Spring 5-9-2020

Ectomycorrhizal Fungi and Effects of Soil Microbes Associated with Slash Pine Encroachment into Native Longleaf Pine Habitat

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ECTOMYCORRHIZAL FUNGI AND EFFECTS OF SOIL MICROBES ASSOCIATED WITH SLASH PINE ENCROACHMENT INTO NATIVE LONGLEAF PINE HABITAT

by
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2020

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Acknowledgements

First and foremost, I would like to thank Dr. Jason Hoeksema for allowing me to research in his lab. I am extremely thankful for the patience and feedback he has given me throughout my research and the writing of this thesis. Thank you to Dr. Stephen Brewer and Dr. Colin Jackson for the feedback and suggestions on my writing. Thank you, again, to Dr. Brewer for helping to collect the data in the field. Special thanks to Dr. Ami Lokhandwala for her role of conducting the laboratory experiment that produced the data I was able to analyze here. Lastly, I would like to thank my friends and family for their unwavering support and encouragement throughout this journey. I am so appreciative of all of you for helping me during this writing process.

ABSTRACT

Ectomycorrhizal Fungi and Effects of Soil Microbes Associated with Slash Pine Encroachment into Native Longleaf Pine Habitat

Biological invasions can cause substantial changes to the environment: indigenous species can be reduced or even eliminated, soil characteristics shifted, and nutrient cycles altered. Ectomycorrhizal (ECM) fungi are thought to be key biological controllers of some plant invasions, especially benefiting Pinus species in invasions at exotic sites, but less is understood about the role of ECM fungi and other soil microbes in encroachment by one plant species into the habitat of another in their native ranges. In this study, soil was collected from three habitats in southern Mississippi, USA: native slash pine maritime forest, longleaf pine savanna encroached by slash pine, and restored longleaf pine savanna where slash pine had been removed. Seedlings of slash pine were grown in the three different soils, which was either sterilized or non-sterilized. After allowing time for ECM fungi to form symbioses on pine seedling roots in the non-sterilized soils, seedling root tips were analyzed to identify ECM fungi present, and plant growth quantified.

Soil microbes and the invasion history of soil both impacted slash pine seedling growth, but this depended on how plant growth was measured. In sterilized soil slash pine seedling growth was much higher than in the non-sterilized soil. This could be due to the

effects autoclaving has on the soil, plants respond better to beneficial and pathogenic microbes being removed from the soil. It could also be due to the Enemy Release hypothesis which states that slash pine escapes the unique pathogens in maritime forest soil when encroaching into longleaf pine savanna. The key to a plant's growth success is the elimination of microbes in the soil.

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LIST OF ABBREVIATIONS

ECM: Ectomycorrhizal

PCR: Polymerase Chain Reaction

ITS: Internal Transcribed Spacer

OTU: Operational Taxonomic Unit

INSD: International Nucleotide Sequence Database

UNITE: User-Friendly Nordic ITS Ectomycorrhizal database

MF: Maritime Forest

RS: Restored Savanna

SI: Savanna Invaded

Tukey HSD test: Tukey's Honestly Significant Difference Test

Introduction

Biological invasions are a driving force of change in the environment, resulting in a species colonizing in a new geographic region. Invasive, nonindigenous species threaten the natural ecosystems in the regions they have invaded, by suppressing and even eliminating species native to the area (Richardson et al., 2000). Invaders have the potential to alter the nutrient cycles, hydrology, and energy budgets of a native ecosystem; even just one invasive species can overrun an ecosystem (Mack et al., 2000). Biotic invaders can wreak havoc on the local biodiversity and to the processes that occur at an ecological level in a region, which will directly impact economic outcomes and ecosystem services, such as crop production, forestry, and livestock grazing range (Mack et al., 2000). We do not fully understand what makes most invasions successful, but there is increasing recognition that mutualisms--mutually beneficial interactions between invaders and other species, may play a key role (Richardson et al., 2000). Here, I explore the role that mutualists such as ectomycorrhizal (ECM) fungi, and other soil microbes, may play in the success of an invading plant species.

ECM associations are mutualistic symbioses that form between soil fungi and the fine root tips of wood plant hosts in a few families, including Pinaceae. In these mycorrhizal partnerships, the fungi typically supply the plant with nutrients and can even protect against pathogens in exchange for photosynthetic carbon (Read, 1986). ECM

fungi influence the establishment of conifers, meaning Pinaceae depend on this symbiosis to survive and colonize in new regions (Nuñez, 2009). The ECM fungi that are most often associated with pine invasion are suilloid fungi, mainly in the genera *Rhizopogon* and *Suillus* (Policelli, 2019). These two genera of suilloid fungi play a critical role in the establishment of pine seedlings in new areas. Because of the nature of this symbiosis, pines have been able to overcome previous barriers, which has unintentionally led to invasion.

Mutualisms, like pollination and seed dispersal by animals and relationships between plants and roots, are often the facilitators of plant invasion. For the most part, mutualisms of plant invaders replicate the partnership of species from their native environment, but on occasion they create new partnerships between previously unassociated species, like mycorrhizal associations between plant roots and fungi (Richardson et al., 2000). Most invasive plant species can only invade in the presence of compatible symbionts, which has been a barrier for some plants that need ECM fungal associations, like *Pinus* species (Nuñez et al., 2009). For *Pinus* species, colonization in nonindigenous areas results from the inoculum of ECM fungal spores (Davis et al., 1996). Although the need to accumulate ECM fungal spores in the soil may be a barrier in the beginning stages of establishing Pinaceae in new habitats (Nuñez et al., 2009), Pinaceae plants are among the world's most successful invaders, usually aided by co-invasion with ECM fungi from their native range (Dickie et al., 2017).

Plants are in constant feedback with soil microbes, including beneficial symbionts, pathogens, and decomposers (Zhang et al., 2016). These feedback interactions

affect the relationship between soil microbes and plant roots, which can alter the plant's ability to compete within the community, and it is increasingly recognized that these feedbacks may play a role in plant invasions (Kardol, 2007). Invasive plants can cause changes in the chemistry of the soil or changes to symbiotic mutualists, both of which may then suppress how native plants perform (Van der Putten, 2007). Because the native plants are now below optimal performance, this increases the likelihood that a nonindigenous species will dominate (Van der Putten, 2010).

The Pinaceae family is one of the most invasive families of plants, and genus Pinus encompasses the majority of the invasive conifers (Richardson & Rejmanek, 2004). Pinus is native to the northern hemisphere, but over the course of many years of introductions by humans, it has been widely established in the Southern Hemisphere. Included in the pine species widely introduced is slash pine (Pinus elliottii), which is native to the southeastern United States. The typical climate for slash pine is warm and humid, with wet summers and a drier fall and spring. Slash pine's native habitat is maritime forest, but now it has been introduced into the Southern Hemisphere as a timber species, and is also encroaching into nearby longleaf pine (Pinus palustris) savanna habitats in its native range. Pinus elliottii is considered invasive and there are serious implications to it invading new habitats.. According to a paper from a study done in Brazil and Mississippi, slash pine invaded sites had a lower plant species richness than non-invaded and restored savanna sites (Brewer et al., 2018). When the density of slash pine is increased, a decrease in groundcover plant diversity is seen (Brewer et al., 2018). It is not clear if ECM fungi or other soil microbes play a role in slash pine invasion.

