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Effects of Invasion by Plants and Beetles on Roots and Mycorrhizal Fungi of Plantation Scots Pines in Poland

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EFFECTS OF INVASION BY PLANTS AND BEETLES ON ROOTS AND MYCORRHIZAL FUNGI OF PLANTATION SCOTS PINES IN POLAND

by

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ABSTRACT

Biological invasions can cause significant changes to the environment in which they occur. One of the main changes that is caused is how invasive species can disrupt mutualisms between native species in ecosystems. The mutualism between mycorrhizal fungi and plants is one of the most important mutualisms that a plant forms. This mutualism is very important because it is the source of many nutrients that the plant needs. This study took place in a Scots pine (*Pinus sylvestris*) plantation in northeastern Poland, and aimed to determine the effects of plant and beetle invaders on ectomycorrhizal (ECM) fungi and roots associated with the pine. Soil was collected from under Scots pine trees at sites that were invaded by beetles (*Phaenops cyanea*), *Quercus rubra, Robinia pseudoacacia, Q. rubra* and *R. pseudoacacia* together, and control (uninvaded) sites. In the lab, pine root length and ECM colonization intensity were quantified, and ECM fungi were identified using Sanger sequencing of colonized pine root tips. Twenty-eight different ECM fungal OTUs were found, but none of the individual OTUs, genera, or families were frequent enough to perform statistical tests of effects of site types. When focusing on the OTUs, Laccaria_1 was the most abundant and Cortinarius_1 was the most frequent. When focusing on each genus, *Laccaria* was the most abundant and *Tomentella, Russula*, and *Lactarius* were the most frequent. When focusing on each family, Russulacae was the most abundant and Thelephoraceae was the most frequent being found in four different samples. The presence of beetles did not affect any of the root or ECM colonization variables. When ignoring the beetles, instead only focusing on whether the site was invaded by *R. pseudoacacia, Q. rubra,* or both, total pine root length was dramatically reduced in sites that contained both plant invaders and there was a similar trend towards reduction of total ECM colonization. Although the exact mechanism for how the invaders affect the native plants is

unknown, it is possible that the invaders affect the native plant in many different ways that could lead to an overall change in reduced soil resources, reduced pine growth and even native pine tree death.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
ECM	Ectomycorrhizal
PCR	Polymerase Chain Reaction
ITS	Internal Transcribed Spacer
OTU	Operational Taxonomic Unit
UNITE	User-Friendly Nordic ITS Ectomycorrhizal
INSD	International Nucleotide Sequence Database

INTRODUCTION

Biological invasions can have a strong impact on ecosystems. However, a complete understanding of the processes that occur during these impacts of invasion is still lacking. It is possible that mutualisms involving introduced species can have a direct detrimental effect on native species, such as the mycorrhizal mutualism between soil fungi and plants (Richardson et al. 2000). This symbiosis benefits both the plants and fungi by providing each with something they need. Fungi found on plant roots provide the plant with minerals, mainly phosphorus (P) and nitrogen (N), to help nourish and grow the plant. The plant, in return, provides the fungi with photosynthates, products made by photosynthesis, which allow the fungi to grow (Smith, S. E. et al. 2010). In this study, we investigated how plant and insect invaders may affect the mycorrhizal fungi of Scots pine (*Pinus sylvestris*) in plantations of northeastern Poland.

Insect invasion is one type of invasion that is very well known, but their effects on mycorrhizal mutualisms have only recently been studied and still not completely understood. There are two main ways that insects affect native trees: they can cause physical harm to the tree (for example by feeding on them and by creating holes or burrows in the leaves, bark, or roots), or chemically alter the trees in some way (Karst et al 2015). It is unclear what the mechanism is, but when beetles attack trees, it changes the physiology of the tree and that can cascade down into the soil and affect the root interaction that the plant has with fungi (Treu et al. 2014). These cascading effects are most probably mediated by the disruption of below-ground mutualists, ectomycorrhizal (ECM) fungi (Karst et al. 2015).

The invasion of native plant communities by exotic plants is one of the leading forms of invasion. Plant invasions are frequently studied, but less is known about how the invasive species

may disrupt native mutualisms. In some cases, the invading plant releases chemicals from its roots that may hinder the beneficial relationships some native plant species have with soil fungi (Roberts et al. 2001). One plant that is known for invading native plants and disrupting mycorrhizal mutualisms is the garlic mustard (*Alliaria petiolata)*. One of the ways that the garlic mustard invades native plants is by putting out chemicals from its roots that actually kill or harm the native mycorrhizal fungi of other plants (Carlson et al. 2014). There are native plants that are dependent on mycorrhizal fungi and the garlic mustard plant comes in and exudes these chemicals from its roots, which hurts the mycorrhizal fungi of these other plants. This action harms those plants as well, which creates a chain reaction, affecting the mycorrhizal fungi in the entire ecosystem. Although studies are still being completed, many studies have shown that Garlic mustard plants have been tied to decreased native herbaceous species richness in invaded forests (NYIS 2019).

Scots pine is the most widely distributed pine in the world, ranging from Scotland east across much of northern Asia and from above the Arctic Circle in Scandinavia to the Mediterranean. These trees have a great economical, social, and ecological importance (Helber et al. 2011). They are usually planted in plantations for lumber production. Scots pine plantations are often invaded by non-native plants and insects, such as beetles (Skilling 2020). Scots pine, like other members of the Pinaceae, rely heavily on mutualisms with diverse ECM fungi, for their growth and survival.

