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Community Ecology Of Soil Microbes In Southeast Usa Oak-Pine Woodlands

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COMMUNITY ECOLOGY OF SOIL MICROBES IN SOUTHEAST USA OAK-PINE WOODLANDS

by

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ABSTRACT

Soil microbial communities can have important effects on plant growth and plant community ecology; however, the relationships between trees and soil microbial communities are still relatively poorly understood. Here I examine several of these relationships. Chapter 1 examines how ecological restoration and environmental conditions affect the community composition and enzyme activities of ectomycorrhizal fungi (EMF). Chapter 2 considers whether host specificity in EMF structures taxa occurrence on oaks and pines, and Chapter 3 investigates how ash addition affects oak seedling growth and the soil bacterial community. I found that while wildfire lowered EMF diversity, prescribed burning did not. Ectomycorrhizal root tips from a plot undergoing regular prescribed burns also showed higher enzyme activity than those from unburned and wildfire plots over a year after burning. Additionally, Russulaceae and Thelephoraceae occurred significantly more often in sites undergoing restoration. Specificity at the host plant family level structures occurrence of EMF, with significant numbers of taxa occurring only on oaks or pines. However, EMF associating with both oaks and pines were usually dominant taxa. Finally, ash addition increased oak seedling growth and soil bacterial diversity. Soil microbial communities play an important

role in structuring plant communities, and better understanding these interactions is important in maintaining and restoring ecosystem health.

LIST OF ABBREVIATIONS

C	Carbon
DWD	Dead woody debris
EMF	Ectomycorrhizal fungi
N	Nitrogen
NAGase	N-acetyl- ß-D-glucosaminidase
OTU	Operational taxonomic unit
P	Phosphorus
SPAC	Strawberry Plains Audubon Center
TEF	Tallahatchie Experimental Forest

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ABSTRACT

Common ecological restoration treatments such as thinning trees and prescribed burning could result in changes to soil fungal communities and changes to the function of those communities. Ectomycorrhizal fungi (EMF) are especially likely to be affected as they are obligate symbionts on plant roots and exhibit host and niche preferences. EMF also produce extracellular enzymes that are important in soil nutrient cycling. To explore the influence of woodland restoration on EMF community composition and function, I conducted a community survey of EMF and assayed ectomycorrhizal root tip enzyme activity using substrate plugs in northern Mississippi upland oak-pine woodland plots differing in restoration history. Restoration treatment was significant in explaining the occurrence of the most common EMF species (*Russula xerampelina*) and the most common EMF family (Thelephoraceae) in the community survey. Highest potential lignin-degradation and chitinase enzyme activity were found in a prescribed burn plot, and the lowest enzyme activities at a wildfire plot, where EMF richness was also lower. Different fungal families displayed significantly different degradation capabilities, with Hydnangiaceae having the highest laccase activity and Tuberaceae having significantly higher peroxidase and chitinase activity than several other families. These results suggest that restoration treatments can affect EMF community composition and function, and better understanding these changes can aid understanding of EMF niches and the impacts of restoration.

INTRODUCTION

Ecological restoration efforts that use prescribed burning and/or thinning to change tree composition are likely to alter associated fungal communities. This alteration may have important consequences for nutrient cycling in forest and woodland ecosystems (Anderson and Cairney 2007). In particular, thinning of trees alters the number and species of hosts available to ectomycorrhizal fungi (EMF), which are symbiotic on the roots of many trees, while burning can damage or kill fungal individuals in the soil, creating empty niches (Bastias et al. 2006, Dahlberg et al. 2001; Dickie et al. 2009). Prescribed burning also directly changes soil nutrient content and availability, and these changes in resource availability may provide a competitive advantage to EMF species with different enzymatic abilities and/or cause EMF to alter their production of extracellular enzymes (Buée et al. 2007, Mosca et al. 2007a). Here, I report results of a study on differences in the EMF communities and changes in potential EMF degradation of lignin and chitin in four types of plots in upland forests of the southeastern United States: thinned plots undergoing frequent prescribed fire, an un-thinned plot that experienced recent wildfire, an unthinned plot undergoing frequent prescribed fire, and untreated control plots.

Although woodlands in the southeastern United States developed with frequent, low-intensity fire, fire has been widely suppressed since the 1920s. As a result, modern forest communities are up to tenfold denser than historic conditions (Nowacki and Abrams 2008). This density has led to mesophication, with forests

that have a darker, cooler, moister understory beneath a closed canopy. Recent research is exploring the effects of attempted restoration of open woodlands in this region on their species composition and ecosystem functions (Boerner and Brinkman 2003, Bowles et al. 2007, Brewer 2016, Brewer et al. 2015, Dickie et al. 2009, Rietl and Jackson 2012, Ryndock et al. 2012).

Prescribed burning is a method of ecological restoration appropriate for previously fire-maintained communities that have experienced fire exclusion (Bowles et al. 2007, Peterson and Reich 2001, Scharenbroch et al. 2012). However, mesophication makes it difficult to create prescribed burns that match even the low historic fire intensity (Nowacki and Abrams 2008). One way to ameliorate the difficulties in creating a fire of sufficient intensity is to thin forests a few years prior to commencing burns, thus lowering humidity, reducing soil moisture, and enabling the growth of native understory plants that will fuel future burns (Johnson et al. 2009).

Fire effects on soil properties and belowground communities are dependent on burn intensity. Prescribed burning treatments in these ecosystems are generally low intensity and result in little immediate change in soil carbon (C) and nitrogen (N), while more intense burns, such as those from a wildfire in an area with heavy fuel loading, can cause C and N losses (González-Pérez et al. 2004, Johnson 1992, Johnson and Curtis 2001, Knicker 2007). Intense burns heat the soil more, burn litter and dead woody debris (DWD) more completely, and are more likely to directly damage soil biota, including root tips and EMF (Neary et al. 1999). Injury or death

of a host plant due to fire can also affect EMF communities (Knicker 2007, Neary et al. 1999). However, heat intense enough to kill EMF and fine roots is often patchy, and the soil niches emptied by a fire can be filled by hyphae from nearby surviving fungi. Thus, restoration would be expected to affect composition of the EMF community.

Through these changes, restoration using thinning and fire may affect the functioning of the EMF community. EMF are obligate symbionts of plants, and obtain the bulk of their fixed C from their hosts in exchange for water and nutrients that the fungi acquire from the soil. EMF are important in nutrient cycling (Anderson and Cairney 2007), and can also have a strong effect on the growth and health of their plant hosts (Hoeksema et al. 2010). Forest EMF communities typically have high species richness, but contain a few common species and many rare ones (Buée et al. 2005, Horton and Bruns 2001). A likely factor in maintaining species diversity is differing abilities of species to exploit soil nutrients, and therefore differing habitat preferences (Bruns 1995, Buée et al. 2007, Dickie et al. 2002, Tedersoo et al. 2003).

In particular, some EMF can produce extracellular enzymes that allow them to degrade lignin and/or chitin—two complex molecules that are relatively resistant to decomposition. Lignin is a major component of wood and the ability to degrade it by using laccases and peroxidases allows EMF access to nutrients in DWD. Chitin is found in exoskeletons of arthropods and in fungal cell walls and is a source of N and C. The activity of a particular EMF enzyme can be dependent on many factors,

including fungal species, season, temperature, soil moisture, host health, and thinning (Buée et al. 2005).

Thinning reduces the number of tree hosts available to EMF, but does not necessarily lead to a decrease in EMF diversity, as the removal of competition can stimulate root growth and create enhanced opportunities for forming mycorrhizae (Mosca et al. 2007b). Thinning can also increase laccase and chitinase activity of EMF (Mosca et al. 2007a). If thinned material is left *in situ*, this DWD is a nutrient resource that can be accessed through lignin-degrading enzymes. Additionally, even low-intensity prescribed burns can reduce the labile C available in litter enough to induce increased EMF laccase activity (Boerner and Brinkman 2003). A previous study in a temperate oak forest showed significantly more activity of the chitinase N-acetyl- ß-D-glucosaminidase (NAGase) on ectomycorrhizal root tips found in DWD than on those found in soils, suggesting that chitinases may have a competitive effect for EMF against other wood-rotting fungi (Buée et al. 2007).

Given this context for the relationship between environment, EMF community composition, and EMF extracellular enzyme activity, I asked three questions:

How does change in the local environment affect EMF community diversity?

We hypothesized that EMF community composition differs when fire and thinning regimes alter the environment, including available host plants, soil organic matter, canopy cover, and other factors. This hypothesis predicted

significant differences in EMF community species composition due to experimental restoration treatment.

How does EMF taxonomy affect enzyme activity?

We hypothesized that EMF in different families have different enzyme activity profiles. This hypothesis predicted significant differences in enzyme activities among families.

How does the environment influence EMF extracellular enzyme activity?

We hypothesized that EMF enzyme activity changes in response to the substrate in which the EMF are growing, and in response to fire history. I predicted that soils from a prescribed burn plot would exhibit higher lignin-degrading enzymatic activity than soils from control or wildfire plots. I also predicted that EMF would exhibit lower enzyme activity in burned DWD than in unburned DWD due to lower N content. Additionally, I predicted that lignin-degrading activity would correlate positively with chitin-degrading activity.

METHODS

Site Descriptions

The study comprised an EMF community survey and an experiment in which substrate cores were used to examine fire effects on EMF enyzme activity. The community survey took place at three sites in northern Mississippi, USA. Two sites, each with paired treated/untreated plots, were located at Strawberry Plains

Audubon Center (SPAC) in Marshall County, Mississippi. A third site at the Tallahatchie Experimental Forest (TEF), part of the Holly Springs National Forest in Lafayette County, Mississippi, contained an additional pair of treated/untreated plots used for the EMF community survey. TEF was also the location of the three plots in which the substrate plug experiment was conducted. Comparison of historic and current tree species composition in the area of the field sites demonstrates that mesophication is occurring in in these forests (Brewer 2001, Surrette et al. 2008).

TEF is a mixed upland forest with second growth stands including *Pinus echinata* (shortleaf pine), *P. taeda* (loblolly pine), *Quercus falcata* (southern red oak), *Q. marilandica* (blackjack oak), *Q. coccinea* (scarlet oak), *Q. stellata* (post oak), and *Q. velutina* (black oak). The terrain is rolling hills and the soil type is Smithdale sandy loams with Lucy loamy sands on slopes (Surrette and Brewer 2008). The EMF community survey at TEF took place in an untreated plot and a plot that was burned in 2005, damaged by a tornado in 2008 (which constituted the thinning treatment), and burned again in 2010 and 2012. The plots used in the substrate plug experiment (all located at TEF) were the untreated plot, an un-thinned plot that experienced prescribed burns in 2005, 2010, and 2012, and a plot in an area burned by a wildfire in July 2012. The same untreated plot at TEF was used in both the community survey and the substrate plug experiment. The wildfire plot had higher burn marks on tree trunks and more charring of downed wood than any of the three prescribed burn plots, indicating greater fire intensity. Previous to these fires, none of the plots had burned since at least the 1980s.

The SPAC sites are similar to TEF, although with fewer pines, which were historically not part of the upland landscape in central Marshall County (Surrette et al. 2008). Soils are less sandy than those at TEF. Site 1 at SPAC consisted of two 70 x 75 m plots, with an untreated plot and a plot that was thinned by cutting or girdling stems of mesophytic tree species, mostly *Nyssa sylvatica* (black gum), *Liquidambar styraciflua* (sweetgum), and *Ulmus alata* (winged elm). The thinning treatment was followed by burning in 2005, 2010, and 2012. Site 2 consisted of two 30 x 30 m plots, one untreated and one burned in 2008, 2010, and 2012, with half of the plot thinned to remove mesophytic species (Rietl and Jackson 2012, Ryndock et al. 2012).

EMF Community Survey

Soil cores 3 cm diameter by 15 cm deep were collected in July 2013. In each of the six plots, a systematic grid was used to collect 28 samples, at least 10 m apart, throughout each plot. This distance was chosen because previous studies found spatial autocorrelation primarily at shorter distances (Bahram et al. 2011, Lilleskov et al. 2004, Pickles et al. 2012). Cores were stored at 4 °C until processing.

Soil from cores was mixed and sub-sampled during processing and assayed for soil organic matter content using a loss-on-ignition method (Davies 1974). After soil sampling, cores were washed over a 2 mm sieve to separate fine roots. Using a dissecting microscope, ectomycorrhizal morphotypes were characterized in each sample, root tips per morphotype counted as a measure of abundance, and three

tips from each morphotype in each sample were selected for molecular identification.