For this experiment, I studied slash pine encroaching from its native maritime forest habitat into longleaf pine (*Pinus* palustris) savanna habitats in southern Mississippi, and the role that ECM fungi and other soil microbes may play in this encroachment. Longleaf pine is indigenous to the southeastern United States, found in the Atlantic and Gulf coastal plains from southeastern Virginia to central Florida and west to eastern Texas (Peet & Allard, 1993). Slash pine began to invade longleaf pine habitat from its nearby maritime forests because of fire exclusion (Brewer et al., 2018). It is important to study slash pine in its native range in order to understand how the soil characteristics of native and invaded habitats play a role in slash pine invasion.

In this study, I sought to answer the following research question: do ECM fungi and other soil microbes play a role in slash pine encroachment into longleaf pine savanna? One hypothesis—"Shared Symbionts"—is that slash pine shares most ECM fungi in common with longleaf pine, and those ECM fungi are equally important for slash pine seedling growth in maritime forest and where it is encroaching into longleaf pine savanna. This hypothesis predicts that in a soil bioassay experiment, ECM fungal colonization levels and the composition of the ECM fungal community would be similar in slash pine maritime forest (MF) and longleaf pine savanna soils. It also predicts that sterilizing soil would be detrimental to slash pine seedling growth, regardless of whether the soil came from slash pine maritime forest (MF), longleaf pine savanna invaded (SI) by slash pine, or restored longleaf pine savanna (RS) where slash pine had been removed. A second hypothesis--"Enemy Release"--is that slash pine has unique pathogens in MF soil that it escapes when encroaching into longleaf pine savanna (Catford et al., 2009).

This hypothesis predicts that slash pine seedlings would generally grow better in RS and SI soils than in MF soils, and that sterilizing soil would especially benefit slash pine seedling growth in MF soil.

Methods

Sample Acquisition

Three types of habitats (two sites each) were sampled at Grand Bay National Estuarine Research Reserve, Moss Point, MS, USA, on 20 July 2016: maritime forest (MF, native slash pine habitat), invaded savanna (SI, native longleaf pine savanna invaded by slash pine), and restored savanna (RS, longleaf pine savanna here invading slash pine had been removed) to obtain soil for a laboratory "bioassay" experiment to identify "spore-bank" ECM fungi compatible with slash pines in each soil, and to measure slash pine seedling growth in sterilized and non-sterilized soils. At each site, approximately 40 soil samples were collected, each in a 1/4 hectare uniform grid. In MF and SI habitats, half of the samples were located randomly, and half were collected within 2 meters of an adult slash pine. At RS sites, half of samples were collected near (less than 2 meters) to stumps of removed slash pine and half were collected far (at least 5 meters) from those stumps. Loose litter was first set aside, and a hand shovel used to collect approximately 240 cm3 of soil from the upper 10 cm. Soil samples were were kept separate, and were transported to the University of Mississippi in coolers. Half of each sample was sterilized by autoclaving twice (at 120°C for two hours), with 24 hours in between each autoclave cycle. After sterilization, sterilized and non-sterilized sub-samples of each soil sample were mixed 50/50 with sterilized playground sand and

used to fill one pot each (bleach-sterilized Ray Leach cone-tainers model SC10, 164 ml; Stuewe & Sons Inc., Tangent, OR).

In 2014, 250 seeds each were provided by the University of Florida's Cooperative Forest Genetics Research Program, from open-pollinated genetic families from baseline seed orchards at four sites in the Gulf Coast of Florida: two each from Taylor County and one each from Okaloosa and Walton Counties. The four genetic families of slash pine seeds represented mixed genotypes from genotypes selected from the Florida Gulf Coast region, each tested to be average for growth and disease resistance compared to wild type trees. The four families were combined together for use in the experiment and stored at 4°C until used in this experiment. Seeds were surface-sterilized with 10% bleach for two minutes, cold stratified for one month at 4°C, and then on October 28, 2016 ten seeds were planted into each pot. Pots were placed in a Conviron growth chamber with a 14:10 hour day:night cycle of light, at a constant temperature of 22°C. As seedlings germinated, they were thinned and transplanted so that only one seedling of similar size was in each pot. Pots were watered to capacity every other day. After approximately 84 days, plants were harvested and separated into shoot and root biomass. Roots were washed free of soil on a 2mm sieve and examined for ECM colonization (see below), and then root and shoot mass were separately dried at 65°C for 48 hours before being weighed.

Molecular Identification of ECM Fungi

Roots were examined using the gridline intercept method to determine the percentage of root length with ECM colonization. ECM root tips in each sample were

examined under a dissecting microscope and were classified into morphological types based on color, texture, branching patterns, and emanating hyphae and rhizomorphs. Replicate root tips from each morphotype in each sample were collected, frozen at -20°C and stored for approximately one year until DNA extraction, PCR, and Sanger sequencing were carried out. DNA extraction of fungal tissue was accomplished by using components from the Sigma Extract-N-Amp Tissue Kit. One root tip from each sample of fungal mycelium was placed in a 96-well plate. In each well, 10 µl of the Sigma Extraction Buffer was added, then heated on the thermocycler at 65°C for 10 minutes, 95°C for 10 minutes, then 30 µl of Neutralization Solution was added.

In order to perform Sanger sequencing of the ECM fungal DNA, the Internal Transcribed Spacer (ITS) regions of the fungal genome were amplified by using the fungi specific forward and reverse primers, ITS1-F and ITS4. The DNA extract was thawed and a mastermix of 2.2 µl PCR grade water + 4 µl of 2X Red Taq Premix + 0.4 µl of each primer (10µM concentration) was made so 7 µl of mastermix could be used per reaction. The amplification process took place in sterile 96-well PCR plates, sealed with a silicon rubber sealing mat, the well was briefly centrifuged, and underwent the following parameters: initial denaturation (1 cycle) at 94°C for 3 minutes, denaturation (30 cycles) at 94°C for 45 s, annealing (30 cycles) at 53°C for 45 s, extension (30 cycles0 at 72°C for 60 s, and a final extension (1 cycle) at 72°C for 10 minutes. A 1% gel was used to check PCR amplification success with SybrSafe DNA stain. Gel was analyzed over UV using Quantity One Software. Enzymatic cleanup of the PCR products was done using ExoSAP-IT by combining 0.25 µl Exo-SAP-IT and 4.75 µl PCR grade water to the 96

well plate of PCR products. The PCR products were then incubated at 37°C for 30 minutes, 80°C for 20 minutes, and 4°C for 5 minutes.

Sanger sequencing of the reaction was accomplished by using the ITS5 primer and BigDye Ready Reaction Mix. The BigDye mastermix contained 0.4 µl Big Dye reaction premix, 1.8 µl Big Dye 5x sequencing buffer, 0.5 µl ITS4 primer at 10 µM concentration, 6.3 µl PCR grade water, and 1 µl of cleaned PCR product. Amplification was performed (ramp speed no greater than 1 degree C per second) under the following conditions: initial denaturation (1 cycle) at 96°C for 1 minute, denaturation (45 cycles) at 95°C for 20 sec, annealing (45 cycles) at 52°C for 20 sec, and an extension (45 cycles) at 60°C for 4 min. Sequencing plates were dried for 30 minutes at 45°C then shipped to the DNA Lab at the School of Life Sciences in Tempe, AZ, for cleaning and reading on a capillary analyzer.

