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RESEARCH

# Fungicide-Mediated Shifts in the Foliar Fungal Community of an Invasive Grass

**Brett R. Lane,[1] Amy E. Kendig,[2] Christopher M. Wojan,[3] Ashish Adhikari,[1] Michelle A. Jusino,[4] Nicholas Kortessis,[5] Margaret W. Simon,[5] Robert D. Holt,[5] Matthew E. Smith,[1] Keith Clay,[3,6] S. Luke Flory,[2] Philip F. Harmon,[1] and Erica M. Goss[1,7,†]**

[1] Plant Pathology Department, University of Florida, Gainesville, FL 32611
[2] Agronomy Department, University of Florida, Gainesville, FL 32611
[3] Department of Biology, Indiana University, Bloomington, IN 47405
[4] United States Department of Agriculture Forest Service, Northern Research Station, Center for Forest Mycology Research, Madison, WI 53726
[5] Department of Biology, University of Florida, Gainesville, FL 32611
[6] Department of Ecology & Evolutionary Biology, Tulane University, New Orleans, LA 70118
[7] Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610

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## ABSTRACT

Invasive plants, which cause substantial economic and ecological impacts, acquire both pathogens and beneficial microbes in their introduced ranges. Communities of fungal endophytes are known to mediate impacts of pathogens on plant fitness but few studies have examined the temporal dynamics of fungal communities on invasive plants. The annual grass *Microstegium vimineum,* an invader of forests and riparian areas throughout the eastern United States, experiences annual epidemics of disease caused by *Bipolaris* pathogens. Our objective was to characterize the dynamics of foliar fungal communities on *M. vimineum* over a growing season during a foliar disease epidemic. First, we asked how the fungal community in the phyllosphere changed over 2 months that corresponded with increasing disease severity. Second, we experimentally suppressed disease with fungicide in half of the plots and asked how the treatment affected fungal community diversity and composition. We found increasingly diverse foliar fungal communities and substantial changes in community composition between timepoints using high-throughput amplicon sequencing of the internal transcribed spacer 2 region. Monthly fungicide application caused shifts in fungal community composition relative to control samples. Fungicide application increased diversity at the late-season timepoint, suggesting that it suppressed dominant fungicide sensitive taxa and allowed other fungal taxa to flourish. These results raise new questions regarding the roles of putative endophytes found in the phyllosphere given the limited number of pathogens known to cause disease on *M. vimineum* in its invasive range.

*Keywords*: *Bipolaris*, invasive species, iprodione, microbiome, *Microstegium vimineum*, mycobiome, phyllosphere, plant pathogen, stiltgrass

The plant microbiome comprises a diverse array of microorganisms, including bacteria, filamentous fungi, and yeasts, among others (Lindow and Brandl 2003). Microbiomes can provide protective effects; fungi, in particular, play a significant role in plant health through the production of antimicrobial compounds (Yu et al. 2010), production of plant hormones that can influence growth of their

[†]Corresponding author: E. M. Goss; emgoss@ufl.edu

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hosts (Chanclud and Morel 2016; Foo et al. 2019), and exclusion of pathogens through resource competition and plant defense priming (Pieterse et at. 2014; Vannier et al. 2019). Pathogenic fungi are also common members of microbiomes. When pathogen infection is not suppressed by host defenses or the plant's microbial community, pathogen establishment can lead to dysbiosis and disruption of beneficial microbial functions (Paasch and He 2021).

Invasive plants are nonnative species that spread rapidly, often with negative ecological and economic impacts (Diagne et al. 2021; Kumschick et al. 2015). In general, invasive plants have fewer pathogens in their introduced range than in their native range (Mitchell and Power 2003), and pathogens establish on invasive plants with increasing time since first introduction (Reinhart and Callaway 2006; Stricker et al. 2016; Sun and He 2010). Although pathogens of invasive plants are often investigated for their potential as biocontrol agents, recent studies have shown that endophytic fungi also colonize invasive plants in their expanded range (Busby et al. 2015; Chen et al. 2020; Currie et al. 2020; Garbelotto et al. 2019; Garnica et al. 2022; Gonzalez Mateu et al. 2020; Mei et al. 2014; Pickett et al. 2022; Shahrtash and Brown 2020; Shipunov et al. 2008). Although these studies indicate the potential importance of endophyte community composition on invasive fitness and biocontrol efficacy, seasonal dynamics of microbial communities have not been considered. Incorporating microbiome dynamics in studies of invasive plants is warranted given our emerging understanding of the role of the microbiome in disease resistance, plant fitness, and plant community diversity (Trivedi et al. 2020).

*Microstegium vimineum* (stiltgrass) is a C4 annual grass originating in Asia that has invaded much of the midwestern and eastern United States, including disturbed areas such as roadsides and crop–forest interfaces, but also intact forests and riparian areas. Although *M. vimineum* was introduced to the United States in the early 1900s, no fungal pathogens were reported on it until 2001 (Lane et al. 2020; Roane 2004). Starting in 2009, multiple *Bipolaris* pathogens were isolated from foliar lesions on *M. vimineum* (Flory et al. 2011; Kleczewski and Flory 2010), and subsequent surveys identified epidemics of disease caused by *Bipolaris* spp. on *M. vimineum* populations across the eastern United States (Lane et al. 2020; Manamgoda et al. 2013; Stricker et al. 2016). Further research showed that *Bipolaris* reduced invader biomass and allowed recovery of native plant populations (Flory and Clay 2013; Stricker et al. 2016; Warren et al. 2011).

