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PLANT MICROBE INTERACTIONS

Plant Host and Geographic Location Drive Endophyte Community Composition in the Face of Perturbation

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Abstract All plants form symbioses with endophytic fungi, which affect host plant health and function. Most endophytic fungi are horizontally transmitted, and consequently, local environment and geographic location greatly influence endophyte community composition. Growing evidence also suggests that identity of the plant host (e.g., species, genotype) can be important in shaping endophyte communities. However, little is known about how disturbances to plants affect their fungal symbiont communities. The goal of this study was to test if disturbances, from both natural and anthropogenic sources, can alter endophyte communities independent of geographic location or plant host identity. Using the plant species white snakeroot (*Ageratina altissima*; Asteraceae), we conducted two experiments that tested the effect of perturbation on endophyte communities. First, we examined endophyte response to leaf mining insect activity, a natural perturbation, in three replicate populations. Second, for one population, we applied fungicide to plant leaves to test endophyte community response to an anthropogenic perturbation. Using culture-based methods and Sanger sequencing of fungal isolates, we then examined abundance, diversity, and community structure of endophytic fungi in leaves subjected to perturbations by leaf mining and fungicide application. Our results show that plant host individual and geographic location are the major determinants of endophyte community composition even in the face of perturbations. Unexpectedly, we found that leaf mining did not impact endophyte communities in white snakeroot, but fungicide treatment resulted in small but significant changes in endophyte community structure. Together, our results suggest that endophyte communities are highly resistant to biotic and anthropogenic disturbances.

Keywords Microbiome · Fungi · Disturbance · Herbivory · Fungicide · *Ageratina altissima*

Introduction

Host-associated microbiotas are an integral part of their host's biology and have been likened to host organs in and of themselves [1]. Consequently, perturbations to a host and to its microbiota cannot be considered independently of one another, and understanding symbiont response to environmental perturbations may be critical to predicting host functional response [2]. For example, antibiotic-induced alterations to the human gut microbiome can increase susceptibility to infection, deregulate metabolism, and alter human homeostasis [3]. Additionally, long-term tolerance of corals to high-salinity levels, including resistance to coral bleaching, has been attributed to a major restructuring of the coral microbiome [4].

Despite similarities in the ecological organization and evolutionary importance of animal and plant microbiomes, we know comparatively less about how disturbance affects the ecology of plant hosts and the composition of their symbiont communities [5]. However, some recent research has begun to investigate how plant-microbe interactions respond to perturbations. For instance, one study of phyllosphere bacteria

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demonstrated that shifts in the bacterial microbiome corresponded to herbivory by a leaf mining fly and a leaf beetle, but the direction and magnitude of these shifts depended on both herbivore and bacteria identity [6]. Another recent study showed that bacterial symbionts of coastal plants exhibited little to no response following the Deepwater Horizon Oil Spill [7]. Interestingly, the same study found that the same plants experienced nearly complete loss of their fungal microbiota compared to plants in unaffected areas [7] although the functional consequences of such a loss are unknown. In general, it seems that the effects of perturbation on the plant microbiome are complex and context-dependent [8, 9].

Endophytic fungi are the functionally dominant members of the plant microbiome that colonize all plant species sampled to date [5, 10]. They are cryptic colonizers, causing no visible damage to plant tissues [11]. Colonization by endophytic fungi can promote increased plant vigor [12], pathogen resistance [13], drought resistance [14], and herbivore defense [15, 16]. Most endophytic fungi (excluding the clavicipitaceous fungi of cool-season grasses and morning glories, and the fungal symbionts of locoweeds [17]) are horizontally transmitted [18] via rain, wind, local leaf litter, or insect vectors [16, 19, 20]. Because a plant can be colonized by endophytic fungi that originate from a neighbor or by spores that persist in the local environment, it is not surprising that endophyte communities found in the same plant host species can show striking geographical differences [10, 21–24]. Additionally, plant host genotype has been shown to shape the foliar fungal microbiome, suggesting that plant genetic identity can influence which endophytes colonize or persist in host tissues [25, 26]. However, it remains unclear if factors such as host identity and location continue to shape the community structure of the fungal microbiome in the face of natural and anthropogenic disturbances. Further, perturbations experienced by a host plant could have cascading effects on its symbiont communities or, alternatively, plants could buffer the effects of perturbation experienced by their fungal microbiome.

Herbivory is one of the most significant biotic pressures faced by plants, and the fungal microbiome can mediate herbivory of its host. For instance, increased colonization by fungal endophytes can negatively affect foraging preferences of leaf-cutter ants [27, 28], and increasing colonization by the endophytes *Fusicladium* sp. and *Melanconium* sp. can be associated with decreasing or increasing pupal mass, respectively, of autumnal moths that feed on birch [29]. There has been a relatively long history studying tri-trophic interactions among plants, fungal endophytes, and leaf mining and galling insects, including aphids, wasps, beetles, moths, and flies. Galling insects induce tumor-like growths (galls) in plant tissue. These galls can be associated with particular species or suites of symbiotic fungi [30, 31]. Leaf mining insects oviposit in leaf tissue, throughout which their larvae then travel and feed [32]. Evidence of this activity is visible on the leaf surface as a tunnel or "mine" that increases in width as the larvae mature and molt. By virtue of their habitat within leaves and stems, both galling and leaf mining insects live in close proximity to fungal endophytes for prolonged periods of time. Thus, these intimate herbivores may not only perturb the plant host but also the plant's foliar fungal microbiota. While several studies have examined perturbations of plants and endophytes in leaf mining and galling systems, this work has been done almost exclusively in a handful of host genera, primarily *Quercus* (oaks) [33–35], and has revealed no consistent differences in the direction and magnitude of the tri-trophic interaction between herbivore, plant, and endophyte species [31]. However, while much attention has been paid to how endophyte colonization affects herbivorous insects, almost no work has been conducted on the reciprocal interaction: does insect herbivory affect the plant-fungal microbiome (but see [31, 36])?

