Linking The Functional Diversity Of Ectomycorrhizal Fungal Species To Soil Carbon And The Genetics Of A Foundational Tree Species

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LINKING THE FUNCTIONAL DIVERSITY
OF ECTOMYCORRHIZAL FUNGAL SPECIES TO SOIL CARBON
AND THE GENETICS OF A FOUNDATIONAL TREE SPECIES

A Thesis

Presented in partial fulfillment of requirements
for the Master of Science Degree
in the Department of Biology
The University of Mississippi

by

NICOLE M. HERGOTT

July 2013
ABSTRACT

Carbon dioxide in the atmosphere continues to increase, leading to an enhanced interest in the potential for ecosystems to sequester carbon. Soil could have a high potential to store carbon in these forests, but there is still a great deal to learn about how carbon storage in soils is influenced by ectomycorrhizal fungi. In this symbiosis, the plant host provides the vast majority of C for fungal growth. As a consequence, ECM fungi may provide one of the major pathways for C from trees to soils. Ectomycorrhizal fungal species produce extracellular enzymes that may degrade soil carbon and may differ in their ability to produce these extracellular enzymes to break down C in the soil. Additionally, tree genetics may control carbon storage in forests through influences on the functional diversity of mycorrhizal fungi, including enzyme activity.

In this study, I examined the composition and functional traits of the ECM fungal community on different genotypes of *Pinus palustris*. I tested seven enzymes: β-1,4-xylosidase, β-1,4-glucosidase, cellobiohydrolase, phosphatase, β-N-acetylglucosaminidase, peroxidase and phenol oxidase to test my hypothesis that tree genetics directly influences particular ectomycorrhizal fungal enzyme activities and soil properties as well as indirectly controlling ectomycorrhizal fungal enzyme activities and soil properties by directly influencing the distribution of mycorrhizal fungal species. I found that ECM fungal community composition and peroxidase activity differed among tree families. Peroxidase activity differed among mycorrhizal fungal species. *Amanita* produced the highest amount of peroxidase activity, suggesting that *Amanita* may have the ability to obtain nutrients by degrading dead woody
debris. N-acetylglucosaminidase activity differed among mycorrhizal fungal species, but not among tree families. *Tomentella*1 produced the highest amount of β-N-acetylglucosaminidase activity, suggesting that *Tomentella*1 may have the ability to obtain nutrients by degrading saprobes. All other enzymes did not differ among mycorrhizal fungal species or tree families. Soil C, N and OM percentage did not vary among the ECM fungal community, tree family, or ECM fungal enzyme activities. Altogether, these results suggest that selection of plant genotypes could allow compositional management of microbial symbionts of trees, including the ECM fungal community, and possibly the traits of those microbial communities.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>C</td>
<td>Carbon</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>DistLM</td>
<td>Distance-Based Linear Model</td>
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<tr>
<td>ECM</td>
<td>Ectomycorrhizal</td>
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<tr>
<td>HEF</td>
<td>Harrison Experimental Forest</td>
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<tr>
<td>INSD</td>
<td>International Nucleotide Sequence Database</td>
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<tr>
<td>ITS</td>
<td>Internal transcribed spacer region between small and large subunit ribosomal genes</td>
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<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
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<tr>
<td>LSU</td>
<td>Large subunit ribosomal genes</td>
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<tr>
<td>NMDS</td>
<td>Nonmetric Multidimensional Scaling</td>
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<tr>
<td>N</td>
<td>Nitrogen</td>
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<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
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<tr>
<td>OM</td>
<td>Organic matter</td>
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<tr>
<td>OTU</td>
<td>Operational Taxonomic Unit</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PERMANOVA</td>
<td>Permutational Multivariate Analysis of Variance</td>
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<tr>
<td>pNp</td>
<td>p-Nitrophenyl</td>
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<tr>
<td>SRL</td>
<td>Specific root length</td>
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<td>SSU</td>
<td>Small subunit ribosomal genes</td>
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<tr>
<td>UAF</td>
<td>University of Alaska, Fairbanks</td>
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<tr>
<td>UNITE</td>
<td>User-Friendly Nordic ITS Ectomycorrhizal database</td>
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ACKNOWLEDGMENTS

First of all, I would like to thank Dr. Jason Hoeksema for all of the support he has given me through this process. Jason has been a mentor and life coach to me. I truly believe that he has given me the tools to succeed in any endeavor. I would also like to thank and acknowledge my committee, Dr. Steve Brewer and Dr. Colin Jackson, for all of their advice and assistance they have given me. Additionally, I would like to thank everyone at the USDA Southern Research Station Institute of Forest Genetics at the Harrison Experimental Forest for their assistance. Finally, I would like to thank Sigma Xi for their financial support.