Here, we report results of an experiment in which we applied fungicide at 1-month intervals to *M. vimineum* to suppress *Bipolaris* spp. and examined dynamics of the *M. vimineum* foliar fungal communities over time. Previous studies have demonstrated that application of the site-specific, trans-laminar penetrant, dicarboximide fungicide iprodione significantly shifts soil fungal and bacterial communities in greenhouse and laboratory experiments (Miñambres et al. 2010; Wang et al. 2004) but less is known about the response of foliar fungal communities to repeated fungicide application, commonly used to study impacts of disease on *M. vimineum* (Flory et al. 2011; Kendig et al. 2021; Stricker et al. 2016). Our objectives were to (i) characterize changes in the fungal community across three timepoints that corresponded to increasing leaf spot disease severity and (ii) determine how monthly applications of iprodione affected the *M. vimineum* fungal community.

We used high-throughput amplicon sequencing of the internal transcribed spacer 2 (ITS2) region to characterize foliar fungal communities 1 month posttreatment to capture persistent responses to fungicide rather than the acute response. We compared fungicide-treated plots with plots that were treated with water only, which served as a control for assembly and succession of *M. vimineum* foliar fungal communities over the growing season. Because the *Bipolaris* epidemic was naturally occurring, we could not include treatments with no disease that were independent of fungicide treatment. Overall, our goal was to describe the *M. vimineum* foliar fungal community as a first step toward incorporating foliar microbiomes into studies of invasive plant–foliar pathogen dynamics and impacts.

## MATERIALS AND METHODS

**Field experiment.** In April 2019, experimental plots were established at three sites in the Big Oaks National Wildlife Refuge in Madison, IN. Each site was invaded by *M. vimineum,* and all populations had shown symptoms of leaf spot caused by *Bipolaris gigantea* the previous year (Lane et al. 2020). Plots (1 $m^2$) were established in the center of a 2-by-2-m area that was cleared of all plants by 4% glyphosate herbicide application (GlyStar Plus, 41% glyphosate concentrate; Albaugh, LLC, Ankeny, IA, U.S.A.) and raking. In total, 20 plots were established at each of the three experimental sites. *M. vimineum* seedlings were grown in a greenhouse at Indiana University for 5 weeks, followed by 1 week of outdoor acclimation prior to transplant. On 7 to 10 May 2019, 6-week-old *M. vimineum* plants were transplanted into each plot. Ten random plots from each site were assigned to receive 1.5% iprodione (Ipro 2SE, 23.8% iprodione; ADAMA, Raleigh, NC, U.S.A.) applications, while the remaining plots were sprayed with water as a control. For both treatments, plants were sprayed with backpack sprayers until runoff. The application of treatments began on 10 May and continued every 4 weeks through the end of the experiment for a total of four applications.

We collected *M. vimineum* leaves from each plot at three timepoints: 2 July, 1 August, and 30 August. Each collection was 4 weeks posttreatment and was conducted immediately prior to the subsequent fungicide or water application. The first collection occurred after two rounds of fungicide applications in May and June. At each timepoint, one leaf (excluding petiole) with median disease severity was selected from each of three random *M. vimineum* tillers per plot, regardless of the position within the canopy. The three leaves from each plot made up one sample. Leaf age was not known and not considered in sampling. Leaves were shipped overnight to the University of Florida on dry ice, where they were immediately stored at –20°C until DNA extraction. At the same time, a random *M. vimineum* tiller was selected from three plants per plot and the total number of leaves on the tiller was counted, as well as the proportion of leaves on the tiller with at least two lesions, to estimate the proportion of infected leaves. One leaf with a median number of lesions for the tiller was collected from each of the three *M. vimineum* plants, separate from the leaves collected for fungal community characterization, to estimate plot disease severity. Images of these leaves were taken with a flatbed scanner and processed with ImageJ version 2.0.0 (Rasband 2014) for percent lesion area as a measure of disease severity. The areas classified as lesions were reviewed manually to improve accuracy. Total disease severity per plot was determined by multiplying the proportion of infected leaf tissue by the proportion of infected leaves per tiller.

**Characterization of the fungal community.** *Molecular methods.* The three *M. vimineum* leaves collected from each plot for microbial analysis were bulked in a single 2-ml bead beating tube (Fisher Scientific, Waltham, MA, U.S.A.) containing three heat-sterilized (250°C for 12 h) 4.5-mm steel beads (Daisy Outdoor Products, Rogers, AR, U.S.A.). Leaves were lyophilized overnight in a FreeZone 2.5 Plus (Labconco Corporation, Kansas City, MO, U.S.A.), then ground for 1 min in a Fastprep-24 bead beater (MP Biomedicals, Solon, OH, U.S.A.). Immediately following bead

beating, 750 µl of sterile cetyltrimethylammonium bromide (CTAB) extraction solution (Teknova, Hollister, CA, U.S.A.) was added to each tube, and tubes were vortexed and placed at –20°C overnight. DNA was extracted using the protocol described by Lindner and Banik (2009). Briefly, tubes were placed in a 65°C water bath for 2 h, cellular debris was then pelleted by centrifugation (10,000 relative centrifugal force) for 1 min, and 100 µl of supernatant was transferred to a strip tube for DNA extraction. DNA was precipitated using 150 µl of isopropanol and washed with 175 µl of 70% ethanol. The DNA was then bound to 2.5 µl of glass milk in 45 µl of molecular-grade water and 135 µl of an NaI solution. Bound DNA was washed with 175 µl of New Wash buffer and eluted in 50 µl of molecular-grade water. The glass milk, NaI solution, and New Wash buffer were obtained from the GeneClean III kit (MP Biomedicals, Irvine, CA, U.S.A.).

