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Tree thinning and fire affect ectomycorrhizal fungal communities and enzyme activities

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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 are especially likely to be affected as they are symbionts on plant roots and exhibit host and niche preferences. Ectomycorrhizal fungi also produce extracellular enzymes that are important in soil nutrient cycling. We conducted a community survey of ectomycorrhizal fungi and assayed ectomycorrhizal root tip enzyme activity using substrate plugs in northern Mississippi upland oak–pine woodland plots differing in restoration history to explore the influence of woodland restoration on ectomycorrhizal fungal community composition and function. Restoration treatment was significant in explaining the occurrence of the most common fungal species (*Russula xerampelina*) and the most common family (Thelephoraceae) in the ectomycorrhizal fungal community survey. Highest potential laccase, peroxidase, and *N*-acetyl-β-D-glucosaminidase enzyme activity were found in a prescribed burn plot, and the lowest enzyme activities at a wildfire plot, where richness of ectomycorrhizal fungi was also lower. Different fungal families displayed significantly different enzymatic 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 ectomycorrhizal fungal community composition and function, and better understanding these changes can aid understanding of the niches of ectomycorrhizal fungi and the impacts of restoration.

Key words: ectomycorrhizal fungi; enzymes; fire; prescribed burning; restoration; wildfire; woodlands.

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INTRODUCTION

While molecular techniques have greatly expanded our understanding of soil fungal diversity, the context of this diversity is still poorly understood (Tedersoo et al. 2014). While placing fungi within broad guilds, such as saprobic vs. ectomycorrhizal fungi (EMF), can explain a fair amount of variation in their traits (Phillips et al. 2013, Talbot et al. 2015), significant variation of function exists within those guilds and within fungal taxonomic lineages (Buée et al. 2007, Koide et al. 2007). Linking composition and function of soil fungal communities, and understanding how those are affected by natural and anthropomorphic habitat changes, is a major current challenge in soil ecology. Here, we report results from a study on how forest disturbances affect the composition and function of EMF.

EMF are particularly interesting because they 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 (Karst et al. 2008). EMF communities typically

have high species richness, comprising a few common species and many rare ones (Horton and Bruns 2001, Buée et al. 2005). One possible mechanism for the maintenance of this richness is niche partitioning—different fungi have differing abilities to exploit soil resources and therefore differing habitat preferences (Bruns 1995, Dickie et al. 2002, Tedersoo et al. 2003, Buée et al. 2007).

One such resource is dead woody debris (DWD). Some EMF produce extracellular laccases and peroxidases that allow them to access nutrients trapped inside DWD. This capability is common in Russulaceae species as well as *Laccaria* species, while members of the Boletales generally lack this ability (Agerer 2001, Burke et al. 2014). Another important soil resource is chitin, a source of nitrogen (N) found in the exoskeletons of arthropods and in fungal cell walls. A previous study in a temperate oak forest showed significantly more activity of the chitinase *N*-acetyl-β-D-glucosaminidase (NAGase) on ectomycorrhizal root tips found in DWD than on ectomycorrhizal root tips found in soils. The authors hypothesize that this finding means EMF NAGase could be induced by the presence of saprobic fungi (Buée et al. 2007). This possibility is supported by previous studies finding that the presence of chitin (Hodge et al. 1995) or the mycelium of saprobic fungi (Mucha et al. 2006) induced chitinase activity in cultured EMF. However, 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).

The availability and types of DWD and woody plant hosts are affected by thinning and prescribed burning, common forest management practices used to reduce the number and severity of wildfires and to restore fire-suppressed woodland communities. They are, therefore, likely to affect the composition and function of EMF communities. Thinning is typically conducted a few years prior to commencing burns, thus lowering stand humidity, reducing soil moisture, and enabling the growth of native understory plants to provide fuel (Johnson et al. 2009). 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. 2007*b*). Thinned material left on site creates a large pool of DWD available to soil fungi.

Prescribed burns are typically less intense than wildfires and affect belowground communities differently. Intense burns heat the soil more, burn litter and 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 (Neary et al. 1999, Knicker 2007). However, heat intense enough to kill trees, fine roots, and/or EMF is often patchy, and the soil niches emptied by a fire can be filled by hyphae from nearby surviving fungi, thus altering fungal community composition. 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 can cause EMF to alter their production of extracellular enzymes (Buée et al. 2007, Mosca et al. 2007*a*). Even low-intensity prescribed burns can reduce the labile carbon available in litter enough to induce increased EMF laccase activity (Boerner and Brinkman 2003).

Here, we report results of two related studies: the first study on differences in the EMF communities in untreated plots vs. those thinned and burned and the second study on differences in EMF community and in potential EMF laccase, peroxidase, and chitinase activity among an untreated plot, an unthinned plot sited at a recent wildfire, and an unthinned plot undergoing frequent prescribed fire. The latter study used transplanted soil cores to separate the effect of site from that of abiotic soil factors. We sought to test three hypotheses:

That EMF community composition differs when fire and thinning regimes alter the environment. This hypothesis predicted significant differences in EMF community species composition due to experimental treatment with thinning and prescribed burning or due to wildfire.