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I: LINKING THE FUNCTIONAL DIVERSITY OF ECTOMYCORRHIZAL FUNGAL SPECIES TO SOIL CARBON AND THE GENETICS OF A FOUNDATIONAL TREE SPECIES

INTRODUCTION

Carbon dioxide (CO$_2$) in the atmosphere has been increasing since the Industrial Revolution (Means and Grow 1985, Canadell et al. 2007, Raupach et al. 2007), largely due to anthropogenic factors. This increase in atmospheric carbon dioxide has led to an enhanced interest in the potential for ecosystems to act as carbon (C) sinks. Plantation forests have the potential to reduce net CO$_2$ emissions via increased carbon sequestration (Goodale et al. 2002, Jackson and Schlesinger 2004). Woody stems of forest trees store a significant amount of carbon, but the belowground processes controlling soil carbon dynamics are poorly understood. Soil could have a high potential to store carbon in these forest ecosystems as the global average C pool in the soil is roughly 3 times the size of the atmospheric pool (Lal 2004), but there is still a great deal to learn about how carbon storage in soils is influenced by key belowground organisms in these ecosystems: ectomycorrhizal (ECM) fungi.

In ECM symbiosis, the plant host provides the vast majority of C for fungal growth. For example, in controlled experiments, it has been estimated that approximately 30% of host net primary productivity can be allocated to ECM fungal symbionts (Nehls and Hampp 2000, Hobbie 2006). The fungi use this C to build hyphal networks in the soil, exploring small spaces in soils and enhancing nutrient uptake (Smith and Read 2008). As a consequence, ECM fungi may provide one of the major pathways for C from trees to soils. Of the C assimilated into
fungal tissues, a majority goes to build chitin, a cell wall component that is relatively recalcitrant to decomposition (Gooday 1994). In addition, ECM fungal species produce extracellular enzymes that may degrade soil carbon and may differ in their ability to use these extracellular enzymes to break down C in the soil (Buée et al. 2007, Courty et al. 2010, Jones et al. 2010). Therefore, ECM fungi may significantly influence soil carbon dynamics. Indeed, using \(^{14}\)C bomb-carbon modeling, a recent study in boreal forest found that 50 to 70% of stored carbon in a chronosequence of islands was derived from roots and root-associated fungi, including ectomycorrhizal fungi (Clemmensen et al. 2013). These results suggest that mycorrhizal fungi play a great role in the regulation of soil organic matter.

Ectomycorrhizal fungal species vary in several traits that can directly affect soil carbon dynamics. First, ECM fungal species differ dramatically in enzymatic capabilities (Courty et al. 2007). Furthermore, ECM fungal species vary in the exploration strategy they use to find nutrients, resulting in distinct phenotypic differences between ECM species (Agerer 2001). These different morphological types correspond to different foraging niches in the soil (Courty et al. 2006), which may correspond to differences in their abilities to control carbon cycling and storage. For example, ECM fungal species of the ‘contact exploration type’ have smooth mantles and receive relatively little carbon from their host, allowing their mycorrhizal structures to be in close contact with the surrounding substrates and leaf litter (Agerer 2001). Furthermore, species from the genera *Russula* and *Lactarius* of the ‘contact exploration type’ revealed a high capability to produce lignin-degrading enzymes such as phenol oxidases (Agerer et al. 2000). On the other hand, ECM species of the ‘long-distance exploration type’ such as *Rhizopogon*, *Tricholoma*, and *Suillus* species have highly differentiated mycelial strands and receive a larger amount of carbon from their hosts, enabling them to build hyphal networks which allow them to
explore for nutrients further from the root (Agerer 2001). Consequently, ECM fungi may exhibit distinct trade-offs between nutrient acquisition strategies and functional traits related to carbon cycling.