Independent of plant-insect interactions, humans are impacting the environment in unprecedented ways, initiating novel sources of disturbance, like pollution, that can then affect both plants and their microbiota. For instance, agricultural practices such as pesticide application and fertilization have been shown to alter communities of endophytic bacteria [8, 9, 37, 38]. Anthropogenic sources of perturbation can similarly affect the fungal microbiome of plants. For example, simulated acid rain significantly reduced the number of fungal endophytes colonizing birch leaves [39]. Furthermore, in light of global change, the increasing frequency and intensity of severe wildfires in many ecosystems has been connected to shifts in foliar fungal microbiomes, including decreases in density, increases in diversity, and changes to the taxonomic composition of endophytes [40]. Fungicide represents another anthropogenic perturbation to plants that is particularly relevant to their fungal microbiome. Used extensively in agricultural and other managed settings, with the potential to pollute natural environments as well, fungicide application is akin to antibiotic use. However, in contrast to responses by the bacterial microbiome in humans and domesticated animals to antibiotic use, we know very little about how the fungal microbiome of plants responds to fungicide application [41].

In the present study, we examined the tension between natural and anthropogenic disturbances and host identity and location, to ask if and how endophytic fungal communities are affected by perturbation. We conducted two experiments in order to test the effect of perturbation on endophyte communities in two different contexts: First, we examined endophyte response to a natural perturbation resulting from leaf mining insect activity. Second, we employed a manipulative study to

test endophyte community response to an anthropogenic perturbation of fungicide application. We conducted both experiments using a perennial wildflower host native to the midwestern USA, *Ageratina altissima* (white snakeroot, Asteraceae). Although we hypothesized that both leaf mining and fungicide application would alter composition and diversity of the fungal microbiome, we found that endophyte communities are strongly buffered from perturbation and that plant host individual and geographic location are major determinants of endophyte community composition even in the face of these perturbations.

Methods

Field Sites and Study System

Studies were conducted in Bloomington, Indiana, at the Indiana University Research and Teaching Preserve sites at Griffy Woods (N 39.191822, W −86.511809) and Bayles Road (N 39.217387, W −86.541081), as well as Dunn's Woods (N 39.215611, W −86.542154) on the Indiana University Bloomington campus. The 75-ha site at Griffy Woods is primarily mature forest, consisting of undisturbed native communities with some invasion by exotic plant species. The 13-ha site at Bayles Road is mostly agricultural land and open fields with a forested perimeter. Dunn's Woods is a 4-ha urban campus woodland, with heavy invasion by exotic plants.

Ageratina altissima (hereafter referred to as white snakeroot) is a perennial wildflower native to the midwestern USA that blooms in the late summer and early fall [42] and is a common understory plant at all of the study sites. White snakeroot is toxic to large herbivores [43], although it is still susceptible to insect herbivory. Specifically, *Liriomyza eupatoriella* is a specialist leaf mining fly that commonly oviposits in white snakeroot leaves and creates extensive leaf mines [44] (Fig. 1).

Experiment 1: Effect of Leaf Mining Perturbation on Endophyte Communities

To investigate the effect of leaf mining on endophyte communities, paired, oppositely arranged, mined and unmined leaves were collected from white snakeroot at all three study sites in the fall of 2014 (Griffy Woods—14 September, Dunn's Woods—16 September, Bayles Road—25 September). Leaves were sampled only if free of visible pathogen and other herbivore damage (excluding mining). Mined leaves met the following requirements: one mine per leaf, fresh appearance of the mine (i.e., not subsequently infected by a foliar pathogen), and completion of the mine (i.e., evidence of insect emergence at the end of the mine). Ten pairs of leaves (mined and unmined) were collected from each of the three sites (total $n = 30$ pairs), with each pair of leaves originating from a different plant individual. Leaves were kept at 4 °C and processed within 24 h of harvest [13].

For experiment 1, samples of mined leaves were taken away from the mine itself. Mines contain less plant tissue available for endophytes to colonize, due to the removal of tissue during larval feeding, so this sampling method was chosen to minimize confounding effects of the amount of tissue available for fungal colonization. Sixteen 4 mm^2 tissue samples were haphazardly removed from the non-mined area of each leaf, and surface sterilized as follows: Tissue fragments were submerged and agitated in 70 % ethanol for 3 min, 10 % sodium hypochlorite for 2 min, and then sterile water for 1 min [45]. Each piece of tissue was then placed in an individual Eppendorf tube containing 2 % malt extract agar (MEA) (i.e., slants) [10]. Slants were sealed with Parafilm and incubated with 12/12 h light/dark cycle at room temperature. Slants were monitored daily for fungal growth. Emergent hyphae were subcultured to 2 % agar plates and allowed to grow until the colony covered the agar plate. Subcultures were grouped into colony morphotypes based on color, mycelial growth patterns, and spore production. At least one

Fig. 1 Leaf mining on a white snakeroot leaf

representative subculture from each morphotype, including all singletons, was identified using Sanger sequencing of the fungal ITS region (see below). For more common morphotypes, more representative subcultures were sequenced. All morphotypes for which multiple subcultures were sequenced were identified as the same operational taxonomic unit, confirming the accuracy of our initial morphotype designations. It is possible that some unsequenced isolates could represent distinct taxa, but this was not apparent when multiple isolates of the same morphotype were sequenced. Vouchers of living mycelia were suspended in sterile water and are stored at room temperature at Indiana University.

Total genomic DNA was extracted directly from fungal isolates using a MO BIO PowerPlant® Pro DNA Isolation Kit. Primers ITS5 and ITS4 [46] were used to amplify the internal transcribed spacer (ITS) region of fungal DNA. PCR amplifications were achieved using a Thermo Scientific Phire Plant Direct PCR Kit. Each 20 μL PCR reaction included 7.1 μL Milli-Q water, 10 μL 2X Phire Plant PCR Buffer (includes dNTPs and $MgCl_2$), 1 μL each primer (5 μM), 0.4 μL Phire Hot Start II DNA Polymerase, and 0.5 μL template DNA. The amplification was run in an MJ Research Tetrad PTC-225 Thermal Cycler [30 s at 98 °C, followed by 30 cycles (5 s at 98 °C, 5 s at 62 °C, 20s at 72 °C), 1 min at 72 °C]. Gel electrophoresis using SYBR Safe produced single bands for all products, and no bands in negative controls. PCR products were cleaned using an Omega Bio-Tek MicroElute® Cycle-Pure Kit and Sanger sequenced for both forward and reverse reads (primers ITS5 and ITS4, respectively) on an ABI3730 at the Indiana Molecular Biology Institute.