DNA was extracted from root tips using components of a Sigma Extract-N-Amp extraction kit (Sigma-Aldrich, St. Louis, MO, USA) as described by Rúa et al. (2015) with the exception that extracts were diluted with 160 µL PCR-grade water and were stored at -20 °C. Some tips not identified by the first round of sequencing were sliced to expose fresh tissue and put through the extraction process a second time in an effort to increase sequence yield. To facilitate Sanger sequencing of EMF species sampled, the Internal Transcribed Spacer (ITS) region of fungal nuclear DNA was amplified using forward primer ITS1-F and reverse primer ITS4 (Gardes and Bruns 1993). Amplification reactions for each sample contained 2.2 µL PCR-grade water, 4 µL of 2X RedTaq Premix (Apex Bioresearch Products, Inc., San Diego, CA, USA), 0.4 µL of each primer at 10 µM concentration, and 1 µL of DNA extract. Reactions occurred in sterile 96-well PCR plates sealed with a sterile silicone sealing mat, briefly vortexted and centrifuged, and amplified as follows: initial denaturation at 94 °C for 3 min; 40 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 53 °C, extension for 72 s at 72 °C; and a final extension for 10 min at 72 °C. Amplification success was checked on a 1% agarose gel with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA).

Successful PCR reactions had excess primer and mononucleotides removed enzymatically, with each reaction containing 0.05 µL ExoI (New England Biolabs, Ipswitch, MA, USA), 0.2 µL Antarctic Phosphatase (New England Biolabs, Ipswitch,

MA, USA), 4.75 µL PCR-grade water, and 5 µL of amplified DNA. Reactions were incubated at 37 °C for 30 min, then 80 °C for 20 min, followed by at least 5 min at 4 °C. Purified DNA was sequenced using the forward primer ITS5 (White et al. 1990) and the Big Dye Terminator Sequencing Kit (v3.1, Invitrogen Corp., Grand Island, NY, USA). Each sequencing reaction contained 0.4 µL BigDye Reaction Premix, 1.8 µL BigDye 5X Sequencing Buffer, 0.5 µL primer at 10 µM concentration, 6.3 µL PCR-grade water, and 1 µL purified DNA. Sequencing reactions were incubated thus: initial denaturation at 96 °C for 1 min; 45 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s, and extension at 60 °C for 4 min. A ramp speed of no more than 1 °C/s was used. Reactions were dried and shipped overnight to the DNA Lab at Arizona State University, Tempe, AZ, where the Big Dye reactions were purified and read on an Applied Bioscience 3730 capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA).

The sequences obtained were edited, assembled into Operational Taxonomic Units (OTUs) at 97% similarity, and identified by comparison to sequences in public databases as described in Rúa et al. (2015), with the exception that matches >99% similarity were assigned a species epithet (or genus if the sequence matched was not identified to species), 95-99% similarity assigned to a genus, and 90-95% assigned to a taxonomic family. OTUs assigned to non-mycorrhizal fungi were discarded. OTUs in the *Cortinarius* genus from the final round of sequencing were excluded because very high levels of *Cortinarius* spp. in re-extracted samples suggested that contamination had occurred in those reactions.

R version 3.2.3 was used for data analysis (R Core Team 2015). To test whether sites, restoration treatments, or the site by treatment interaction influenced EMF community composition, OTU richness was measured and Shannon-Weiner diversity was calculated using the diversity() command in *vegan* (Oksanen et al. 2016). Groups were compared with Fisher-Pitman permutation tests using the function oneway_test() in *coin* (Hothorn et al. 2012).

OTUs occurring in ≥5 soil cores and the families occurring in ≥10 soil cores were also analyzed individually in univariate analyses. Generalized linear models using a logit link function were fit to presence/absence data for taxa of interest using glm() in *stats* and evaluated using Anova() from *car* with type II sums of squares and likelihood ratio test statistics (Fox and Weisberg 2011, R Core Team 2015). All models included site, treatment, the interaction of site and treatment, and soil organic matter as factors.

Substrate Plug Experiment

Four mature canopy oak trees were selected along a gradient of slope positions (top, top-middle, bottom-middle, and bottom) in each of the unburned, prescribed-burn-unthinned, and wildfire plots at TEF. All oaks selected were historic upland species (*Quercus coccinea, Q. falcata, Q. marilandica, Q. rubra, Q. stellata,* and *Q. velutina*). EMF communities and their potential enzymatic activity were studied by installing around the focal trees plugs of five experimental substrates: unburned soil, prescribed burn soil, wildfire soil, unburned DWD, and

burned DWD. Substrate plugs were used to separate the effect of substrate from plot effects on EMF enzyme activities. In May 2013, two replicates of each substrate were planted around each tree under the drip line, for a total of 120 substrate plugs (3 plots x 4 focal trees x 5 substrates x 2 replicates). Plugs were placed in a stratified random manner around each tree, with each substrate represented on the north half and on the south half of each tree to control for the effects of aspect (Figure 1). Each plug was 15 cm deep by 10 cm diameter.

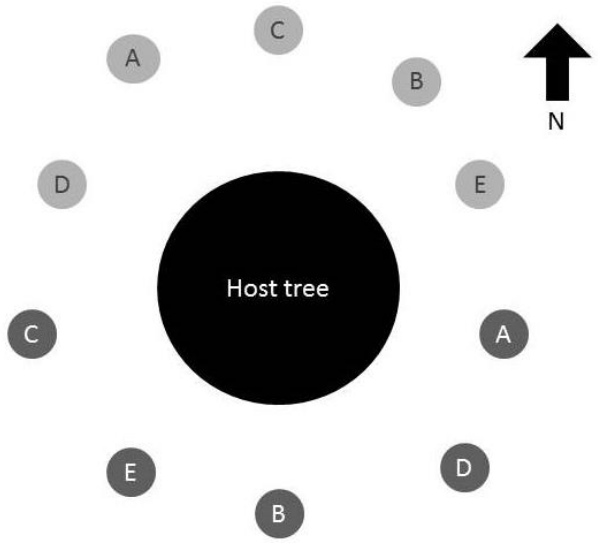

Figure 1. Arrangement of soil plugs around host trees. Five substrates (unburned soil, prescribed burn soil, wildfire soil, unburned dead woody debris (DWD), and wildfire DWD; represented by A-E) were randomly assigned to a position on the north side and a position on the south side of each host tree. Each plug was 15 cm deep and 10 cm in diameter, and was located under the canopy edge of the focal tree (generally about 2 m from the trunk).

DWD that was not visibly decomposed was collected from litter in unburned and wildfire plots for use in substrate plugs. The collected debris was chipped, with a typical chip <5 cm long and <1 cm thick. Soil substrates were taken from holes dug for insertion of soil plugs, and within each treatment all soil was mixed and sieved to remove roots and other large organic matter. Experimental plugs were harvested just before leaf drop in late October and early November 2013, approximately six months after they were installed in the field.

Plugs were collected into coolers in the field, returned to the laboratory, and stored at 4 °C for up to two weeks. Each plug was processed as for the community survey soil cores above, with three root tips from each EMF morphotype collected for enzyme assays. Samples were processed less than 24 h before running enzyme assays, to minimize effects of storage on potential enzyme activity (Pritsch et al. 2011). Enzyme substrates used were methylumbelliferone-N-acetyl-ß-D-glucosaminide to test NAGase activity (Pritsch et al. 2004, Pritsch et al. 2011), L-3,4-dihydroxyphenylalanine (L-DOPA) alone to test laccase activity, and L-DOPA with hydrogen peroxide to test combined laccase and peroxidase activity (Jackson et al. 1995). Laccase activity was subtracted from the combined activity to calculate peroxidase activity. The root tips used for enzyme assays were photographed and their projected area measured. As projected area is linearly correlated with entire surface area, this measurement was used to scale enzyme activity per unit area of the mycorrhizal root tip (Talbot et al. 2013). DNA was extracted from these same tips, amplified, Sanger sequenced, and the sequences processed using the same

methods as for the community survey. All *Cortinarius* OTUs were discarded because that genus was found only in samples processed with the contaminated community survey samples.

Although the primary focus of this experiment was collection of enzyme activity data, analyses on community composition were also carried out. Species richness and Shannon-Weiner diversity were compared across plots, substrates, and slope positions using Fisher-Pitman permutation tests as in the community survey.

Generalized linear models with a logit link relating plot, substrate, canopy cover, and slope position to presence/absence data were run using glm() in *stats* for OTUs found in ≥4 cores and families found in ≥10 cores (R Core Team 2015). Taxa without enough data to fit the model were excluded from results.

Soil samples from a subset of cores (n = 52) were assayed for enzyme activity as a control to test whether EMF root tip assays were actually measuring the enzymatic activity of residual free-living soil microbes on the samples. To calculate a plug-level measurement of EMF enzyme activity to compare with activity estimates from the bulk soil samples, the per-unit area activity of each morphotype in a plug was multiplied by the area of mycorrhizal root tips from that EMF morphotype found in the plug, then the results were summed across morphotypes within each plug to create a total enzyme activity for the plug. Bulk soil enzyme activity was tested for correlation with the EMF enzyme activity of a plug using

Spearman's rank correlation, with separate analyses for each of the three focal enzymes.

Root tip phosphatase activity was measured by colorimetric assays using PNP-linked substrates as described in in Jackson et al. (2006). This measurement was taken on a subset of morphotypes (n = 44) as a proxy for high overall EMF enzymatic activity. Per-unit-area values of potential phosphatase activity were compared with per-unit-area measurements for each focal enzyme using Spearman's rank correlation to test whether high activities of NAGase, peroxidase, or laccase were due to an unmeasured condition that created rapid nutrient cycling overall. Correlations among the focal enzyme activities within each morphotype were also tested using three Spearman's rank correlation tests.

Separate Fisher-Pitman permutation tests using the function oneway_test() in *coin* were used to test the effect of taxonomic family, plot, substrate, slope position, and the plot by substrate interaction on NAGase, laccase, and peroxidase (Hothorn et al. 2012). Families with fewer than three identified morphotypes were excluded from family-level analyses. The robust post-hoc test mcppb20() in *WRS* was used to compare enzyme activities between pairs of families (Wilcox and Schönbrodt 2015). Because this test uses the same data over multiple comparisons, an adjusted "critical p" is calculated that indicates a likelihood equivalent to $\alpha = 0.05$, against which p values for individual comparisons are evaluated.

RESULTS

Community Survey Results

Species richness of EMF communities was not significantly different across sites ($X^2_{2, 163} = 1.684$, p = 0.431), treatments (Z = -1.353, p = 0.176), or the site by treatment interaction ($X^2_{5, 160} = 11.057$, p = 0.087). Shannon-Weiner diversity was also not significantly different across sites ($X^2_{2, 163} = 1.129$, p = 0.569) or treatments (Z = -1.667, p = 0.095), and was marginally significant for the site by treatment interaction ($X^2_{5, 160} = 10.249$, p = 0.068).

187 EMF OTUs were identified across the experiment. The most commonly detected OTUs, each occurring in at least five soil cores, were *Russula xerampelina* (16 cores), *R. pectinatoides* (8 cores), *R. californiensis* (5 cores), *Phylloporus rhodoxanthus* (6 cores), and *Clavulina* 1 (6 cores). The most commonly detected families were Russulaceae (in 81 cores), Thelephoraceae (30 cores), Boletaceae (14 cores), Amanitaceae (13 cores), Gloniaceae (12 cores), and Cortinariaceae (11 cores).

Univariate analysis of each of these most common OTUs and families using generalized linear models and presence/absence data found that the occurrence of *Russula xerampelina* was significantly explained by site ($X^2_{2, 158} = 7.609$, p = 0.022) and treatment ($X^2_{1, 158} = 4.737$, p = 0.030), with most frequent occurrence at SPAC 1 sites and in treatment plots. The occurrence of *Clavulina* 1 was significantly explained by the site by treatment interaction ($X^2_{2, 158} = 8.739$, p = 0.013) and was found most commonly overall in the SPAC 1 treatment plot, but was more common in control plots at the other two sites. *R. pectinatoides* occurrence showed a trend in

occurrence connected to the site by treatment interaction ($X^2_{2, 158} = 5.225$, $p = 0.073$), with the most occurrences at the SPAC 2 treatment site. *P. rhodoxanthus* occurrence was near-significantly affected by site ($X^2_{2, 158} = 5.133$, $p = 0.077$), with no occurrences at TEF. None of the chosen variables significantly explained occurrence of *R californiensis* ($p > 0.12$).

At the family level, Russulaceae occurrence was significantly explained by the site by treatment interaction ($X^2_{2, 158} = 9.092$, $p = 0.011$), with the family occurring most commonly at the TEF control site but more frequently in the treatment plots at the other two sites. Thelephoraceae occurrence was significantly explained by soil organic matter ($X^2_{1, 158} = 5.543$, $p = 0.019$) and treatment ($X^2_{1, 158} = 4.315$, $p = 0.038$), with this family occurring more commonly at untreated plots and in cores with higher soil organic matter. Occurrences of Boletaceae were significantly explained by site ($X^2_{2, 158} = 6.024$, $p = 0.049$), with only one occurrence at TEF compared to six and seven at SPAC 1 and SPAC 2, respectively. The occurrence of Amanitaceae was significantly explained by site ($X^2_{2, 158} = 11.368$, $p = 0.003$), and this family was not detected at SPAC 2. The occurrence of Gloniaceae showed a trend of treatment on occurrence ($X^2_{1, 158} = 3.381$, $p = 0.066$), with nine occurrences in treatment plots versus three in untreated plots. None of the chosen variables significantly explained occurrence of Cortinariaceae ($p > 0.13$).