ECM Fungal Sequence Data Analysis

The cleaned DNA sequences obtained from Arizona were edited manually using the software Geneious to correct any ambiguous bases in the fungal sequences. Sequences were edited to <3% ambiguity or less and no less than 200 base pairs; all sequences not fitting within this criterion were discarded. The sequences remaining underwent OTU assembly using CAP3 software using default settings with these changes: maximum overhang percent length: 60, match score factor: 6, overlap percent identity cut-off: 97, and clipping range: 6. Sequences >97% similar were considered the same OTU. Representative sequences from each OTU were checked using BLAST

searches on INSD and UNITE databases to determine the best taxonomic classification of the OTUs. OTUs matching 99% or better to database sequences from named, cultured fungi were considered the same species. Sequences with matches of 98% similarity or less were assigned to a genus based on the recommendations of Tedersoo and Smith (2017), and were assigned a number (e.g., *Russula*_1). If sequence matches among the sequence repositories showed equal affinity or similarity to multiple genera within a family, priority was given to the vouchered specimens residing on the UNITE database. Any taxon known to be strictly non-mycorrhizal was eliminated from the data set.

Data analysis

Relative growth rate (RGR) of needle production, height, and basal diameter were calculated using the following equation:

$$(ln\frac{variable\ 2}{variable\ 1}) / \#\ of\ days\ = RGR$$

where "variable" stands for either needle number, height, or basal diameter. The number of days varied depending on the variable: RGR for number of needles was measured over 40 days, height over 20 days, and basal diameter over 20 days.

In order to analyze the effect of habitat on root colonization by ECM fungi, and the effects of habitat and soil sterilization on plant growth, R statistical software, Version 1.1.463 was used. To examine effects of habitat type on frequency of different fungi, no individual OTUs were frequent enough for analysis, so Chi-squared tests were applied to test the effect of habitat type on the frequencies of the two most abundant fungal genera,

Rhizopogon and *Suillus*. When assumptions of low cell counts were violated, randomization procedures were used to obtain P-values for these tests.

Analysis of plant growth variables was performed using a mixed-model ANOVA with habitat, soil sterilization, and their interaction as fixed effects and with site as a random effect to account for non-independence of the multiple replicates from each of the two sites per habitat type. These tests in R were performed using the *lmerTest* function. Significant effects of habitat or habitat by sterilization interactions were followed by Tukey HSD post-hoc tests to separate means. The *sciplot* function (Scientific Graphing Functions for Factorial Designs) was used to graph results.

Results

ECM *Fungal Composition*

At the individual OTU level, twelve different unique OTUs were found across all the habitats combined. *Suillus decipiens* and *Rhizopogon _sp4* were found in five samples, *Rhizopogon_sp5* was found twice, and all other OTUs were found once each (Fig. 1). *Helotiales_sp1* was found solely in the MF habitat. (Fig. 2). Multiple *Rhizopogon* OTUs were found there, as was *Suillus decipiens*, but none of the OTUs occurred more than once. In RS habitat, *Suillus decipiens* was the most prevalent OTU, found in four samples, followed by *Rhizopogon_sp4,* found three times; other OTUs of *Rhizopogon* and *Suillus* were found once (Fig. 3). In SI, three *Rhizopogon* OTUs and one *Suillus* OTU were found here and they occurred once each (Fig. 4).

At the genus level for the ECM fungi, three fungal genera were found to be present across the three different habitats studied: *Rhizopogon*, *Suillus*, and a member of the order Helotiales (Fig. 5). *Rhizopogon* occurred the most with thirteen of the twenty-one samples containing *Rhizopogon*. *Suillus* was next with seven of the twenty-one samples containing the *Suillus* genus. The least frequent fungal genus was the member of the Helotiales with one occurrence. In the MF habitat, *Rhizopogon* was found most frequently with four samples, followed by *Suillus* and Helotiales each with one (Fig. 6). In the RS habitat, *Rhizopogon* and *Suillus* occured almost equally frequently, at six

and five respectively (Fig. 7). In the SI habitat, *Rhizopogon* occurred the most with three samples and *Suillus* only occurred once (Fig. 8). Pearson's Chi-squared test of habitat type on *Rhizopogon* and *Suillus* frequency were tested, but there were no significant differences in frequency among the three habitats (*Rhizopogon*: P = 0.625, *Suillus*: P = 0.093), although there was a trend for *Suillus* being more frequent in RS than in the other habitats.

Plant Growth and Total Mycorrhizal Colonization

When analyzing root colonization by ECM fungi, a trend was found among habitats (P = 0.1022): colonization tended to be lower in MF soil than in foreign soil (Fig. 9, Table 1). For shoot dry weight, there was a main effect of habitat (P<0.0001) and sterilization (P = 0.00081) individually, but no interaction (Table 1). MF had higher shoot dry weight than the other habitats (Fig. 10, P<0.0001), and was greater in the sterilized soil compared to non-sterilized (Fig. 11, P=0.00081). Root dry weight was not significantly affected by sterilization of soil or by soil from different habitats (Table 1). Similarly, for Height RGR, we found no significant effect of habitat or soil sterilization (see Table 1). The effect of soil sterilization was dependent on habitat for Needle RGR (P=0.0001703). Specifically, within the RS habit, needle RGR was significantly lower in the sterilized soil than in the non-sterilized soil (Fig. 12). For Basal Diameter RGR, growth was better in sterilized soil (P<0.0001, Fig. 13).

Table 1. Results from R statistical analyses of plant growth and ECM fungal colonization.

Response	**Source**	**$F_{df1,df2}$**	**P**
Root dry weight (g)	Habitat Sterilization Habitat × Sterilization	$0.36_{2,103}$ $0.13_{1,103}$ $1.5_{2,103}$	0.70 0.72 0.23
Shoot dry weight (g)	Habitat Sterilization Habitat × Sterilization	$14_{2,103}$ $12_{1,103}$ $0.60_{2,103}$	P<0.0001 P<0.0001 0.55
RGR of height	Habitat Sterilization habitat × Sterilization	$0.02_{2,190}$ $2.5_{1,190}$ $1.0_{2,190}$	0.98 0.11 0.36
RGR of needles	Habitat Sterilization Habitat × Sterilization	$3.0_{2,3.14}$ $11_{1,187}$ $9.1_{2,187}$	0.19 P<0.0001 P<0.0001
RGR of diameter	Habitat Sterilization Habitat × Sterilization	$0.13_{2,189}$ $18_{1,189}$ $0.61_{2,189}$	0.88 P<0.0001 0.54
Root colonization	Habitat	$7.8_{2,2.2}$	0.1