CodonCode Aligner v5.0.1 (CodonCode Aligner Company) was used to make base calls, perform quality assessments, and assemble consensus sequences according to 95 % ITS sequence similarity, with a minimum of 40 % overlap [47]. Identification of consensus sequences was performed using the Ribosomal Database Project (RDP) Bayesian Classifier with the Warcup ITS training set [48], and archived at GenBank under accession numbers KX271283-KX271333.

Experiment 2: Effect of Fungicide Application on Endophyte Communities

To investigate the effect of fungicide perturbation on FEF communities, 20 white snakeroot individuals were selected at the Griffy Woods field site in the fall of 2014. One healthy, fully developed leaf from each plant was harvested before treatment (17 September) to examine initial endophyte communities. Healthy leaves were defined as having no apparent symptoms of herbivory (including leaf mining) or disease. Immediately following the collection of one leaf from each plant, a small paintbrush was used to apply Spectracide Immunox® Multipurpose Fungicide (1.5 % in sterile water, diluted according to manufacturer's instructions) to the front and back of every leaf on each plant in the experimental group ($n = 10$). The active ingredient is myclobutanil, which inhibits the enzyme that catalyzes the production of ergosterol, a compound necessary for fungal cell wall formation, arresting fungal growth and development [49]. Concurrently, sterile water was applied to all leaves of the control plants ($n = 10$) using a clean paintbrush. One leaf from each plant was subsequently collected 2 days post-treatment and 2 weeks post-treatment. Samples were collected 2 days post-treatment to observe the immediate effects of fungicide application on fungal endophytes. Samples were subsequently collected 2 weeks post-treatment to observe longer-term effects as the fungicide was advertised to maintain effectiveness for up to 2 weeks after application. Following sampling, all leaves were maintained at 4 °C and processed within 24 h of harvest. Sixteen 4 mm^2 tissue samples from each leaf were removed and surface sterilized, and fungi were cultured, sequenced, identified, and archived following methods described in experiment 1.

Statistical Analyses of Fungal Communities

For both experiments, operational taxonomic units (OTUs), as designated by the fungal ITS region, were used for ecological analyses. Species accumulation curves and estimates of total richness were inferred using EstimateS 9.0.1 [50]. Rarefaction curves were scaled by the number of accumulated samples (i.e., number of leaves sampled) to depict species density [51].

All other ecological analyses were performed using R v.3.1.2 [52]. Isolation frequency was calculated as the percentage of tissue segments containing cultivable fungi as a proxy for degree of host tissue colonization. The Shannon Diversity Index was measured using the VEGAN package v.2.3-5 [53]. ANOVA was used to compare isolation frequency and diversity across treatments. To examine the effects of leaf mining, fungicide treatment, and time on endophyte community composition, the VEGAN package was used to calculate permutational multivariate analysis of variance (PERMANOVA) using distance matrices with the Bray-Curtis dissimilarity index (VEGAN package, function *adonis*) on all non-singleton OTUs. The ordination goodness-of-fit was measured by the stress value [54]. NMDS ordinations were created in VEGAN used to visualize community similarities across treatments and time [22].

Results

Of the 1779 fungal endophytes isolated across both experiments, 287 representative cultures were sequenced, which resulted in 70 OTUs clustered at 95 % similarity. The remaining 1492 isolates were classified based on sequence data from their representative isolate(s). Overall, 32 OTUs (40.32 %) were singletons (i.e., represented by only one isolate) and 38 OTUs were isolated multiple times (Table 1). High quality

Table 1 List of endophyte OTUs isolated across both experiment 1 and 2, in order of prevalence (OTU 1 = most common), with number of isolations in both experiments, accession number, and best match (with confidence) according to the Warcup Fungal ITS Trainset