This study took place in a Scots pine plantation in northeastern Poland, and aimed to determine the effects of both beetle and plant invasion on roots and ECM fungi associated with Scots pine. In prior years before this study took place, invasion of Scots pine plantations was observed by researchers that showed invasions by both beetles (*Phaenops cyanea*), which bore

into the bark causing damage to the tree, and by two non-native plants, *Quercus rubra* (hereafter, "*Quercus*") and *Robinia pseudoacacia* (hereafter, "*Robinia*"). A collaboration of students and faculty from the University of Mississippi and Bialystok University of Technology collected soil samples containing roots and ECM fungi from Scots pine trees at a plantation site near the city of Bialystok, in stands invaded by beetles, stands invaded by one or both plant species, and uninvaded control stands. For my Honors thesis project, I completed sequencing of ECM fungal DNA, processed fungal DNA sequence data, and analyzed the data to investigate how the invaders may have affected the roots and ectomycorrhizal fungi of Scots pine.

METHODS

Field sampling

Scots pine roots were sampled on 12 July 2017 in a plantation site near Bialystok, Poland, where an open field had been vegetated with Scots Pine trees 60-65 years prior. We sampled seven Scots pine plantation sites that varied in their history of invasion by plants and beetles including two control uninvaded sites, sites invaded by *Robinia*, *Quercus*, or both, and beetle-invaded sites (Table 1.1).

Transect lines (30 m long, 10 m apart) were established at each site in an orientation perpendicular to a stand edge. Along each transect, soil was collected from under the nearest Scots Pine tree, approximately every 10 m, with the first sample being 10 m from the boundary of the forest. Three samples were taken from each site except for site five where five samples were taken from under beetle-invaded trees in site 5a and two samples were taken from under control (uninvaded) trees in site 5b. Altogether, 22 individual tree rhizosphere soil samples were sampled over the transects. Beneath each tree, a small shovel was used to collect soil samples (30

cm diameter x 15 cm wide x 50 mm deep). Litter in the forest primarily consisted of several centimeters of dead pine needles and hardwood leaves, which was removed before sampling occurred. Soil samples were brought back to the laboratory in insulated coolers, stored at 4 °C, and processed within 1-7 days of collection.

Table 1.1: Scots pine (*P. sylvestris*) plantation sites, including Site Type (invasion history), Polish forestry map codes, and geographic coordinates

SITE	SITE TYPE	FORREST MAP CODE	LATITUDE	LONGITUDE
1a	*Quercus*	134a	N 53º10’04.54”	E 23º10’18.73”
1b	Control	134c	N 53º10’06.46”	E 23º10’25.05”
2	*Robinia*	139a	N 53º10’01.3”	E 23º10’33.9”
3	*Quercus*	140c	N 53º09’57.07”	E 23º10’28.85”
4	*Quercus* and *Robinia*	139b	N 53º10’04.38”	E 23º10’45.45”
5a	Beetles	264d	N 53º14’58.4”	E 23º13’48.8”
5b	Control	264a	N 53º14’37.3”	E 23º13’27.5”

Initial Laboratory Processing of Root Samples

On 13 July 2017 through 19 July 2017 the lab work for ECM morphotyping and DNA extraction took place at Bialystok University of Technology. Roots were hand-washed over a 2

mm sieve to remove rhizosphere soil. Using a dissecting microscope, the number of root tips with apparent ECM colonization (based on thickness and morphology) was counted, and each root tip was classified into a morphotype based on characteristics such as color, texture, branching patterns, and emanating hyphae or rhizomorphs.

Two ECM root tips from each morphotype from each sample were collected, and DNA extraction was performed immediately. DNA was extracted from root tips from each sample using components of a Sigma Extract-N-Amp extraction kit (Sigma-Aldrich, St. Louis, MO, USA). 10 µL of the Sigma Extraction Buffer was added to each root tip, which was heated to 65 °C for 10 min, 95 °C for 10 min, and then received 30 µL of Sigma Neutralization Solution and 60 µL PCR-grade water. DNA extracts were frozen at −20 °C, shipped frozen to the University of Mississippi, and stored at −20 °C for approximately two months until PCR was performed.

PCR and Sanger sequencing of fungal DNA

To facilitate Sanger sequencing of ECM fungal species colonizing root samples, the Internal Transcribed Spacer (ITS) region of the fungal nuclear genome was amplified using the fungal-specific combination of forward and reverse primers, ITS1-F and ITS4 (White et al. 1990). Amplification reactions for each sample consisted of 2.2 µL PCR-grade water, 4 µL of 2X RedTaq Premix (Apex Bioresearch Products, Inc., San Diego, CA, USA), 0.4 µL of each primer (10 µM stock concentration), and 1 µL of DNA extract for a total of 8 µL per reaction. Amplification occurred in sterile 96-well PCR plates that were sealed with a sterile silicone sealing mat, centrifuged briefly, and amplified under the following conditions: initial denaturation for 3 min at 94 °C; 30 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 53 °C and extension for 60 s per cycle at 72 °C; and a final extension of 10 min at 72 °C.

Amplification success was checked on a 1% agarose gel with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). Excess primer and unincorporated nucleotides were removed enzymatically using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) by adding 0.25 μL ExoSAP-IT and 4.75 μL sterile PCR-grade water to 5 μL of the PCR product. Reactions were incubated at 37 °C for 45 min, then 80 °C for 20 min, and finally 4 °C for at least 5 min.