An aliquot of extracted DNA was diluted 1:20 for PCR amplification. A partial segment of the ITS2 was amplified using primers fITS7 and ITS4 (Ihrmark et al. 2012; White et al. 1990) modified with Illumina Nextera v2 adapters (Illumina Inc., San Diego, CA, U.S.A.). Each reaction contained 7.5 µl of 2× GoTaq Green Master Mix (Promega Corp., Madison, WI), 0.12 µl of bovine serum albumen (BSA) (New England Biolabs, Ipswich, MA, U.S.A.), primers to a final concentration of 0.2 µM, 2 µl of diluted DNA, and molecular-grade water to a final volume of 15 µl. The PCR conditions were 94°C for 3 min, followed by 37 cycles of 94°C for 30 s, 60°C for 30 s, stepping down 0.5°C each cycle until reaching 55°C, and an extension at 72°C for 1 min (Gilmartin et al. 2022; Palmer et al. 2018). The final extension was conducted at 72°C for 7 min. Amplification was confirmed by agarose gel visualization. A second step of PCR amplification was conducted to dual index the above product using Illumina Nextera v2 indices and adapters (Illumina Inc.). The reactions were identical to the preceding PCR, except for annealing at 55°C for eight cycles. Indexed samples were quantified by comparison of bands on agarose gels in comparison with Low DNA Mass Ladder (Fisher Scientific).

In addition to our samples, we also included negative controls in lyophilization, DNA extraction, and both sequence amplification and indexing (each carried through subsequent steps) and two positive controls in PCR amplification. The first positive control comprised 14 fungal species which we anticipated to be present in our samples (Supplementary Table S1). The second positive control was the synthetic mock control SynMock (Palmer et al. 2018), a nonbiological ITS community comprising sequences of varying length. Both positive controls were amplified with Nextera modified fITS7 and ITS4 primers and were indexed as described above. Samples and controls of similar concentration were combined into sets of three for the removal of primers, dNTPs, salts, and other post-PCR contaminants using Zymo Select-A-Size spin columns (Zymo Research, Irvine, CA, U.S.A.). Cleaned products were quantified using a Qubit 4.0 fluorometer and 1× double-stranded DNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and combined into a single amplicon library in equimolar concentrations. Because the negative control samples contained little to no detectable DNA, these samples were included in the library using the median volume of all other samples. The amplicon library was shipped overnight to the University of California–Riverside, Institute for Integrative Genome Biology, for sequencing using 2 × bp sequencing on an Illumina MiSeq.

**Sequence processing.** We processed our high-throughput amplicon data using AMPtk version 1.5.1, a bioinformatics pipeline that employs SynMock, for improved analysis of variable-length amplicons by reducing the effects of tag-switching in multiplexed sequencing runs (Palmer et al. 2018). In this pipeline, paired reads were merged using USEARCH version 9.2.64 (Edgar 2010); then, the forward and reverse ITS primers were removed. Any reads shorter than 125 bp were discarded. Reads longer than 300 bp were trimmed to 300 bp to address known biases in the UPARSE pipeline which arise from the use of reads of unequal length (Palmer et al. 2018). Samples containing fewer than 10,000 reads were removed prior to clustering; control samples were maintained regardless of read number. The cluster function within AMPtk is a wrapper that combines quality filtering and read clustering using the UPARSE algorithm. First, reads were quality filtered with expected errors <1.0. Expected errors is calculated as a function of Phred scores and read length. Then, duplicate reads were removed and sorted by abundance. Reads were then clustered into operational taxonomic units (OTUs) at 97% similarity and duplicated reads were mapped back to OTUs. Finally, the hybrid taxonomy algorithm in AMPtk was used for taxonomic assignments of OTUs using the UNITE database version 8.2 (Nilsson et al. 2019). In brief, the AMPtk hybrid taxonomy algorithm performs a global alignment for each read against the UNITE database using USEARCH. If the global alignment is >97% identity, that hit is retained. If the global alignment is <97% identical, then the best hit of the Bayesian classifiers UTAX (Edgar 2013) and SINTAX (Edgar 2016) is retained. OTUs not identified as fungi were removed. Because many fungal species share >97% sequence identity to other members of their respective genus, OTUs were not taxonomically assigned beyond the genus level. The *decontam* package was used to identify contaminating OTUs using negative control samples (Davis et al. 2018). No contaminating OTUs were identified.

**Data analysis.** All statistical analyses were conducted in R version 4.0.3 (R Core Team 2020) using the packages *car, lme4, lmerTest, microbiome, phyloseq,* and *vegan* (Bates et al. 2015; Fox and Weisberg 2019; Kuznetsova et al. 2017; Lahti and Shetty 2019; McMurdie and Holmes 2013; Oksanen et al. 2020). Data visualizations were produced using the package *ggplot2* (Wickham et al. 2016).

Adequate sequencing depth was confirmed by visual inspections for saturation of rarefaction curves. For analysis of α-diversity metrics, samples were rarified to the depth of the sample with the smallest number of reads. We calculated the observed species richness and Shannon diversity index using the *estimate_richness* function on rarified data. α-Diversity metrics were transformed and normality was confirmed through the Shapiro-Wilk test of normality and by visual inspection of residuals for normality and homogeneity of variance (Shapiro and Wilk 1965). The observed species richness was transformed using the natural log while Shannon diversity and evenness were squared. To test for differences in α-diversity metrics among three timepoints and between two treatments, we fit a linear mixed-effects model using the *lmer* function with time point, treatment, and their interaction as fixed effects. To test for statistical significance of fixed effects, we performed an analysis of variance with type III sum of squares. To account for block effects from the three sites and repeated measures of the 20 plots within each site, we included plot and site as crossed random intercepts. Pairwise comparisons of treatments within timepoints were conducted as described above with a linear mixed-effects model, using treatment as a fixed effect and site as a random intercept.

For analyses of community composition, OTUs were scaled to relative abundances within samples without rarefaction to prevent the loss of read data. Principal coordinates analysis ordination plots were calculated using the *ordinate* function in *vegan* using Bray-Curtis dissimilarity. The homogeneity of group variance for timepoint and timepoint–treatment was tested with the *betadisper* function in *vegan*. Significance was determined using the *permutest* function with 10,000 permutations. Posthoc analyses were conducted using Tukey's honestly significant difference (HSD).