That EMF in different families have different enzyme activity profiles, predicting that Russulaceae and Hydnangiaceae would have relatively high laccase and peroxidase activities and Boletaceae would have little laccase or peroxidase activity compared to other EMF.

That EMF enzyme activity changes in response to the substrate in which the EMF are growing

and in response to fire history. We predicted that soils from a prescribed burn plot would exhibit higher laccase and peroxidase enzymatic activity than soils from control or wildfire plots. We also predicted that EMF would exhibit lower enzyme activity in burned DWD than in unburned DWD due to lower N content in the burned DWD. Additionally, we predicted that laccase and peroxidase activity would correlate positively with NAGase activity.

Methods

EMF community survey

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. Comparison of historic and current tree densities and species composition in the area of the field sites demonstrated that current density is much higher and that mesic, fire-sensitive plant species are present that were historically confined to lowlands (Brewer 2001, Surrette et al. 2008). Thinning and burning in treated plots are part of an ongoing effort to study the process and consequences of restoration of low-density oak woodlands in this area.

Tallahatchie Experimental Forest is a mixed upland forest with mature (>120 yr old) second-growth stands and no evidence of recent agricultural activity. Dominant trees included *Pinus echinata* (shortleaf pine), *P. taeda* (loblolly pine), *Quercus coccinea* (scarlet oak), *Q. falcata* (southern red oak), *Q. marilandica* (blackjack 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 an EF4-intensity tornado on 5 February 2008 (which constituted the thinning treatment), and burned again in 2010 and 2012. The tornado initially reduced canopy cover to an average of 40%, recovering to 55% by 2012 (Brewer 2016) compared to canopy cover in the untreated plot averaging 88%. All plots at TEF were 70 × 75 m. Previous to these fires, none of the plots had burned since at least the 1980s.

Compared to the TEF site, the SPAC sites contained fewer pines, which were historically not part of the upland landscape in central Marshall County, likely due to differences in soil texture (Surrette et al. 2008). The soil at SPAC is Providence silt loam and Cahaba loam, which is finer textured than the sandier soils at TEF. The Providence silt loam at SPAC also contains a moderate amount of loess and has a fragipan below the surface, which impedes drainage relative to TEF (Surrette and Brewer 2008, NRCS Soil Survey Staff 2016). The TEF plots are on steeper slopes than the SPAC plots, which also promote drainage at TEF. The loess in the Providence silt loam makes it likely to have higher calcium, magnesium, and iron content than the non-loessial soils in the study (Caplenor et al. 1968). Soil types at both sites typically have an approximate pH of 5, with the soils at TEF tending to be slightly more acidic (Caplenor et al. 1968). Like TEF, SPAC 1 contained a mature (>120 yr old) second-growth stand with no evidence of recent agricultural activity. It consisted of two 70 × 75 m plots, with an untreated plot and a plot that was thinned in 2004 by cutting or girdling stems of mesophytic tree species, mostly *Nyssa sylvatica* (black gum), *Liquidambar styraciflua* (sweetgum), and *Ulmus alata* (winged elm). Initially, girdle wounds were treated with 8% triclopyr. To attain better control of resprouting, girdled trees were treated with Pathway (5.4% picloram, 20.9% 2-4-D) from Dow Agrosciences, beginning in 2007 (Ryndock et al. 2012). The thinning treatment was followed by burning in 2005, 2010, and 2012. SPAC 2 contained some old oak trees (>100 yr old), but unlike at TEF and SPAC 1, agriculture was not abandoned at this site until the 1950s, possibly contributing to more eroded soils at this site. It consisted of a 60 × 30 m treatment plot, burned in 2008, 2010, and 2012, with half of the plot thinned to remove mesophytic species, and a 60 × 30 m untreated plot (Rietl and Jackson 2012, Ryndock et al. 2012). Because girdled trees were not removed from these sites, canopy cover remained relatively high on treated plots, with SPAC 1 averaging 82% cover on the treated plot vs. 91% on the untreated plot, and SPAC 2

averaging 85% cover on the treated plot vs. 88% on the untreated plot.

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 (Lilleskov et al. 2004, Bahram et al. 2011, Pickles et al. 2012). Cores were stored at 4°C for up to two weeks until processing. A previous study sequencing the plant hosts of ectomycorrhizal root tips in these plots found that 75% of root tips sampled were from oaks (Craig et al. 2016).