Plant host genetics may directly or indirectly influence soil carbon dynamics. Host plant genetics may indirectly affect soil carbon dynamics by favoring different ECM fungal species. Studies have suggested that forest tree species have genetic variation in their compatibility with particular ECM fungal species (Korkama et al. 2006, Leski et al. 2010) and can influence soil C dynamics (Hoeksema and Classen 2012). For example, *Pisolithus tinctorius* varied in its intensity of colonization among loblolly pine genotypes (Dixon et al. 1987). The genetic selection of plant genotypes could lead to indirect selection of the microbial symbionts of trees, including the ECM fungal community (Hoeksema and Classen 2012). Consequently, this indirect selection of the ECM fungal community could influence extracellular enzymatic activities and other traits of the ECM fungi, which could impact soil C dynamics. Soil carbon dynamics may also be directly influenced by plant genotypes. For example, Courty et al. (2011) inoculated 38 genotypes from a *Populus deltoides* and *Populus trichocarpa* controlled cross with the ECM fungus *Laccaria bicolor* and found that ECM fungal enzyme activities of N-acetylglucosaminidase, β-glucuronidase, cellobiohydrolase, β-glucosidase, β-xylosidase, and acid phosphatase differed among *Populus* genotypes. Since the plant genotypes were inoculated with only one ECM fungal species, the level of enzymes secreted by the ectomycorrhizal root tips is under the genetic control of the host and can influence ECM fungal enzymatic activities regardless of ECM fungal species (Courty et al. 2011).

Currently, loblolly pine (*Pinus taeda*) represents the most common target of efforts to sequester increased C from the atmosphere in U.S. forests due to its rapid growth rate and wide
deployment as a plantation species (Johnsen et al. 2001). However, longleaf pine (*Pinus palustris*) has been suggested to be a superior alternative to loblolly pine for soil C sequestration due to its greater longevity, wood density, disease resistance, fire tolerance, and wind resistance (Kush et al. 2004). In addition, there is increasing interest in ecological restoration of *P. palustris* ecosystems, which are among the most biologically diverse ecosystems in the United States (Means and Grow 1985, Noss 1989), but currently occupy only a small fraction of their pre-modern distribution (Noss 1989, Frost 1993). Hence, using *P. palustris* as a target of C sequestration efforts has the potential to combine biodiversity conservation with effective C sequestration. Thus, *P. palustris* is an ideal system in which to test whether the genetics of foundational tree species in forests control important ecosystem processes, such as carbon cycling and storage, through influences on the functional diversity of ECM fungi.

In this study, I examined the composition and functional traits of the ECM fungal community on different genotypes of *Pinus palustris* to investigate the question: What are the relationships among tree genetics, ECM fungal enzyme activities, the ECM fungal community, and soil properties? My overall hypothesis was that tree genetics would directly influence particular enzyme activities and indirectly influence soil properties through the ECM fungal community and ECM fungal enzyme activities (Figure 1)
MATERIALS AND METHODS

Study site

The study sites were located at the Harrison Experimental Forest (HEF) in Saucier, Mississippi, USA, located 32 km north of Gulfport, Mississippi, USA (30.65N, 89.04W, elevation 50 m). The HEF is in the Gulf Coastal Plain within the historic range of longleaf pine. This region receives ~1650 mm of annual precipitation. The forest soil consists mainly of fine sandy loam, which is well-drained and low in nutrients (Samuelson et al. 2012).

A longleaf pine complete half diallel cross experiment, where 13 parents were crossed in all combinations (except self-pollinating) to produce 78 replicated full-sib families (13 parents and 78 crosses x 8 replicates), was planted in 1960 at two separate sites (4 replicates each) located 2 miles apart. All parents were of local origin (within Harrison County, MS) and were randomly selected. Each replicate consisted of 78 familial eight-tree row plots, each containing 8 full-sib progeny from one of the 78 crosses (Figure 2). Trees were spaced 12 feet apart, at the apexes of equilateral triangles. Four replicates from the north site and one replicate from the south site were utilized in this study. Three replicates from the south site have been withdrawn from plot maintenance due to extensive tree damage inflicted by Hurricane Katrina in 2005. Families were chosen based on previous data on tree survival from Hurricane Katrina.

Root and ectomycorrhizal collection

In May, June and August of 2011, in each of the 5 replicates, I collected and pooled a total of 6 soil cores (15 cm deep x 1.5 cm diameter) from under 3-6 trees within each of 15
familial 8-tree row plots, each of which had at least 3 surviving trees. The cores were collected <0.25m from the base of each tree trunk. When fewer than 6 trees were alive in a given plot, I collected and pooled more than one soil core under each tree. Soil cores were collected from one replicate at a time in the north site on May 11, May 26, and June 18 (3 replicates total). On August 26, I collected soil cores from the fourth replicate in the north site and from one replicate in the south site.