Current nonparametric statistical methods for microbial community composition data are limited in their ability to handle repeated measures, which were included in our experimental design that sampled the same plots at three timepoints. Thus, we limited our analysis of variation over time to visualization of fungal communities via ordination plots and direct pairwise comparisons. We tested the effect of treatment on community composition summarized by both the Bray-Curtis and Jaccard dissimilarity metrics on relative abundances within sample. Comparisons were conducted at each timepoint using permutational multivariate analysis of variance (PERMANOVA) with the *adonis* function using 10,000 permutations (Anderson 2001).

We identified OTUs of interest by two approaches. First, analysis of compositions of microbiomes with bias correction (ANCOM-BC) was used to identify OTUs which were differentially abundant between treatments (Lin and Peddada 2020). Our second approach was to use the *core_members* function of the *microbiome* package to identify OTUs which were core for a treatment at a given timepoint (i.e., detected in at least 90% of samples). As before, ANCOM-BC was used to test core OTUs shared between treatments for significant differences in relative abundance. The above approaches were also used at the family level, the lowest level at which at least two-thirds of the OTUs were identified.

## RESULTS

**Sequence processing.** After quality filtering, 7.0 million reads were clustered into 3,989 OTUs. Using the filter function in AMPtk, 675 singleton OTUs, 6 chimera OTUs, and 12 synthetic mock OTUs were removed. The remaining 3,296 OTUs, containing 6,744,155 reads, underwent taxonomic assignment where 3,257 OTUs were identified. Of these, 32 OTUs (332 reads) were assigned to the clade Viridiplantae and were discarded. In total, 2,644 OTUs were assigned to at least the order level, comprising 96.0% of the reads in 110 orders and 322 families. There were 16 OTUs that each exceeded 1% of the total reads, 97 OTUs each with least 0.1% of reads, and 303 OTUs with at least 0.01% of reads. An analysis of our positive control samples revealed that they were assigned to the expected taxa. No contaminating taxa were identified in the negative controls using the *decontam* package.

**Fungal communities changed through the growing season.** Fungal communities of *M. vimineum* leaves changed markedly over time, with time playing a larger role in community composition than treatment. The detected fungal community on *M. vimineum* leaves was least diverse in July and most diverse in late August, regardless of diversity measure (Fig. 1). Late August samples were significantly more diverse, when measured using Shannon diversity, than July and early August samples. Late August samples were distinct in composition from earlier samples when analyzed by Bray-Curtis dissimilarity and projected onto two dimensions (Fig. 2A). Although the bulk (90%) of the July samples differed in OTU composition from the other timepoints, four July samples were more like the early August samples. Results for Jaccard dissimilatory were consistent with Bray-Curtis dissimilarity. Dispersion from the centroid differed significantly among timepoints (Betadisper Permutest); July samples were significantly more dispersed than early or late August samples (Tukey's HSD), indicating more variation in fungal communities among samples collected at the July timepoint than at later timepoints.

We also analyzed the core OTUs, defined as OTUs detected in at least 90% of the samples. In total, 11 and 13 core OTUs were detected in early and late August when samples were analyzed by timepoint. Among July samples, there were no core OTUs detected. Only a single core OTU, assigned to the genus *Pseudopithomyces*, was shared between early and late August. When all months were combined, no core OTUs were detected.

Core families, which were families detected in at least 90% of samples, totaled 13, 31, and 37 in July, early August, and late August, respectively. Most core families were shared between at least two timepoints and no core family was unique to July. When all timepoints were combined, there were 21 core families.

**Fungicide treatment effects on fungal communities.** Our analysis of α-diversity metrics across timepoints revealed that fungicide-treated samples had significantly higher Shannon's diversity overall (Table 1). Comparison of treatments within timepoints revealed a significant increase in Shannon's diversity in the fungicide treatment in late August but not in earlier timepoints (Fig. 1). There was not a significant effect of treatment on the number of observed OTUs (Table 1). Fungicide treatment altered community composition, as measured by dissimilarity metrics using relative

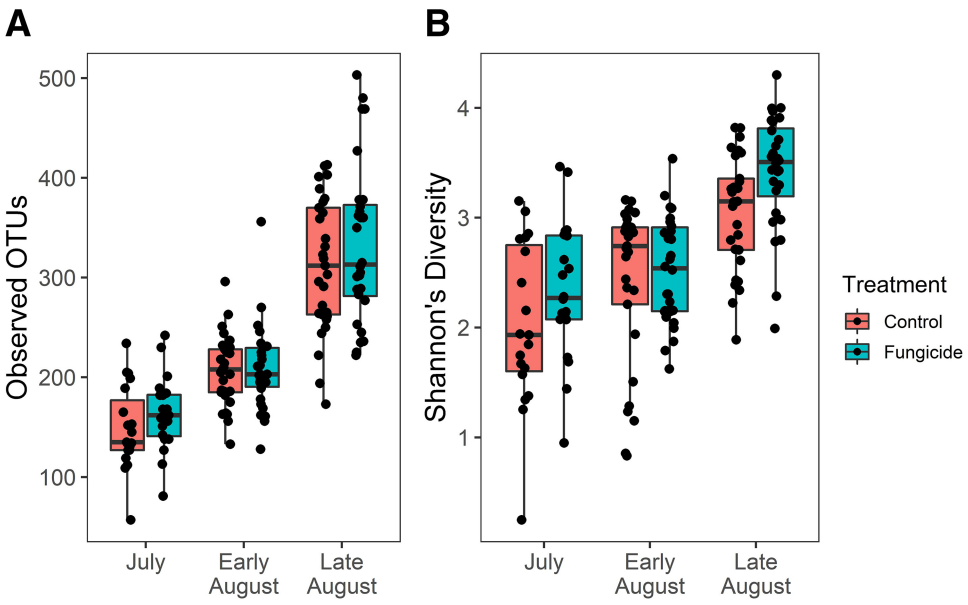


**Fig. 1.** Microbial richness and diversity on leaves from control and fungicide-treated plots over time. **A,** Observed operational taxonomic units (OTUs) and **B,** Shannon's diversity. OTUs were formed at a 97% similarity threshold. Observed OTUs were significantly different between all timepoints. Shannon's diversity was significantly higher in late August than at earlier timepoints. There were significant differences between treatments only for Shannon's diversity in late August. Posthoc comparisons were conducted using Tukey's honestly significant difference.