Sequencing was performed using the forward primer ITS5 (Gardes et al. 1993) and the Big Dye Terminator Sequencing Kit (v3.1, Invitrogen Corp., Grand Island, NY, USA). Each Big Dye reaction contained 0.4 μL Big Dye Reaction Premix, 1.8 μL Big Dye 5× sequencing buffer, 0.5 μL of the forward primer at 10 μM concentration, 6.3 μL of PCR-grade water, and 1 μL of the cleaned PCR product. Amplification conditions were 96 °C for 1 min; followed by 45 cycles of 95 °C for 20 s, 52 °C for 20 s, and 60 °C for 4 min. Reactions were dried and shipped overnight to the DNA Lab at Arizona State University, in Tempe, Arizona, where the Big Dye reactions were purified and read on an Applied Bioscience 3730 capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA).

The fungal DNA sequences obtained were edited manually in Geneious software (Biomatter Ltd., Auckland, New Zealand), correcting ambiguous bases associated with dye blobs and elsewhere when possible. All sequences with >3% ambiguous bases or <200 base pairs long were deleted. Remaining sequences were subjected to operational taxonomic unit (OTU) assembly (at 97% similarity) using CAP3 software (Huang 1999), as described previously (Taylor et al. 2007) using default settings except the following: maximum overhang percent length = 60, match score factor = 6, overlap percent identity cut-off = 96, clipping range = 6. Grouping homologous sequences that are >97% similar as a specific OTU is a conservative approach employed by previous studies (O'Brien et al. 2005, Izzo et al. 2005, Smith et al. 2007)

that assumes a 0.2%–1.2% error rate produced by PCR and unidirectional sequencing, as well as ~1.5% divergence of the ITS region that may occur within some species at small spatial scales (Horton 2002). Representative fungal sequences from each OTU were checked using BLAST (nucleotide) searches on the International Nucleotide Sequence Database (INSD) and the User-Friendly Nordic ITS Ectomycorrhizal (UNITE) database to obtain best matches for taxonomic affiliation of OTUs. The ultimate decision on the best match to a sequence was based on both similarity and length of the match (Table 1.2). 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 et al. 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 species known to be strictly non-mycorrhizal was eliminated from the data set.

Data Analysis

For ECM fungi, none of the individual OTUs, genera, or families were frequent enough to perform statistical tests of effects of site types.

When performing statistical analysis on the three root and mycorrhizal colonization variables--total pine root length (cm), total number of ECM root tips on pine roots, and ECM colonization intensity (ECM root tips per cm pine root length)--two different approaches were used to analyze the data, which differed in the way of defining the sites. In the first approach to classify the sites, there were five site types: Control, beetles, *Robinia*, *Quercus*, and both *Robinia* and *Quercus*. The second approach was to ignore the beetles, instead using a factorial design to classify whether each site was invaded by *Robinia* or *Quercus* or both *Robinia* and *Quercus*. In

both approaches, mixed-effect linear models were used to analyze each of the three response variables separately, with Site as a random effect to model non-independence among samples from the same site. In the first approach to analysis, site type (with 5 levels) was the only fixed factor. In the second approach to analysis, presence/absence of *Quercus* and presence/absence of *Robinia*, as well as the *Quercus* x *Robinia* interaction, were included as fixed effects.

RESULTS

Twenty-eight different ECM fungal OTUs were found, but none of the individual OTUs, genera, or families were frequent enough to perform statistical tests of effects of site types. When focusing on the OTUs, Laccaria_1 was the most abundant, having 710 root tips in sample 8 (Fig. 1) and Cortinarius_1 was the most frequent being found in 3 different samples (Fig. 2). All of Laccaria_1 was found in site 2 which is a site with only *Robinia* present. When focusing on each genus, *Laccari*a was the most abundant, having 710 root tips in sample 8 (Fig. 3) and *Tomentella*, *Russula*, and *Lactarius* were the most frequent, all being found in 3 different samples (Fig. 4). *Tomentella* was only found in site 3, which is a site with only *Quercus* present. When focusing on each family, Russulacae was the most abundant, having 734 root tips across samples 3, 4, 7, 17, and 19 (Fig. 5) and Thelephoraceae was the most frequent being found in 4 different samples (Fig. 6). Thelephoraceae was mainly found in site 3, which is a site with only *Quercus* present.

Table 1.2: ECM fungal OTUs, length of query sequences, identity and accession # of best matches on public databases, and match percentage and length.

OTU-group name	bp	Best Match (Accession #)	% match/bp
Amanita pantherina	636	Amanita pantherina (AB080776)	99%/634
Archaeorhizomyces_1	350	Archaeorhizomyces (JN006470)	99%/347
Cenococcum_1	232	Cenococcum geophilum (LC013710)	97%/225
Cenococcum_geophilum	618	Cenococcum geophilum (LC013710)	99%/611
Chaetomium_1	470	Chaetomium (MH517679)	97%/455
Cortinarius_1	639	Cortinarius (MF352699)	99%/633
Elaphomyces_granulatus	618	Elaphomyces granulatus (KR029768)	99%/611
Imleria_1	469	Imleria (KT334688)	99%/465
Laccaria_1	645	Lactarius (MF352719)	99%/640
Laccaria_laccata	659	Laccaria laccata (UDB015789)	98%/646
Lactarius_1	202	Lactarius rufus (MG597391)	97%/196
Lactarius_rufus	687	Lactarius rufus (KF617345)	99%/686
Lactarius_tabidus	680	Lactarius tabidus (KT165310)	99%/671
Mycena_cinerella	469	Mycena cinerella (KT900146)	98%/458
Mycenaceae_1	237	Mycenaceae (JF300825)	99%/234
Phialocephala_1	526	Phialocephala (KX440117)	98%/515
Podosphaera_1	368	Podosphaera eriderontis (MH249992)	98%/362
Podosphaera_erigerontis-canadensis	326	Podosphaera eriderontis (MH249992)	99%/325
Russula_clavipes	606	Russula clavipes (MG687340)	99%/602
Russula_firmula	543	Russula firmula (UDB011315)	99%/537
Russula_vinosa	573	Russula vinosa (MH248055)	99%/566
Sistotrema_1	374	Sistotrema (EU668935)	98%/368
Sistotrema_alboluteum	601	Sistotrema alboluteum (JN889865)	99%/595
Thelephora_1	483	Thelephora terrestris (KX438349)	97%/469
Thelephoraceae_1	224	Thelephoraceae (U83486)	97%/217
Tomentella_1	299	Tomentella sublilacina (KY693713)	97%/290
Tomentella_2	592	Tomentella sublilacina (KY693713)	97%/574
Tomentella_3	618	Tomentella sublilacina (KY693713)	97%/601
Tomentella_4	326	Tomentella sublilacina (MF352799)	99%/323
Tomentella_5	604	Tomentella sublilacina (KY693713)	98%/592
Tomentella_sublilacina	627	Tomentella sublilacina (KY693713)	99%/619