Substrate Plug Experiment Results

EMF species richness varied by plot ($X^2_{2, 118} = 8.581$, $p = 0.014$). As the data was not strongly skewed, aov() and TukeyHSD() in *stats* were used to evaluate contrasts, showing that the wildfire plot had lower richness than the unburned plot (adjusted $p = 0.013$), with other contrasts not significant. Observed OTU richness at the wildfire plot was 28, compared to 40 at the prescribed burn plot and 44 at the unburned plot. Species richness did not vary by substrate ($X^2_{4, 116} = 4.750$, $p = 0.314$) or slope position ($X^2_{3, 117} = 2.893$, $p = 0.408$). Shannon-Weiner diversity did not vary by plot ($X^2_{2, 118} = 4.5387$, $p = 0.103$), substrate ($X^2_{4, 126} = 5.633$, $p = 0.228$), or slope position ($X^2_{3,117} = 2.920$, $p = 0.404$). The number of mycorrhizal root tips found per core was the same across all three plots ($X^2_{2, 118} = 0.495$, $p = 0.781$).

Ninety-seven EMF OTUs were identified in this experiment, with the most commonly detected OTUs being identified as *Laccaria* 51 (present in 15 cores), Thelephoraceae 52 (6 cores), *Cenococcum* 53 (4 cores), *Cenococcum* 54 (4 cores), *Russula californiensis* (4 cores), *Tomentella* 55 (4 cores), and *Tomentella* 59 (4 cores). The most commonly detected families were Thelephoraceae (59 cores), Hydnangiaceae (31 cores), Gloniaceae (16 cores), and Russulaceae (15 cores).

Univariate analyses based on generalized linear models with presence/absence data and a logit link could not be fit for Thelephoraeceae 52, *Cenococcum* 54, *Russula californiensis*, *Tomentella* 55, and *Tomentella* 59 due to separation of variables. Analysis of *Laccaria* 51 and *Cenococcum* 53 occurrence

found suggestive evidence for the effects of substrate on the occurrence of both taxa. *Laccaria* 51 ($X^2_{4, 101} = 8.676$, $p = 0.070$) had no occurrences in unburned DWD, and *Cenococcum* 53 ($X^2_{4, 101} = 8.064$, $p = 0.089$) had three occurrences in unburned soil and one in wildfire soil, with none in the other substrates.

At the family level, the occurrence of Gloniaceae was significantly explained by substrate ($X^2_{4, 101} = 9.749$, $p = 0.011$), with no occurrences of this family in unburned DWD. Thelephoraceae occurrence was inversely related to canopy cover ($X^2_{1, 101} = 10.411$, $p = 0.001$). Hydnangiaceae occurrence was not significantly affected by plot ($X^2_{2, 101} = 2.606$, $p = 0.272$), substrate($X^2_{4, 101} = 5.171$, $p = 0.270$), canopy cover($X^2_{1, 101} = 0.007$, $p = 0.933$), or slope position ($X^2_{3, 101} = 0.453$, $p = 0.929$). Russulaceae occurrence was also not explained by plot ($X^2_{2, 101} = 2.447$, $p = 0.294$), substrate($X^2_{4, 101} = 3.261$, $p = 0.515$), canopy cover($X^2_{1, 101} = 1.161$, $p = 0.281$), or slope position ($X^2_{3, 101} = 3.416$, $p = 0.332$).

There was no correlation between bulk soil and core average root tip enzymatic activity for any of the three focal enzymes (NAGase: $\rho_{50} = -0.159$, $p = 0.260$; peroxidase: $\rho_{50} = 0.050$, $p = 0.725$; laccase: $\rho_{50} = -0.039$, $p = 0.782$). Phosphatase activity per unit area was also not significantly correlated with the activity of these enzymes (NAGase: $\rho_{38} = 0.080$, $p = 0.623$; peroxidase: $\rho_{38} = 0.103$, $p = 0.529$; laccase: $\rho_{38} = -0.238$, $p = 0.140$). Activities of peroxidase and NAGase per unit area were positively correlated ($\rho_{161} = 0.363$, $p < 0.001$), while laccase activity

per unit area was not correlated with either NAGase ($\rho_{161} = 0.107$, $p = 0.175$) or peroxidase ($\rho_{161} = -0.087$, $p = 0.271$) activity.

Activity per unit area of all three focal enzymes varied significantly by EMF family (Figure 2). NAGase activity per unit area varied by family ($\chi^2_{12, 143} = 24.075$, $p = 0.033$), with Tuberaceae having higher activity than Cantharellaceae, Amanitaceae, and Strophariaceae. Peroxidase activity per unit area also varied at the family level ($\chi^2_{12, 143} = 55.742$, $p = 0.002$), with Tuberaceae having higher activity than Strophariaceae, Thelephoraceae, and Cantharellaceae. Laccase activity varied by family ($\chi^2_{12, 143} = 76.652$, $p < 0.001$), with Hydnangiaceae having higher activity than Boletaceae, Gloniaceae, Sebacinaceae, Cantharellaceae, Strophariaceae, and Thelephoraceae.

The plot by substrate interaction influenced activities of all three enzymes in these univariate models (Figure 3): NAGase ($\chi^2_{28, 168} = 47.828$, $p < 0.001$), laccase ($\chi^2_{28, 168} = 29.202$, $p < 0.009$), and peroxidase ($\chi^2_{28, 168} = 36.398$, $p < 0.002$). Robust post-hoc testing found no significant contrasts between groups for laccase activity. Wildfire soil in the wildfire plot had the lowest NAGase activity, while wildfire DWD at that plot had the lowest peroxidase activity. Peroxidase activity was high in the prescribed burn plot for unburned and prescribed burn soils and unburned and wildfire DWD.

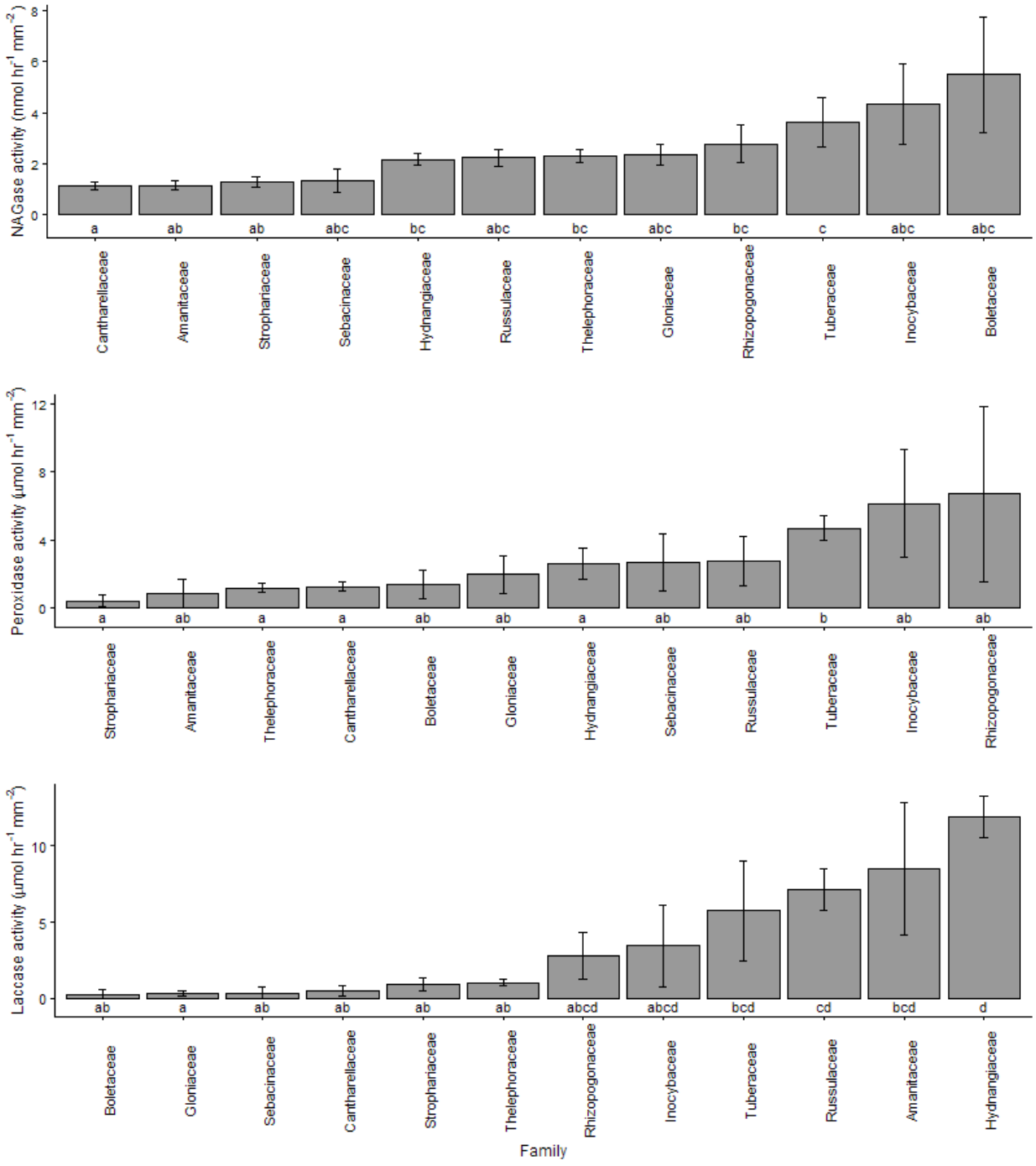

Figure 2. Variation among fungal families in potential ectomycorrhizal fungal enzyme activity per unit area of mycorrhizal root tip. Samples were taken from soil and dead woody debris plugs in a temperate oak-pine woodland. Note overlap between families with high NAGase activity and high peroxidase activity.

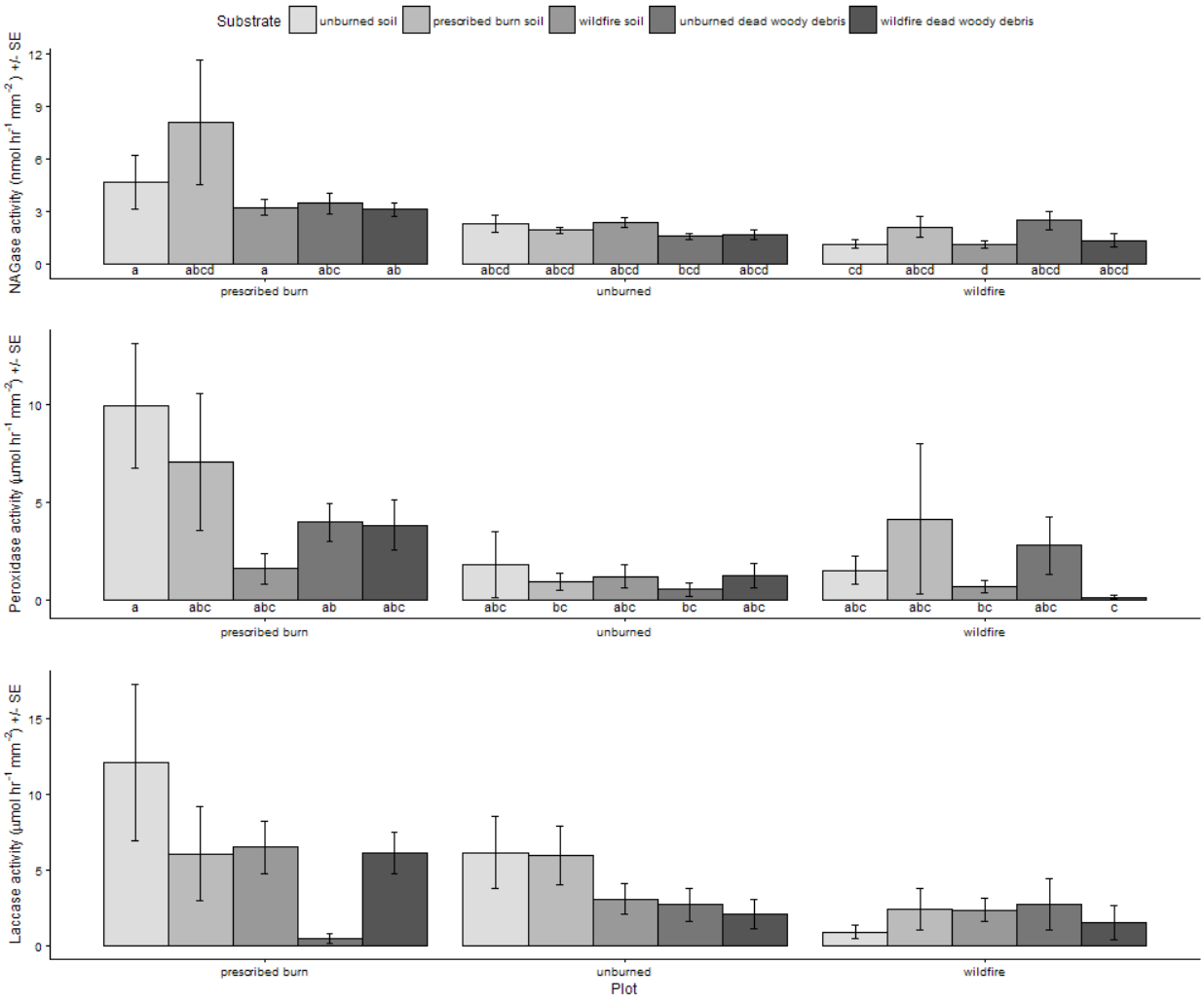

Figure 3. Variation among plot-substrate combinations in potential ectomycorrhizal fungal enzyme activity per unit area of mycorrhizal root tip in a temperate oak-pine woodland. Bars are standard error and contrasts were compared using means trimming and bootstrapping. Top panel: NAGase varied with site-substrate combinations, with highest activity in soils in the prescribed burn site. Middle panel: peroxidase activity was highest in unburned soil at the prescribed burn site and lowest in wildfire dead woody debris at the wildfire site. Bottom panel: no significant differences among site-substrate combinations were found for laccase activity.