abundances, in early and late August (PERMANOVA, $P < 0.001$) but not July (Fig. 2B). Similar results were observed at the family level, where treatment had a significant effect on family composition in early and late August (Table 2; Fig. 3). The $r^2$ values were higher in early August than late August (Table 2), suggesting that treatment explained more variation in relative abundances of OTUs in early August. *Bipolaris* taxa (11 total OTUs) increased in relative abundance in each subsequent timepoint, comprising 0.36% (6 OTUs), 0.76% (9 OTUs), and 1.34% (8 OTUs) of the total reads in July, early August, and late August, respectively, and were significantly more abundant in the control samples than in fungicide-treated samples in early and late August (Table 3). We did not observe a significant difference between treatments in *Bipolaris* relative abundance in our July samples. Disease severity in *M. vimineum* plots was estimated to be just over 1% at the time of the July sampling, and there was no significant difference between fungicide-treated and control plots (Table 3). Disease severity increased throughout the experiment, and the control samples were significantly more diseased than fungicide-treated plots, on average, at both August timepoints.

**Fungicide application resulted in differentially enriched OTUs.** We found that fungicide application was associated with differential abundance of individual OTUs and families in early and late August (Supplementary Tables S2 and S3). We did not observe this same pattern in July, where there were no significant differences between fungal communities, likely due to the large variability between samples within treatments.

We found 15 OTUs that were significantly more abundant in the control samples in early August, all of which belonged to the order Pleosporales (Supplementary Table S2). The majority of these belonged to the families Pleosporaceae or Didymellaceae. Five OTUs were significantly more abundant in the fungicide samples in early August, all of which were assigned to the family Nectriaceae in the order Hypocreales. In late August, 12 OTUs were significantly enriched in the control samples, the majority of which were assigned to the families Mycosphaerellaceae and Phaeosphaeriaceae. Ten OTUs were significantly more abundant in the fungicide samples, of which nine were assigned to the division Basidiomycota. Only a single OTU in the division Ascomycota was determined to be more abundant in the fungicide samples, identified as an *Aureobasidum* sp. Of the nine OTUs assigned to the division Basidiomycota, five were identified as members of the order Tremellales.

At the family level, six families were determined to be differentially abundant between the control and fungicide samples in early August (Supplementary Table S3). Four families, all of which were members of the order Pleosporales, were significantly more abundant in the control samples. Two families, Nectriaceae and Sporidiobolaceae, were significantly more abundant in the fungicide samples. By late August, only two families (Phaeosphaeriaceae and Gomphillaceae) were significantly more abundant in the control samples, while eight families were significantly enriched in the fungicide samples (Supplementary Table S3; Fig. 3).

**Fungicide application caused shifts in core fungi.** Fungicide and control samples were also analyzed for changes in the core

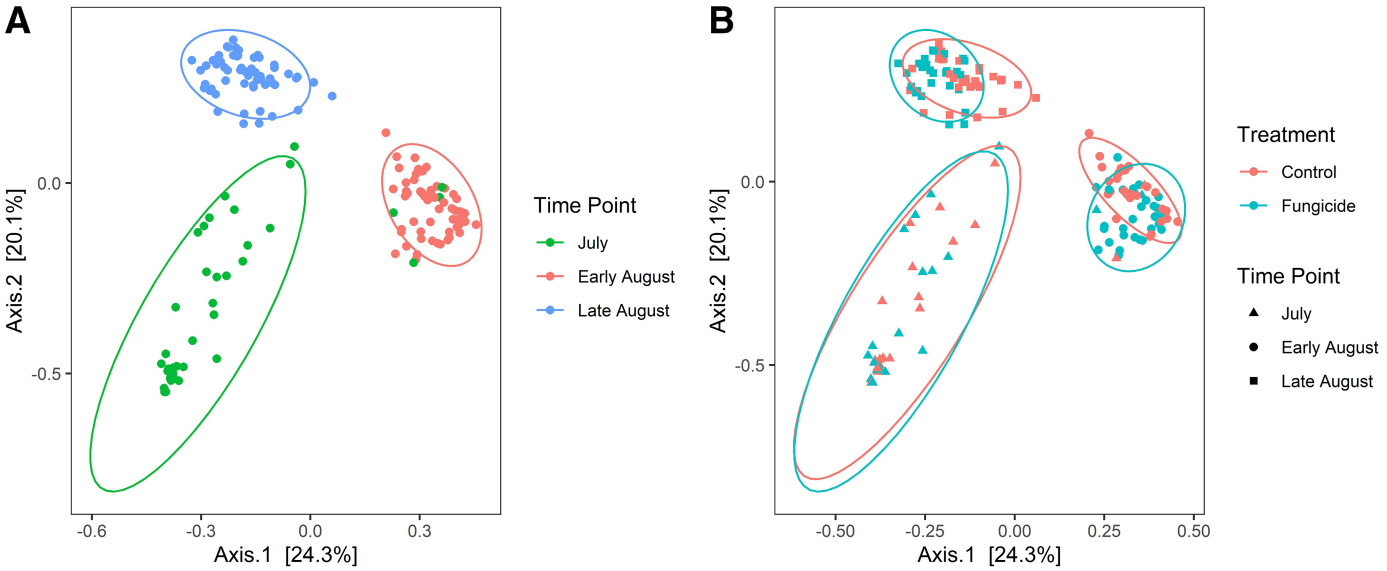


**Fig. 2.** Principal coordinate analysis of foliar fungal communities by plot using Bray-Curtis dissimilarity. Ellipses show grouping of samples by **A,** timepoint and **B,** treatment.