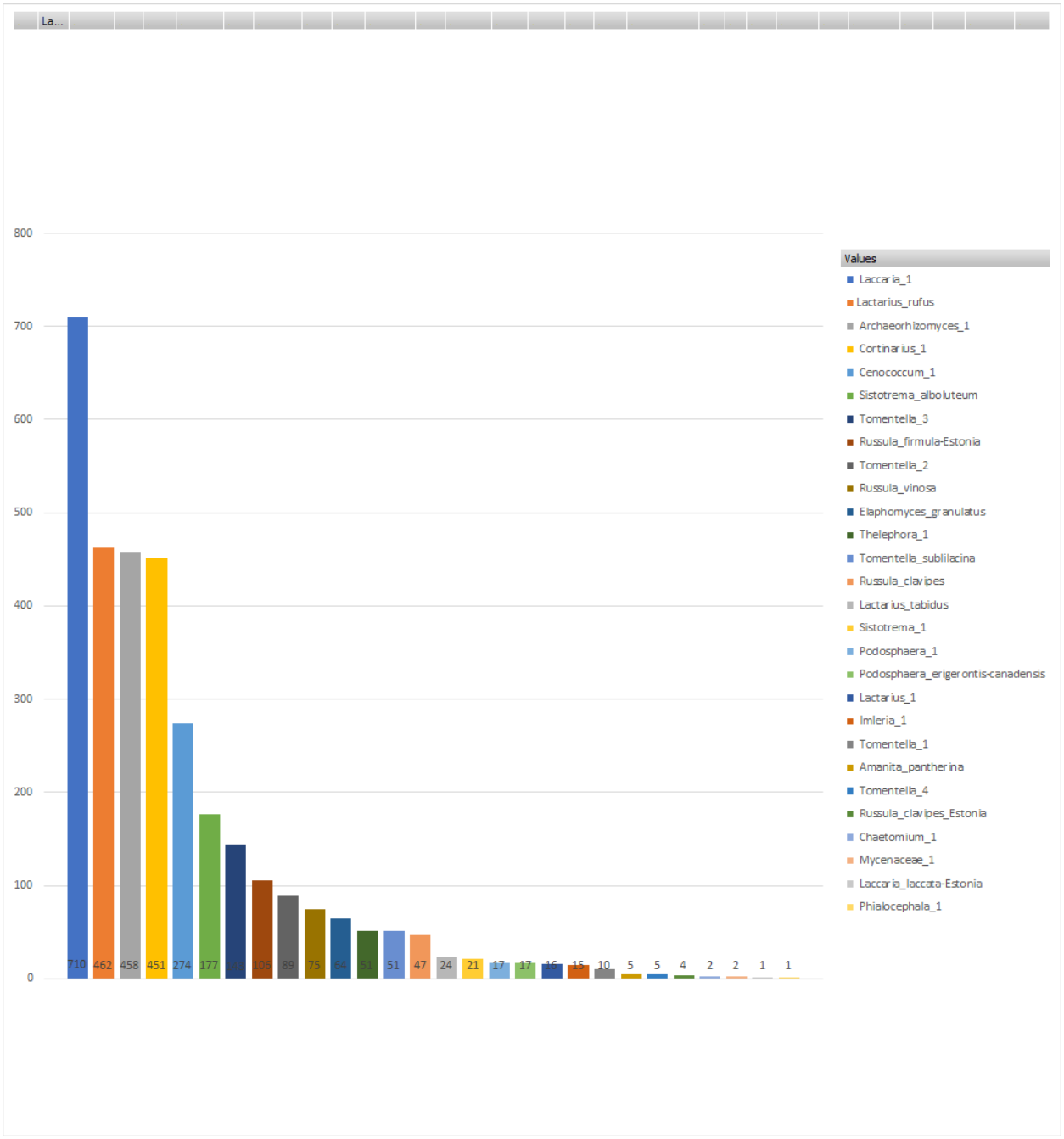

Figure 1: The abundance of each ECM fungal OTU from all samples taken. The height of the bar indicates the amount of ECM root tips on which each OTU was found across all sites studied.