Soil cores were put on ice and brought back to the University of Mississippi, where they were stored at 4°C and were processed within 14 days. Using 2 mm sieves, soil was separated from coarse fragments and roots. Root samples were analyzed under a dissecting microscope for determination of ECM morphotypes, and exploration strategies of each morphotype (contact, short-distance, medium-distance, and long-distance) were described using the classifications from Agerer (2001). Two root tips per morphotype per sample were removed and placed in individual wells in a 96-well PCR plate for DNA extraction (see below). In addition, dominant ECM morphotypes in each sample were collected more intensively and three to five root tips per morphotype for each of 7 planned enzyme assays (see below) were placed in wells of 96-well plates (3-5 root tips per enzyme assay + 1-2 sample replicates + 1-2 sample controls x 7 enzyme assays = 21-36 tips per morphotype per sample).

Soil analysis

Soil samples were analyzed for soil organic matter percentage (%OM) and total carbon (C) and nitrogen (N) contents to help link ECM functional diversity to soil carbon storage. Soils were sieved (2 mm) to remove any coarse fragments and roots and were dried in an oven at 60°C for 48 hours, and subsamples of soil were ground using a mortar and pestle. Organic C and N
percentages were determined using an ECS 4010 CHNSO Analyzer (Costech Analytical Technologies). The oven dried soil was weighed, ashed (500 °C, 2 h), and reweighed to determine organic matter (OM) content for each soil sample as ash free dry mass.

Molecular identification of ECM fungi

DNA was extracted from ectomycorrhizal root tips using Extraction Buffer from Sigma Extract-N-amp Tissue Kits (Sigma-Aldrich, St. Louis, MO). One fresh root tip was placed in a well of a 96-well PCR plate. For each root tip, 10μl of Sigma Extraction Buffer was added, then heated at 65°C for 10 minutes and at 95°C for 10 minutes. Thirty μl of Sigma Neutralization Solution was then added to each well, along with 60 μl of PCR-grade water for dilution.

Polymerase Chain Reaction (PCR) was performed using the fungal-specific forward and reverse primers, ITS1-F and ITS4 (Gardes and Bruns 1996). These primers amplify the internal transcribed spacer regions between the small subunit (SSU) and large subunit (LSU) ribosomal genes. Each 8 μl reaction contained 2.7 μl of sterile PCR-grade water, 0.4 μl (10 μM concentration) of each primer, 4 μl of 2X Red Taq Premix and 0.5 μl of DNA sample. Thermal cycling was performed with the following parameters: 1 cycle for 3 minutes at 93°C for initial denaturation; 35 cycles of 45 seconds at 94°C for denaturation, 45 seconds at 58°C for annealing, and 72 seconds at 72°C for extension; and 1 cycle for 10 minutes at 72°C for final extension.

Excess primer and unincorporated nucleotides were removed enzymatically using ExoSAP-IT (USB Corporation, Cleveland, OH, USA). Five μl of ExoSAP-IT mastermix (0.25 μl of ExoSAP-IT with 4.75 μl of sterile water) were combined with five μl of PCR product and were maintained at 37°C for 45 minutes, 80°C for 20 minutes, and 4°C for at least 5 minutes. Each sample was then included in a 10 μL Sanger sequencing reaction, which included 0.4 μl of
Big-Dye Reaction Premix (Life Technologies, Inc.), 1.8 μl Big-Dye 5X Sequencing Buffer, 0.5 μl of 10 μM Primer, 6.3 μl of PCR-grade water, and 1 μL of cleaned PCR product. Thermocycling was performed with the following parameters: 96°C for 1 minute, followed by 35 cycles of 30 seconds at 95°C for denaturing, 20 seconds at 50°C for annealing and 4 minutes at 60°C for extension. Reactions were dried and shipped overnight to the DNA Lab at Arizona State University, in Tempe, Arizona, where the sequenced reactions were purified and read on a capillary genetic analyzer.