Horizons of each soil core were 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 using components of a Sigma Extract-N-Amp extraction kit (Sigma-Aldrich, St. Louis, Missouri, 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, 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). Thermal cycling was 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, and extension for 72 s at 72°C; and a final extension for 10 min at 72°C. Successful amplifications had excess primer and mononucleotides removed enzymatically, with each reaction containing 0.05 μL ExoI (New England Biolabs, Ipswitch, Massachusetts, USA), 0.2 μL antarctic phosphatase (New England Biolabs), 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, Grand Island, New York, USA). Sequencing reactions had an 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, Arizona, USA, where the Big Dye reactions were purified and read on an Applied Bioscience 3730 capillary genetic analyzer (Applied Biosystems, Foster City, California, USA). The sequences obtained were edited, assembled into operational taxonomic units (OTUs) at 97% similarity, and identified by comparison with sequences in the UNITE and NCBI sequence 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. Operational taxonomic units assigned to non-mycorrhizal fungi were discarded. Operational taxonomic units 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. Sequences used in analysis have GenBank accession numbers KX816049–KX816235.

R version 3.2.3 was used for data analysis (R Core Team 2016). To test whether sites, treatments, or the site-by-treatment interaction influenced EMF community composition as a whole, species richness was measured and Shannon diversity index 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). Non-metric multidimensional scaling and distance-based redundancy analyses were performed, but no useful ordinations were produced.

Operational taxonomic units occurring in ≥5 soil cores and the families occurring in ≥10 soil cores were analyzed individually in univariate analyses. Generalized linear models using a logit link function were fit to the 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 2016). All models included site, treatment, the interaction of site and treatment as factors, and soil organic matter as a covariate.

Substrate plug experiment

Tallahatchie Experimental Forest, as described above, was also the location of the three plots in which the substrate plug experiment was conducted. The plots used in the substrate plug experiment (all located at TEF) were an untreated plot; an unthinned plot that experienced prescribed burns in 2005, 2010, and 2012; and a plot in an area burned by a wildfire in July 2012, all measuring 70 × 75 m. The same untreated plot at TEF was used in both the community survey and the substrate plug experiment. This lack of site replication means that results cannot be generalized beyond the site from which they were obtained, but the opportunity to compare the effects of a wildfire with existing research plots was a unique opportunity. The wildfire plot had higher burn marks on tree trunks and more charring of downed wood than the thinned and unthinned prescribed burn plots, indicating greater fire intensity. Four mature canopy oak trees were selected along a gradient of slope positions (top, upper middle, lower middle, and bottom) in each of the unburned, prescribed-burn-unthinned, and wildfire plots at TEF. All oaks selected were historic upland species (*Q. 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. Dead woody debris (burned and unburned) that was not visibly decomposed was collected from litter in wildfire and unburned 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.

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 × 4 focal trees × 5 substrates × 2 replicates). Plugs were placed in a stratified random manner around each tree, with one replicate of each substrate represented on the north half and one on the south half of each tree to control for any effects of aspect. Each plug was 15 cm deep × 10 cm diameter. Canopy photographs were taken in June 2013 using a camera with a fisheye lens on a leveled tripod and analyzed using Gap Light Analyzer (Cary Institute of Ecosystem Studies; Simon Fraser University, Millbrook, New York, USA). 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 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 <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-β-D-glucosaminide to test NAGase activity (Pritsch et al. 2004, 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 was 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, the ITS region amplified and sequenced, and the sequences processed using the same methods as for the community survey. *Cortinarius* OTUs were discarded because that genus was found only in samples processed with the contaminated community survey samples. Sequences used in analysis have GenBank accession numbers KX816236–KX816332.

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 diversity index 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 the presence/absence data were run using glm() in stats for OTUs found in ≥4 cores and families found in ≥10 cores (R Core Team 2016). Taxa without enough data to fit the model were excluded from results.

As a control to test whether EMF root tip assays were actually measuring the enzymatic activity of residual free-living soil microbes, samples from a subset of cores ($n = 52$) were assayed for bulk soil enzyme activity. At SPAC, another study found that soil NAGase activity increased after burning (Rietl and Jackson 2012), and we wanted to ensure our measurements were not conflating EMF enzyme activity with soil enzyme activity. 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, and 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.

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

Operational taxonomic unit richness of EMF communities was not significantly different across sites, treatments, or the site-by-treatment interaction ($\chi^2_{2, 163} = 1.684$, $P = 0.431$; $Z = -1.353$, $P = 0.176$; $\chi^2_{5, 160} = 11.057$, $P = 0.087$). Operational taxonomic unit richness for each plot was as follows: TEF treatment, 35; TEF control, 48; SPAC 1 treatment, 33; SPAC 1 control, 39; SPAC 2 treatment, 31; SPAC 2 control, 34. Shannon diversity index was also not significantly different across sites, treatments, or the site-by-treatment interaction ($\chi^2_{2, 163} = 1.129$, $P = 0.569$; $Z = -1.667$, $P = 0.095$; $\chi^2_{5, 160} = 9.622$, $P = 0.087$).

