere to the foliar tissues even after our surface washing.

Even though our community-wide PERMANOVAs provided no support for shifts in the foliar fungal communities, our indicator taxon analyses revealed several OTUs that were either more abundant in one host or occurred more frequently at one site than at the others before correction for multiple testing. OTUs associated with *A. gerardii* remained unclassified below the level of order and represented fungal groups that include many taxa commonly associated with plant tissues (OTUs assigned only to Sordariomycetes and Pleosporales). In contrast, the indicator OTUs for *A. canescens* included OTUs assigned to genus *Coniothyrium*, *Dictyosporella*, and *Mycena*. In general, these *A. canescens* indicators represent common leaf-associated fungi. For example, genus *Coniothyrium* includes plant-associated Pleosporalean mycoparasites (e.g., Whipps et al. 2008) and plant endophytes, antagonists, and pathogens (e.g., Peters et al. 1998; Berg 2009). Further, species of *Mycena* have been generally considered saprotrophic (Læssøe et al. 1996; Emmett et al. 2008), or when colonizing plant tissues, considered latent saprotrophs (Osono 2010; Kohout et al. 2018). However, recent research has documented some as endophytes that associate with roots of many plant hosts (see, e.g., Roy et al. 2021; Thoen et al. 2020). The foliar *Mycena* spp. may indeed represent either latent foliar saprotrophs or indicate presence of leaf spot disease caused by some species of *Mycena* (e.g., Avelino et al. 2007). Why these taxa might be overrepresented in *A. canescens* remains unclear, but may reflect host preference for a landscape position, differences in host susceptibility, and/or quality of plant tissue available for fungal colonization. We also observed indicator OTUs (assigned to ascomycete genera *Coniothyrium*, *Phaeosphaeria*, and *Sclerostagonospora*, and the basidiomycete genus *Marasmiellus*) that were more abundant at sites in the mesic end of our gradient (KNZ and WEL). These OTUs are not unexpected as they represent common foliar tissue associates, plant endophytes, pathogens and saprobes (e.g., Peters et al. 1998; Phookamsak et al. 2014; Oliveira et al. 2019). It may be helpful to consider these OTUs infrequent or near absent in arid sites, suggesting that the more arid end of our gradient may lie at the edge of their environmental tolerances.

Our study is a snapshot of the phyllosphere fungal communities during one summer. Our sampling alternated between arid and mesic sites to avoid temporally confounding sampling as it was stretched between the months of June and July. The lack of support for our MAP hypothesis may be attributable to seasonal dynamics of fungal communities. Jumpponen and Jones (2010) dissected foliar fungal communities and sampled *Quercus macrocarpa* leaves in northeastern Kansas six times during one growing season. They identified clear seasonal shifts in these phyllosphere communities, suggesting that 1) we might have detected greater variation among sampling areas and/or between hosts had we collected specimens over a longer period and 2) the seasonal heterogeneity in the fungal communities may have masked the hypothesized community differences. In addition to the seasonal variability, plant-associated fungal communities may have interannual dynamics and vary among years. For example, Farner, Spear, and Mordecai (2020) sampled the perennial bunchgrass, *Stipa pulchra* Hitchc., in California and documented interannual dynamics in culturable fungal pathogen communities.

Although our sampling covered nearly a two-fold range in precipitation, sampling over a greater span of precipitation could have been beneficial. Our observed (though often nonsignificant) trends suggest that sampling further into the drier west and further into the wetter southeast parts of the precipitation gradient might have provided a clearer separation of the communities as a function of MAP. However, locating co-occurring populations of the two target species became increasingly more challenging the further into the arid sites we traveled. Although our data did not support our hypotheses, our study contributes towards a better understanding of the hyperdiverse foliar fungal communities in prairie ecosystems. When considering the microbial communities in prairie restoration, this information is useful for understanding where inoculant soils could possibly be obtained for transfer to restored locations (Koziol and Bever 2016).