Sequences were edited manually using Geneious (v5.6.6, Biomatter Ltd.) software, where ambiguous bases associated with dye blobs and elsewhere were corrected. All sequences containing <200 base pairs and >3% of ambiguous bases were deleted. The sequences were subjected to operational taxonomic unit (OTU) assembly (at 97% similarity) using CAP3 software (Huang and Madan 1999) on the University of Alaska Fairbanks (UAF) informatics server. Sequences from each OTU were submitted using BLAST searches on the International Nucleotide Sequence Database (INSD), User-Friendly Nordic ITS Ectomycorrhizal (UNITE) database and the curated fungal ITS dataset on the UAF Informatics Portal to obtain the best matches for taxonomic assignment of OTUs. Sequences >99% similar in composition to database sequences from named, cultured fungi were considered identified to species. Sequences with matches <99%, but >95% similarity to a database sequence with an assigned taxonomic unit were identified only to genus. Sequences with matches <95%, but >90% were identified only to family. Finally, sequences with matches <90% were excluded. Any species known to be strictly non-mycorrhizal was also eliminated from the data set. If an OTU was a >99% unambiguous match to a trustworthy specimen with a species, name (such as *Cenococcum geophilum*), then that OTU was given that name followed by ‘gr’ for ‘group,’ e.g., *Cenococcum geophilum gr.*
For matches at higher taxonomic levels, names where with numbers added onto the end, e.g., *Russula1, Russula2, Thelephoraceae1, and Thelephoraceae2*.

**Assays for ectomycorrhizal extracellular enzyme activity**

The most dominant ectomycorrhizal fungal species in the system were assayed for the activities of 7 extracellular enzymes using and L-3,4-dihydroxyphenylalanine (L-DOPA) and p-Nitrophenyl (pNP)-linked substrates, following a protocol modified from Jackson et al. (2006). The specific enzyme families detected by the assays are involved in the degradation of organic phosphate (phosphatase), cellulose (β-1,4-glucosidase and cellbiohydrolase), chitin (β-N-acetylglucosaminidase), hemicellulose (β-1,4-xylosidase), and lignin and polyphenolic compounds (phenol oxidase and peroxidase). Substrates for the pNP-linked substrates were 5 mM pNP-phosphate, 5 mM pNP-β-glucopyranoside, 2 mM pNP-cellobioside, 5 mM pNP-β-xylopyranoside and 2 mM pNP-β-N-acetylglucosaminide. 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA) was the substrate used for the phenol oxidase and peroxidase assays.

Reaction incubation times were 2 hours (phosphatase, cellbiohydrolase, β-xylosidase and β-N-acetylglucosaminidase) or 4 hours (β-glucosidase, phenol oxidase and peroxidase), during which microplates were mixed for 2-3 minutes every 30 minutes using a Microplate Genie. After the incubation period, microplates were centrifuged briefly and 281 μl of reaction mixture was transferred to a new 96-well microplate containing 19 μl of 1 M NaOH (for assays using pNP-linked substrates) or 200 μl H₂O (for assays using L-DOPA substrates). The absorbance was read using a Synergy HT microplate reader (Biotek, Winooski, VT) at 410 nm (pNP-linked substrates) or 460 nm (for L-DOPA substrates). Final absorbance was adjusted according to sample and substrate controls and enzyme activity calculated as described by Jackson et al. (2006).
Microplates were sealed using AirPore Tape Sheets (Qiagen, Hilden, Germany) and placed in an oven at 60°C to dry ectomycorrhizal root tips. ECM root tips were weighed to determine dry sample weight (g), so that enzyme activities could be expressed per gram of mycorrhizal dry weight. Although a proportion of this weight is made up of plant mass, mycorrhizal weight is proportional to the surface area of root occupied by ECM fungi. Total enzyme activity per soil core volume was also estimated by multiplying the total weight of a root tip of a particular ECM fungal species by enzyme activity per unit mass.

**Statistical Analysis**

All data were analyzed using the *lm* function from the *nlme* package in R (v.2.15.1, R Foundation for Statistical Computing, Vienna Austria) for univariate analyses of variation in enzyme activities, soil properties, and single ECM fungal species, or using Primer (v6, PRIMER-E, Plymouth, UK) for multivariate analyses of ECM fungal community composition. Response variables in univariate analyses were log transformed to fit model assumptions of normality and homogeneity of variances when necessary. Details for each analysis are described separately below.

*Do ECM fungi vary among tree families and/or soil properties?*

Data were standardized and a matrix of Bray-Curtis distances was generated using the resemblance feature in Primer. The ectomycorrhizal fungal community composition was ordinated using Nonmetric Multidimensional Scaling (NMDS) to visualize longleaf pine genetic families’ variation in relative compatibility with particular ECM fungal species. A permutational multivariate analysis of variance (PERMANOVA) was used to determine if fungal species composition varied among longleaf pine genetic families or among replicates.
To test if soil properties (C, N and OM percentage) influenced the distribution of ECM fungal species, a Distance-Based Linear Model (DistBL) was used, where the continuous explanatory variables were C, N and OM percentage and the response was ECM fungal community composition.