A total of 187 EMF OTUs were identified across the experiment (see Appendix S1: Table S1). 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 the presence/absence data found that the occurrence of *R. xerampelina* was greater in treated plots ($\chi^2_{1, 158} = 4.737$, $P = 0.030$). The occurrence of *Clavulina* 1 was significantly explained by the site-by-treatment interaction and was found most commonly overall in the treatment plot at SPAC 1, but was more common in control plots at the other two sites ($\chi^2_{2, 158} = 8.739$, $P = 0.013$). *R. pectinatoides* occurrence showed a trend in occurrence connected to the site-by-treatment interaction, with the most occurrences at the SPAC 2 treatment site ($\chi^2_{2, 158} = 5.225$, $P = 0.073$). *P. rhodoxanthus* occurrence was near-significantly affected by site, with no occurrences at TEF ($\chi^2_{2, 158} = 5.133$, $P = 0.077$). None of the chosen variables significantly explained occurrence of *R. californiensis* ($P > 0.12$).

At the family level, occurrence of Russulaceae was significantly explained by the site-by-treatment interaction, with the family occurring most commonly at the TEF control site but more frequently in the treatment plots at the other two

sites ($\chi^2_{2,\ 158} = 9.092$, $P = 0.011$). Occurrence of Thelephoraceae was significantly explained by soil organic matter and treatment, with this family being found more commonly at untreated plots and in cores with higher soil organic matter ($\chi^2_{1,\ 158} = 5.543$, $P = 0.019$; $\chi^2_{1,\ 158} = 4.315$, $P = 0.038$). Occurrence of Boletaceae was significantly explained by site, with only one occurrence at TEF compared to six and seven at SPAC 1 and SPAC 2, respectively ($\chi^2_{2,\ 158} = 6.024$, $P = 0.049$). The occurrence of Amanitaceae was significantly explained by site, with this family not found at SPAC 2 ($\chi^2_{2,\ 158} = 11.368$, $P = 0.003$). Gloniaceae showed a trend of treatment on their occurrence, being found nine times in treatment plots vs. three in untreated plots ($\chi^2_{1,\ 158} = 3.381$, $P = 0.066$). None of the chosen variables significantly explained occurrence of Cortinariaceae ($P > 0.13$).

Substrate plug experiment results

EMF species richness varied among the three plots at TEF ($\chi^2_{2,\ 118} = 6.0974$, $P = 0.047$) and was significantly lower in the wildfire plot than in the unburned plot (Tukey HSD adjusted $P = 0.017$). Observed OTU richness at the wildfire plot was 28, compared to 40 at the prescribed burn plot and 44 at the unburned plot. Operational taxonomic unit richness did not vary by substrate or slope position ($\chi^2_{4,\ 116} = 4.750$, $P = 0.314$; $\chi^2_{3,\ 117} = 2.893$, $P = 0.408$). Shannon diversity index did not vary by plot, substrate, or slope position ($\chi^2_{2,\ 118} = 4.539$, $P = 0.103$; $\chi^2_{4,\ 126} = 5.633$, $P = 0.228$; $\chi^2_{3,\ 117} = 2.920$, $P = 0.404$). The number of mycorrhizal root tips found per core was similar across all three plots ($\chi^2_{2,\ 118} = 0.495$, $P = 0.781$).

Ninety-five EMF OTUs were identified in this experiment (see Appendix S1: Table S2), with the most commonly detected OTUs being identified as *Laccaria* 51 (present in 15 cores), Thelephoraceae 52 (six cores), *Cenococcum* 53 (four cores), *Cenococcum* 54 (four cores), *Russula californiensis* (four cores), *Tomentella* 55 (four cores), and *Tomentella* 59 (four 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 the 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 near-significant effects of substrate on the occurrence of both taxa. *Laccaria* 51 had no occurrences in unburned DWD ($\chi^2_{4,\ 101} = 8.676$, $P = 0.070$). *Cenococcum* 53 had three occurrences in unburned soil and one in wildfire soil, with none in the other substrates ($\chi^2_{4,\ 101} = 8.064$, $P = 0.089$).

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

There was no correlation between the bulk soil controls 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: $\rho_{50} = -0.039$, $P = 0.782$). Activities of peroxidase and NAGase per unit area of mycorrhizal root tips were positively correlated, while laccase activity per unit area was not correlated with either NAGase or peroxidase activity ($\rho_{161} = 0.363$, $P < 0.001$; $\rho_{161} = 0.107$, $P = 0.175$; $\rho_{161} = -0.087$, $P = 0.271$).