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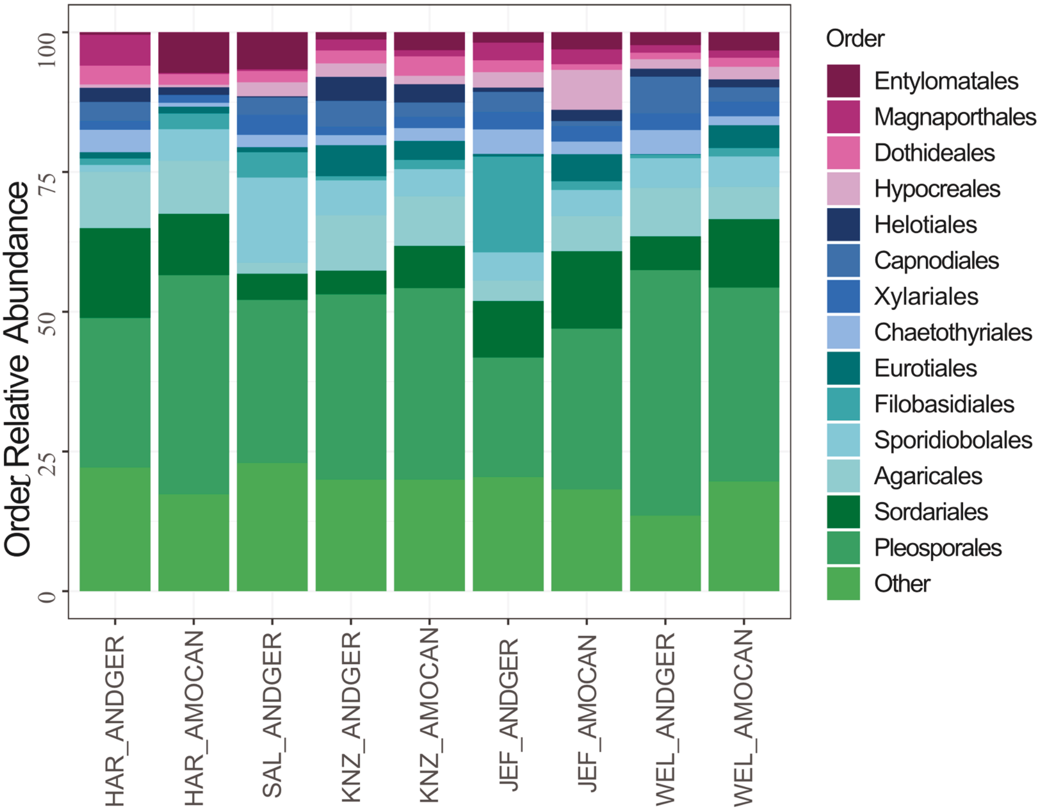
APPENDIX

Appendix Table 1. Indicator Taxon Analysis results from analyses run on 3 different subsets of the data: the top 100 most abundant OTUs in all sites where both species were found (SAL was excluded) listed as “All Sites”, the top 100 most abundant OTUs in the terminal sites (HAR and WEL) listed as “Terminal”, and all OTUs assigned exclusively to the guild “Plant Pathogen” by FunGuild (64 OTUs total) in all sites where both species were found. Analyses were run comparing the plant hosts or sites. Included also are OTU number, Group indicated, statistic, P-value, significance of P-value, P-value after correction for multiple testing (p.FDR), taxonomic resolution (Level), and Taxon name.