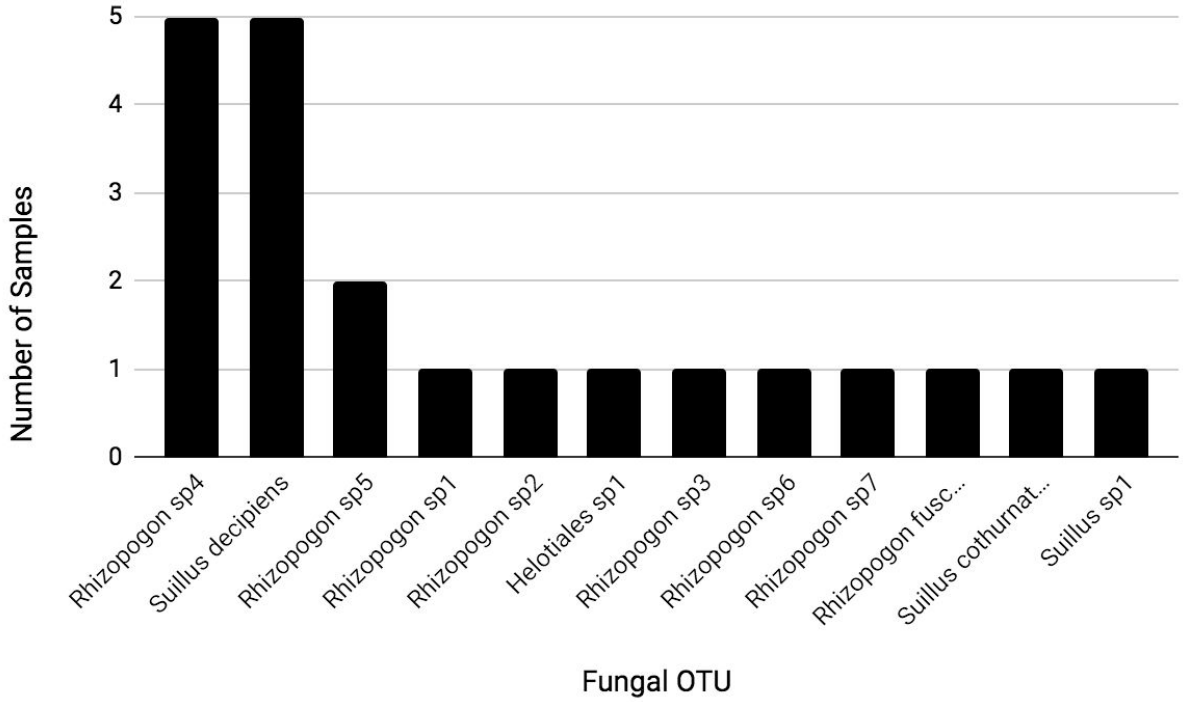

Figure 1. Frequency of OTUs of ECM fungi found in all soil samples across the three habitats, MF, RS, and SI.

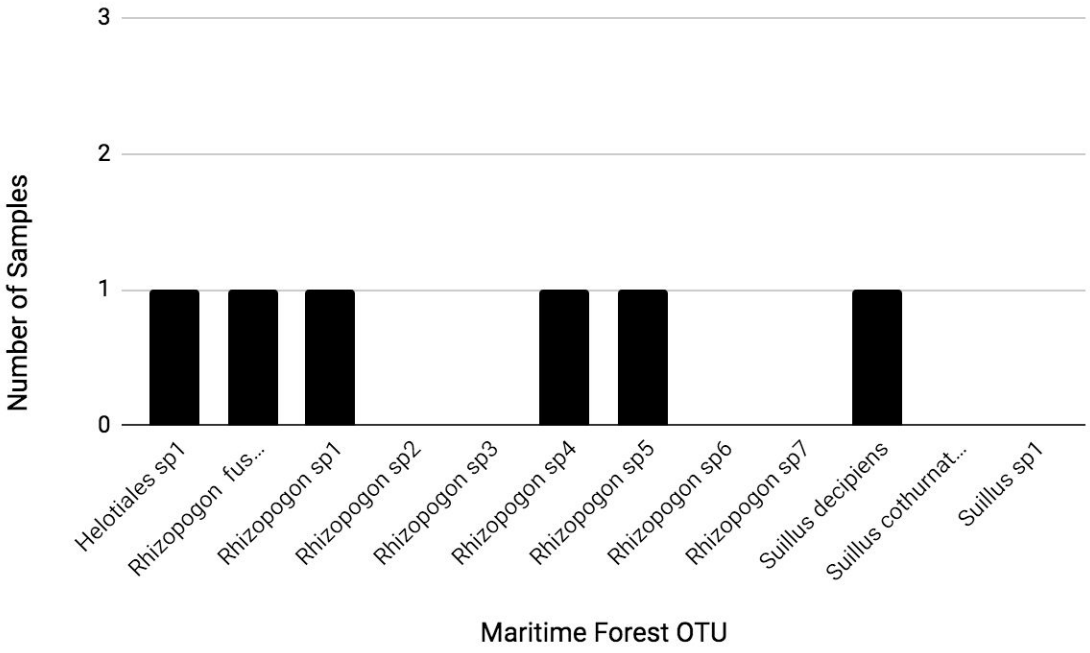

Figure 2. Frequency of OTUs of ECM fungi found in soil samples of MF habitat.

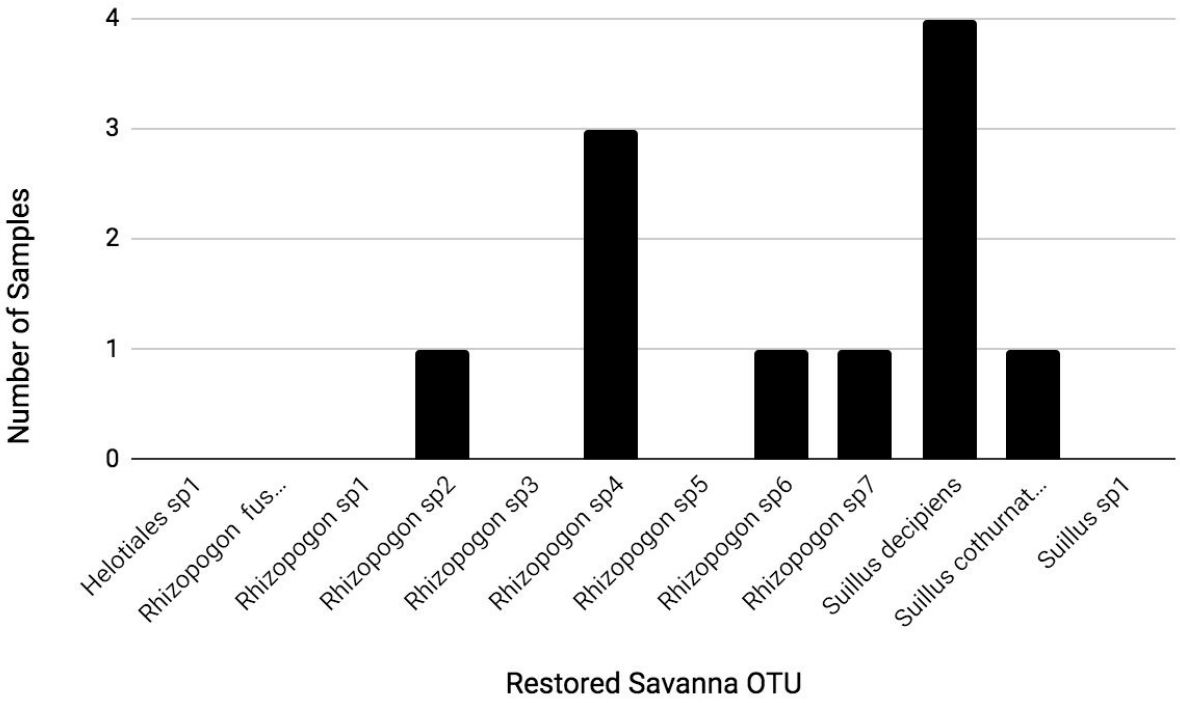

Figure 3. Frequency of OTUs of ECM fungi found in soil samples of RS habitat.