*Does enzymatic activity differ among mycorrhizal fungal species or among tree families?*

To determine if tree families, different ECM fungal species or replicates varied in ECM fungal extracellular enzymatic activities, a separate analysis of variance (ANOVA) was performed for each of the seven enzyme families assayed using the dominant fungal species. In each analysis, ECM fungal species, tree family, and replicate were the categorical explanatory variables and the continuous response variable was ECM fungal enzyme activity. ECM fungal species were excluded from each separate ANOVA for each of the seven enzymes if that fungal species did not have at least two observations for that enzyme assay.

*Do ECM fungal communities, tree family or ECM fungal enzyme activities influence soil C, N and OM percentage?*

Separate univariate ANOVAs were performed to determine if tree families influenced each of three soil properties (C, N and OM percentage), where the categorical explanatory variable was tree family and the continuous response variables were the soil properties. A Distance-Based Linear Model (DistLM) in Primer was used to test if enzyme activities (per core) influenced soil properties (C,N, and OM percentage), where the categorical explanatory variable was enzyme activity and soil properties were continuous response variables.
To test if the ECM fungal community influenced soil C, N and OM percentage, ECM fungal species data were standardized and a matrix of Bray-Curtis distances among core samples generated using the resemblance feature in Primer (v6, PRIMER-E, Plymouth, UK). Nonmetric Multidimensional Scaling (NMDS) was used to ordinate the samples with respect to ectomycorrhizal fungal community composition among longleaf pine families, to generate values for NMDS Axis 1 and 2 to be used as the explanatory variables in a Distance-Based Linear Model (DistLM) in Primer, where soil C, N and OM percentage were used as continuous response variables.
RESULTS

Do ECM fungi vary among tree families and/or soil properties?

A total of 50 distinct ectomycorrhizal OTUs (hereafter, species) were represented in this study, including 24 OTUs only observed once each. ECM fungal community composition differed among 15 tree families ($F_{14,46} = 1.332, P = 0.0022$, Figure 3) and tended to differ among replicates ($F_{4,46} = 1.270, P = 0.068$). However, OM content ($F_{1,63} = 0.856, P = 0.657$), $%N$ ($F_{1,63} = 0.946, P = 0.536$) and $%C$ ($F_{1,63} = 1.1534, P = 0.279$) did not influence the distribution of ECM fungal species.

Does enzymatic activity differ among mycorrhizal fungal species or among tree families?

Peroxidase activity of 14 dominant ECM fungal species within each plot differed among tree families ($F_{14,16} = 3.106, P = 0.028$), with only 4 families out of 15 exhibiting significant peroxidase activity (Figure 4), and peroxidase activity differed among these 14 mycorrhizal fungal species ($F_{13,16} = 3.125, P = 0.029$) (Figure 5). *Amanita* produced the highest amount of peroxidase activity (Figure 5). N-acetylglucosaminidase activity differed among the 14 mycorrhizal fungal species that had at least two observations for that assay ($F_{13,16} = 2.811, P = 0.027$), but not among tree families ($F_{14,16} = 1.142, P = 0.396$) or replicates ($F_{4,16} = 1.170, P = 0.361$). *Tomentella* produced the highest amount of $\beta$-N-acetylglucosaminidase activity (Figure 6)
Phosphatase, β-glucosidase, phenol oxidase, xlyosidase, and cellobiohydrolase fungal enzymatic activity did not differ among mycorrhizal fungal species or among tree families (Table 1).

*Do ECM fungal communities, tree family or ECM fungal enzyme activities influence soil C, N and OM percentage?*

The ECM fungal community did not influence soil %C, %N or %OM (F\(_{1,63}\) = 0.048, P=0.981). ECM fungal enzyme activities did not influence soil %C, %N and %OM percentage (Table 2). Longleaf pine genetic families did not differ in %N (F\(_{14,46}\) = 0.963, P = 0.504), %C (F\(_{14,46}\) = 1.453, P = 0.168, and % OM (F\(_{14,46}\) = 1.715, P=0.085). However, %N (F\(_{4,46}\) = 4.062, P = 0.007), %C (F\(_{4,46}\) = 7.712, P < 0.0001, and % OM (F\(_{4,46}\) = 8.865, P < 0.0001) varied among replication sites.
DISCUSSION