The main effect of plot was a significant factor explaining the per-unit-area activity of each enzyme, with highest activity levels in the prescribed burn plot (Figure 4). For NAGase, all three plots had significantly different enzyme activities, with the highest activity at prescribed burn plot highest and lowest at the wildfire plot (critical $p = 0.017$, contrasts against the prescribed burn plot both $p < 0.001$, unburned vs. wildfire contrast $p = 0.008$). Peroxidase activity was higher at the prescribed burn plot than wildfire or unburned plots (critical $p = 0.017$, contrasts against prescribed burn plot both $p < 0.001$), which were similar. Laccase activity at the prescribed burn plot was higher than at the wildfire plot (critical $p = 0.017$, $p = 0.011$), while the unburned plot had laccase activity that was not different from either of the other two plots.

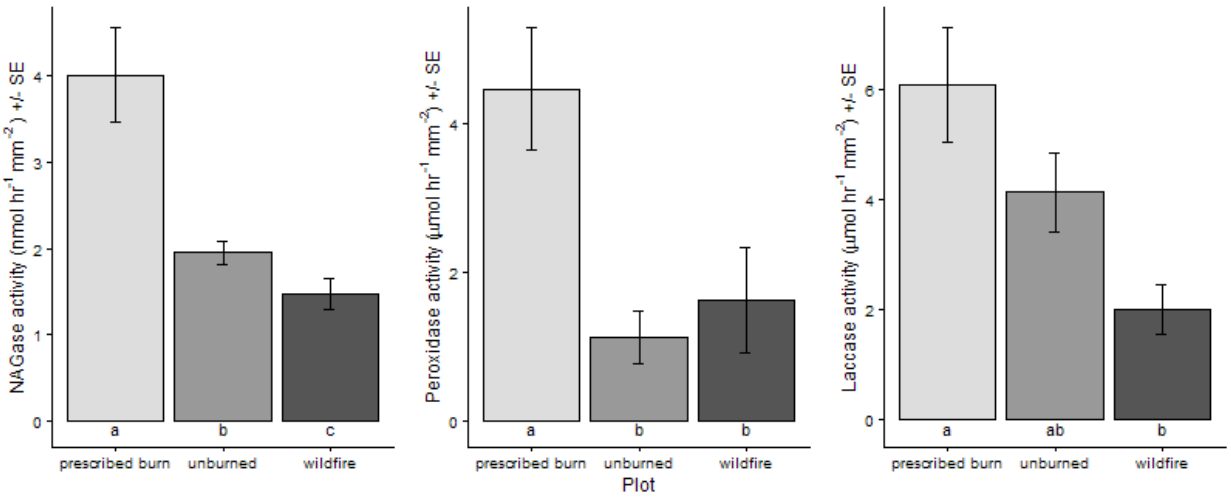

Figure 4. Potential ectomycorrhizal fungal enzyme activity per unit area of mycorrhizal root tip averaged by plot. Bars are standard error, and contrasts were compared using trimmed means and bootstrapping. Left panel: NAGase activity is significantly different for all three plots. Center panel: peroxidase activity was higher in the prescribed burn plot. Right panel: laccase activity was significantly higher in the prescribed burn plot than the wildfire plot.

DISCUSSION

How does local environmental change affect EMF community diversity?

The most common OTU (*Russula xerampelina*) and most common family (Thelephoraceae) showed a response to restoration treatments in the community survey. Site, which was significant in explaining the occurrence of *Russula xerampelina,* Amanitaceae and Boletaceae, and the site by treatment interaction, which was significant in explaining *Clavulina* 1 and Russulaceae occurrence, are both best interpreted as an effect of distance in the community survey. The expected spatial autocorrelation in EMF community surveys is around 3 m (Bahram et al. 2011, Lilleskov et al. 2004, Pickles et al. 2012), which is much smaller than plot and site sizes, so detecting distance effects is expected.

Restoration did not negatively affect diversity or abundance of EMF, suggesting that thinning and prescribed burning is not negatively affecting EMF at these sites. Thelephoraceae occurrence was positively associated with soil organic matter content, supporting the idea that different taxa have different preferred niches.

A previous EMF community survey was conducted at these same sites four years prior to ours and only one year after prescribed burning was established in the treatment plots. That study found variation in EMF community composition among sites, but not in response to restoration treatments (Craig et al. 2016). Overall, EMF OTU richness (173 found by Craig et al. (2016), 187 here) and

composition at the family level were similar for both surveys, with Russulaceae and Thelephoraceae making up the largest shares of OTUs detected; however the current study found higher abundance of Amanitaceae and Boletaceae, compared to more representatives of Sebacinaceae and Cantharellaceae in the Craig et al. (2016) survey. In both studies, well over half of the taxa found were detected in only one core.

In the substrate plug experiment, the lower richness of EMF at the wildfire plot suggests that the wildfire, which was more intense than typical prescribed burns, may have partially reset fungal succession. In a common pattern of EMF succession after fire, taxa such as Tuberaceae and Rhizopogonaceae that form resistant propagules are more likely to remain after severe fire, and species in Amanitaceae, Russulaceae, and Thelephoraceae characterize mature communities (Gardes and Bruns 1996, Horton and Bruns 1998, Taylor and Bruns 1999). My observations agree with this pattern: out of seven Rhizopogonaceae and Tuberaceae OTUs detected, only one occurred in the unburned plot, and four occurred exclusively in the wildfire plot. Of 45 OTUs from Amanitaceae, Russulaceae, and Thelephoraceae, only 10 were observed in the wildfire plot, while 19 were found in the prescribed burn plot and 25 were found in the unburned plot. Different composition of woody tree species may also have affected EMF at the wildfire plot; this area contained more *L. styraciflua* than the other plots, and *L. styraciflua* does not commonly form ectomycorrhizae.

How does EMF identity affect enzyme activity?

Despite high variability within some groups, there were significant differences among families in ability to degrade lignin. Laccase activity in EMF can correlate with morphology, with fungi with very little extraradical mycelium, such as *Russula* species, often having the highest laccase activity (Tedersoo et al. 2012), a pattern corroborated here. In culture, laccase activity can even be higher in some *Russula* species than in some saprotrophs (Burke et al. 2014). EMF enzyme activity in general is more strongly related to exploration type than fungal lineage, suggesting that similar morphotypes may experience convergent evolution (Tedersoo et al. 2012). Unfortunately, exploration type data were not collected during sample processing, and due to variation in exploration type within families and genera, this study was unable to examine these links more closely.

In line with the overall correlation between NAGase and peroxidase activities, Tuberaceae, Rhizopogonaceae, and Inocybaceae had some of the highest activities for both enzymes. Ectomycorrhizae produce little NAGase relative to saprotrophs (Burke et al. 2014), but even though their enzyme activity levels are low, their activity is important because EMF represent a direct path of N to trees (Cullings and Courty 2009).

How does the environment influence EMF extracellular enzyme activity?

The prescribed burn plot had the highest enzyme activity for all three enzymes tested, whereas the wildfire plot was consistently lowest (Figure 4),

supporting my hypothesis of higher lignin degradation in the prescribed burn plot. This result suggests that frequent, low-intensity burns play a different ecological role than infrequent higher-intensity burns in this ecosystem. It is also suspected that repeated prescribed burning can select for a microbial community that doesn't suffer long-term effects after fire (Johnson and Curtis 2001).

My predictions about how substrates would affect extracellular enzyme activity were not supported, although plot by substrate interactions were significant (Figure 3), and differences among plots were especially strong in unburned soil and prescribed burn soil. Being in the highest slope position positively affected NAGase and peroxidase activities.

Although EMF in burned DWD had lower enzyme activities than those in unburned DWD, this difference was not significant. Counter to the findings of Buée et al. (2007), where high NAGase activity was found only in DWD-enhanced samples, NAGase activity was not higher in DWD, nor were Russulaceae and Thelephoraceae more likely to occur in DWD. As these are facultative enzymes, environmental conditions may explain the differing expression of extracellular enzymes by these taxa in other studies. The Buée study took place in an oak forest, and the presence of pine in the DWD collected, as well as sampling in late fall vs. winter in Buée et al. (2007), are possible explanations of the difference.

The prediction that lignin and chitin degradation would be correlated was supported by the correlation between peroxidase and NAGase activities, however

this correlation was consistent across substrate types, which questions the notion that EMF chitinase activity is serving a competitive function against saprobic wood-decay fungi to claim wood resources. EMF may still inhibit saprotrophs through other mechanisms, such as nutrient uptake from organic forms (Dickie et al. 2014). Also, since EMF may not completely degrade wood on their own, but rather finish degrading wood that saprotrophs have begun to break down (Cullings and Courty 2009), EMF competition against saprotrophs may be dependent on the stage of wood decay.

Overall, this study found that wildfires can have negative effects on EMF and their role in soil nutrient cycling, whereas prescribed burning preserved EMF richness and positively affected their degradative activity. Ultimately, a better understanding of the links between community diversity and functional diversity will benefit a broad range of both basic science and applied research. In addition to using niche preference and enzymatic capability to help resolve species complexes and understand how high diversity is maintained in EMF communities, this work can also help to select appropriate species for inoculation of nursery trees and enhance models of nutrient cycling.

CHAPTER II: EFFECTS OF ASH ADDITION ON *QUERCUS COCCINEA* (SCARLET OAK) SEEDLING GROWTH AND THE ASSOCIATED SOIL BACTERIAL COMMUNITY

ABSTRACT

Prescribed burning is a common woodland restoration treatment used to reduce fuel loads and promote understory grasses and forbs. The impacts of burning can be separated into immediate soil heating and ash addition and understanding how these two factors individually affect plant and belowground communities will aid in understanding the effects of prescribed burning as a whole. In this study, I investigated the effects of leaf litter ash addition on *Quercus coccinea* (scarlet oak) seedling growth and on the composition of the soil bacterial community. Relative growth rate of oak seedling height, diameter, stem volume, and leaf area was not affected by ash addition. Ash addition increased the Shannon diversity index and species richness of the soil bacterial community although the overall composition of the bacterial communities in ash-treated and untreated samples were similar.

INTRODUCTION

Prescribed burning is a common treatment in ecological restoration and fire protection efforts, and it affects both aboveground plant communities and belowground microbial communities. Disturbance from fire may have important consequences for nutrient cycling in forest and woodland ecosystems (Anderson and Cairney 2007). Understanding functional changes in soil microbial communities following disturbance should be a priority of forest ecology and management (Leckie 2005). Despite the importance of soil microbes, their response to restoration treatments is underrepresented in the literature (Rietl and Jackson 2012). One consequence of prescribed burning is addition of ash to upper soil layers, which directly changes soil pH, nutrient content, and nutrient availability. These changes in resource availability in turn alter bacterial communities (Augusto et al. 2008, Bååth and Arnebrant 1994, Noble et al. 1996, Noyce et al. 2015, Peltoniemi et al. 2016) and plant growth and communities (Augusto 2008). Plant productivity and diversity may also be indirectly affected through the changes in bacterial communities (Panke-Buisse et al. 2014, van der Heijden et al. 2008, Zak et al. 2014).

As prescribed burning to meet restoration and fuel management goals becomes more common, it is important to understand its effects on soil microbial communities. Fire may affect bacterial communities for years post-burn (Perkiömäki and Fritze 2002). While prescribed burning may be necessary to achieve other management goals, frequent burns can result in lower litter quality

and alter the microbial community, possibly creating further negative effects on decomposition rates, soil C storage, and soil erosion (Williams et al. 2012). The biogeochemistry of soil affects its post-fire trajectory, so having studies from a range of ecosystems is important in understanding context dependence (Hamman et al. 2007).