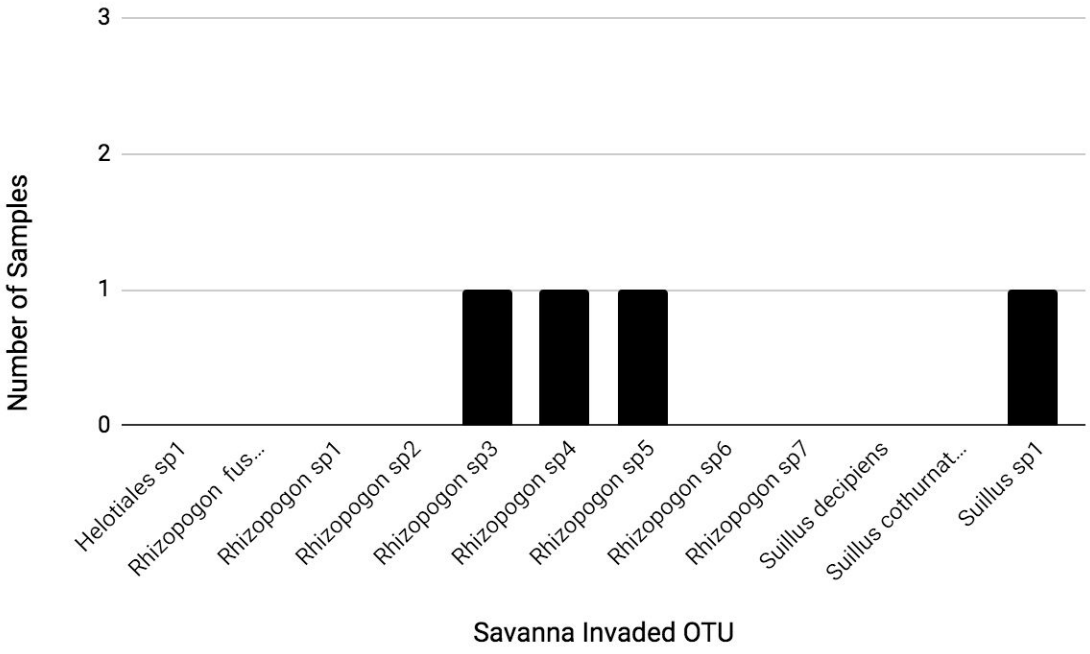

Figure 4. Frequency of OTUs of ECM fungi found in soil samples of SI habitat.

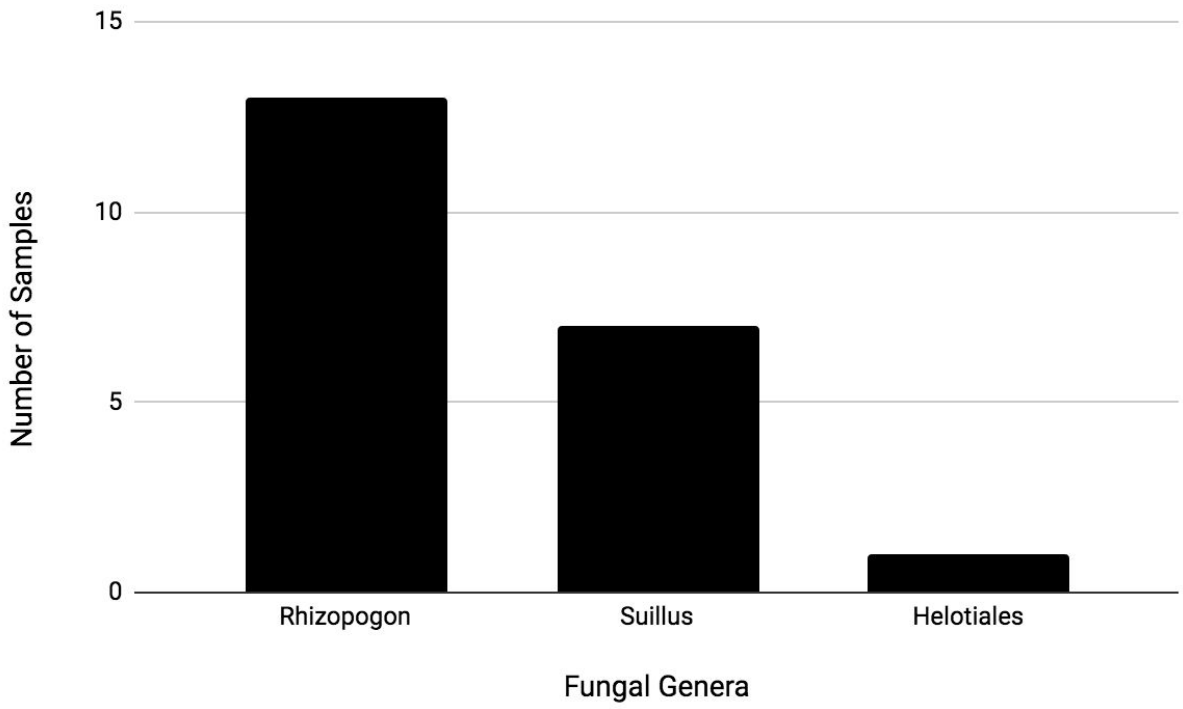

Figure 5. Types of ECM fungi aggregated by genus, across all three habitats. “Helotiales” represents an unknown genus in the order Helotiales.

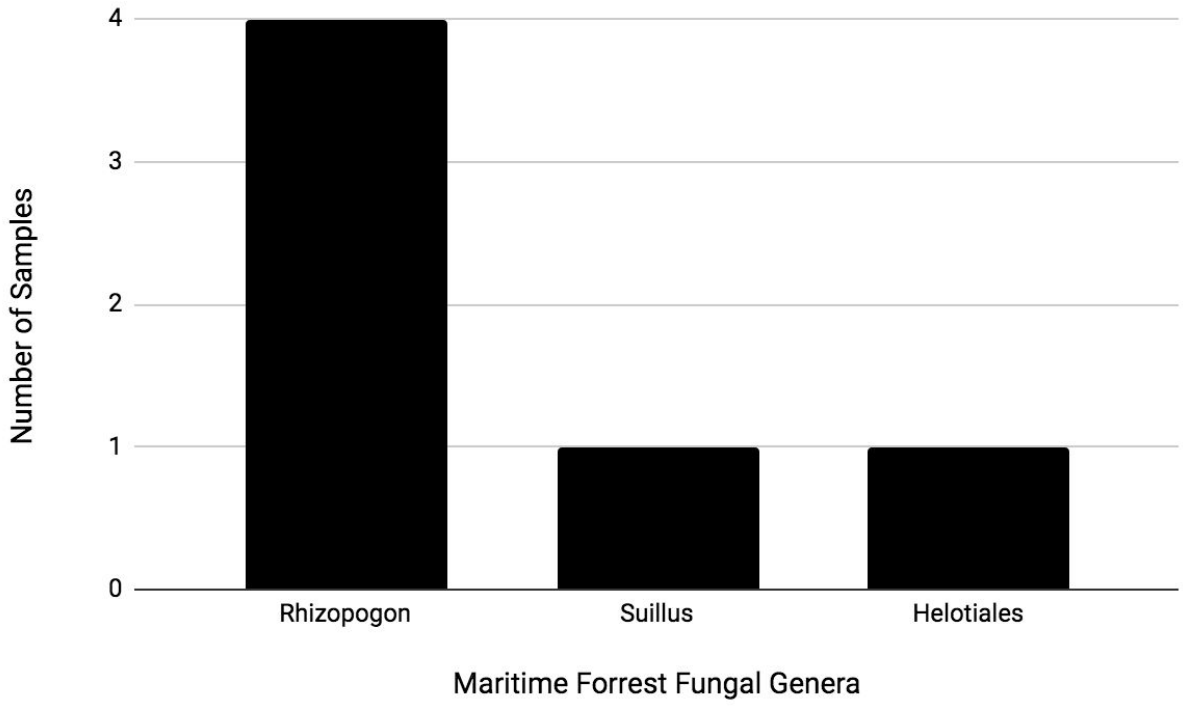

Figure 6. Types of ECM fungi aggregated by genus, in MF habitat. “Helotiales” represents an unknown genus in the order Helotiales.