Activity per unit area of all three focal enzymes varied significantly by EMF family (Fig. 1). Tuberaceae had higher NAGase activity than Cantharellaceae, Amanitaceae, and Strophariaceae ($\chi^2_{12,\ 143} = 24.075$, $P = 0.033$). Tuberaceae had higher peroxidase activity than Strophariaceae, Thelephoraceae, and Cantharellaceae ($\chi^2_{12,\ 143} = 55.742$, $P = 0.002$). Hydnangiaceae had higher laccase activity than Boletaceae, Gloniaceae, Sebacinaceae, Cantharellaceae, Strophariaceae, and Thelephoraceae ($\chi^2_{12,\ 143} = 76.652$, $P < 0.001$).

The plot-by-substrate interaction influenced activities of all three enzymes in these univariate models (NAGase $\chi^2_{28,\ 168} = 47.828$, $P < 0.001$; laccase $\chi^2_{28,\ 168} = 29.202$, $P < 0.009$; and peroxidase $\chi^2_{28,\ 168} = 36.398$, $P < 0.002$, see Fig. 2). Robust

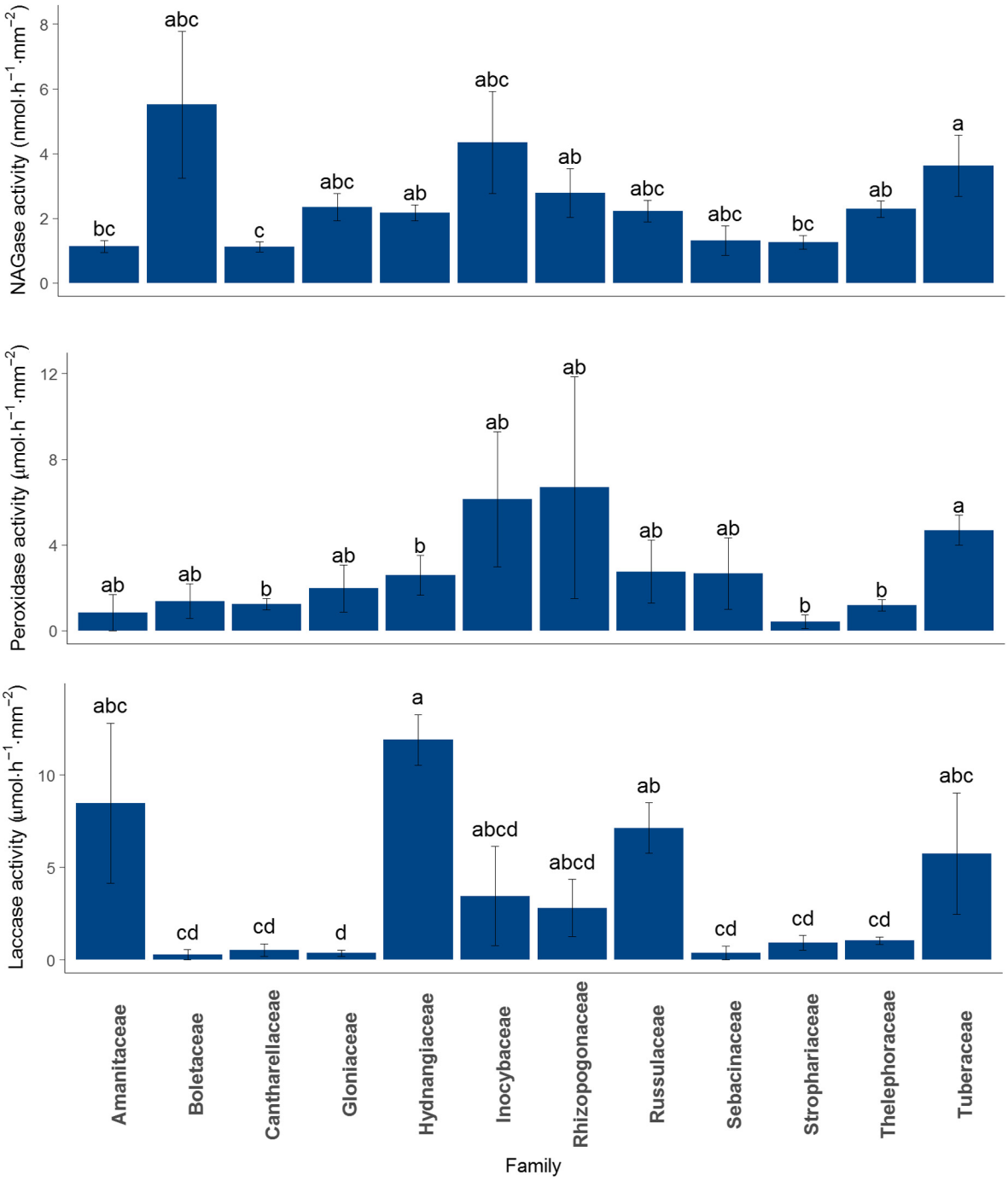

Fig. 1. Variation among fungal families in potential ectomycorrhizal fungal enzyme activity per unit area of mycorrhizal root tip. Samples were taken from soil and dead woody debris plugs in a temperate oak–pine woodland. Note overlap between families with high NAGase activity and high peroxidase activity.