OTU	# isolations experiment 1	# isolations experiment 2	Total # isolations	Accession number	Warcup Fungal ITS Trainset 1 Best Match	Confidence (%)
OTU 1	142	349	491	KX271283	*Colletotrichum phormii* / *Colletotrichum salicis*	62
OTU 2	1	255	256	KX271284	*Colletotrichum tropicale*	74
OTU 3	200	17	217	KX271285	*Colletotrichum lineola*	100
OTU 4	115	62	177	KX271286	*Daldinia childiae*	100
OTU 5	50	46	96	KX271287	*Whalleya microplaca*	100
OTU 6	72	7	79	KX271288	*Glomerella cingulata var brevispora*	45
OTU 7	17	31	48	KX271289	*Phomopsis magnoliae*	78
OTU 8	38	2	40	KX271290	*Daldinia loculata*	58
OTU 9	29	4	33	KX271333	*Xylaria longipes*	45
OTU 10	25	7	32	KX271291	*Hypoxylon perforatum*	100
OTU 11	26	–	26	KX271292	*Colletotrichum karstii*	58
OTU 12	23	1	24	KX271320	*Pestalotiopsis crassiuscula* / *Pestalotiopsis photiniae*	84
OTU 13	12	12	24	KX271293	*Nigrospora oryzae*	100
OTU 14	18	5	23	KX271294	*Daldinia loculata*	17
OTU 15	23	–	23	KX271295	*Hypoxylon investiens*	100
OTU 16	22	–	22	KX271296	*Diaporthe aspalathi* / *Diaporthe meridionalis*	24
OTU 17	19	1	20	KX271297	*Menispora ciliata*	57
OTU 18	3	11	14	KX271298	*Diaporthe melonis var. brevistylospora*	60
OTU 19	14	–	14	KX271321	*Pestalotiopsis mangifolia*	56
OTU 20	–	11	11	KX271322	*Calonectria pseudospathiphylli*	34
OTU 21	4	6	10	KX271323	*Mycoleptodiscus indicus*	87
OTU 22	9	–	9	KX271299	*Phacellium veronicae*	52
OTU 23	1	6	7	KX271300	*Xylaria bambusicola*	31
OTU 24	7	–	7	KX271324	*Hypocrea koningii* / *Hypocrea viridescens*	33
OTU 25	2	2	4	KX271325	*Nemania diffusa*	100
OTU 26	4	–	4	KX271326	*Gaeumannomyces cylindrosporus*	42
OTU 27	1	2	3	KX271301	*Nemania sp. JJP-2009a*	100
OTU 28	3	–	3	KX271302	*Xylaria longipes*	100
OTU 29	3	–	3	KX271327	*Cercospora apii*	89
OTU 30	–	3	3	KX271303	*Diaporthe caulivora*	99
OTU 31	–	2	2	KX271304	*Anthostomella eucalyptorum*	37
OTU 32	–	2	2	KX271305	*Rosellinia corticium*	100
OTU 33	–	2	2	KX271306	*Pseudovalsaria ferruginea*	90
OTU 34	2	–	2	KX271307	*Podospora intestinacea*	83
OTU 35	2	–	2	KX271308	*Nigrospora sphaerica*	96
OTU 36	1	–	1	KX271309	*Acremonium kiliense*	57
OTU 37	1	–	1	KX271310	*Hypoxylon rubiginosum*	79
OTU 38	1	–	1	KX271328	*Arthrinium phaeospermum*	100
OTU 39	–	1	1	KX271311	*Creosphaeria sassafras*	100
OTU 40	–	1	1	KX271312	*Annulohypoxylon annulatum*	100
OTU 41	–	1	1	KX271329	*Ramularia collo-cygni*	53
OTU 42	–	1	1	KX271313	*Annulohypoxylon truncatum*	100
OTU 43	–	1	1	KX271314	*Phyllosticta pyrolae*	98
OTU 44	–	1	1	KX271315	*Daldinia petriniae*	19
OTU 45	–	1	1	KX271330	*Fusarium arthrosporioides* / *Fusarium torulosum*	50

Table 1 (continued)

OTU	# isolations experiment 1	# isolations experiment 2	Total # isolations	Accession number	Warcup Fungal ITS Trainset 1 Best Match	Confidence (%)
OTU 46	–	1	1	KX271316	*Mycoleptodiscus indicus*	100
OTU 47	–	1	1	KX271331	*Discosia sp. 2 KT-2010*	93
OTU 48	–	1	1	KX271317	*Pezicula cinnamomea*	94
OTU 49	–	1	1	KX271318	*Nemania plumbea*	100
OTU 50	–	1	1	KX271319	*Lecythophora fasciculata*	100
OTU 51	–	1	1	KX271332	*Botryosphaeria berengeriana*	25

sequence data was not recovered from 16 singletons and three other OTUs (representing a total of 14 isolates). These isolates without high quality sequence data constituted only 1.7 % of the dataset and were excluded from analyses. In both experiment 1 and 2, at least one isolate was recovered from every leaf. The best hit for the five most commonly isolated endophytes across the entire dataset, respectively, were *Colletotrichum phormii*/*Colletotrichum salicis* (28.07 % of all isolates), *Colletotrichum tropicale* (14.64 %), *Colletotrichum lineola* (12.41 %) *Daldinia childiae* (10.12 %), and *Whalleya microplaca* (5.49 %). For both experiment 1 and experiment 2, species accumulation curves, scaled by the number of leaves sampled, remained non-asymptotic, but the estimated richness fell within the 95 % confidence interval for both curves (Fig. 2). Thus, the datasets were considered sufficient for the community analyses described below [47].

Experiment 1: Effect of Leaf Mining Perturbation on Endophyte Communities

For experiment 1, 919 isolates were recovered from 960 tissue segments collected from 60 leaves (one mined and one unmined leaf from each of 30 plants across three different sites; isolation frequency = 95.73 %). Endophyte isolation frequency differed significantly between sites (df = 2, $F = 12.0086$, $p = 4.572e\text{-}05$, Fig. 3a). Specifically, fewer endophytes were isolated per tissue sample from plants located at the Dunn's Woods site. Shannon diversity also differed among sites, and was lowest at Dunn's Woods (df = 2, $F = 6.8198$, $p = 0.002$, Fig. 3b). By contrast, there was no difference between mined and unmined leaves in isolation frequency (df = 1, $F = 0.0793$, $p = 0.7793$) and endophyte diversity (df = 1, $F = 0.0071$, $p = 0.933$). Location was a significant predictor of endophyte community composition ($F_{2,59} = 19.3570$, $R^2 = 0.28479$, $p = 0.001$), as was individual plant ($F_{27,59} = 2.5069$, $R^2 = 0.49791$, $p = 0.001$) (Figs. 4a, 5a, b), but leaf mining was not, accounting for less than 1 % of the variation ($F_{1,59} = 0.5408$, $R^2 = 0.00398$, $p = 0.838$, Figs. 4b, 5c).