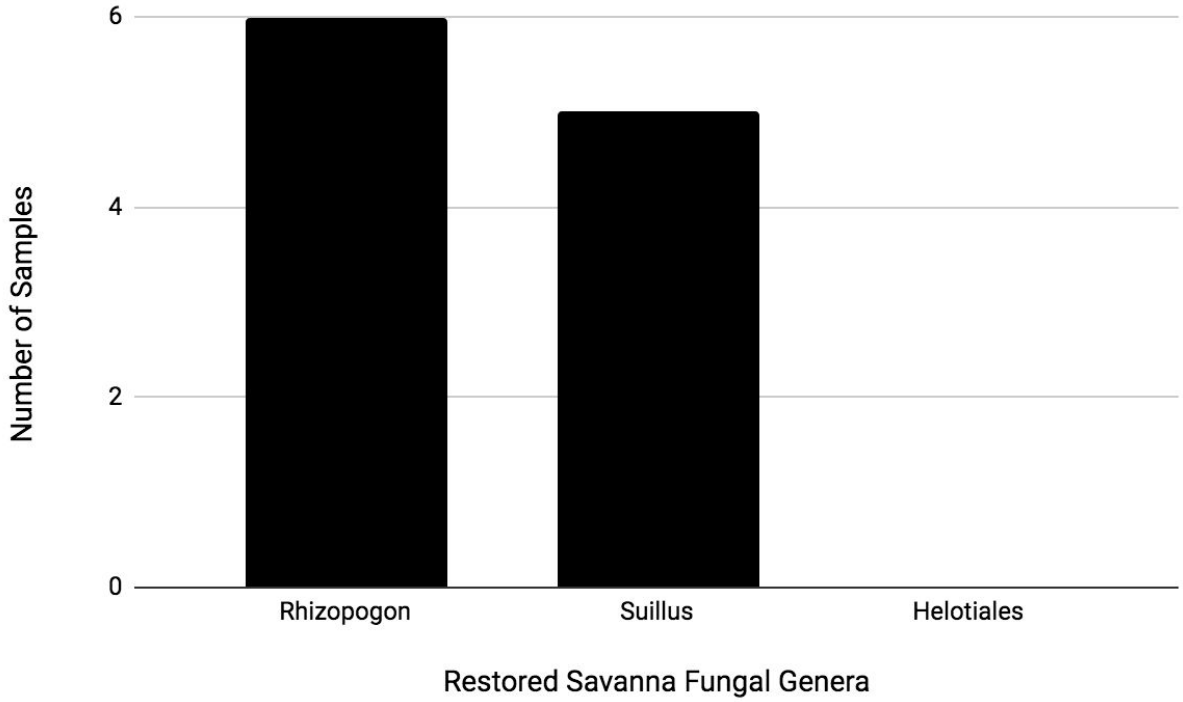

Figure 7. Types of ECM fungi aggregated by genus, in RS habitat. “Helotiales” represents an unknown genus in the order Helotiales.

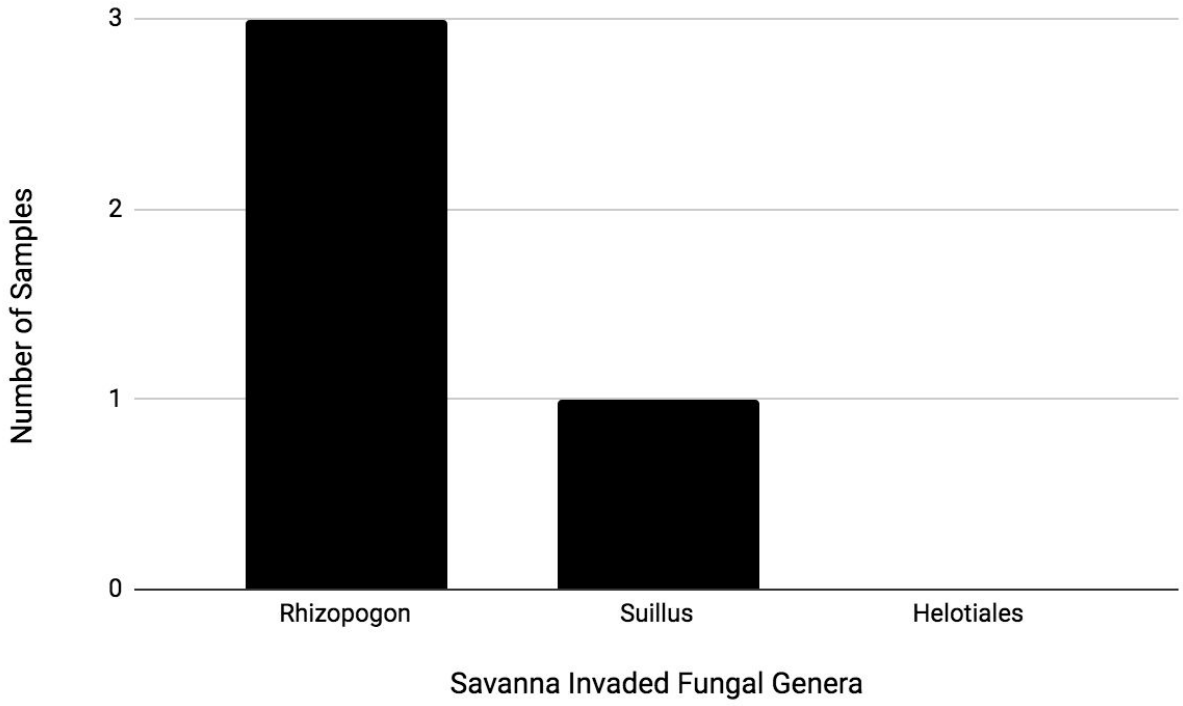

Figure 8. Types of ECM fungi aggregated by genus, in SI habitat. “Helotiales” represents an unknown genus in the order Helotiales.

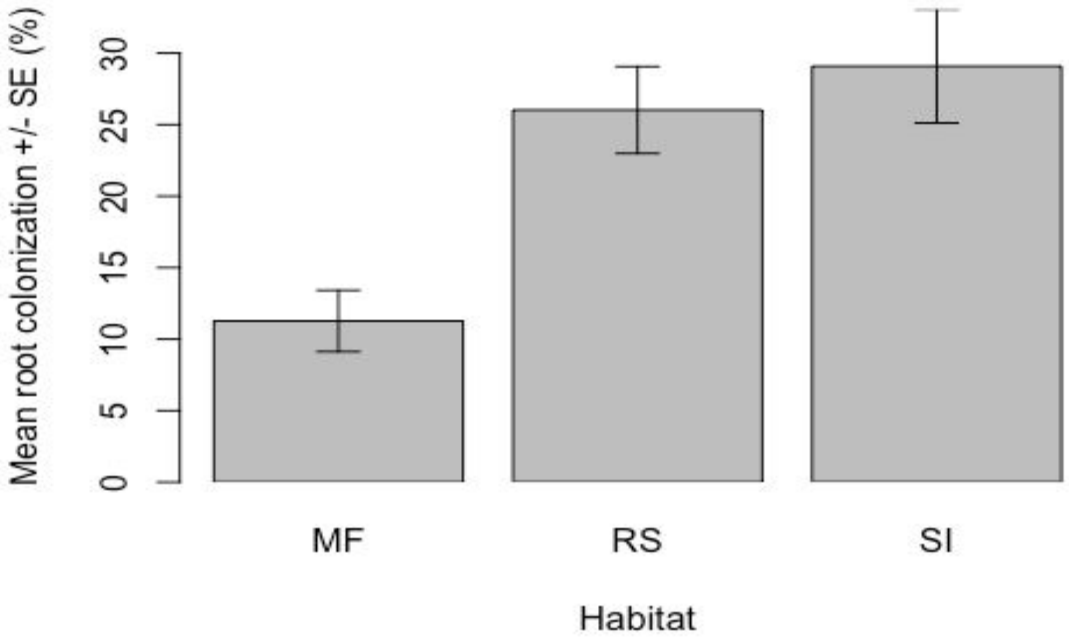

Figure 9. Effect of habitat and sterilization on root colonization (%) by ECM fungi.