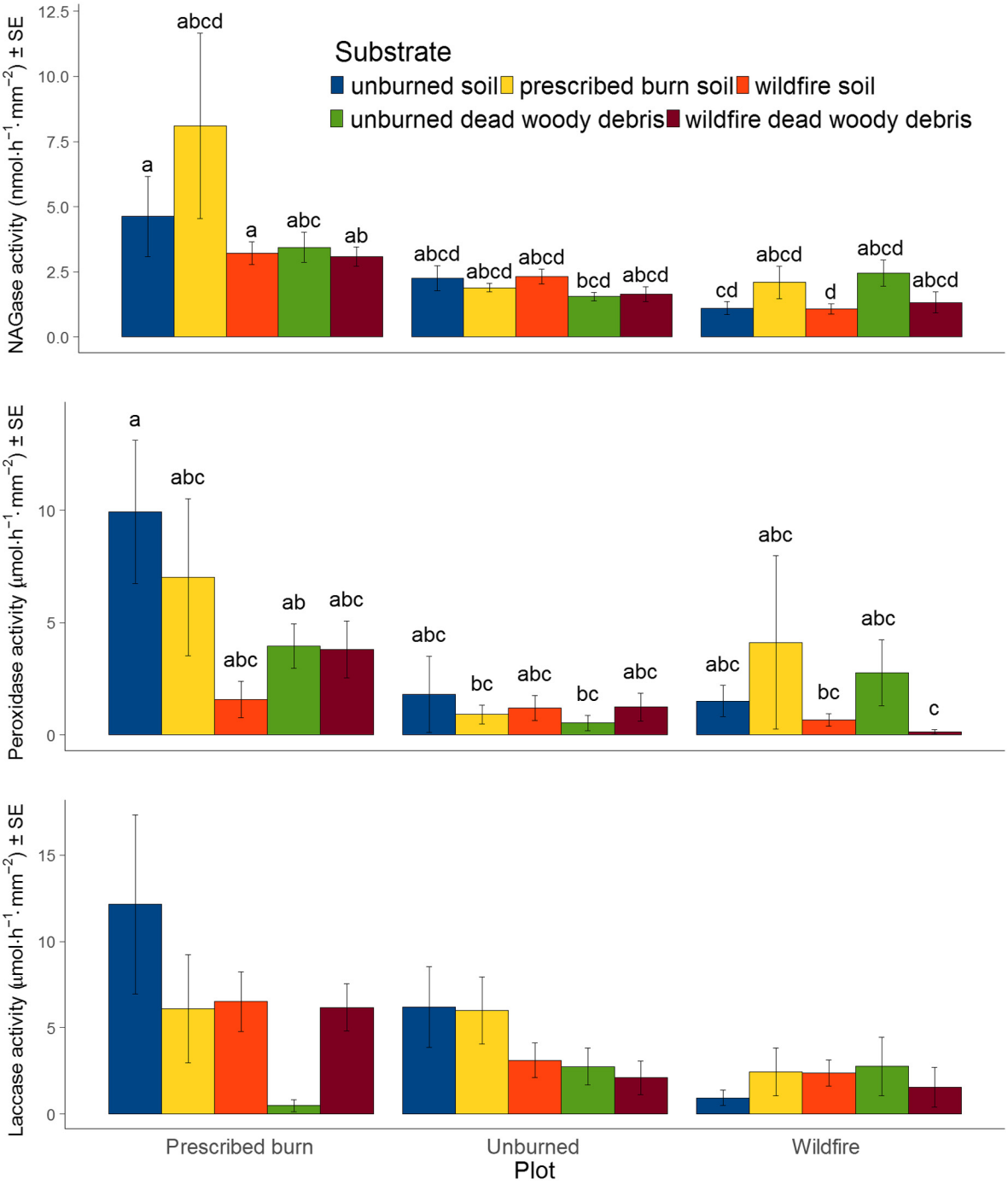

Fig. 2. Variation among plot–substrate combinations in potential ectomycorrhizal fungal enzyme activity per unit area of mycorrhizal root tip in a temperate oak–pine woodland. No significant differences among site–substrate combinations were found for laccase activity.

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 same 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.

The main effect of plot was significant in explaining the activity of each enzyme per unit area of mycorrhizal root tip. The highest activity levels were found in the prescribed burn plot (Fig. 3). For NAGase, all three plots had significantly different enzyme activities, with the highest activity at prescribed burn plot 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, which were similar (critical $P = 0.017$, contrasts against prescribed burn plot both $P < 0.001$). 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.