Experiment 2: Effect of Fungicide Application on Endophyte Communities

For experiment 2, 859 isolates were recovered from 960 tissue segments collected from 60 leaves, representing ten fungicide-treated plants and ten control plants at three time points (isolation frequency = 89.48 %). Endophyte isolation frequency did not differ significantly between treatments (df = 2, $F = 0.7587$, $p = 0.473$), over time (df = 1, $F = 0.0000$, $p = 1.0$), or due to a treatment*time interaction (df = 1, $F = 0.1480$, $p = 0.702$). Endophyte community diversity also did not differ between treatments (df = 2, $F = 0.8067$, $p = 0.452$) or over time (df = 1, $F = 0.0003$, $p = 0.986$, and there was no treatment*time interaction (df = 1, $F = 0.1800$, $p = 0.673$). However, treatment (fungicide vs. control) significantly predicted endophyte community structure ($F_{2,59} = 2.0523$, $R^2 = 0.04712$, $p = 0.043$, Figs. 6a, 7a, b). Specifically, OTU 13 (df = 2, $F = 3.0692$, $p = 0.054$) and OTU 30 (df = 2, $F = 3.3529$, $p = 0.042$) differed significantly among treatments, as did the number of singletons (df = 2, $F = 5.7914$, $p = 0.005$). Sampling time also had a marginally significant effect on endophyte community structure ($F_{1,59} = 2.3577$, $R^2 = 0.02707$, $p = 0.058$, Figs. 6b, 7b, c). As in experiment 1, plant individual was the most significant predictor of community structure of endophytes ($F_{9,59} = 3.7380$, $R^2 = 0.38622$, $p = 0.001$, Fig. 7d).

Discussion

Our results demonstrate that host individual and geographic location are the dominant drivers of the fungal microbiome of white snakeroot (*Ageratina altissima*), even in the face of leaf mining and fungicide perturbations. This study provides insights into microbiome community ecology and response to natural and anthropogenic disturbances. Additionally, it represents the first characterization of fungal endophytes in white snakeroot, as well one of the few studies that has examined endophytes in the Asteraceae family, or other wild, native understory plants in general (but see [23, 36, 55]). The three most commonly isolated OTUs across both experiments match most closely to species in the genus *Colletototrichum.*

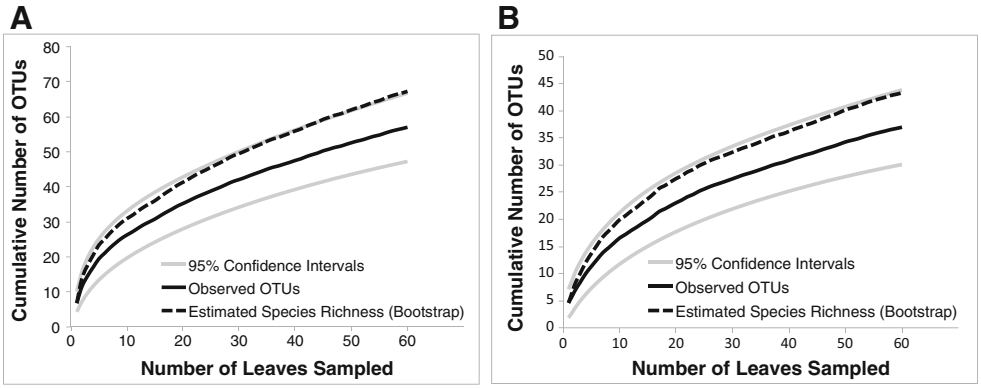

Fig. 2 Species accumulation curves of cultivable fungal endophytes in white snakeroot for **a** experiment 1 and **b** experiment 2. *Solid black line*: number of observed OTUs (based on 95 % sequence similarity); *grey lines*: upper and lower bounds of the 95 % confidence interval around observed richness; *dashed line*: bootstrap estimate of total species richness

Colletotrichum is a common genus of both asymptomatic endophytic fungi and fungal pathogens [56]. In fact, the best hit for the three most common OTUs (C. *phormii*, *C. tropicale*, *and C. lineola*) have all been reported to cause disease in various plant hosts [57–59]. In this experiment however, these potentially pathogenic fungi were isolated from apparently healthy, asymptomatic leaves, suggesting that the effects of particular endophytes on plant health may be host species-dependent or may vary with other environmental conditions. Future work characterizing the functional roles of common endophyte species across plant hosts or environmental gradients would help delineate the pathogen to mutualist spectrum across which endophytes exist [18].

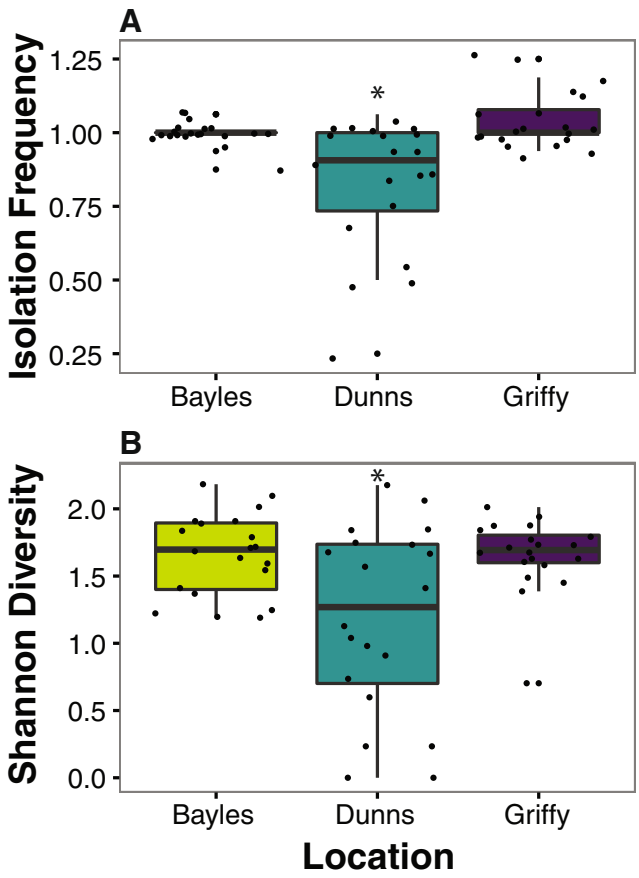

Fig. 3 **a** Isolation frequency and **b** Shannon diversity differed significantly across sites in experiment 1. Both isolation frequency ($p = 4.572e\text{-}05$) and diversity ($p = 0.002$) were significantly lower at the Dunn's Woods site