I found that after 50 years of longleaf pines conditioning the soil beneath them, plant genetic variation can affect mycorrhizal community composition and potential peroxidase activities of ECM fungi, but not soil properties such as total carbon content. The results reported here support the hypothesis that plant genotype is an important ecological factor affecting mycorrhizal dynamics, including ECM community composition and enzyme activities. I found that peroxidase activity also varied among ECM fungal species, supporting the hypothesis that plant genotypes and ECM fungal species may control ECM enzyme activities simultaneously. However, some ECM fungal enzymes may only be controlled by ECM fungi. For example, I found that only ECM fungal species, and not tree genotypes, differed in N-acetylglucosaminase activity. In total, these results demonstrate that ECM fungal community composition and peroxidase vary by plant genotype, which suggests that plant genotype not only influences the surrounding biotic community, but also can directly select for particular ECM fungal characteristics.

Ectomycorrhizal fungal community composition

Longleaf pine families influenced ECM fungal community structure (Figure 3). Other studies from numerous systems have shown that plant host genetics can influence associated communities, including arthropods, soil bacteria and herbivores. For example, *Populus* genotypes growing in a common garden were shown to influence soil microbes and even explained 70% of microbial community composition (Schweitzer et al. 2008). More specifically,
studies have suggested that forest tree species have genetic variation in their compatibility with particular ECM fungal species (Korkama et al. 2006, Leski et al. 2010). For example, *Pisolithus tinctorius* varied in its intensity of colonization among loblolly pine genotypes (Dixon et al. 1987). However, this is the first study of which we are aware to show that plant genotypes can alter mycorrhizal fungal community composition in a long-term field experiment. This observation suggests that genetic selection of plant genotypes could allow compositional management of microbial symbionts of trees, including the ECM fungal community, and possibly the traits of those microbial communities (Hoeksema and Classen 2012).

Replication site also tended to influence the distribution of ECM fungal species, indicating that there may be some environmental variation between sites affecting the ECM fungal community. Although ECM community structure was not influenced by %N, %C, %OM in our analyses, other soil chemical and physical properties (such as water availability, total P, soil pH, and soil texture) could also determine the distribution of ECM fungal species.

*Relationships between longleaf pine genetics and ECM enzyme activities*

In this study, longleaf pine genetic families varied dramatically in their average peroxidase activity of ECM root tips, with most families exhibiting little or no activity and others showing substantial activity (Figure 4). Other studies have also found that ECM fungal enzyme activities may be under the direct genetic control of the host. For example, *Populus* genotypes were found to differ in all ECM enzyme activities measured (including N-acetylglucosaminidase, β-glucuronidase, cellobiohydrolase, β-glucosidase, β-xylosidase, and acid phosphatase) except for laccase activity (Courty et al. 2011). The implications of the results reported here are that genotype selection has the potential to directly influence the degradation of woody debris and recalcitrant materials through control of peroxidase activity in ECM fungal species. More
specifically, in this study Family 1 displayed particularly high ECM peroxidase activity (Figure 4). This genetic family may be associated with degradation of woody debris and may influence the breakdown of recalcitrant C compounds in the soil. The families that did not display any ECM peroxidase activity may be associated with storage of C in soil.

ECM fungal species also varied dramatically in their potential activity of N-acetylglucosaminidase, an enzyme involved in the degradation of chitin (Figure 6). *Tomentella*2, a dominant genus within this study, displayed high N-acetylglucosaminidase activity (Figure 6). The finding that *Tomentella* produces N-acetylglucosaminidase is consistent with the enzymatic assay study conducted by Buée et al. (2007), which found that the ECM morphotypes belonging to the genera *Tomentella* expressed strong chitinase activity (Buée et al. 2007). Buée et al. (2007) also found that several ECM fungal species exhibited elevated chitinase activity when growing in dead woody debris that was also colonized by white-rot saprobic fungi, suggesting that some ECM fungal species may have the ability to degrade tissue of other fungi and interfere with the decomposition of woody debris by saprobic fungi (Buée et al. 2007). Therefore, these ECM fungal species may interfere with the breakdown of C in the soil.