Considering the effects of soil heating and ash addition separately may allow us to draw from the significant literature that considers leaf litter ash and wood ash addition as soil amendments and as a way to dispose of waste products from logging, pulp mills, and bioenergy (Bååth and Arnebrandt 1994, Huotari et al. 2015, Noble et al. 1996, Noyce et al. 2015, Ohno and Erich 1990, Peltoniemi et al. 2016, Perkiömäki and Fritze 2002). As soil heating is difficult to measure accurately in the field because of the patchiness of burns and variation in soil moisture, separating the effects of soil heating from ash addition may also allow for more accurate predictions of the effects of fires (Pietikäinen et al. 2000). Changes induced by ash addition can affect the soil microbial community regardless of site fertility (Perkiömäki and Fritze 2002). Characterizing the microbial response to ash addition will assist in understanding the overall forest health effects of ash as a soil amendment (Noyce et al. 2015).

We sought to answer two fundamental questions about the influence of ash in oak-pine woodlands. First, does ash addition increase the growth rate of oak seedlings? Second, does ash addition affect bacterial diversity and/or community structure? I predict that ash addition will increase oak seedling growth rate and

bacterial community diversity, and significantly change bacterial community composition at the phylum level.

METHODS

Site Description

Soil and leaf litter used in this study was obtained from the Tallahatchie Experimental Forest (TEF), part of the Holly Springs National Forest in northern Mississippi, USA. TEF is a mixed upland forest with second growth stands including *Pinus echinata* (shortleaf pine), *P. taeda* (loblolly pine), *Quercus falcata* (southern red oak), *Q. marilandica* (blackjack oak), *Q. coccinea* (scarlet oak), *Q. stellata* (post oak), and *Q. velutina* (black oak). The terrain is rolling hills and the soil type is Smithdale sandy loams with Lucy loamy sands on slopes (Surrette and Brewer 2008). The study site had not been burned since at least the 1980s. Prescribed burning that combusts the litter layer is an appropriate restoration treatment for this ecosystem, and nearby stands under active management are burned approximately every 2-3 years. Soil pH on site ranges from 5.3-6.1 (NRCS Soil Survey Staff 2016).

Litter, Soil, and Acorn Collection

Litter was collected within 21 20 x 20 cm quadrats on upper slopes of TEF prior to collection of the underlying soil. Litter was allowed to air dry at 20 °C and then completely burned in the base of a kettle-style grill lined with aluminum foil. Amount of ash addition was determined by dividing total weight of burned ash (369

g) by total surface area collected (8400 cm^2) and then multiplying by the surface area of a conetainer used for planting (12.57 cm^2), yielding 0.55 g of ash addition per conetainer. An ash sample was sent to the Soil, Water and Plant Laboratory at the University of Georgia (Athens, GA) for elemental analysis using total acid digestion. Leaf ash contained 14.6% total C, 2.71% Ca, 0.68% N, 0.45% Al, 0.43% manganese (Mn), 0.35% Fe, 0.28% magnesium (Mg), 0.20% K, 0.16%P, and 0.12% sulfur (S). All other elements were found at levels <0.01%.

The top 15 cm of field soil was collected from each quadrat for preparing a microbial filtrate and for use as a substrate for growing seedlings. This soil depth approximated the depth of the conetainers used, after accounting for empty space at the top and bottom.

Acorns were collected from beneath a mature *Quercus coccinea* tree in Oxford, MS, USA, in October 2014. Unsound acorns were excluded by removing those that floated in water and by visual inspection for damage. Acorns from which it was difficult to remove the cap were discarded as immature seeds. Sound, mature acorns were stored in high humidity at 4°C until planting, 180 days. Shortly before planting, acorns were allowed to air dry, washed in a 0.53% sodium hypochlorite solution, and rinsed in deionized water for two minutes.

Microbial Filtrate Preparation

A microbial filtrate was prepared on the day of planting by adding 1 L of field soil to 1L reverse-osmosis water and allowing it to soak for two hours. The slurry was then sieved through a 38 μm filter and the filtrate retained and used to inoculate all seedlings. Four subsamples of filtrate were centrifuged briefly, the supernatant was removed, and the pellet was frozen at -20°C for subsequent determination of bacterial community composition.

Soil Preparation and Planting

Wet field soil and commercial play sand were placed in separate autoclave bags and autoclaved at 121°C for 1 h. Material was allowed to cool for 24 h, the bags opened briefly for stirring with a plastic spatula, and autoclaved again to kill any germinating spores. Using gloved hands, conetainers were filled to within 3 cm of the top with autoclaved soil, and then an acorn was gently pressed into the pot with the cap scar up and covered with 2-3 cm of autoclaved sand. All conetainers were then inoculated with 10 mL microbial filtrate and were placed in a growth chamber, set to 12 h of daylight at 18 °C to encourage optimal growth (Larson 1970).

Watering, Ash Addition, and Harvesting

Seedlings were watered with DI water as needed 2-3 times per week and conetainer positions were randomized weekly until most seedlings had 2-4 true leaves, a period of 42 d. At this point, 0.55g ash was added to half of the seedlings, selected by randomization. Seedling height and basal diameter were measured on

all seedlings before ash addition and again 27 d later, at harvest. Acorns that did not sprout and seedlings that were highly stunted were removed from the analysis, leaving 136 samples, 69 of which received ash addition and 67 of which did not. When more than one stem was present, the tallest stem was used. Photographs to assess leaf area were taken before ash addition and at harvest.

Initial leaf area measurement was conducted using the Fiji distribution of ImageJ (Schindelin et al. 2012), as the young oak leaves were very variable in color. Easy Leaf Area (Easlon and Bloom 2014) was used for final leaf area measurements as it performed well on those images. A subsample of final leaf area photos was also measured using Fiji to ensure that measurements were consistent between methods.

The pH of an ash sample and three pre-ash-addition soil samples was measured by mixing sample 1:1 with distilled water, waiting 5 minutes, and evaluating the resulting solution using a pH meter with a glass electrode. Ash pH was 8.0, and pH of soil samples ranged from 6.1-6.3.

Molecular Methods

DNA was extracted from the four samples of filtrate and from bulk soil sampled from each of 35 randomly selected seedlings using a PowerSoil extraction kit (MoBio, Carlsbad, CA) following the included procedures. A dual barcoding approach was used for sequencing, targeting the V4 region of the 16S ribosomal RNA gene (Kozich et al. 2013), following the protocols in Jackson et al. (2015) and

Stone and Jackson (2016). Amplification products were standardized using SequalPrep Normalization Plates (Life Technologies, Grand Island, NY) and products were pooled prior to sequencing. The multiplexed library was sequenced using the Illumina MiSeq platform at the Molecular and Genomics Core Facility at the University of Mississippi Medical Center (Jackson, MS).

Data Analysis – seedling growth

Relative growth rate was calculated ((ln(final) – ln(initial)) / days) for leaf area, height of tallest stem, diameter of tallest stem, and stem volume modeled as a cylinder. Ash-addition and no-ash groups were compared in R version 3.2.5 (R Core Team 2015). Where the distribution of data was approximately normal, the Welch's unequal variances t-test was performed using the command t.test(). For variables with a non-normal distribution, the Mann-Whitney rank sum test was performed using the command wilcox.test().

Data Analysis – molecular data

Raw data files (FASTQ) were processed using the Mothur bioinformatics pipeline (Schloss et al. 2009) following the procedures recommended by Schloss et al. (2009) and Kozich et al. (2013). After removal of chimeras, other potentially erroneous data, and mitochondrial and chloroplast sequences, one sample had <1,500 valid bacterial sequences and was removed from the dataset. All diversity analyses were conducted using operational taxonomic units (OTUs) defined by 97 %

sequence similarity and by subsampling (1,000 iterations) the number of reads to that in the lowest remaining sample (4,087 sequences).

Good's coverage, inverse Simpson, Chao, Shannon, and ACE were calculated using the summary.single() command in mothur, and distance matrices using the Jaccard index and Yue and Clayton's theta were calculated using the dist.shared() command. Rarefaction curves for samples with coverage <0.9 were produced using rarefaction.single(). Non-metric multidimensional scaling was also performed using both distance metrics in mothur, using the nmds() command. Coordinates were then plotted in R using *ggplot2* (Wickham 2009) for two-dimensional data and *plot3D* (Ligges and Mächler 2003) for three-dimensional data. The ash-addition and no-ash groups were tested for differences in bacterial community composition using amova() and verified using homova() and anosim() in mothur. Indicator species analysis was performed using the mothur command indicator().

Differences in bacterial community diversity indices between ash-addition and no-ash groups were tested for significance in R using Welch's t-test and the Mann-Whitney rank sums test as above.

RESULTS

Seedling growth

There were no significant differences between ash-amended and no-ash seedlings in relative growth rate of leaf area ($W = 2012$, $p = 0.457$), height of tallest stem ($W = 2390$, $p = 0.622$), diameter of tallest stem ($t_{130.7} = 1.0445$, $p = 0.298$), or

stem volume (W = 2418, p = 0.536). Relative growth rate of stem diameter was highly correlated with relative growth rate of stem volume (S = 55852, ρ = 0.864, p < 0.001). Relative growth rate of stem height was also highly correlated with relative growth rate of stem volume (S = 167060, ρ = 0.593, p < 0.001). Relative growth rate of stem diameter and stem height were significantly correlated (S = 314960, ρ = 0.232, p = 0.007).

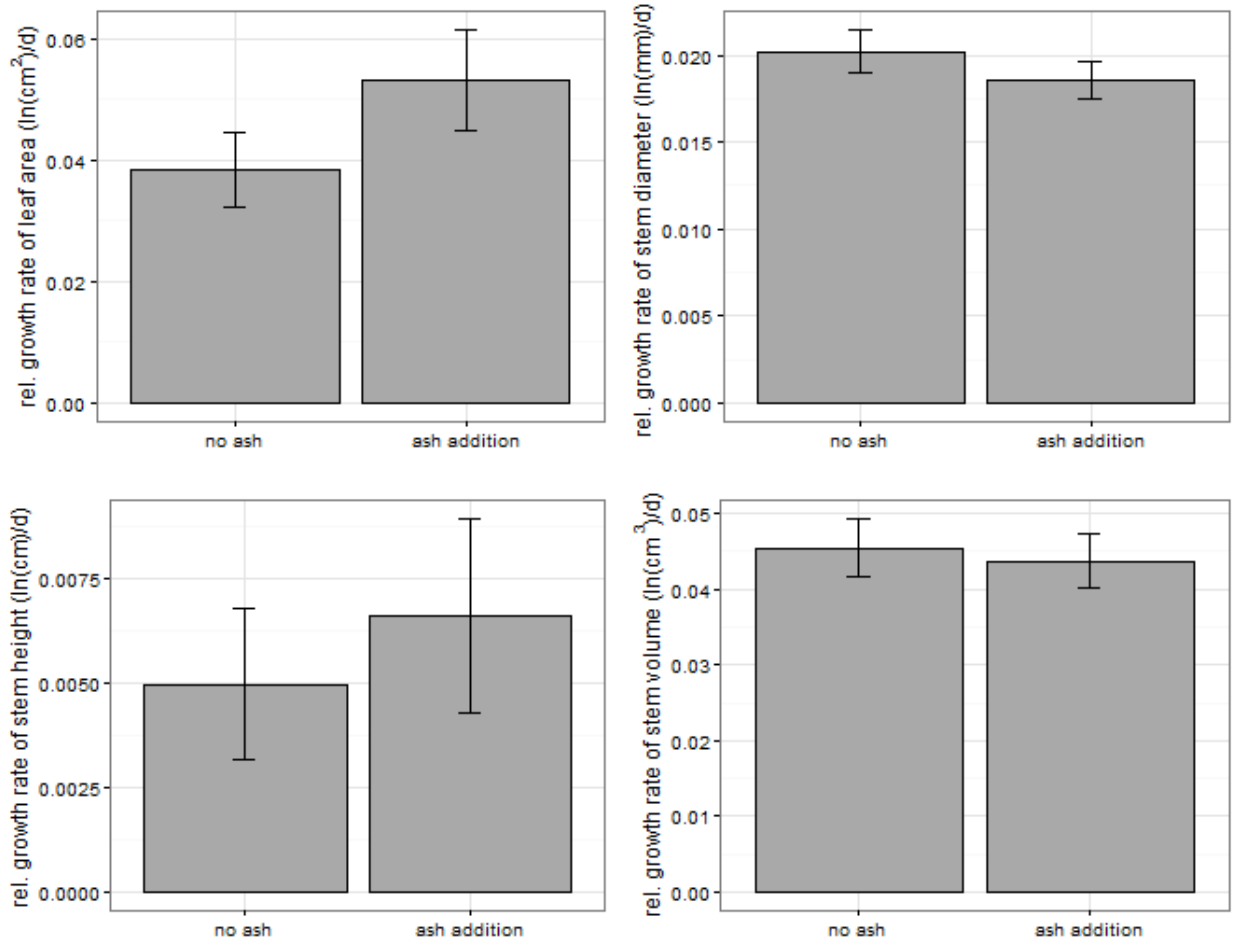

Figure 5. Relative growth rates of seedlings with standard error bar. No significant differences between groups.