Several previous studies have explored the importance of geographic location on endophyte community structure, abundance, and diversity. Endophyte communities isolated from a single host species can vary according to geographic location. This has been shown to occur both at a large scale, such as across the continent of North America [10], and a small scale, on the order of just hundreds of meters [21]. Our results are consistent with the latter; we found very distinct endophyte communities within the leaf tissues of a single host species separated by only a few kilometers. One of the sites, Dunn's Woods, exhibited lower endophyte abundance and diversity compared to the other two sites. Dunn's Woods is a forested site located on the Indiana University campus, and as such is more heavily managed and more frequented by people than the other two sites. It is also the only urban site and is the smallest of the three sites. It is possible that over time, the higher levels of human disturbance or isolation from other forested areas have reduced spore sources at this site, leading to lower levels of endophyte colonization. Previous work has demonstrated that environmental fragmentation can affect species distribution and frequency of fungal endophytes [60]. Specifically, as fragment size decreases, so does endophyte colonization, in concordance with the theory of island biogeography [60]. More surprisingly, our results show that individual plant identity controlled a large amount of the variation seen in endophyte community composition across both experiments (50 and 39 % for experiments 1 and 2, respectively). Previous studies in other systems have focused on and demonstrated significant variation in endophyte communities across communities [10], populations [21], and genotypes [25, 26]. Future studies would benefit from

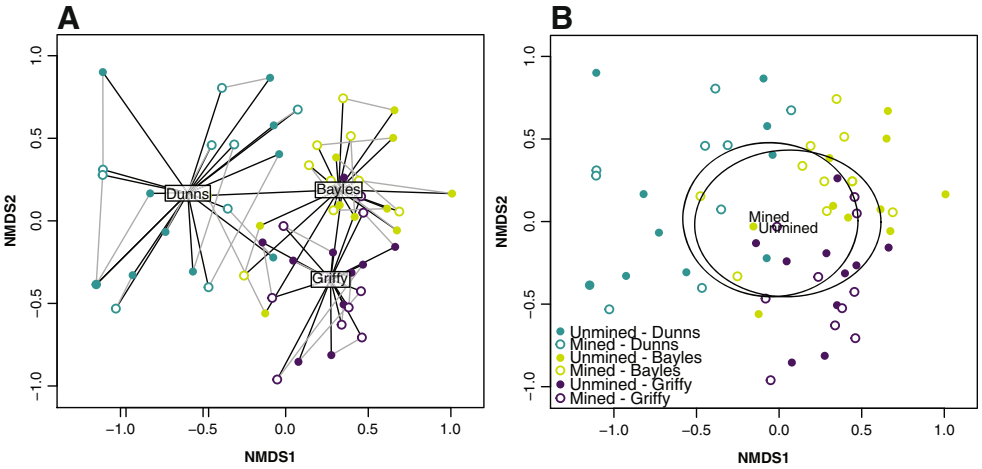

Fig. 4 NMDS illustrating community structure of endophytes in experiment 1. **a** PERMANOVA showed that community structure differed significantly among sites ($R^2 = 0.28479$, $p = 0.001$) and individual plant ($R^2 = 0.49791$, $p = 0.001$), **b** but not between mined and unmined leaves ($R^2 = 0.00398$, $p = 0.838$)

repeated sampling of individual plants over time, or sampling multiple leaves from individual plants, in order to further inform under what contexts individual plant hosts exert strong influence over resident endophyte communities [5].

Our results suggest that leaf mining does not alter endophyte community composition or diversity in white snakeroot. This is contradictory to much of the previous literature, which has, for the most part, reported negative relationships between

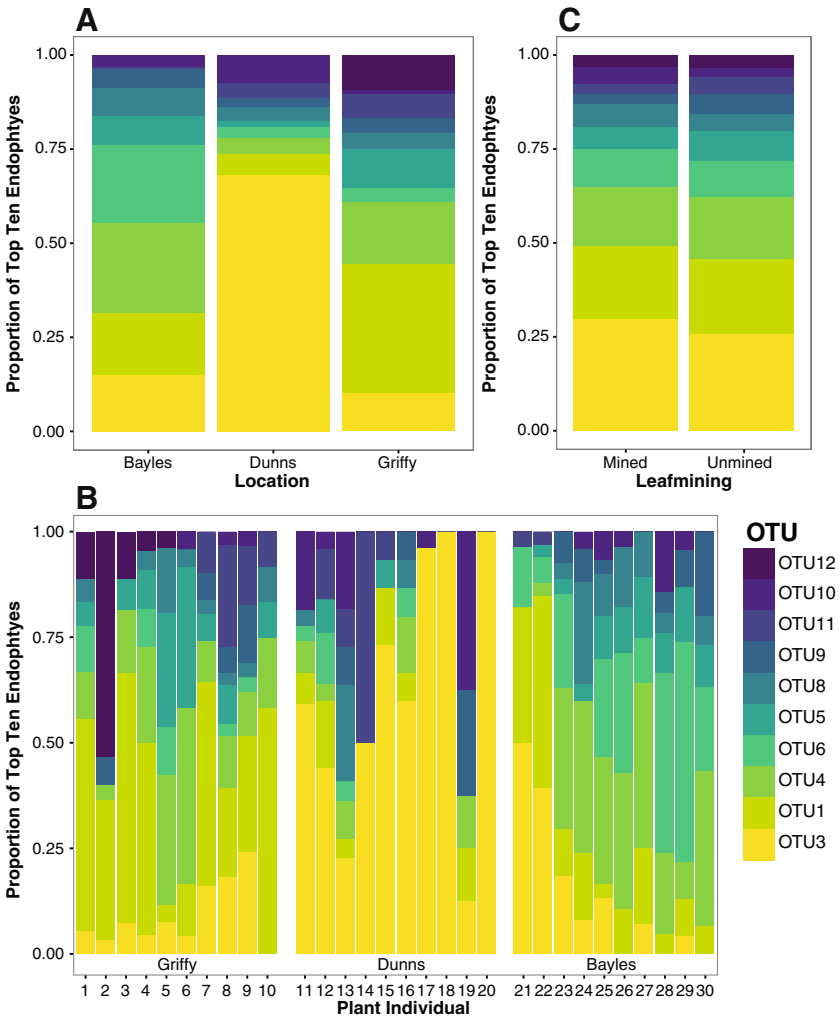

Fig. 5 Distribution of the 10 most abundant OTUs isolated in experiment 1, across **a** sites, **b** mined vs. unmined leaves, and **c** individual plants. Abundance is scaled as the percentage of total abundance of the 10 most common OTUs