None of the focal enzymes were significantly affected by the main effect of substrate in univariate tests (NAGase: $\chi^2_{4,\ 159} = 3.653$, $P = 0.479$; peroxidase: $\chi^2_{4,\ 159} = 5.789$, $P = 0.208$; laccase: $\chi^2_{4,\ 159} = 6.884$, $P = 0.138$). NAGase activity varied by slope position ($\chi^2_{3,\ 160} = 36.398$, $P < 0.002$), with significantly higher activity around trees at the top of slopes compared to those in the upper-middle position (crit $P = 0.009$, top vs. upper-middle $P = 0.002$). Slope position also affected peroxidase activity ($\chi^2_{3,\ 160} = 13.555$, $P = 0.002$), with higher activity at trees at the top of slopes compared to other slope positions (crit $P = 0.009$, top vs. bottom $P < 0.001$, top vs. lower-middle $P = 0.003$, top vs. upper-middle $P = 0.001$). Slope position did not significantly affect laccase activity ($\chi^2_{3,\ 160} = 6.884$, $P = 0.140$).

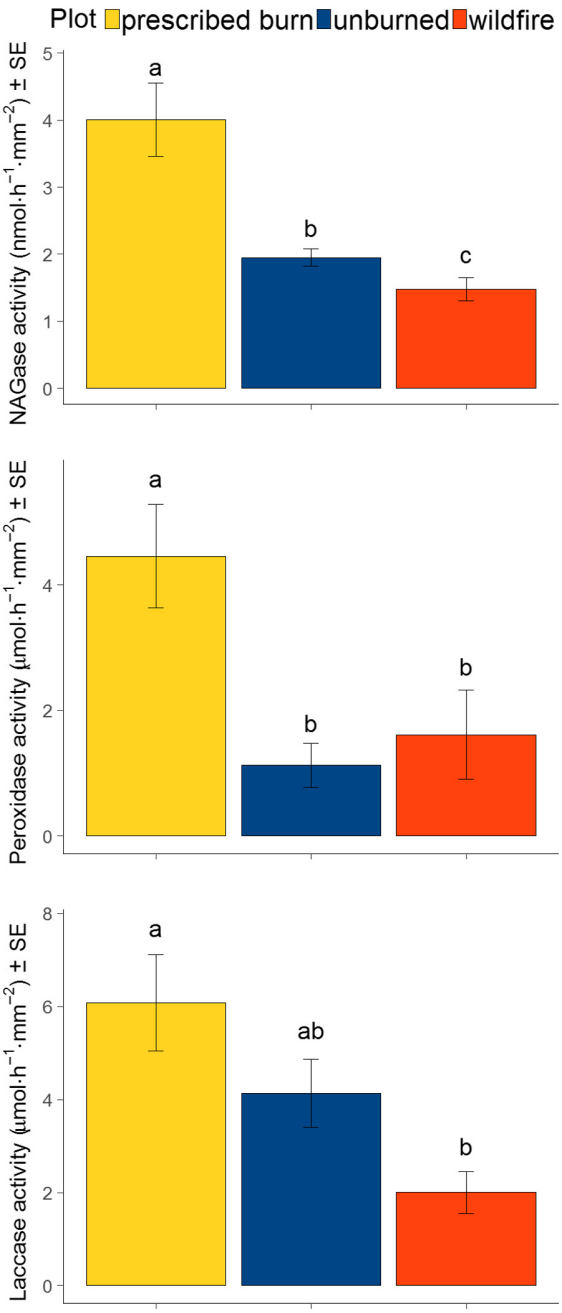

Fig. 3. Variation among plots in potential ectomycorrhizal fungal enzyme activity per unit area of mycorrhizal root tip.

Discussion

Considering the effect of environment on EMF community, the most common OTU, *Russula xerampelina*, was more common in plots that were burned and thinned than in control plots, whereas the most common family, Thelephoraceae, was more common at untreated plots. In contrast, a previous EMF community survey conducted at these same sites four years earlier, and only one year after prescribed burning was

established in the treatment plots, found similar levels of Thelephoraceae in treatment and control plots (Craig et al. 2016), suggesting that Thelephoraceae has since responded negatively to thinning and/or repeated prescribed burning. 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. The differences in analysis between the studies and the plot-level supplemental data available permit only broad comparisons. Site, which was significant in explaining the occurrence of 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 and the patchy occurrence of EMF. The Craig et al. (2016) study found that the two SPAC sites were different in EMF composition from the TEF site, the pattern followed here by abundance of Boletaceae. The expected spatial autocorrelation in EMF community surveys is around 3 meters (Lilleskov et al. 2004, Bahram et al. 2011, Pickles et al. 2012), which is much smaller than plot and site sizes, so detecting distance effects is expected. Thelephoraceae occurrence was positively associated with soil organic matter content in the community survey and negatively associated with canopy cover in the substrate plug experiment, supporting the idea that different taxa have different preferred niches.

Despite reducing the number of available hosts and heating upper soil layers, management treatments did not negatively affect diversity or abundance of EMF in either study, suggesting that thinning and prescribed burning is not negatively affecting EMF at these sites. 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). Our 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 *Liquidambar styraciflua* than the other plots, and *L. styraciflua* does not commonly form ectomycorrhizae.