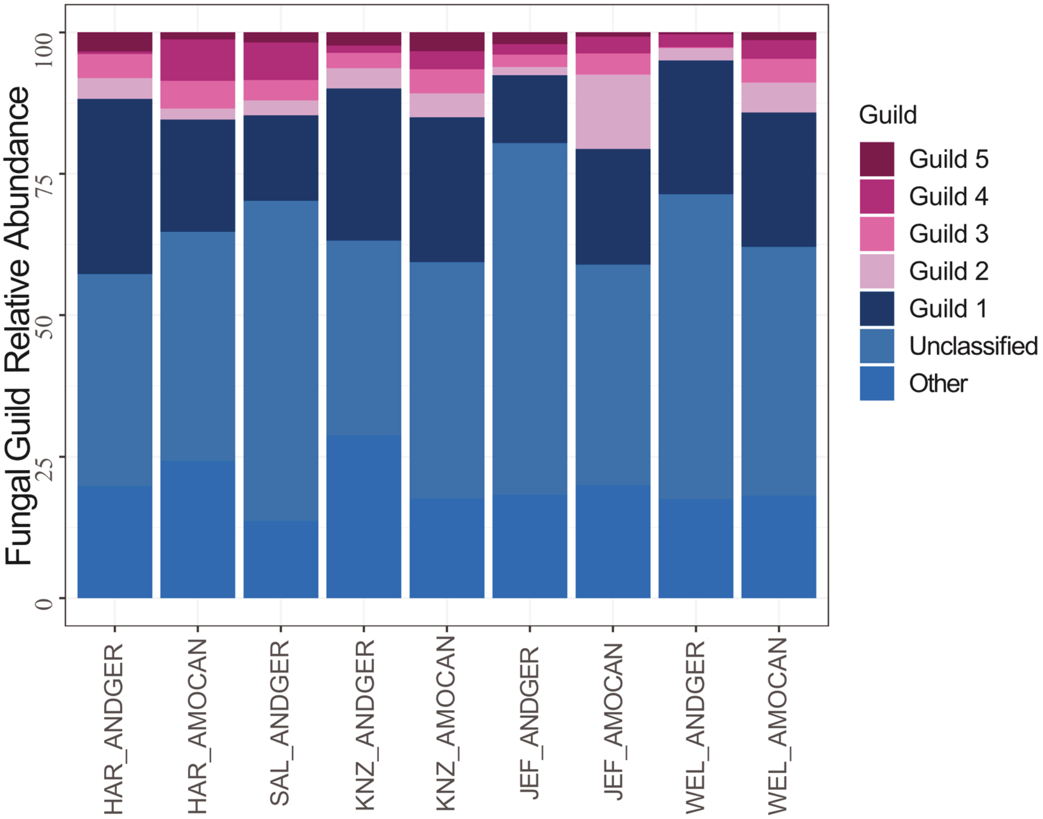
Data subset	Compare	OTU	Group	Stat	p.value	sig	p.FDR	Level	Taxon
All Sites	Hosts	Otu0077	*A. gerardii*	0.56	0.026	*	0.65	Order	*Pleosporales*
All Sites	Hosts	Otu0100	*A. canescens*	0.53	0.02	*	0.65	Genus	*Coniothyrium*
All Sites	Hosts	Otu0057	*A. canescens*	0.71	0.021	*	0.65	Genus	*Fusarium*
All Sites	Hosts	Otu0087	*A. canescens*	0.6	0.023	*	0.65	Species	*Mycena olida*
All Sites	Hosts	Otu0204	*A. canescens*	0.47	0.045	*	0.9	Genus	*Dictyosporella*
All Sites	Site	Otu0083	KNZ	0.63	0.008	**	0.2	Family	*Mycosphaerellaceae*
All Sites	Site	Otu0058	KNZ	0.71	0.017	*	0.34	Genus	*Sclerostagonospora*
All Sites	Site	Otu0043	KNZ	0.66	0.025	*	0.417	Species	*Marasmiellus tricolor*
All Sites	Site	Otu0022	WEL	0.76	0.003	**	0.2	Genus	*Phaeopoacea*
All Sites	Site	Otu0077	WEL	0.7	0.004	**	0.2	Order	Pleosporales
All Sites	Site	Otu0100	WEL	0.67	0.007	**	0.2	Genus	*Coniothyrium*
All Sites	Site	Otu0025	WEL	0.64	0.031	*	0.443	Species	*Phaeosphaeria microscopica*
Terminal	Hosts	Otu0077	*A. gerardii*	0.75	0.042	*	0.85	Order	Pleosporales
Pathogens	Hosts	Otu0092	*A. canescens*	0.6	0.016	*	0.398	Genus	*Curvularia*
Pathogens	Hosts	Otu0349	*A. canescens*	0.47	0.044	*	0.398	Genus	*Drechslera*
Pathogens	Hosts	Otu0433	*A. canescens*	0.47	0.045	*	0.398	Genus	*Monosporascus*
Pathogens	Hosts	Otu0488	*A. canescens*	0.47	0.045	*	0.398	Genus	*Monosporascus*
Terminal	Hosts	Otu0100	*A. canescens*	0.79	0.015	*	0.85	Genus	*Coniothyrium*
Terminal	Hosts	Otu0160	*A. canescens*	0.71	0.031	*	0.85	Order	Pleosporales
Terminal	Hosts	Otu0028	*A. canescens*	0.76	0.034	*	0.85	Genus	*Psathyrella*
Terminal	Hosts	Otu0092	*A. canescens*	0.71	0.05	*	0.85	Genus	*Curvularia*