Bacterial communities

Analysis began with 2,399,539 sequences, with 842,437 remaining after removing any sequences with ambiguous bases and sequences over 275 bases. After aligning sequences and removing sequences with uncommon start or end points or 8 identical bases in a row, 835,244 sequences remained, 114,781 unique. After using UCHIME to remove chimeras, 819,449 sequences remained. Sequences identified as belonging to chloroplasts, mitochondria, Archaea, or Eukaryotes were removed, leaving 809,087 sequences, representing 36, 287 unique sequences. Finally, one sample was represented by only 1,446 sequences and was excluded from analyses. Mean number of OTUs per sample, standardized by subsampling to 4,087 sequences, was 537.7 for the ash-amended soil samples, 472.3 for the soil that did not receive ash, and 1076.2 for the filtrate samples. Coverage was > 0.90 for all soil samples; however, the filtrate samples had lower coverage, ranging from 0.84-0.86.

Two-dimensional NMDS ordinations (stress = 0.21) based on theta dissimilarity scores that account for the proportions of taxa separated filtrate samples from soil samples but did not separate ash-amended soil samples from soil samples that did not receive ash (see Figure 2). Ordinations based on presence-absence data of OTUs (Jaccard index) were unclear in two dimensions (stress = 0.387) but more resolved in three dimensions (stress = 0.286) and suggested the same pattern. AMOVA (Jaccard $F_{1,32} = 1.232$, $p = 0.058$; theta $F_{1,32} = 1.160$, $p = 0.268$), HOMOVA (Jaccard $B = 0.005$, $p = 1$; theta $B = 0.5146$, $p = 1$), and ANOSIM (Jaccard $R = 0.060$, $p = 0.198$; theta $R = -0.067$, $p = 0.782$) confirmed these findings,

suggesting no significant differences in overall community structure between ash-treated and untreated samples.

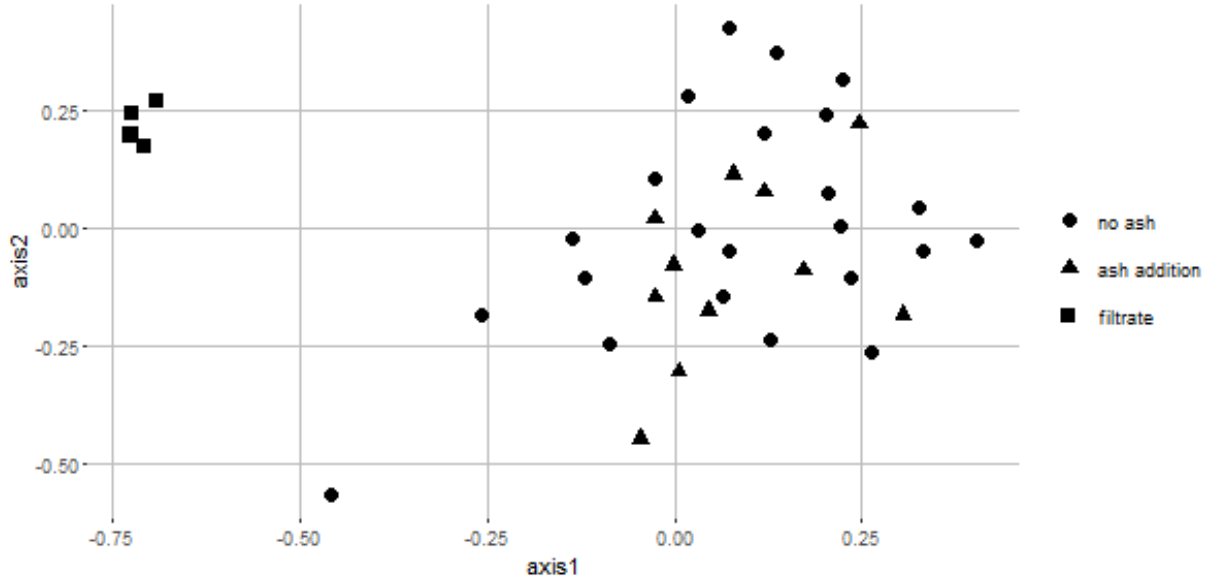

Figure 6. Ordination of bacterial community samples using Yue and Clayton's Theta and non-metric multidimensional scaling.

This difference between filtrate and soil samples is borne out when looking at the proportions of the phlya that made up more than 1% of pooled sequences each for filtrate, ash-amended soil, and no-ash soil (Figure 3). The no ash and ash amended samples have very similar composition, whereas the filtrate samples have a more even distribution of phyla, and more phyla are common.

While there appeared to be no overall differences in community composition between the soil sample groups, the diversity of the bacterial community in the ash-treated samples was higher than that in the no-ash samples. Chao's diversity index

was significantly higher for ash-addition samples than no-ash samples ($t_{29.42}$ = -2.087, p = 0.046), and the Shannon index (W = 66, p = 0.026) and ACE (W = 73, p = 0.050) corroborated this finding. Inverse Simpson diversity did not differ between groups (W = 86, p = 0.143).

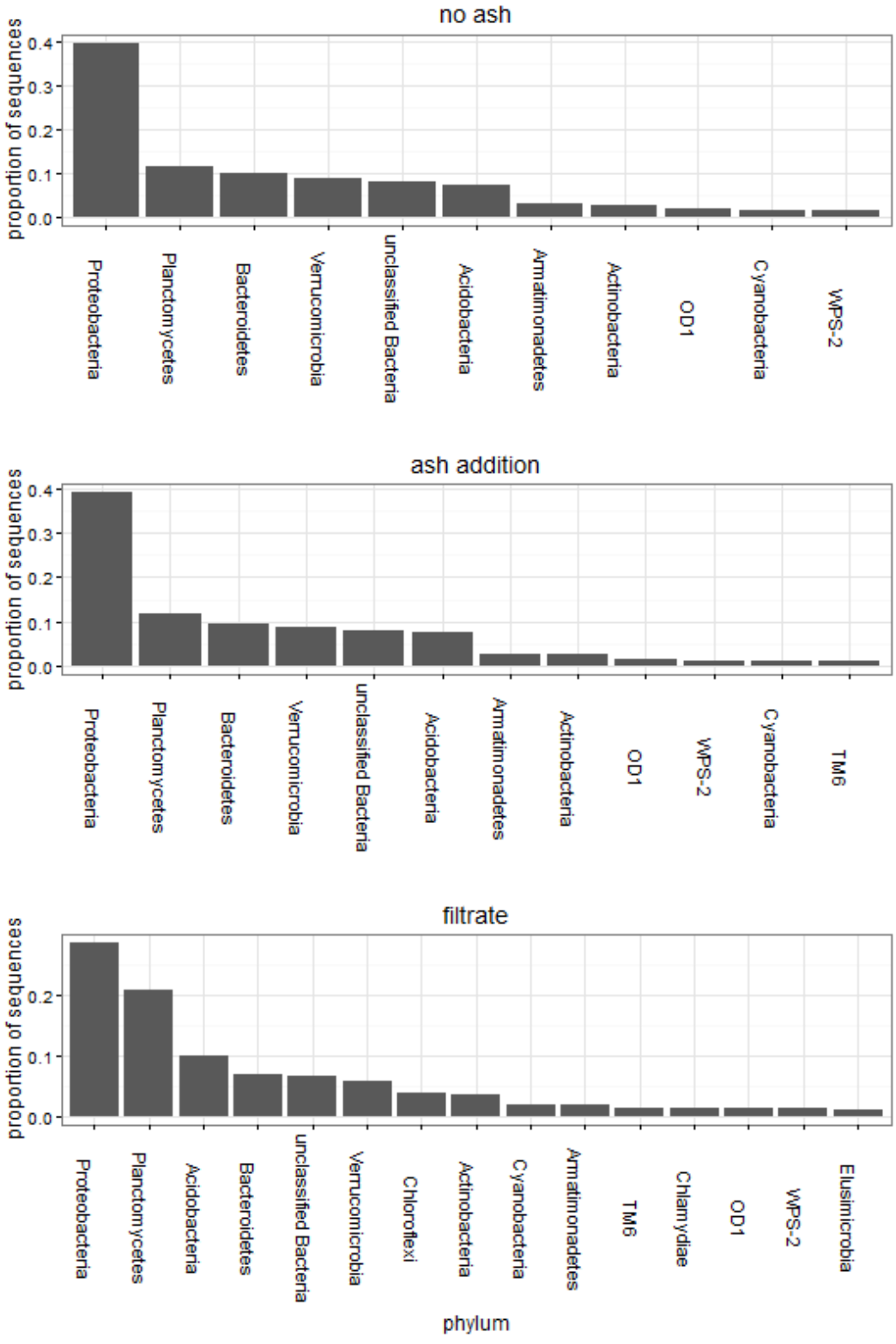

Figure 7. Barplots of most common phyla (> 10% of sequences) in sample groups. Based on 23 no-ash samples, ten ash-amended samples and five filtrate samples.

DISCUSSION

Contrary to my prediction, plant growth was not affected by ash addition. Litter ash content was lower in nutrients compared to reported values for leaf litter ash and wood ash (Augusto et al. 2008, Bååth and Arnebrant 1994, Materechera and Mkhabela 2002, Ohno and Erich 1990, Perkiömäki and Fritze 2002, Singh et al. 2010), which may explain the lack of an effect. Also, the soil used was not only moderately acidic, which may have reduced the benefits of addition of higher-pH ash on N availability. The primary benefit of ash addition to plant growth comes from the "liming" or alkalinization of acid soils, which can improve tree growth significantly on organic soils, where N is present in the soil but not available due to soil acidity (Augusto et al. 2008). Finally, due to the size of the conetainers, the seedlings were allowed to grow only 17 d after ash addition. The roots of many seedlings had reached the bottom of their containers and additional growth would have been complicated by negative effects from becoming root bound. A longer growth period may have allowed the seedlings to respond to ash addition.

Comparing filtrate and soil samples, the filtrate clearly had a different bacterial community composition and much higher diversity than the soil samples; not all taxa present in the filtrate persisted in the soil. The overall composition of the bacterial communities did not differ between the ash addition and no ash groups, although ash addition increased some measures of soil bacterial diversity. This may be due to the addition of ash increasing the pH of the soil (Rousk et al. 2010). This observation is consistent with findings that the low soil pH under oaks

can be a constraint on soil microorganisms (Hackl et al. 2005) and that increased pH due to burning altered bacterial communities in a boreal spruce forest (Smith et al. 2008).

Another possible explanation for the increased bacterial diversity is that ash addition increased both the diversity and availability of substrates available for bacterial growth, as ash commonly contains aromatic compounds that are otherwise uncommon in soil (Hart et al. 2005). This could have increased the growth of more diverse bacteria (Carney and Matson 2005) or at least allowed them to persist in the community.

Although ash addition comprises an important part of the effects of fire, it is important to consider that it does not stand alone. Microbial communities are likely to show greater changes in diversity and composition at sites exposed to the full effects of wildfire rather than just ash addition. Fire can result in dramatic changes to the vegetation which may override effects of nutrient amendment from ash (Noyce et al. 2015). Vegetation change can also drive changes in the soil and bacterial communities after prescribed burns (Williams et al. 2012).

In addition to its relevance to restoration, wood ash addition to soils is a common practice to dispose of ash waste from commercial logging (Noyce et al. 2015), pulp and paper mills (Ohno and Erich 1990), and the use of wood as a source of bioenergy (Huotari et al. 2015, Peltoniemi et al. 2016). Although studies on wood ash are more common, the leaf litter ash of a few tree species has been studied as a

possible soil amendment because it is available and inexpensive. Urban environments produce a large amount of leaf litter that needs to be disposed of, and as urban leaves show some promise as a bioenergy fuel stock (Piepenschneider et al. 2016), leaf litter ash addition to soils may become more common. Understanding the effects of this practice on plant growth and the soil microbial community is important in determining which areas are suited for ash addition.

CHAPTER III: HOST SPECIFICITY OF ECTOMYCORRHIZAL FUNGI IN MIXED PINE-OAK WOODLANDS

ABSTRACT

Most plant genera that form ectomycorrhizas have at least some fungal partners that are specific to that host genus, but many ectomycorrhizal fungi (EMF) are broad generalists. Because shared mycorrhizal fungi mediate plant community interactions, host specificity has implications for plant succession and competition. Considering host specificity against phylogeny and geography also allows insight into coevolution. I studied the EMF of oaks (*Quercus* spp.) and pines (*Pinus* spp.) in a north Florida, USA, forest, focusing on symbionts shared with longleaf pine (*P. palustris*). Longleaf pine is an increasingly important species in the southeast U.S., both as a commercial replacement for loblolly pine (*P. taeda*) and for restoring decimated savanna and woodland habitat. However, I was unable to find research on the identities of naturally-occurring EMF colonizing longleaf pine roots. The proportion of EMF operational taxonomic units (OTUs) found on oaks, pines, or both showed evidence of host specificity. Although most EMF were detected only on oaks or only on pines, the few OTUs found on both tended to be widespread and dominant. These results suggest that common mycorrhizal networks may be an important pathway for interaction between oaks and pines.