**TABLE 1**
**Results of two-way analysis of variance of microbial richness and diversity, measured as observed operational taxonomic units (OTUs) and Shannon's diversity, by timepoint and treatment[a]**

| | Observed OTUs | | | Shannon's diversity | | |
|---|---|---|---|---|---|---|
| | df | *F* | *P* value | df | *F* | *P* value |
| Timepoint | 2 | 138.7348 | **<0.001** | 2 | 53.9128 | **<0.001** |
| Treatment | 1 | 2.2018 | 0.144 | 1 | 6.1913 | **0.016** |
| Timepoint × treatment | 2 | 0.6087 | 0.546 | 2 | 2.2647 | 0.109 |

[a] Experimental site and plot were included as random effects to account for block effects and repeated measures, respectively. Numbers in bold indicate significant effects based on *P* values less than 0.05.

community when separated by treatment (Fig. 4; Supplementary Table S4). No core OTUs were detected in either treatment for the July timepoint. In early August, eight OTUs were core in both treatment groups and six of these belonged to the order Pleosporales. An additional 15 of the 17 core OTUs unique to the control group were members of the order Pleosporales. In late August, the core OTUs unique to the control samples were dominated by Pleosporales and Mycosphaerellaceae species. In early August, two of the three core OTUs unique to the fungicide samples belonged to the genus *Fusarium*; the third was an unidentified member of the same family, Nectriaceae. In contrast, by late August, 6 of the 10 core OTUs unique to the fungicide samples were in division Basidiomycota.

Taxonomic assignment to the family level revealed 8 families core to both treatments out of 11 overall at the July timepoint. In early August, 28 core families were shared between the treatments; only 3 and 1 core families were unique to the fungicide or control treatments, respectively. By late August, the number of shared core families increased to 35, with no families unique to the control group.

## DISCUSSION

We documented diverse foliar fungal communities associated with the invasive grass *M. vimineum* that shifted in composition over the course of the growing season and an epidemic caused by *Bipolaris* spp. Large changes in community composition between monthly timepoints occurred across samples, suggesting that seasonal dynamics were the primary driver of community composition. We also observed that monthly fungicide application caused shifts in fungal community composition in the phyllosphere relative to control samples. Fungicide-treated samples had higher Shannon's diversity at the late-season timepoint but not more observed OTUs, suggesting that fungicide treatment suppressed the dominance of iprodione-sensitive taxa in the microbial community and allowed other fungal taxa to flourish.

**TABLE 2**
**Permutational multivariate analysis of variance analysis testing effect of fungicide treatment on microbial community composition, as described by Bray-Curtis and Jaccard dissimilarity, segregated by timepoint[a]**

| | OTUs | | | | Family | | | |
|---|---|---|---|---|---|---|---|---|
| Month | df | *F* | $R^2$ | *P* value | df | *F* | $R^2$ | *P* value |
| Bray-Curtis | | | | | | | | |
| July | 1 | 0.633 | 0.0163 | 1 | 1 | 0.930 | 0.0241 | 1 |
| Early August | 1 | 15.905 | 0.1693 | **0.001** | 1 | 27.525 | 0.2751 | **0.001** |
| Late August | 1 | 7.736 | 0.1004 | **0.001** | 1 | 9.696 | 0.1276 | **0.001** |
| Jaccard | | | | | | | | |
| July | 1 | 0.700 | 0.01798 | 1 | 1 | 0.700 | 0.01798 | 1 |
| Early August | 1 | 9.507 | 0.11585 | **0.001** | 1 | 9.507 | 0.11585 | **0.001** |
| Late August | 1 | 5.123 | 0.07394 | **0.001** | 1 | 5.123 | 0.07394 | **0.001** |

[a] OTUs = operational taxonomic units. *P* values were corrected for multiple comparisons using a false discovery rate correction (FDR). Adonis, as integrated by vegan, limits *P* values to a minimum value of 0.001. Thus, an FDR correction was not utilized on *P* values of 0.001. Numbers in bold indicate significant differences between treatments.