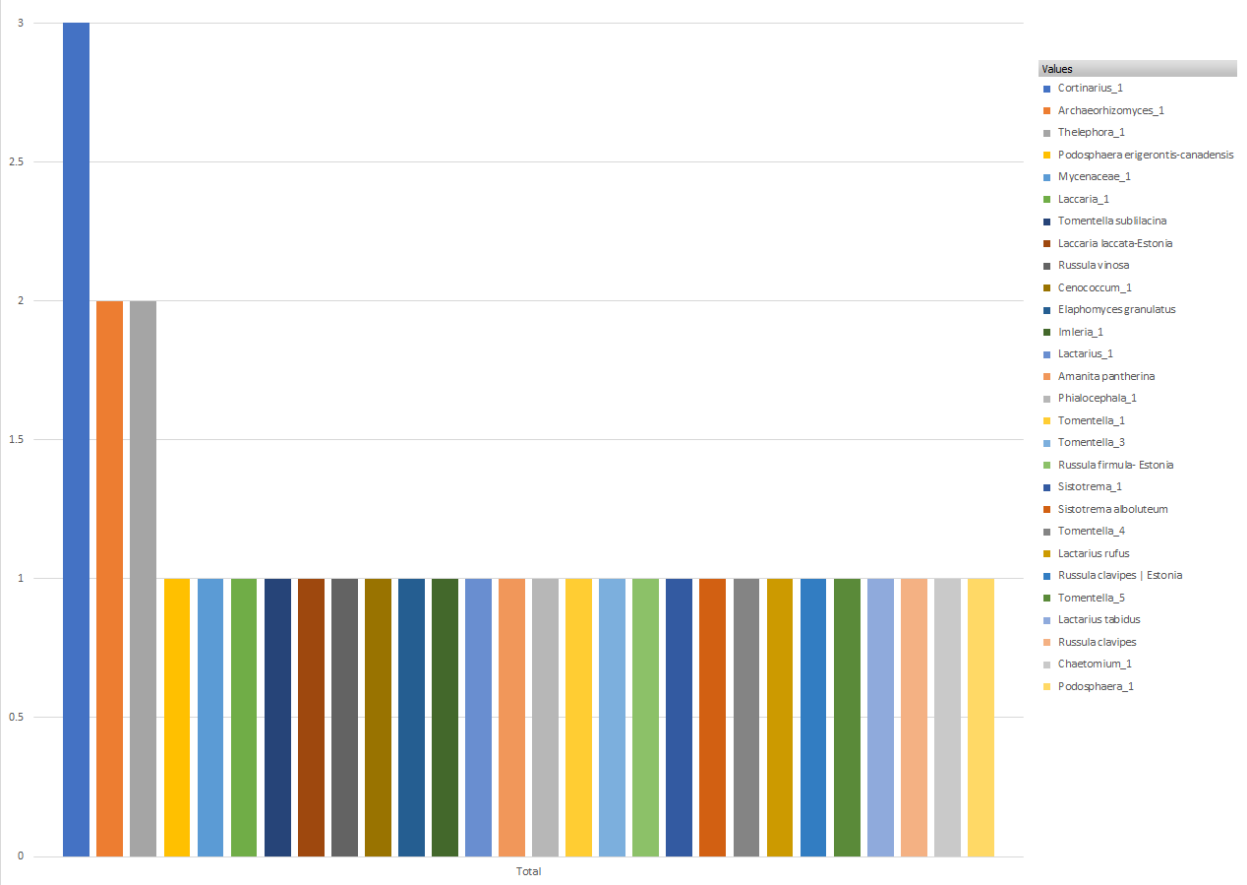

Figure 2: The frequency of each ECM fungal OTU from all samples taken. The height of the bar indicates the number of samples in which that OTU was found throughout all sites studied.

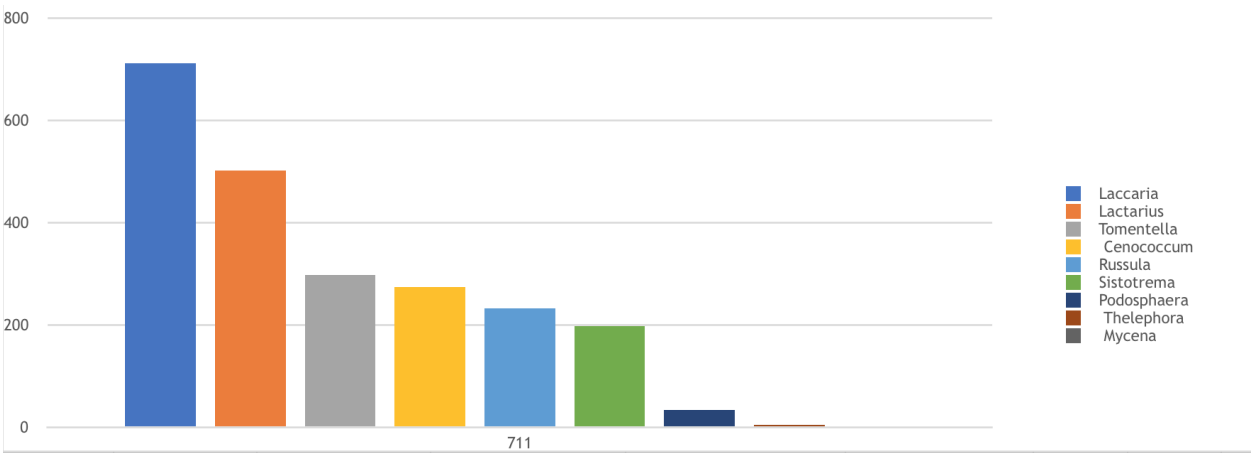

Figure 3: The abundance of each ECM fungal genus from all samples taken. The height of the bar indicates the amount of ECM root tips on which each genus found across all samples studied.