ECM fungal species also showed a trend towards variation in peroxidase activity (Figure 5). *Amanita*1 produced strong potential peroxidase activity involved in the degradation of recalcitrant organic substrates (Figure 5). The expression of high potential peroxidase activity from these ECM fungal species suggests that they have significant saprobic capabilities that can potentially access nutrients from recalcitrant soil organic matter and degrade C in the soil. The finding that *Tomentella* and *Amanita*1 produce peroxidase is consistent with the enzymatic assay study conducted by Buée et al. (2007), which found that *Tomentella* species expressed strong
ligninase activity involved in the degradation of recalcitrant organic substrates (Buée et al. 2007). The expression of high potential peroxidase activity from Tomentella and Amanita suggests that some ectomycorrhizal fungi have significant saprobic capabilities that can potentially access nutrients from recalcitrant soil organic matter. Interestingly, Tomentella is characteristic of the “contact exploration type” with a smooth mantle and few hyphae that was described by Agerer (2001) and has been described in woody debris (Tedersoo et al. 2003). However, Amanita, which also produced high peroxidase activity in this study, is described as the “medium distance exploration type by Agerer (2001), which is characterized by forming rhizomorphs, suggesting that it receives more carbon from its plant host. These results suggest that although ECM fungi differ in their mycelial structures and soil exploration strategies, ECM fungi may be able to change their abilities to break down soil organic matter, depending on the environment. For example, Lactarius quietus produced cellobiohydrolase in leaf litter and chitinase when it grew in dead woody debris (Courty et al. 2006). In this study, ECM fungi such as Russulaceae3 (Figure 5) did not produce peroxidase. Therefore the ECM fungal species may not be able to access nutrients from recalcitrant soil organic matter and C may be sequestered in the soil.

However, phosphatase, β-glucosidase, phenol oxidase, xlyosidase, and cellobiohydrolase did not differ in activity among plant genotype or ECM fungal species. One possible explanation for this lack of variation is that all samples were collected in the summer, and previous studies have found that enzyme activities of ectomycorrhizal fungal species change seasonally. For example, Courty et al. (2007) found that laccase, β-glucuronidase, cellobiohydrolase, and β-glucosidase activity were related to tree reactivation and climate.
Influences on soil properties

Although longleaf pine genetics did influence ECM fungal community composition and potential enzyme activities of ECM roots, my overall hypothesis that these genetic effects would influence soil C, N and organic matter percentage was not supported. Thus, it is possible that tree genetic variation simply does not have significant consequences for soil nutrient dynamics and carbon storage. Alternatively, it is possible that my approach to measuring these processes failed to adequately describe them for this system, and that more elaborate methods could be used to measure soil C storage. For example, many studies are using stable isotopes to measure soil C sequestration (Clemmensen et al. 2013). Also, important C storage may take place in deeper profiles of the soil than I measured. Carbon sequestration was found to increase with depth, where root-associated fungi play a greater role in the regulation of organic matter (Clemmensen et al. 2013).
Figure 1: Diagram of possible relationships among tree family, enzyme activities, the ECM fungal community, and soil properties. (1) Tree genetic families influence the structure of the ECM fungal community. (2) Tree genetic families directly influence ECM fungal enzyme activities. (3) ECM fungal species vary in ECM fungal enzyme activities. (4) Tree families directly influence soil properties. (5) ECM fungal enzyme activities influence soil properties. (6) ECM fungal community can affect soil properties.
Figure 2: Diagram of a familial eight-tree row plot, containing 8 full-sib progeny from one of the 78 crosses
Figure 3: Nonmetric Multidimensional Scaling of the ECM fungal community composition among longleaf pine families using Bray-Curtis distances. The numbers above the symbols represent sample plots.
Figure 4: ECM fungal peroxidase activity among longleaf pine genetic families. Means with different letters are significantly different from each other (p<0.05) according to Tukey HSD post-hoc tests.
Figure 5: Peroxidase activity of dominant ECM fungal Operational Taxonomic Units (OTUs). Means with different letters are significantly different from each other (p<0.05) according to Tukey HSD post-hoc tests.
Figure 6: N-acetylglucosaminidase activity of dominant ECM fungal Operational Taxonomic Units (OTUs). Means with different letters are significantly different from each other (p<0.05) according to Tukey HSD post-hoc tests.
<table>
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<th>Enzyme</th>
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<th>P &gt; F</th>
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Table 1: Results of ANOVA of ECM fungal enzyme activity as a function of Operational Taxonomic Unit, Tree Family and Replication for phosphatase, β-glucosidase, phenol oxidase, xlyosidase, and cellobiohydrolase. P value less than 0.05 indicates significant effect.
<table>
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Table 2: Results of DistLM of soil properties (%N, %C, and %OM) as a function of ECM enzyme activity. P value less than 0.05 indicates significant effect.


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