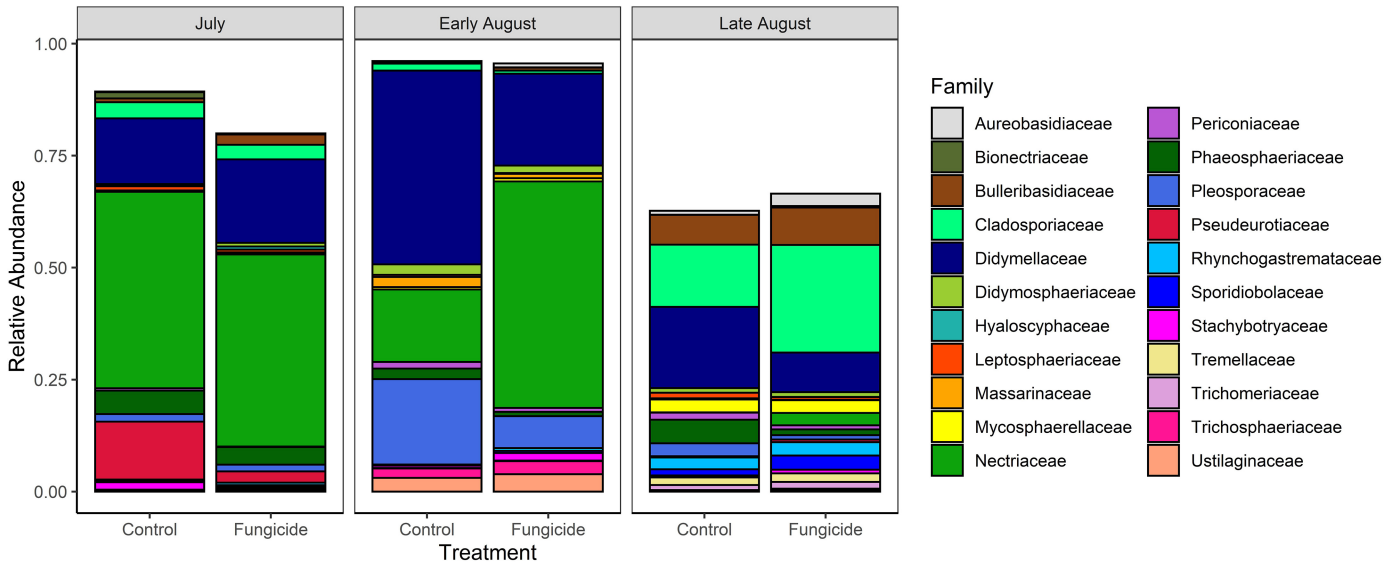


**Fig. 3.** Relative abundance of families by timepoint and treatment. Families displayed were among the top 10 most abundant families for at least one timepoint or treatment.

Early-season foliar fungal communities, represented by our July samples that were collected 8 weeks after planting, were less diverse in overall fungal richness and were significantly more variable among plots than fungal communities in early or late August. In July, fungal communities also lacked a detectable core group of taxa. These results indicate that fungal communities on *M. vimineum* were still undergoing recruitment and had not established their core mycobiome by early July. Disease severity was also low, suggesting that *Bipolaris* pathogens did not play a major role in community assembly up to early July. Our results are consistent with previous studies that have found that the microbial community of early vegetative phase growth in crop plants is highly variable before convergence and stabilization during late-phase vegetative and reproductive growth (Copeland et al. 2015; Edwards et al. 2018; Wei et al. 2019). Microbial assemblages are formed by a continuum of stochastic and deterministic processes. Stochastic processes often play their largest role in periods of primary succession, such as the initial formation of a foliar microbiome. During this time, the influx of early colonizers can overshadow deterministic processes such as environmental conditions, carbon and nutrient availability, and microbial interactions (Aydogan et al. 2018; Hassani et al. 2018; Leveau 2019). Following the initial establishment of a microbial community, deterministic processes can play an increasingly larger role in community assembly (Dini-Andreote et al. 2015).

Indeed, by early August, the assemblages of fungi across plots had converged in composition. Both August timepoints had core communities of 11 and 13 OTUs, respectively, but with a near complete turnover in core OTUs from early to late August; only a single core OTU was shared between these timepoints. Previous studies have established that the microbial communities of annual plants are transient throughout the growing season (Comeau et al. 2020; Moroenyane et al. 2021). Changes in community composition may be driven by plant growth stage, temperature, and sunlight availability, among other factors (Dumbrell et al. 2011; Unterseher et al. 2007). Although we did not control for leaf age, *M. vimineum* produces new tillers throughout the summer that were included in our samples. Although we expect that variation in leaf age caused variation in fungal community composition within samples, it is likely that this was only one of several factors responsible for shifts in fungal communities among timepoints. When we analyzed changes in fungal communities at the family level to examine the phylogenetic structure of the shift in community composition over time, we observed that over half of the core families were shared among timepoints. The contrast in the number of shared core OTUs versus core families between timepoints suggests that some of the shifts in fungal taxa over time were occurring within families. Because phylogenetically related species are likely to have shared similar niches through evolution, they often share traits for establishment and growth in a given host (Brown and Jumpponen 2015; Philippot et al. 2010). Thus, we hypothesize that these within-family shifts are a result of biotic and abiotic factors that select for taxonomic groups.

**TABLE 3**
**Disease severity and relative abundance of sequencing reads classified as *Bipolaris*[a]**

| | Disease severity | | | Relative abundance of *Bipolaris* reads | | |
|---|---|---|---|---|---|---|
| Month | Control (SE) | Fungicide (SE) | *P* value | Control (SE) | Fungicide (SE) | *P* value |
| July | 1.3% (0.33) | 1.2% (0.35) | 0.971 | 0.03% (0.02) | 0.67% (0.49) | 0.609 |
| Early August | 19.7% (3.4) | 9.4% (2.14) | **0.039** | 1.41% (0.37) | 0.14% (0.04) | **0.006** |
| Late August | 36.7% (3.94) | 22.7% (2.14) | **0.009** | 2.13% (0.65) | 0.52% (0.28) | **0.042** |

[a] Disease severity per plot was determined from ImageJ analysis of leaf scans and compared between treatments using Student's *t* test. *P* values were corrected for multiple comparisons using a false discovery rate correction. SE = standard error. Significant differences in relative abundance of *Bipolaris* reads between treatments were determined using analysis of compositions of microbiomes with bias correction. Numbers in bold indicate significant differences between treatments within disease severity or relative abundance of *Bipolaris* reads.