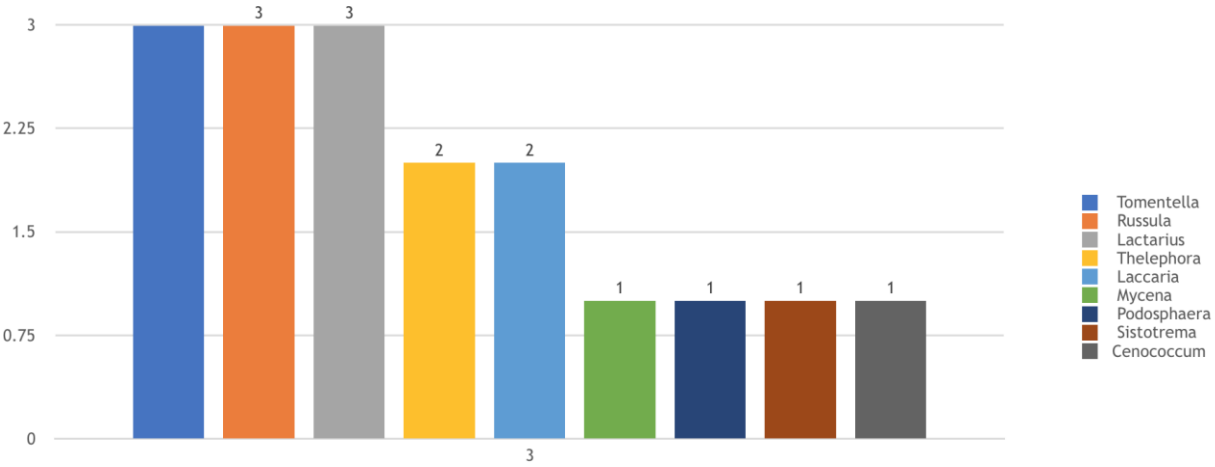

Figure 4: The frequency of each ECM fungal genus from all samples taken. The height of the bar indicates the number of samples in which that genus was found throughout all sites studied.

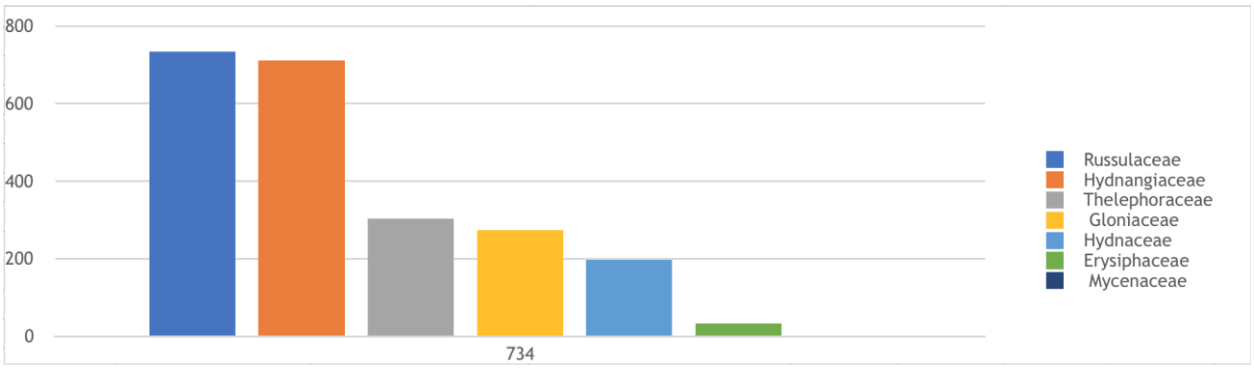

Figure 5: The abundance of each ECM fungal family from all samples taken. The height of the bar indicates the amount of root tips on which that family was found across all sites studied.

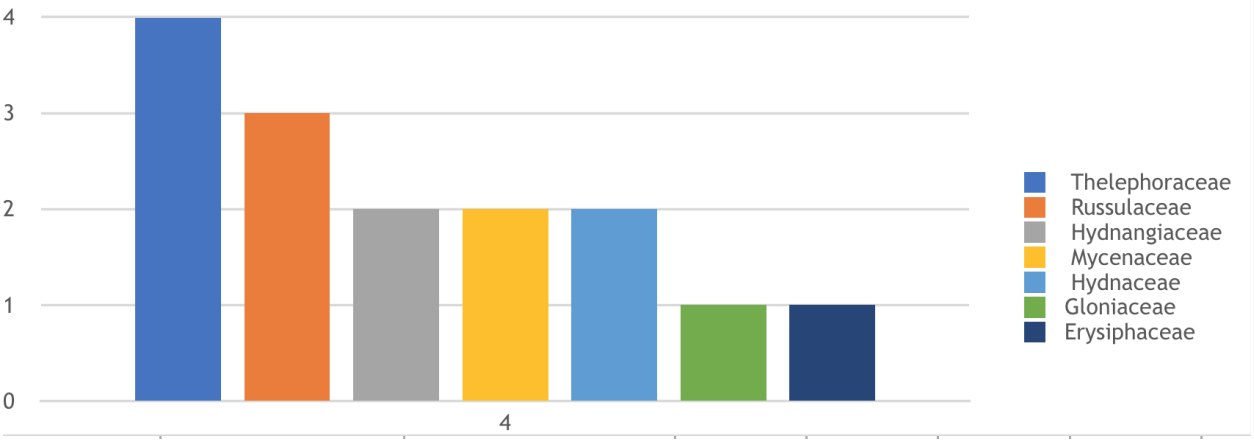

Figure 6: The frequency of each ECM fungal family from all samples taken. The height of the bar indicates the number of samples on which that family was found throughout all sites studied.