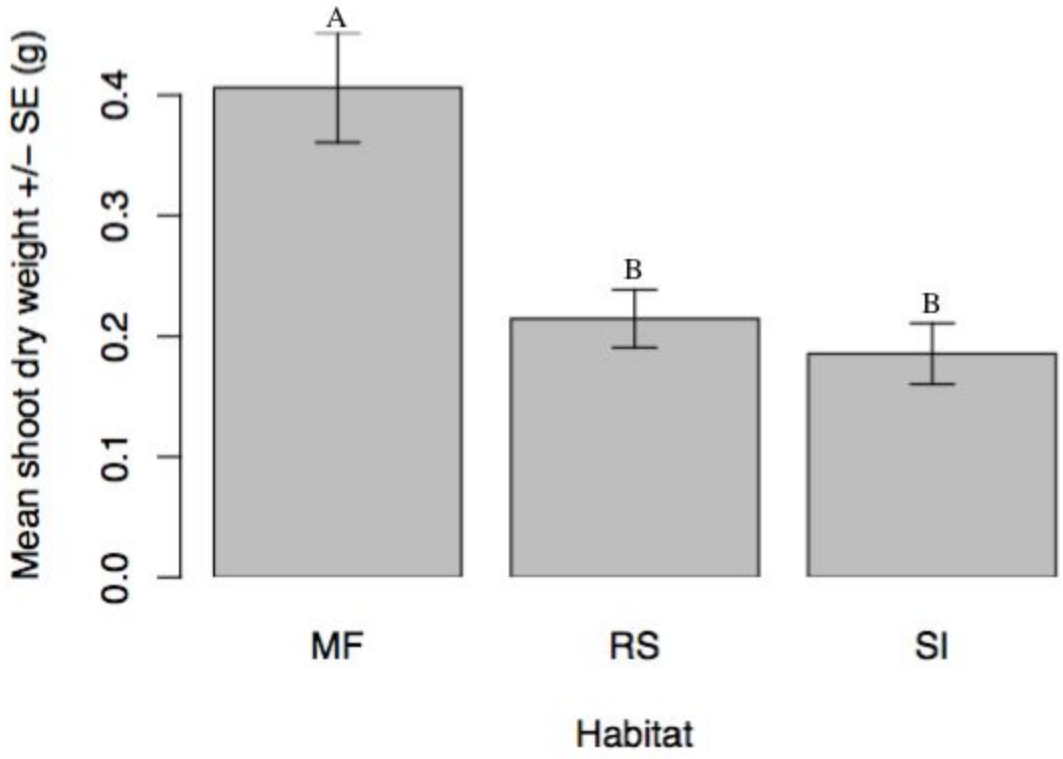

Figure 10. Variation in shoot dry weight among habitat types. Means that share letters were not different according to Tukey HSD post-hoc tests.

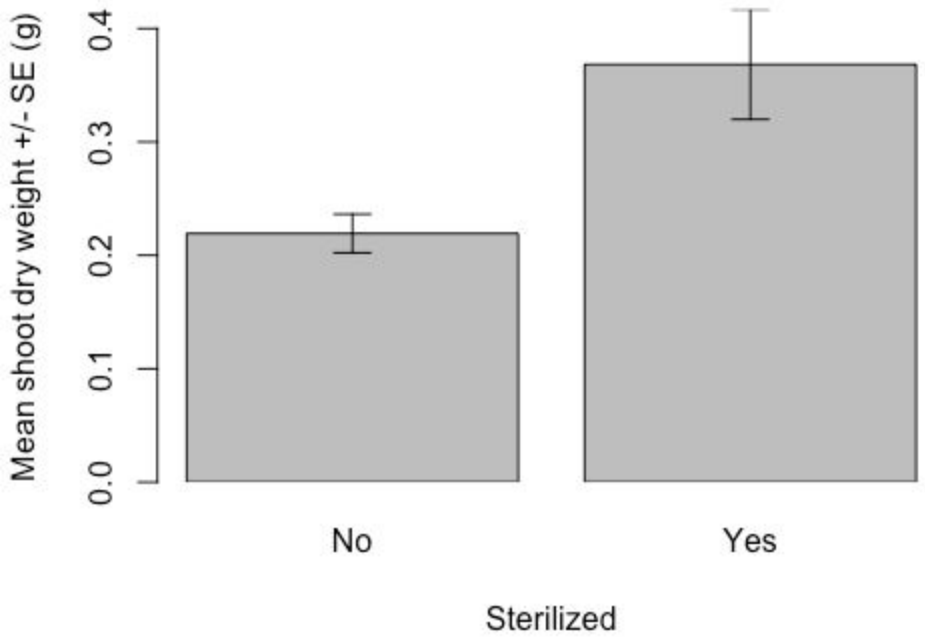

Figure 11. Variation in shoot dry weight among habitat and sterilization according to Tukey HSD post-hoc test