INTRODUCTION

Specificity in species interactions is important in understanding coevolution, community ecology, and even predicting extinction risk (Bruns et al. 2002, Devictor et al. 2010, Molina et al. 1992, Thompson 2009). Ectomycorrhizal fungi (EMF) show a range of specificity in which plant hosts they colonize. Although many EMF have broad host specificity, there seem to be at least some EMF specific to most ectomycorrhizal host genera (Molina et al. 1992, Toju et al. 2013). Members of the Pinaceae, in particular, have many family- and genus- restricted EMF partners (Bruns et al. 2002, Molina et al. 1992). However, relatively little information on host specificity of EMF comes from field studies on the roots of multiple distantly related plant taxa at the same site (Craig et al. 2016, Horton et al. 1999, Kennedy et al. 2003, Richard et al. 2005, Tedersoo et al. 2008, Twieg et al. 2007). This limits my understanding of how EMF specificity manifests in community patterns at the local scale. Here, I report results of a study on EMF found on neighboring pine and oak trees. I focused on sites containing longleaf pine, *Pinus palustris*, which is an important species for both commercial plantations and for restoring a rapidly disappearing ecosystem in the southeastern United States (Glitzenstein et al. 2003, Noss et al. 1995, Van Lear et al. 2005). However, despite some research on the mycorrhizal associations of longleaf pine, no studies have investigated the range of EMF taxa they associate with in the field.

Specificity in species interactions goes far beyond successful inoculation (Devictor et al. 2010, Thompson 2009). In addition to specificity being part of the

description of an organism's niche, specificity itself can also be conceived of as an "n-dimensional hypervolume" (Hutchinson 1957), with many factors affecting fundamental and realized specificity. Whether a symbiont will associate with a partner in the field can be affected by environmental conditions and the presence or absence of other species (Molina et al. 1992). Symbiosis formation may be assisted by a third party, such as with mycorrhizal helper bacteria (Garbaye 1994). The geographic facet of specialization is also important – host use may change across a species' range (Poulin et al. 2011). However, mutualisms can also expand niches, creating apparent generalism in environmental needs of their hosts (Poisot et al. 2011). Considering spatial and temporal scales is important when defining specificity, as the scale of environmental change, local genetic adaptations, and phenotypic plasticity of individuals are all important in understanding the underlying dimensions of specificity (Devictor et al. 2010).

Ecological specialization, or adaptation to a subset of possible environments, is a particularly important facet of specificity (Devictor et al. 2010, Molina et al. 1992, Poisot et al. 2011). Sampling only similar environments can give a false impression of specificity, which becomes particularly problematic if a range of environmental conditions are sampled at one experimental unit, but only similar conditions are sampled at another, creating variation in realized specialization (Devictor et al. 2010). However, a coarse scale of environmental variation relative to the dispersal area of a species is expected to lead to specialization, meaning that field conditions to test specificity may not be possible and a common garden

experiment would be needed (Poisot et al. 2011). Specificity is not a binary concept, but rather it is often governed by many interacting factors.

Specificity is also interesting because it can be a precursor to or a product of coevolution (Hoeksema 1999, Thompson 2009). Evolution of specificity requires constraints on adaptive evolution plus covariation of genotype and performance in an environment. This potential specificity is then further narrowed to realized specificity, which includes the effect of ecology and chance events on compatibility (Poisot et al. 2011). For specialization to evolve, Futuyma and Moreno (1988) predict that environmental constancy and either the ability of specialists to exclude less-efficient generalists or a change in resource use due to competition are necessary.

When interpreting patterns of specificity, it is also important not to assume it is driven by coevolution. A potential indicator of coevolution in specific interactions is phylogenetic tracking, whereby speciation in one partner is matched by speciation in another, leading to strong patterns of host specificity, but this is not a necessary condition for coevolution, nor an inevitable outcome of it (Thompson 2009). A species may be selecting specific partners due to another, non-phylogenetic trait, and non-reciprocal selection between species can lead to patterns of specificity and/or phylogenetic tracking. Alternatively, one species range may have expanded too recently for reciprocal evolutionary change to have occurred (Thompson 2009).

Evidence suggests that ectomycorrhizal symbioses are usually beneficial to both partners (Hoeksema et al. 2010, Karst et al. 2008). Thus, hypotheses on specificity and coevolution of mutualisms, as opposed to negative interactions, are most relevant to understanding specificity of EMF. For example, it is generally true that allocation of more resources to the most beneficial mutualists can be an evolutionary advantage and lead to specificity (Poisot et al. 2011). However, if a large number of mutualistic partners are available, the effect of the most beneficial partner may be swamped by the combined effect of the other partners, precluding specialization (Thompson 2009). On the other hand, a common pattern is for free-living mutualisms to accumulate species on one or both sides that share core traits governing the interaction (Thompson 2005), and coevolutionary selection may happen in various ways among guilds of interacting species (Hoeksema 2010). Shared EMF also allow the potential for formation of common mycorrhizal networks among plant species (Kennedy et al. 2003, Twieg et al. 2007), which can significantly alter the outcomes of plant-plant interactions by transferring water, nutrients, hormones, and allelochemicals between plants (Newmann 1988, Simard et al. 2012). For example, EMF networks associated with canopy *Pinus radiata* transferred water to conspecific seedlings, allowing increased drought tolerance and offsetting the negative effect of root competition on the seedlings (Booth and Hoeksema 2010). Between genera, stressed Douglas-fir (*Pseudotsuga menziesii*) transferred photosynthetic carbon to ponderosa pine (*Pinus ponderosae*) via mycorrhizal networks (Song et al. 2015).

Host specificity of EMF is especially compelling because interspecies mycorrhizal interaction is a mediator of plant community ecology. The availability of compatible fungi can drive plant succession and guild formation (Molina et al. 1992). For example, *Arcstaphylos* chaparral has been shown to provide EMF inoculum that drives *Pseudotsuga* succession in California central coast chapparal (Horton et al. 1999). In contrast, selective pressure on ruderal EMF such as some *Rhizopogon* may explain why many of them are specialized to early successional trees – the need to locate and colonize a host when only a few seedlings are available may conflict with the ability to colonize a broad host range (Bruns et al. 2002). Understanding whether specialization is a benefit or a detriment to the symbiotic partners will help drive applied decisions about types of inoculum to use in nurseries as well as resolve theoretical questions about the evolution of specificity.

Ectomycorrhizal interactions are also unique from a theoretical standpoint because they combine elements of both intimate and diffuse symbiosis (Bruns et al. 2002). Intimate mutualisms tend to have a range of specificities (Bruns et al. 2002). However, while the fungus is obligately tied to the plant root, plant and fungus reproduce and disperse separately from one another and can both form symbioses with multiple partners. These characteristics of diffuse symbiosis are predicted to reduce specificity and cheating in the interaction (Bruns et al. 2002). Preferential allocation of resources by the host to the most beneficial mutualists would be expected to lead to specificity (Poisot et al. 2011), although as both partners

generally need to form a mycorrhizal association to thrive, specialization may be a risky strategy on the part of either partner (den Bakker et al. 2004). Low specialization in many EMF taxa would therefore suggest that there either aren't significant differences in the amount of benefit that they provide, that the large number of EMF a host can interact with is swamping its ability to selectively reward the best partners, and/or that the risk of finding no specialized partner outweighs the cost of accepting sub-optimal partners.

In practice, however, host specificity among EMF ranges from relatively narrow to quite broad. Host specificity at the genus-to-genus or genus-to-family level has been documented in *Alnicola* and *Alpova* (both genera associate only with *Alnus*); *Leccinum* (fungal species to genus or family in Pinaceae); and Suillaceae (almost all fungal species restricted to subsets of Pinaceae) (Bruns et al. 2002, den Bakker et al. 2004, Rochet et al. 2011). The degree of specialization can vary, however; the EMF genus *Leccinum* shows evidence of a specialized common ancestor and speciation occurring in conjunction with host shifts, including the evolution of a generalist, *Leccinum aurantiacum* (den Bakker et al. 2004). At the other end of the spectrum many common species in the Amanitaceae, Boletaceae, Cortinariaceae, and Russulaceae are broad generalists. Host shifts and host specificity have contributed to EMF evolution, and in turn cycles of niche expansion and contraction over geologic time explain speciation and host specificity patterns in EMF (den Bakker et al. 2004, Kretzer et al. 1996).

Multihost fungi do tend to be the dominant species in mixed stands (Kennedy et al. 2003, Richard et al. 2005, Tedersoo et al. 2008, Twieg et al. 2007). However, Smith et al. (2009) found that EMF communities were structured by host even when the fungi known to show host specificity were excluded – understanding host preference and the ecological context of interactions is necessary. Some question the common assumption that most EMF are multihost, and the regular discovery of cryptic species in EMF may also uncover unknown pockets of specificity (Roy et al. 2008).

Longleaf pine, *P. palustris*, is important commercially and ecologically in the southeast United States. Longleaf pine is resistant to many diseases that affect other pines grown commercially in the area (Otrosina et al. 1999) and can grow on poor soils that often make the most common commercial species, *P. taeda* or loblolly pine, weakened and more susceptible to disease (Coyle et al. 2015, Eckhardt et al. 2010). Longleaf pine is also more resistant to windfall than loblolly, an increasing concern as hurricane frequency and severity increase along the Gulf Coast (Gresham et al. 1991, Johnsen et al. 2009). Finally, longleaf pine is a keystone species in longleaf pine savannas, a critically endangered habitat that supports extremely high species diversity (Frost 1993, Glitzenstein et al. 2003, Van Lear et al. 2005, Mitchell et al. 2006).

While research has examined the amount of EMF mycelium found in longleaf pine stands (Hendricks et al. 2006, McCormack et al. 2010, Runion et al. 1997, Sims et al. 2007), I was unable to find surveys of the EMF taxa present beyond

observation of a single *Thelephora terrestris* sporocarp. *Pisolithus tinctorius* has been trialed as a possible inoculum on longleaf, with varying results (Kais et al. 1981, Cram et al. 1999). EMF are expected to be important to longleaf pine success, as their typical habitat is sandy, fire-maintained communities, where acquisition of water is important and the minimal organic layer may make nutrient acquisition difficult (Hendricks et al. 2006).

This research examines host specificity of EMF in oaks and pines, with a focus on *P. palustris*, an important timber species in the southeastern United States. Specifically, I set out to answer the question of whether EMF with broad host range or narrow host range dominate in longleaf-dominant oak-pine forests. I hypothesized that EMF with broad host range will be more commonly detected and constitute a higher proportion of colonized root tips. I further hypothesized that due to the dominant nature of multihost EMF, the proportion of taxa colonizing oaks, pines, or both would be consistent with an assumption of no host specificity.

METHODS

Site Description

Samples were collected from the Jackson Guard section of Eglin Air Force Base near Niceville, FL, USA (30.5247, -86.4921). The area includes pine plantations as well as areas with more varied vegetation that support a variety of wildlife. Six sites within the base were selected in consultation with base staff to include all pine species found on base and a variety of oak species. Soils ranged

from very sandy and well-drained to saturated soils with high organic matter content to soils with high clay content to Dorovan muck (NRCS Soil Survey Staff 2016). The predominant soil type in the area is Lakeland sand (NRCS Soil Survey Staff 2016). All sampling sites were located within approximately 30 km of each other. *P. palustris* is common on the site, as is *P. taeda*. *Quercus laevis* is the most frequent oak species, although others are also common, including *Q. geminata* and *Q. incana*. Apparent oak hybrids were also common and were excluded from sampling. Soil pH ranged from about 4-6, with 5 being a typical value (NRCS Soil Survey Staff 2016).