Appendix Table 2. Niche Preference Analysis results from analyses run on 3 different subsets of the data: the top 100 most abundant OTUs in all sites where both species were found (SAL was excluded) listed as "All Sites", the top 100 most abundant OTUs in the terminal sites (HAR and WEL) listed as "Terminal", and all OTUs assigned exclusively to the guild "Plant Pathogen" by FunGuild (64 OTUs total) in all sites where both species were found. Analyses were run comparing the plant hosts or sites. Included also are OTU number, Group indicated, statistic, P-value, significance of P-value, P-value after correction for multiple testing (p.FDR), taxonomic resolution (Level), and Taxon name.

Data Subset	Compare	OTU	Group	Stat	p.value	sig	p.FDR	Level	Taxon
All Sites	Hosts	Otu0077	*A. gerardii*	0.26	0.02	*	0.7	Order	*Pleosporales*
All Sites	Hosts	Otu0047	*A. gerardii*	0.28	0.029	*	0.7	Class	*Sordariomycetes*
All Sites	Hosts	Otu0087	*A. canescens*	0.35	0.022	*	0.7	Species	*Mycena olida*
All Sites	Hosts	Otu0100	*A. canescens*	0.25	0.024	*	0.7	Genus	*Coniothyrium*
All Sites	Hosts	Otu0204	*A. canescens*	0.17	0.046	*	0.7	Genus	*Dictyosporella*
All Sites	Site	Otu0043	KNZ	0.51	0.01	**	0.73	Species	*Marasmiellus tricolor*
All Sites	Site	Otu0063	KNZ	0.37	0.031	*	0.73	Class	Sordariomycetes
All Sites	Site	Otu0022	WEL	0.47	0.022	*	0.73	Genus	*Phaeopoacea*
All Sites	Site	Otu0077	WEL	0.39	0.023	*	0.73	Order	Pleosporales
All Sites	Site	Otu0100	WEL	0.42	0.05	*	0.73	Genus	*Coniothyrium*
Pathogens	Hosts	Otu0092	*A. canescens*	0.26	0.037	*	0.438	Genus	*Curvularia*
Pathogens	Hosts	Otu0358	*A. canescens*	0.25	0.042	*	0.438	Species	*Curvularia inaequalis*
Terminal	Hosts	Otu0100	*A. canescens*	0.38	0.005	**	0.5	Genus	*Coniothyrium*
Terminal	Hosts	Otu0160	*A. canescens*	0.28	0.022	*	0.833	Order	Pleosporales
Terminal	Hosts	Otu0092	*A. canescens*	0.35	0.025	*	0.833	Genus	*Curvularia*
Terminal	Hosts	Otu0159	*A. canescens*	0.38	0.049	*	0.904	Genus	*Phaeosphaeria*
Terminal	Site	Otu0029	*WEL*	0.53	0.02	*	0.88	Order	Pleosporales
Terminal	Site	Otu0108	*WEL*	0.43	0.034	*	0.88	Species	*Rachicladosporium*
Terminal	Site	Otu0023	*WEL*	0.33	0.045	*	0.88	Family	Gomphillaceae



Appendix Figure 1. Relative abundance of fungal orders within each site-host combination with two letter identifiers for plant host (ANDGER = *Andropogon gerardii* and AMOCAN= *Amorpha canescens*). Orders that made up <2% total sequences were grouped into the "Other" category.



Appendix Figure 2. Abundance of fungal orders within each site-host combination with two letter identifiers for plant host (ANDGER= *Andropogon gerardii* and AMOCAN = *Amorpha canescens*). See Appendix Table 2 for taxonomic information. Guild 1 = Undefined Saprotroph; Guild 2 = Endophyte, Guild 3 = Plant Pathogen; Guild 4 = Endophyte-Litter Saprotroph-Undefined Saprotroph; Guild 5= Animal Pathogen-Endophyte-Epiphyte-Plant Pathogen-Undefined Saprotroph; Guilds that made up <2% total sequences were grouped into the “Other” category.