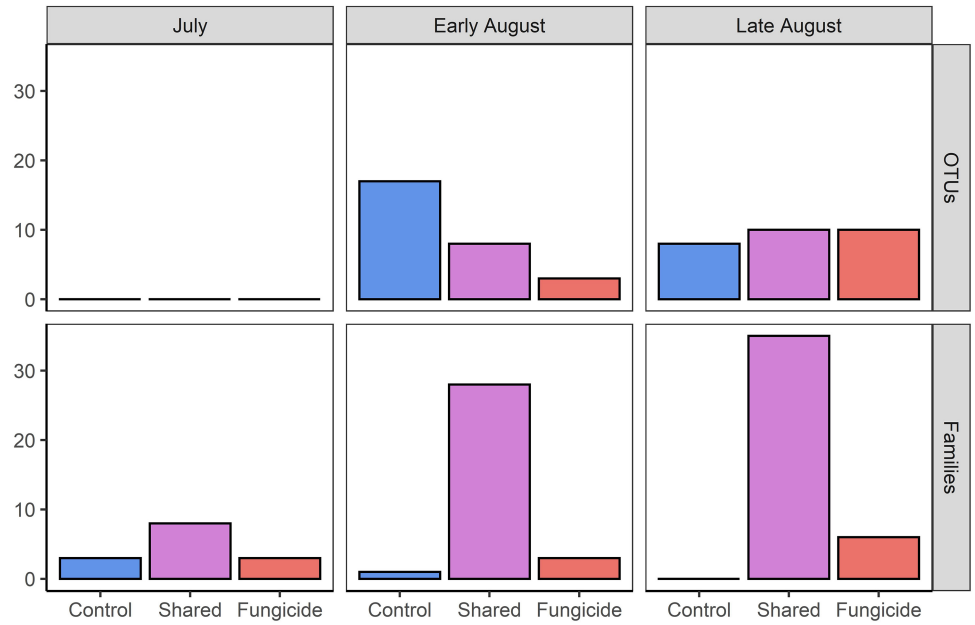


**Fig. 4.** Core operational taxonomic units (OTUs) and core families by timepoint and treatment. Shown are the number of core OTUs or core families specific to treatment or shared between treatments. Core OTUs and core families were defined as those detected in at least 90% of the samples. There were no core OTUs for the July timepoint.

Iprodione is a dicarboximide fungicide labeled for the treatment of numerous pathogens within the family Pleosporaceae. It is commonly used for the management of fungal diseases by soil drenching or foliar application (Katsoula et al. 2020). The iprodione product used in this study and similar products are labeled and used to manage diseases of crops and turfgrass caused by *Bipolaris* spp., with reapplication intervals between 14 and 28 days. Although our application of iprodione was intended to suppress the *Bipolaris* epidemic on *M. vimineum*, iprodione has been demonstrated to be effective on numerous species within the order Pleosporales (Forcelini and Reis 1988; Maude et al. 1984; Wetzel and Butler 2001) among other labelled taxa such as *Botrytis* (order Helotiales), *Monilinia* (order Helotiales), and *Rhizoctonia* (order Cantharellales). One month post fungicide application, the species in order Pleosporales were diminished in the samples from fungicide-treated plots, as expected. Although we likely observed the amplification of DNA from nonviable cells, the growth of fungicide-tolerant or -insensitive, viable cells would most likely be reflected in the differential enrichment of OTUs. In early August, OTUs enriched in the fungicide-treated samples were identified as *Fusarium* spp. or other members of the family Nectriaceae. Although iprodione is labeled for the treatment of some plant-pathogenic *Fusarium* spp., other fusaria are insensitive or resistant to the fungicide (Smiley and Craven 1979). Iprodione has also been used as selective agent for the isolation of some *Fusarium* spp. (Abildgren et al. 1987; Thrane 1996). Katsoula et al. (2020) showed that iprodione application was associated with an increase in the relative abundance of species of class Sordariomycetes, including family Nectriaceae and genus *Fusarium*, on pepper plants. Similar to species of the order Pleosporales, many members of the family Nectriaceae have adopted endophytic lifestyles (Kuldau and Yates 2000). Thus, we hypothesize that, when the application of iprodione resulted in the reduction of the order Pleosporales, the vacated niche became colonized by members of the family Nectriaceae. In our late August samples, 9 of the 10 OTUs significantly more abundant in the fungicide-treated samples were identified as members of division Basidiomycota. The most frequent of these were members of the order Tremellales, an order of dimorphic fungi that are well-known mycoparasites (Kurtzman et al. 2011). The single Ascomycota species enriched in the late August fungicide-treated samples was identified as a species of genus *Aureobasidium*, a well-known colonizer of the phylloplane which is known for its suppression of fungal postharvest pathogens (Fonseca and Inacio 2006; Freimoser et al. 2019). As mycoparasites, members of the order Tremellales and genus *Aureobasidium* could reduce disease and pathogen transmission (Adhikari et al. 2018; Konsue et al. 2020) or survival of fungal tissue in overwintering debris, lowering the initial inoculum in the following year, potentially to the benefit of the invasive plant (Fraser et al. 2021). The enrichment of putatively mycoparasitic taxa in the fungicide samples may suggest that these organisms were suppressed by iprodione-sensitive taxa. Alternatively, as potential inhabitants of healthy tissue, putative mycoparasites may be indirectly enriched in fungicide-treated plots due to the lower disease severity in these plots. Comparisons with nondiseased *M. vimineum* would clarify the prevalence and role of mycoparasites in this invasive plant.

In this study, we found a diverse and dynamic fungal community in the phyllosphere of the widespread invasive grass *M. vimineum* in eastern U.S. forests. The lack of significant loss of fungal richness and Shannon's diversity throughout the season when we sprayed fungicide monthly after planting suggests strong recruitment of fungi to the phyllosphere. However, like iprodione, most fungicides have a broad spectrum of activity and indiscriminately inhibit both pathogenic and nontarget fungi. Suppression of nontarget fungi may have indirect effects on plant health. For example, Henriksen and Elen (2005) found that the application of the mixture of fungicides cyprodinil and propiconazole (anilinopyrimidine and ergosterol synthesis inhibitor, respectively; product Stereo 312.5 EC) to barley and wheat too early preceding flowering suppressed *Fusarium* antagonists or competitors and resulted in an increased prevalence of Fusarium head blight. The ubiquitous but iprodione-sensitive species *Epicoccum nigrum* has antagonistic effects on several *Fusarium* spp., including *Fusarium graminearum* and *F. verticillioides*, two major cereal pathogens that also act as endophytes of various grass species (Jensen et al. 2016; Lofgren et al. 2018; Luongo et al. 2005; Ogórek and Pląskowska 2011). Genus *Epicoccum* was among the most frequently identified taxa from the *M. vimineum* phyllosphere and is one of multiple potential foliar endophytes identified in this study. Given the limited number of pathogens known to cause disease on *M. vimineum* in its invasive range and the diversity of putative endophytes found in its phyllosphere, the role of endophytes in protecting *M. vimineum* from pathogens shifting to this abundant host may be of interest.

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