By the first approach to classify the sites (with 5 site types: Control, beetles, *Robinia*, *Quercus*, and both *Robinia* and *Quercus*), there was no effect of site type on total root length, mycorrhizal tips, or tips per cm root. Moreover, when we classified site type this way with beetles as a separate site type, we saw that beetles were not having a negative effect on plant root or mycorrhizal variables; if anything, the trend was towards having a slightly positive (although insignificant) effect (Table 2.1).

The second approach was to ignore the beetles, instead only focusing on whether the site was invaded by *Robinia* or *Quercus* or both *Robinia* and *Quercus*, treating sites with beetles as uninvaded controls. With this approach, we saw that the plant invaders had no effect on ECM colonization intensity (tips/cm of root) (Fig. 8), but they did affect total pine root length (Table 2.2). Specifically, total pine root length was dramatically reduced by the combination of *Quercus* and *Robinia* (Fig. 7). There was also a trend (P=0.07) toward *Robinia* reducing the total number of ECM root tips (Table 2.2, Fig. 9).

Table 2.1: Statistical Analysis using approach 1

Response	Source	$F_{df1,df2}$	P
Pine Root Length (cm)	Site Type	$5.90_{4,2}$	0.17
Tips per cm root	Site Type	$0.06_{4,17}$	0.99
ECM Tips Total	Site Type	$1.8_{4,17}$	0.17

Table 2.2: Statistical Analysis using approach 2

Response	Source	$F_{df1,df2}$	P
Pine Root Length (cm)	*Quercus*	$1.26_{1,18}$	0.28
	Robinia	$9.16_{1,18}$	0.01
	Quercus x *Robinia*	$7.75_{1,18}$	0.01
Tips per cm root	*Quercus*	$0.02_{1,18}$	0.89
	Robinia	$0.01_{1,18}$	0.92
	Quercus x *Robinia*	$0.14_{1,18}$	0.72
ECM Tips Total	*Quercus*	$2.09_{1,18}$	0.17
	Robinia	$3.59_{1,18}$	0.07
	Quercus x *Robinia*	$1.36_{1,18}$	0.26