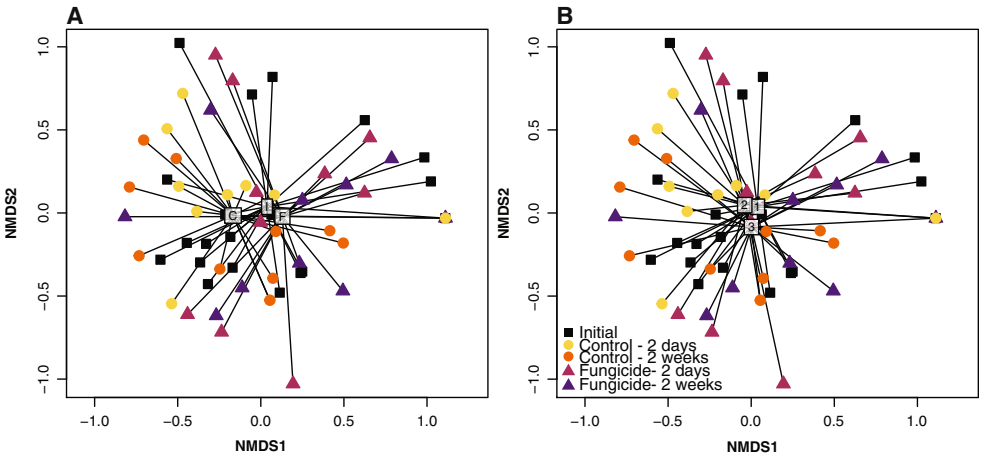

Fig. 6 NMDS illustrating community structure of endophytes in experiment 2. **a** PERMANOVA showed that community structure differed significantly among treatments ($R^2 = 0.04712$, $p = 0.043$) and **b** marginally significantly across time points ($R^2 = 0.02707$, $p = 0.058$). Individual plant was a highly significant predictor of endophyte community structure ($R^2 = 0.38622$, $p = 0.001$)

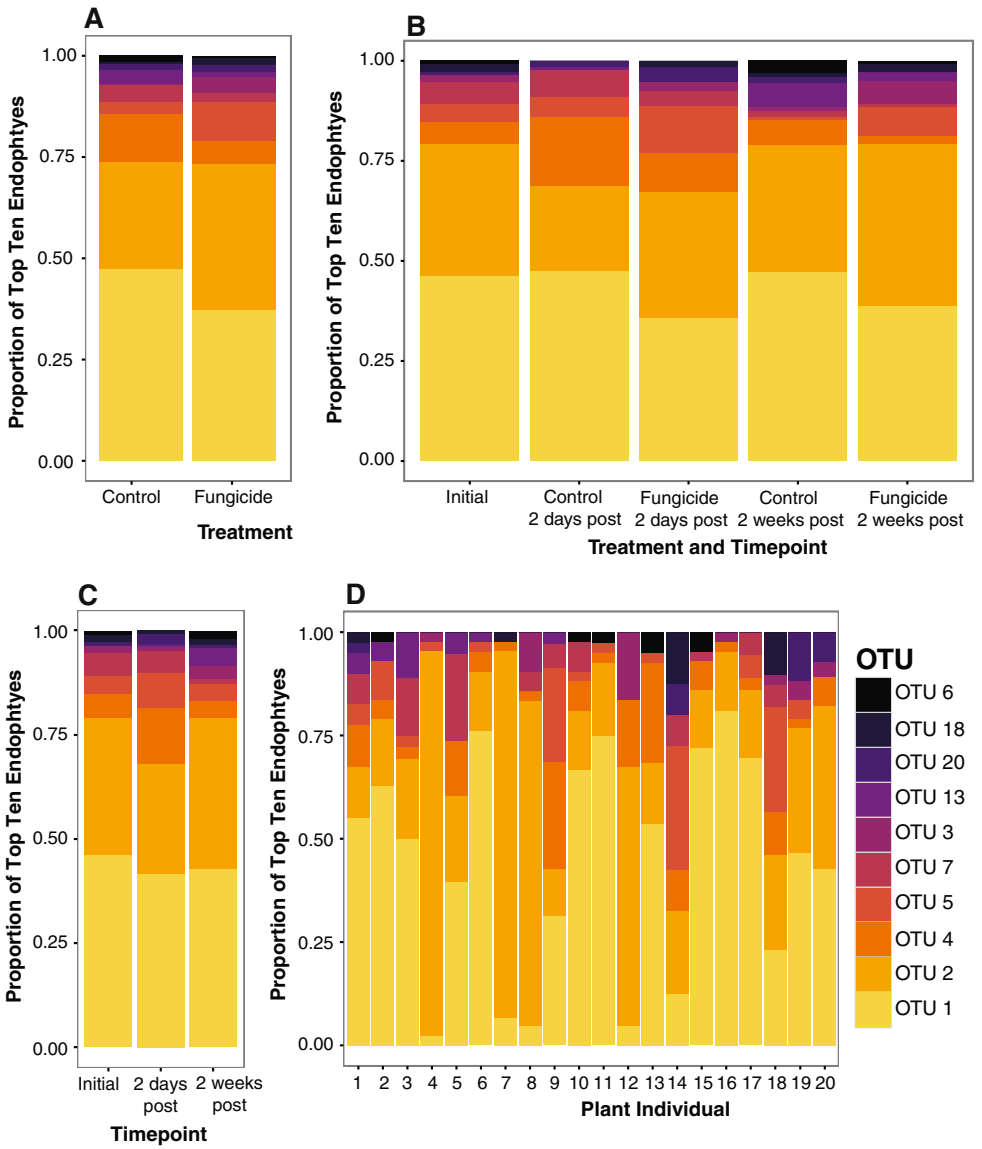

Fig. 7 Distribution of the 10 most abundant OTUs isolated in experiment 2, across **a** treatments, **b** treatment*time interaction, **c** time points, and **d** individual plants. Abundance is scaled as the percentage of total abundance of the 10 most common OTUs