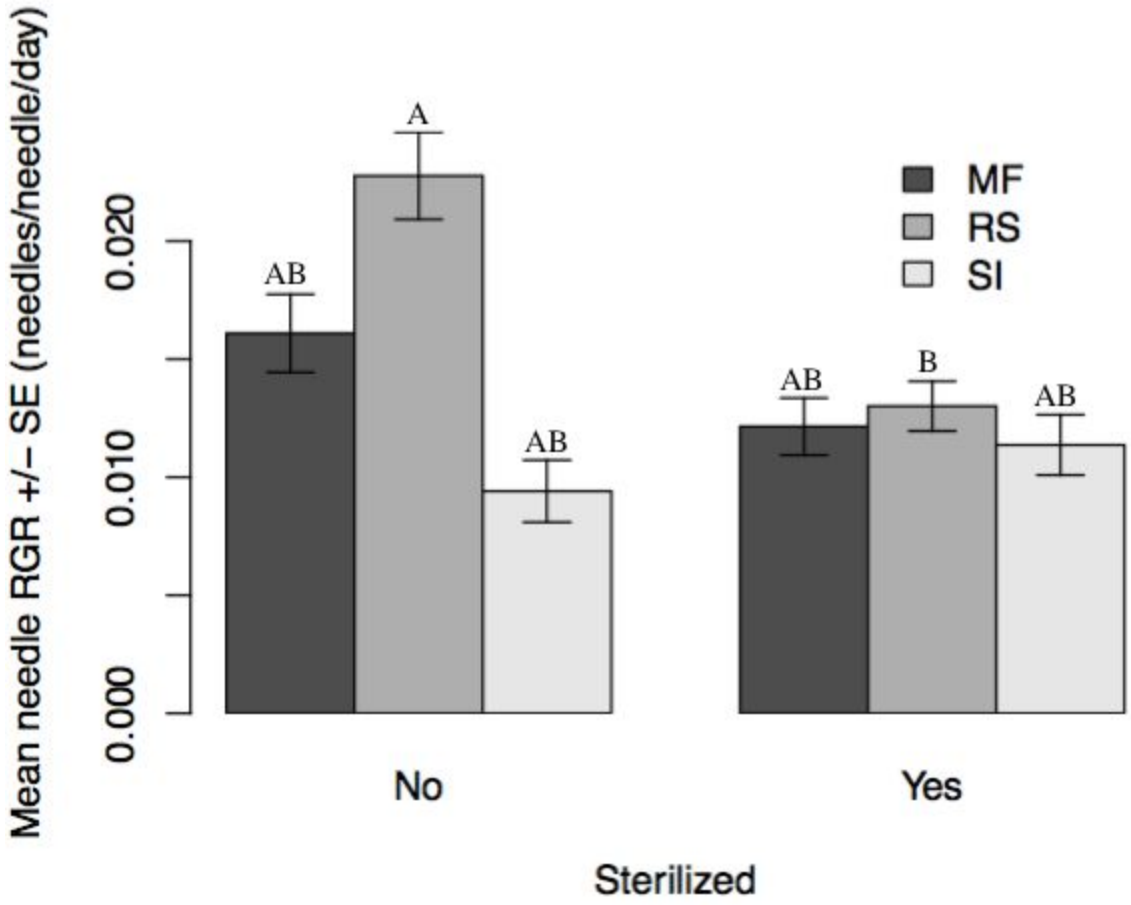

Figure 12. Variation in RGR of needles among habitats with or without sterilization. Means that share letters were not different according to Tukey HSD post-hoc tests

Figure 13. Variation in RGR of diameter among habitat and sterilization according to Tukey HSD post-hoc test

Discussion

In this study, I sought to answer the question, do mycorrhizal fungi and other soil microbes play a role in slash pine encroachment into longleaf pine savanna? I tested two alternative hypotheses: Shared Symbionts, whereby slash pine encroachment is facilitated by sharing most ECM fungi in common with longleaf pine, and Enemy Release, whereby slash pine has unique pathogens in maritime forest soil that it escapes when encroaching into longleaf pine savanna. Neither hypothesis was clearly supported by the data I gathered. The slash pine seedlings generally grew better in the sterilized soil, in terms of shoot biomass and diameter RGR (Fig. 11 and 13); this pattern is opposite of the prediction from the Shared Symbionts hypothesis, and was not more true in MF soil than the other habitats as predicted by the Enemy Release hypothesis. This result does, however, suggest a key role for soil pathogens in the system. That slash pine seedlings grew best in MF soil (Fig. 10) does not clearly support either hypothesis. Below, I elaborate on results for ECM fungi associated with slash pine seedlings growing in the three soils, as well as how plant growth was influenced by soil sterilization and soil history, and what all these results imply about the roles of ECM fungi and other soil microbes for slash pine invasion.

ECM fungal composition and colonization were similar across habitats

According to this experiment, slash pine and longleaf pine habitats share many of the same spore-bank ECM fungi. This result supports one prediction of the Shared Symbionts hypothesis, that slash pine shares most ECM fungi in common with longleaf pine. Our ECM fungal community was dominated by *Rhizopogon* and *Suillus* species, which is not surprising because many of them are specialists on pines (Fig. 5, Policelli et al., 2019) and are very common constituents of spore-bank ECM fungal communities of pines elsewhere (Kjøller & Bruns, 2003). Moreover, suilloid fungi (*Rhizopogon* and *Suillus*) have been shown to be important in pine invasions worldwide (Policelli et al. 2019). The root tips found in all three habitats shared the fungal OTU *Rhizopogon*_sp4 (Fig. 2,3,4). The MF and RS habitats also shared the fungal OTU *Suillus decipiens* (Fig. 2,3). The MF and SI habitats shared the fungal OTU *Rhizopogon*_sp5 as well as *Rhizopogon*_sp4 (Fig. 2,4). Because the root tips found in all three habitats share at least one fungi OTU in common, this supports the idea that slash pine and longleaf pine share symbionts. Although there was a trend towards *Suillus* being more common in the Restored Savanna habitat (Fig. 3), this trend was insignificant.

I also observed that in the soils from longleaf pine habitats, savanna restored and invaded (RS and SI), a lack of mycorrhizal fungi was not limiting on slash pine growth (Fig. 9), which also somewhat supports the Shared Symbionts hypothesis. In fact, there was a trend observed that overall ECM root colonization in MF soil was lower than in foreign soil (RS and SI) (Fig. 9). One potential explanation for that result is that slash

pine actually prefers some of the different ECM fungi in longleaf savanna soils. However, the plant growth results did not support this idea.

Plant growth was influenced by both soil microbes and habitat

For shoot dry weight, slash pine grew better in MF soil (Fig. 10), supporting neither hypothesis. Rather, I hypothesize that this growth difference was due to differing soil properties such as pH, available nutrients, or preferred symbiotic mutualists; maritime forest might have had more organic nutrients associated with the tides. Also, unlike the two pine savannas, there was no history of recent fire in the maritime forest, which perhaps could have volatilized nitrogen in the savannas.The type of habitat did not impact RGR of height or RGR of diameter, and for RGR of needle growth the effect of habitat depended on soil sterilization (Fig. 12), implying differences among habitats in the effects of soil microbes (see below). The difference in results between needle RGR and basal diameter RGR could be caused by using different periods of time when analyzing the growth rate, since needle RGR was dependent on soil sterilization and habitat.

The effects of soil sterilization depended on which plant growth measure was analyzed, and provided mixed support for the two hypotheses. The plant growth metrics that were not impacted by soil sterilization were root dry weight and RGR of height. The results on shoot biomass (Fig. 11) and diameter RGR (Fig. 13) do not seem to support either hypothesis because they show that sterilizing the soil allows for better growth, but these patterns were not stronger in MF soil as predicted. This higher growth could be due

to a chemical release experienced after autoclaving. On the other hand, the results on needle RGR (Fig. 12) support the Shared Symbionts hypothesis by showing that sterilization is detrimental to needle growth, especially in the RS habitat.

Overall, plant growth was better in sterilized soil, possibly because of the elimination of beneficial microbes, decomposers, and pathogenic microbes. An alternative possibility is that autoclaving changed the physical and/or chemical properties of the soil (Berns, 2008), which altered plant growth. For example, particular macronutrients could be made more available by autoclaving, which could improve plant growth, and/or heavy metals could be released, which could harm plant performance. The bigger key to slash pine seedling growth in this system may not be the beneficial soil microbes, but rather the elimination of all soil microbes that could lead to stunting seedling growth. When slash pine encroaches into longleaf pine habitat, it leaves behind the pathogenic microbes of the MF soil and is able to thrive in colonization because there are no pathogenic microbes interfering with the seedling growth. Using the Enemy Release hypothesis, we can predict that invasive plants thrive in foreign habitats because there are fewer harmful "enemies" in the soil. This study provides indirect evidence that slash pine growth in native habitats could be halted by negative soil pathogens.

Conclusions

This study indirectly shows slash pine growth is impacted soil sterilization. Presumably, sterilizing the soil killed all of the microbes present, including beneficial mutualists, decomposers, and pathogenic microbes. Thus, plant growth may have been

higher in autoclaved soil because plants are responding to beneficial or harmful microbes being removed and because autoclaving makes the nutrients in the soil more available. This result implies that slash pine seedling growth in native and foreign habitats may be inhibited by soil microbes, so conditions that reduced microbe populations in the soil, such as fire, could allow slash pine to successfully encroach into longleaf pine habitat.

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