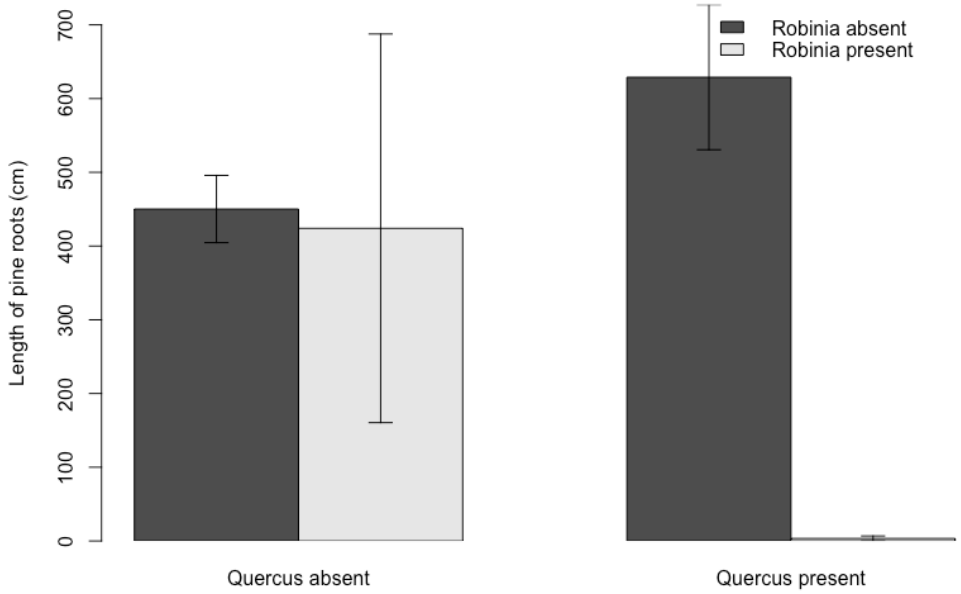

Figure 7: Length of Pine Root vs *Quercus* and *Robinia*

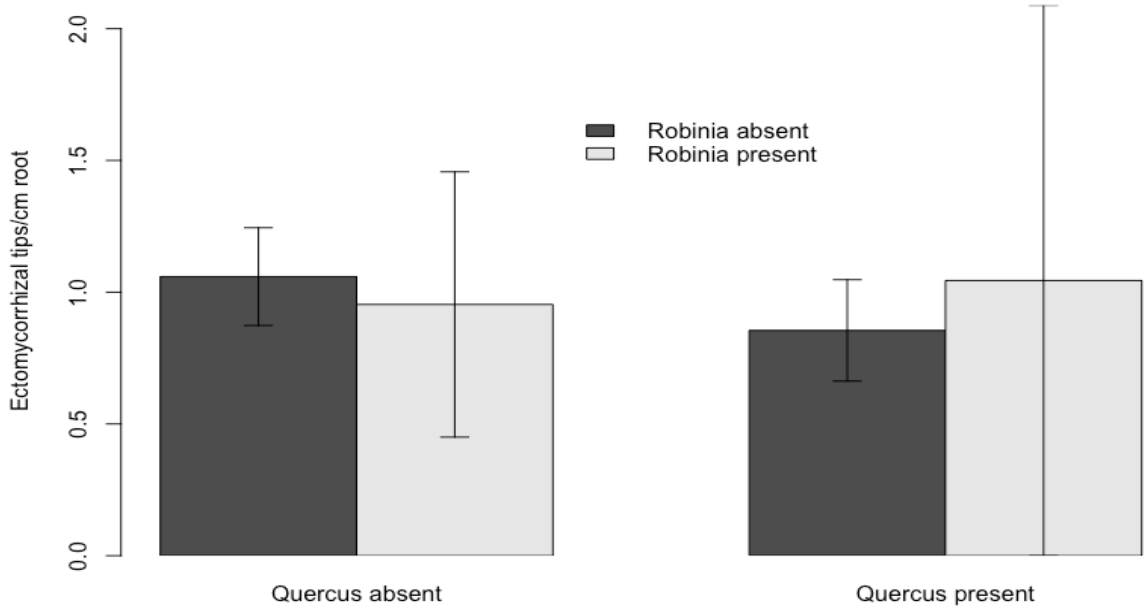

Figure 8: Ectomycorrhizal tips per cm root vs *Quercus* and *Robinia*

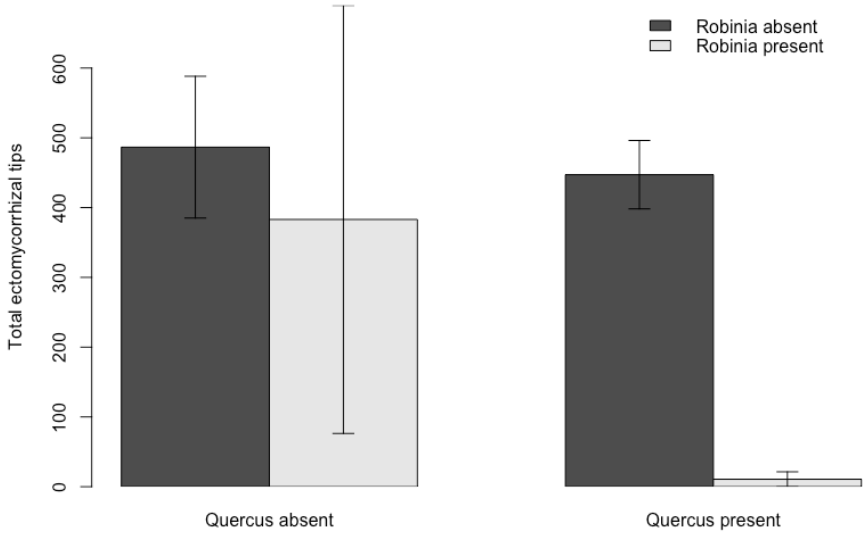

Figure 9: Total ectomycorrhizal root tips vs *Quercus* and *Robinia*

DISCUSSION

One particular phenomenon that remains poorly understood is how invasive species can disrupt mutualisms between native species in ecosystems (Stinson et al. 2006), such as the mycorrhizal mutualism between soil fungi and plants. Effects of invasions by plants and beetles on mycorrhizal fungi of Scots pine were complex in this study. While there were no overall patterns of how the invaders may have affected the ECM fungi themselves, and no evidence of beetles having any effect, it is clear overall that the plant invaders had a negative effect on pine root growth, and may have also reduced the total abundance of ECM fungi.

ECM fungal community

In this study, the total number of OTUs, or OTU richness, was found to be 28. Even with this number, none were frequent enough to provide evidence for differences in composition due

to the invaders. It was interesting to see that at the OTU, genus, and family levels, the family Thelephoraceae was among the most frequent taxa. Thelephoraceae were often found in Quercus-invaded sites. Although the fungal data found in this study did not directly match another study, there were many similarities between the ECM fungal communities present here and in other studies. According to many studies done on the ECM fungi on *Pinus* roots, *Tomentella* is a genera found in many studies (Aucina 2007). Several studies suggest the presence of multiple small genets of ECM fungi on *Pinus* roots such as *Laccaria, Lactarius, Russula* and these were all dominant in this study (Jones 2003).

Effects of invaders on pine roots and abundance of ECM fungi

The most significant result found in this study was the dramatic negative effect of *Quercus* plus *Robinia* on total pine root length in the soil (Fig. 7). This could have happened due to how *Quercus* and *Robinia* together are really effective competitors for space in the soil, occupying space and depleting nutrients, preventing pine roots from proliferating. Another mechanism that could have caused this is *Quercus* and *Robinia* could be producing one or more allelopathic chemicals that may hinder pine root growth and/or the beneficial relationship the pine roots have with soil fungi (Roberts et al. 2001).

There was a lack of an effect of the invaders on ECM fungal colonization intensity (tips/cm root) (Fig. 8), but there was a trend towards *Robinia* negatively affecting the total ECM fungal colonization (Fig. 9). It was seen that the more *Robinia* present, the less colonization of ECM fungi on the pine roots. This could simply be an indirect effect of the reduction in pine root length. If pine roots are less abundant, there are fewer colonization sites for ECM fungi. Although this could also be a more direct result. *Robinia* could release a chemical from its roots that actually kills or harms the native mycorrhizal fungi of the pine roots (Carlson et al. 2014).

Overall, the reduction in pine root length is a major effect in response to the plant invaders. Plant invaders, such as *Robinia* and *Quercus* as seen in this study, could ultimately lead to reduced soil resources available for the pines, and ultimately reduced pine growth.

After completing this study, it was clear to see that the effects of invasions by plants on the roots and mycorrhizal fungi of the Scots pine are significant. Because of the invaders, changes were made to the ECM fungi and especially the pine roots themselves. Although the exact mechanism for how the invaders affect the native plants is unknown, it is possible that the invaders affect the native plant in many different ways that could lead to an overall change in reduced soil resources, reduced pine growth and even native tree death.

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