There were significant differences among EMF families in NAGase, peroxidase, and laccase activity, although there was high variation within some families. The prediction that Russulaceae and Hydnangiaceae would have high laccase and peroxidase activity was partially supported, with both families having high laccase activity. Also as predicted, Boletaceae had low peroxidase and laccase activity. Driving the overall correlation between NAGase and peroxidase activities, Tuberaceae, Rhizopogonaceae, and Inocybaceae had some of the highest activities for both enzymes.

Laccase activity in EMF often correlates with morphology, with fungi with very little extraradical mycelium, such as many *Russula* species, often having the highest laccase activity (Tedersoo et al. 2012, Hupperts et al. 2016), a pattern corroborated here. EMF enzyme activity was also seen to be more strongly related to exploration type than fungal lineage in a tropical forest (Tedersoo et al. 2012) and more strongly related to exploration type than host phenology in a boreal forest (Hupperts et al. 2016). *Laccaria*, the principal genus represented in the Hydnangiaceae, typically have medium- or short-distance exploration types (Agerer 2001) and here had high laccase activity consistent with short-distance exploration types. However, the Sebacinaceae are expected to form short-distance mycorrhizae based on *Sebacina* (Agerer 2006) and were found here to exhibit low laccase activity and moderate peroxidase activity. Also, Rhizopogonaceae, a family that typically forms long-distance type mycorrhizae, also here produced the highest mean peroxidase activity, contrary to expectations based on its morphology.

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). Boletaceae here had high NAGase activity but low phenol-degrading activity, consistent with Hupperts et al. (2016). This family consistently produces rhizomorphs characteristic of a long-distance exploration type (Agerer 2001). Also, Tuberaceae, which typically form short-distance mycorrhizae (Agerer 2006), had high NAGase activity as well as moderately high laccase and peroxidase activity, suggesting that factors beyond exploration type are important in understanding enzyme production. Although we did not measure soil nitrogen, a worthy endeavor in future research would be to quantify N cycling in this system and ask whether changes in EMF composition and enzyme activities may drive changes in N cycling.

Some families had fairly low enzyme activity for all three enzymes tested. Gloniaceae data are likely principally from *Cenococcum geophilum*, which has a short-distance exploration type, but did not have notably high activity for any enzyme. It is possible that the dense hyphae characteristic of *C. geophilum* mycorrhizae led to higher measurements of root tip size and thus lower relative activity, but these hyphae would also create a much larger surface area. A recent study in French oak forests found that enzyme activity of *C. geophilum* varied significantly by site for five of eight enzymes tested, so context may be particularly important in determining enzyme activity of this taxon (Courty et al. 2016).

Supporting our hypothesis of higher laccase and peroxidase activity in mycorrhizal roots from the prescribed burn plot, the mycorrhizal root tips in the prescribed burn plot had the highest potential enzyme activity for all three enzymes tested, whereas those in the wildfire plot were consistently the lowest (Fig. 3). This result, combined with the lower EMF species richness in the wildfire plot, suggests that frequent, low-intensity burns have a different effect on EMF community richness and function than infrequent higher-intensity burns in this ecosystem. It is also suspected that repeated prescribed burning can select for a microbial community that does not suffer long-term effects after fire (Johnson and Curtis 2001).

Our predictions that soils from a prescribed burn plot would exhibit higher laccase and peroxidase enzymatic activity than soils from control or wildfire plots and that EMF would exhibit lower enzyme activity in burned DWD than in unburned DWD were not supported, although plot-by-substrate interactions were significant (Fig. 2), 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. The expectation of drier soil at the top of slopes suggests that lower soil moisture may be an underlying variable in this effect.

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 et al. (2007) study took place in a forest composed entirely of oaks, and the presence of pine may have affected the wood composition of the DWD collected in our study.

The prediction that phenol-degrading and chitinase activity 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. It is possible that 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.

Conclusions

In summary, this study found that a wildfire was associated with lower EMF species richness and a reduced role of EMF in soil nutrient cycling, whereas prescribed burning preserved

EMF richness and increased their potential enzyme activity. Five years after initialization of burning and thinning, although not affecting EMF diversity, restoration treatments are beginning to alter composition and function of EMF. Additional studies in similar ecosystems are necessary to generalize the results to other sites. Studies using next-generation sequencing techniques to provide good detection of mycorrhizal fungi could also increase the power of observations of which environmental conditions favor certain species, although at the expense of understanding their ecological functioning.

Ultimately, more information on the links between community diversity and functional diversity will aid understanding of EMF natural history and community ecology. This work is a step toward using niche preference and enzymatic capability to help resolve species complexes, reveal functional variation in diverse EMF communities, and understand how high diversity is maintained in EMF communities.

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