Sample Collection and Processing

Sampling was conducted May 12-14, 2014. To maximize the likelihood of finding fungal species shared by different hosts, samples were taken between pairs of mature trees. Because of the site management's interest in increasing the use of *P. palustris* for plantations, each pair of trees was composed of a *P. palustris* and another tree. Other tree species included the other pine species present at Eglin (*P. clausa* (n = 8)*, P. elliotii* (n = 8)*, and P. taeda* (n = 8)) and a variety of red and white oaks chosen due to on-site abundance (red oaks: *Q. arkansana* (n = 5)*, Q.hemisphaerica* (n = 5)*, Q. incana* (n = 6)*,* and *Q. laevis* (n = 7); white oaks: *Q. geminata* (n = 7) and *Q. margaretta* (n = 6)). Between each pair of trees, four 7 cm diameter by 15 cm deep cores were taken in the root zone of each tree and these eight cores were compounded into one sample per tree pair. Sixty pairs of trees were sampled. Where they could be reached, leaves were also collected from sampled

trees to provide a reference for analysis of plant DNA in roots. Additional pine needles sampled from trees in other locations along the Gulf Coast were also used to create reference sequences. Soil was kept in coolers in the field to prevent samples heating in the sun and refrigerated at the end of the sampling day. Upon return to the lab, samples that could not be processed within two weeks of harvest were frozen at 0 °C until processing. Soil was sieved using a 2 mm mesh, debris was removed, and roots were washed with tap water and placed in a Petri dish. Samples with large quantities of roots were subsampled. Colonized root tips were classified into morphotypes based on color, surface texture, and branching pattern under a dissecting scope, and the number of root tips corresponding to each morphotype was counted. Three tips from each morphotype in each sample were saved for molecular identification. Sieved soil from each sample was saved for soil texture and soil organic matter assays. Soil texture was measured using a LaMotte soil texture test (LaMotte Company, Chestertown, MD, USA). Soil organic matter content was measured using a loss-on-ignition method. Soil was dried to a steady weight at 100 °C, then a subsample was placed in a tin of known weight, weighed, heated in a muffle furnace for two hours at 360 °C, and reweighed when cool enough to handle (Davies 1974).

Molecular Methods

DNA was extracted from all sampled root tips on the day the soil sample was processed. Components of Sigma Extract-N-Amp extraction kits (Sigma-Aldrich, St. Louis, MO, USA) were used as described by Rúa et al. (2015) with the exception

that extracts were diluted with 160 µL PCR-grade water and were stored at -20 °C. To facilitate Sanger sequencing of EMF species sampled, the Internal Transcribed Spacer (ITS) region of fungal nuclear DNA was amplified using forward primer ITS1-F and reverse primer ITS4 (Gardes and Bruns 1993). Amplification reactions for each sample contained 2.2 µL PCR-grade water, 4 µL of 2X RedTaq Premix (Apex Bioresearch Products, Inc., San Diego, CA, USA), 0.4 µL of each primer at 10 µM concentration, and 1 µL of DNA extract. Reactions occurred in sterile 96-well PCR plates sealed with a sterile silicone sealing mat, briefly vortexted and centrifuged, and amplified as follows: initial denaturation at 94 °C for 3 min; 40 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 53 °C, extension for 72 s at 72 °C; and a final extension for 10 min at 72 °C.

PCR to identify the plant host amplified the psbA-trnH chloroplast DNA locus using a touchdown PCR program due to difficulties in finding an optimum annealing temperature. The amplifcation parameters started with 3 minutes at 94 °C, then 15 cycles were run, over which the annealing temperature was decreased from 55 °C to 51 °C in 0.2 °C increments, followed by 25 cycles with an annealing temp of 51 °C. The cycles were denatured for 40 s at 94 °C, 40 s at the annealing temp, and 45 s at 72 °C. The cycling was followed by a final extension of 10 min at 72 °C. The psbA-trnH locus was selected because of its relatively high variability, particularly in oaks (Simeone et al. 2013) and common use as a plant barcoding locus (Hollingsworth et al. 2011). The trnL-trnF locus (Taberlet et al. 1991) was also tested and did not provide additional resolution in identifying plant species.

Success of PCR was evaluated on a 1% agarose gel with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). Successful PCR reactions had excess primer and mononucleotides removed enzymatically, with each reaction containing 0.05 μL ExoI (New England Biolabs, Ipswitch, MA, USA), 0.2 μL Antarctic Phosphatase (New England Biolabs, Ipswitch, MA, USA), 4.75 μL PCR-grade water, and 5 μL of amplified DNA. Reactions were incubated at 37 °C for 30 min, then 80 °C for 20 min, followed by at least 5 min at 4 °C. Purified fungal DNA was sequenced using the forward primer ITS5 (White et al. 1990) and purified plant DNA was sequenced using the psbA forward primer. All sequencing used the Big Dye Terminator Sequencing Kit (v3.1, Invitrogen Corp., Grand Island, NY, USA), with each sequencing reaction containing 0.4 μL BigDye Reaction Premix, 1.8 μL BigDye 5X Sequencing Buffer, 0.5 μL primer at 10 μM concentration, 6.3 μL PCR-grade water, and 1 μL purified DNA. Sequencing reactions were incubated thus: initial denaturation at 96 °C for 1 min; 45 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 20 s, and extension at 60 °C for 4 min. A ramp speed of no more than 1 °C/s was used. Reactions were dried and shipped overnight to the DNA Lab at Arizona State University, Tempe, AZ, where the Big Dye reactions were purified and read on an Applied Bioscience 3730 capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA).

The fungal sequences obtained were edited, assembled into operational taxonomic units (OTUs) at 97% similarity, and identified by comparison to sequences in public databases as described in Rúa et al. (2015), with the exception

that matches >99% similarity were assigned a species epithet (or genus if the sequence matched was not identified to species), 95-99% similarity assigned to a genus, and 90-95% assigned to a taxonomic family. Plant DNA sequences were aligned and compared to sequences from collected leaves, and compared to sequences on the GenBank database using the BLAST utility.

Data Analysis

Because of low genetic diversity in both pines and oaks, the locus sampled could only resolve the plant hosts into three groups: red oaks (*Quercus* section *Lobatae*), white oaks (*Quercus* section *Quercus)*, and pines (genus *Pinus*). As only 3 fungal OTUs were identified on white oaks, red and white oaks were pooled for analysis. Data was analyzed using R version 3.2.5 (R Core Team 2016). A list of fungal species associated with each identifiable plant group was compiled and compared to determine the amount of overlap within and among groups. A venn diagram showing the number of OTUs associated with oaks, pines, or both was created using *gplots* (Warnes et al. 2016). A chi-squared test was conducted to determine if the occurrence of OTUs associated with oaks, pines, both, or an unidentified host was consistent with a null hypothesis of no specificity. As data were non-normal, Spearman's rank correlation was used to compare the occurrence (presence/absence) and abundance (count of colonized root tips) of common taxa with soil organic matter and soil texture, using the cor.test() function.

RESULTS

164 EMF OTUs were identified, with these OTUs representing 16,290 ectomycorrhizal root tips. The OTUs occurring in the most samples were *Cenococcum geophilum* (in 15 samples), *Russula* 7 (15 samples), *Russula* 2 (11 samples), *Rhizopogonaceae* 1 (8 samples), *Hebeloma* 1 (7 samples), *Russula* 1 (7 samples), and *Lactifluus piperatus* (6 samples).

At least one plant host was identified for 120 of these OTUs. Of OTUs with an identified host, 85 OTUs were found only with pines, 25 OTUs were found only with oaks, and 10 OTUs were detected on both hosts (Figure 1).

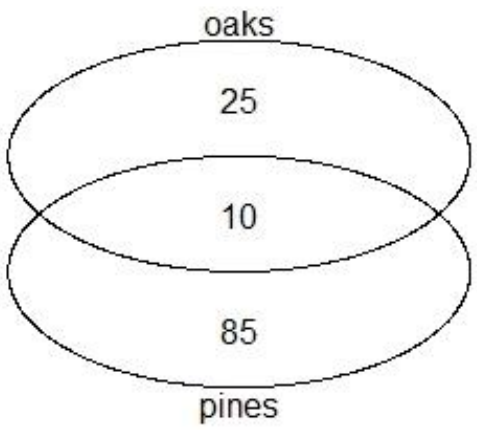

Figure 8. : Venn diagram of EMF OTUs by host genus. Eighty-four oak trees and 36 pine trees were sampled.

A chi-squared test found that this distribution of OTUs is significantly different from the expected distribution ($X_1^2 = 14.239$, $p < 0.001$), with most of the

chi-squared value coming from fewer OTUs than expected found on both oak and pines ($X^2 = 5.114$), and more OTUs than expected found on oaks but not pines ($X^2 = 6.940$).

However, the OTUs shared by oaks and pines included dominant fungi composing five out of seven of the most commonly detected OTUs and substantial proportions of the total number of root tips, including *Cenococcum geophilum* (3.4% of total root tips including samples lacking host plant identity), *Russula* 7 (3.0% of root tips), *Russula* 2 (4.2% of root tips), *Hebeloma* 1 (2.1% of root tips), and *Lactifluus piperatus* (1.2% of root tips). Common EMF found only on pines were Rhizopogonaceae 1 (detected in 8 cores and representing 2.1% of root tips), and *Russula* 1 (detected in 7 cores and representing 1.6% of root tips).

A few other taxa also represented similarly high percentages of total root tips, despite being detected in fewer cores: *Hygrophorus* 1 (3.1% of root tips), *Lactarius* 3 (3.1% of root tips), *Lactarius corrugis* (2.8% of root tips), *Rhizopogon* 3 (2.6% of root tips), Rhizopogonaceae 1 (2.1% of root tips), Amanitaceae 1 (2.1% of root tips).

Comparing taxa at the family level, Russulaceae dominated the EMF community, colonizing 41% of identified root tips and occurring in 49 out of 60 samples. Gloniaceae, the family that includes *C. geophilum*, was detected in 21 samples, representing 7.4% of total root tips. Rhizopogonaceae was found in only 11 samples, but colonized the second-highest number of root tips, 8.5%.

Russula 2 was negatively correlated with the percent silt in the soil (occurrence $\rho = -0.303$, $p = 0.19$; abundance $\rho = -0.310$, $p = 0.016$). Russulaceae occurrence was negatively correlated with soil organic matter, although the relationship with Russulaceae abundance was not significant (occurrence $\rho = -0.289$, $p = 0.026$; abundance $\rho = -0.155$, $p = 0.236$). Russulaceae occurrence was also positively correlated with percentage sand in the soil, and the relationship between abundance and sand was near-significant (occurrence $\rho = 0.316$, $p = 0.014$; abundance $\rho = 0.221$, $p = 0.089$). No other common OTUs or families were significantly correlated with soil organic matter or soil texture.

DISCUSSION

Finding *Rhizopogon* and Rhizopogonaceae species only on pines is consistent with what is already known about host specificity in EMF (Horton and Bruns 1998, Molina et al. 1992). The distribution of OTUs among oaks and pines also demonstrates that host specificity affects EMF community structure. However, most commonly-occurring taxa and abundant taxa were found on both oak and pine, and this dominance of multihost fungi is typical of EMF communities (Kennedy et al. 2003, Richard et al. 2005, Roy et al. 2008, Toju et al. 2013). The patchiness of occurrence leading to uncommon OTUs representing a large number of root tips is also consistent with typical EMF community structure (Horton and Bruns 2001). However, another possible explanation for these data is that apparently host-specific fungi were simply uncommon enough that they were not detected on both hosts despite being compatible with both. There is also evidence that environment

plays a role in defining niche for EMF, as Russulaceae occurred more often in sandier soils.

We found no evidence that coevolution between trees and EMF has resulted in dominance of host-specific symbionts. The pattern found better fits a highly diverse community of compatible EMF swamping the host plant's ability to preferentially reward the most beneficial fungus (Thompson 2009), or the EMF guild coevolving relatively uniformly with their host plants (Hoeksema 2010). Alternately, it could be that the need for both partners to quickly form a symbiosis to successfully compete for resources drives the lack of specificity found in dominant EMF (den Bakker et al. 2004).

The dominance of generalist EMF also suggests that common mycorrhizal networks (CMNs) may form between pines and oaks. As both genera are widespread in the northern hemisphere, interactions between them may be an interesting test case for studying intergeneric CMNs. The variety of habitats in which *Quercus* and *Pinus* are found together will provide useful information about how the context of these interactions affects the direction and amount of flow of various resources.

Lack of plant DNA barcodes with resolution at fine taxonomic levels is a barrier in investigating host specificity of plant symbionts at the plant species level (Shaw et al. 2005). The plant taxa used for this study were particularly difficult to resolve because oaks have both narrowly-defined species and hybridize extensively, and pines also hybridize, leading to difficulties in using chloroplast loci

(Hollingsworth et al. 2011, Piredda et al. 2011, Simeone et al. 2013). Improving the available loci for identifying plants will make identification of root tips to plant species possible without building custom libraries of nucleotide polymorphisms for each project.

Overall, however, the decreasing cost of sequencing is facilitating studies of specificity and coevolution. While ectomycorrhizal root tips are still important as functional units of symbiosis, high-throughput sequencing of soil allows for detection of many more organisms than other sampling methods, enhancing ability to detect specificity (Öpik et al. 2009). Advances in sequencing also make it easier to detect cryptic species and construct phylogenies to investigate patterns of specialization (Kretzer et al. 1996, Rochet et al. 2011, Roy et al. 2008).

Understanding specificity and the selective pressures important in mycorrhizal evolution is a promising path to increased understanding of symbiosis, coevolution, and plant community ecology. While many mycorrhizal fungi have an apparently broad host range, host and ecological specificity likely have consequences for plant invasions, succession, and competition.

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Effects of prescribed burning and thinning on composition and function of the ectomycorrhizal fungal community in an oak woodland 2014
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