leaf endophytes and mining insects (summarized in [31]). Specifically, endophytic fungi have been reported to have negative fitness consequences on leaf mining insects, such that insects will seek out low-endophyte space in which to oviposit [35]. However, almost all of this previous work has been conducted using tree hosts, which may not be representative of all plant hosts. Moreover, the outcome of the interaction in a given tripartite system is not always consistent [31]. For example, interactions between the host species *Quercus emoryi*, leaf mining insect *Cameraria* sp., and endophyte species *Ophiognomia cryptica* and *Asteromella* sp., have been shown to result in both negative [35] and neutral [34] outcomes. Furthermore, in another oak species, *Quercus gambelii*, the endophyte *Gnomonia cerastis* had a positive effect on the leaf mining moth *Phyllonorycter* sp. [61] With such variation in outcome within one host genus, it is clear that it is not appropriate to generalize patterns across plant host species, including the perennial wildflower described in this study. There are clearly direct effects of these herbivores on their plant hosts (via consumption of tissue) but we have less understanding of the indirect functional effects of leaf mining on host plant health. For instance, leaf miners may indirectly affect plant hosts by changing the production or function of chemical production by the endophytes or by the host plants themselves. Future work should examine tri-trophic interactions between endophyte communities and insects such as leaf miners, gall-formers, and bark beetles, across a wider range of understudied plant hosts.

Unlike leaf mining, the application of fungicide at the Griffy Woods site had a significant effect on endophyte community composition. For example, OTU 13 was significantly less abundant in the fungicide-treated plants. The best match for OTU 13 is *Nigrospora oryzae*, which can be pathogenic on plants such as rice [62], but is also used as a biocontrol agent and antimicrobial drug [63–65]. Depending the priorities of researchers or land managers (e.g., pathogen control vs. bioprospecting), application of fungicide could have positive or negative consequences. Another endophyte, OTU 30, was present initially but then was never isolated from either fungicide-treated or control plants. Thus, the shifts in this endophyte could also be attributed to time, or stochasticity. Further, both control and fungicide-treated plants had significantly more singletons than did corresponding initial communities. The increase in number of singletons could also be a function of time, representing a temporal diversification of endophyte communities. Alternatively, the wetting of the leaf surface that occurred in both control and fungicide treatments could have created conditions that promoted colonization by more rare fungi. However, the amount of variation in community structure explained by the fungicide treatment (~5 % of variation) was low in comparison to that explained by individual plant host (~39 % of variation). Moreover, it is surprising that fungicide application did not alter the isolation frequency of endophytic fungi from plant tissue. The particular fungicide used in this experiment acts on fungal pathogens by inhibiting fungal cell wall formation. Application of different types of fungicide that eradicate fungi rather than prohibit cell growth and division might alter fungal abundance in different ways. Regardless of treatment, endophyte communities experienced a small shift in community composition across the three time points, demonstrating that endophyte communities change temporally. However, the variance explained by time (~3 %) was also minimal in comparison to the effect of the plant host.

Although perturbation by fungicide elicited a small but significant change in community composition, implications of these community changes for endophyte function and subsequent effects on plant host health remain unknown. This unexplored area has economic and agricultural, as well as ecological, implications. When plants are treated with broad-spectrum fungicide to suppress fungal pathogens, endophyte communities could change in ways that are unpredictable and perhaps antagonistic to host health. Shifts in endophyte community structure could open niches and new opportunities for pathogens. This unintended consequence is analogous to the use of insecticides, which can reduce predatory insect populations, allowing herbivorous insect populations to increase and do more damage to plants. This "paradox of fungicides" would thereby represent a new facet of the "Paradox of Pesticides" [66, 67]. Future research should test multiple active ingredients and concentrations of fungicide, on a wide range of both agricultural crops and wild plants, and over different time periods, for their impacts on FEF community structure and functional implications for plant hosts [68]. Studies that couple shifts in endophyte community composition with endophyte gene expression or metabolite production would be particularly informative.

White snakeroot is itself a chemically interesting plant. It produces tremetone and other benzofuran ketones which are toxic to large herbivores [43]. The particular chemistry of white snakeroot could therefore be important in shaping the community and spatial structure of endophytes within its plant tissues. Additionally, endophytes in culture have been shown to produce myriad biologically active secondary metabolites which can potentially defend plant hosts from herbivores and pathogens [69]. The question remains whether these antagonistic substances are also synthesized when endophytes engage in symbiosis within the plant. In other systems (i.e., fungal symbioses of cool-season grasses, locoweeds), chemicals are produced that are not plant toxins per se, but rather are produced by the fungal symbiont. Alternatively, endophyte infection could stimulate plant secondary metabolite production. In either case, fungal colonization could potentially mediate chemical interactions between plants and their attackers [70]. Future work should examine how colonization by endophytes affects, and is affected by, host plant

chemistry, and what roles changes in chemistry have in shaping plant and endophyte responses to perturbation.

Conclusions

This study examined the effects of perturbation on fungal endophyte communities using two different experiments and three different sites. Our results provide robust and consistent evidence that endophyte communities are primarily shaped by characteristics of their individual host plants and their local environment, but do still respond to some perturbations. Additionally, we found that endophyte communities demonstrate strong resistance and/or resilience to disturbance. Few studies have systematically examined the effects of perturbation on plant microbial communities. This study focused on identifying culturable endophytes, and as such may represent a possible bias against those fungi that will not grow in culture. Deeper identification using culture-independent sequencing methods would strengthen the conclusions drawn in this study. As we accumulate more knowledge of how and in what environmental contexts perturbations affect endophyte community dynamics, we will be able to conduct functional tests of how these disturbances mediate plant host health.

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Compliance with Ethical Standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest The authors declare that they have no conflict of interest.

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