Final Report

Project Title: Investigating the decomposition of disease-resistant transgenic American chestnut (*Castanea dentata*) leaf litter and the colonization of the litter by ectomycorrhizal fungi

A project conducted by Amanda Gail Gray as a component of her M.S. research

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1. Objectives

We investigate the effect of transgenic American chestnut litter on the diversity of fungal species and functional groups colonizing litterbags during a five month field trial in Syracuse, NY. We predict that there will be no significant difference in the diversity of species or functional groups between types of litter, since previous work has demonstrated that the litter quality of each type is similar during 2.5 years of *in situ* decomposition in almost every aspect.

2. Methods

Site description

The area used for this experiment is located at the SUNY ESF Lafayette Road Experiment Station in Syracuse, NY (42.991174°, -76.132039°). The general climate for New York State is humid continental. The mean annual temperature for Syracuse, NY is 9.3°C, and the mean annual precipitation is 102.6 cm (NOAA, 2013).

The 4047 m^2 plot is located within a naturally regenerated 100 year old oak-hickory stand, which was once used for agriculture and is adjacent to a major highway. The soil map unit for the area including the plot is Benson-Wassaic-rock outcrop (BNC), which is typically found on the broad flat tops or on the sides of limestone bedrock-controlled landforms (Hutton and Rice, 1977). The relative stand density of the plot is 50% and the total basal area is 14.7 m^2 per hectare (unpublished data, C. Nowak). The 50% relative stand density was chosen for the plot to facilitate American chestnut seedling establishment and growth. The soil profile at this site lacks an O horizon, due to rapid decomposition. We analyzed characteristics of the Ap layer, which are as follows: pH 5.4, 5.9 % SOM, 59 % sand, 6 % silt, and 35 % clay (sandy loam).

Field methods

Three litter types were selected for this study: a wild-type litter from the Zoar genotype American chestnut, Darling 4 from the transgenic event Darling 4 and Hinchee 1 from the transgenic event Hinchee 1. Litterbags were made of 44μm nylon mesh (Plastok Associates Limited); senesced leaves were picked off of seedlings and 3 g dry-weight of senesced leaf litter from one of the three litter types were placed into each bag. A hot-glue gun was used to seal the nylon bags. Ten litter bags were prepared for each tree type, for a total of 30 bags. The litter bags were then placed on the surface of the Ap layer of soil, adjacent to a plant of the same type. Litterbags were left in the field from mid-June 2014 to mid-November 2014.

Sample preparation and molecular analysis

Litterbags were placed in petri dishes with water, and were opened under a dissecting microscope. Using forceps, hyphae were pulled out of the litter and placed into separate petri dishes based on morphological characteristics such as color, thickness, and hyphal proliferation.

Using a compound light microscope, hyphae were also sorted by presence of septa, and presence of rhizomorphs. Hyphal morphotypes from litterbags were placed into separate 1.5 ml Eppendorf® tubes containing CTAB (without the β–mercaptoethanol) for storage before DNA extraction (Gardes and Bruns, 1993). DNA was extracted from morphotypes of every litterbag as described in Gardes and Bruns (1993) with minor alterations. PCR was carried out using the primers ITS1-F (Gardes and Bruns, 1993) and either ITS-4B (Gardes and Bruns, 1993) or ITS-4 (White et al., 1990) for 46 cycles.

The fungal community was characterized using restriction fragment length polymorphism (RFLP) followed by sequencing one candidate of each RFLP type. Species-level matching was determined by identical RFLP matches with digests of two enzymes, *Hinf*I, and *Dpn*II. Representative ITS PCR products of each RFLP type were purified with the QIAquick® PCR purification kit (Qiagen Inc.,Valencia, California USA) and sequenced using the Applied Biosystems Automated 3730 DNA Analyzer at the Cornell University Biotechnology Resource Center. Sequences were examined and hand-corrected and were compared to the GenBank (NCBI) database using the BLASTN algorithm (Altschul et al., 1997) for approximate identification. Operational taxonomic unit (OTU) names were assigned based on top NCBI matches to determine species, genus, or family level identity depending on available data and similarity of sequences in the database. Identifying OTUs to species allows for a more precise characterization of the communities on each litter type, but it should be understood that it does not necessarily mean that the OTU is that species. All sequences were submitted to GenBank (accession #s KT336208 – KT336226). When submitting sequences to GenBank, sequences that were named to species based solely on NCBI-BLASTn, are assigned the term cf. for "confer", meaning that the sample I have is most likely the same species as the sample submitted to

GenBank. For example, the OTU identified as *Suillus sibiricus* in this study is listed as *Suillus* cf. *sibiricus* in GenBank.

Diversity and data analysis

Species diversity values for each litter type were computed in Estimate S (Colwell, 2013) using estimated species richness, Shannon, and inverse Simpson indices. Absolute frequency is defined as the proportion of bags in which the OTU was observed. We use RFLP type diversity as a proxy for species diversity. To assess how completely fungal communities were sampled for each type, species rarefaction curves were constructed in Excel using Chao 2 and Jackknife 2 values calculated in Estimate S (Colwell, 2013). Differences in species diversity among litter types were determined by analysis of variance (ANOVA) at $\alpha = 0.05$ followed by a Tukey test at $\alpha = 0.10$ using Minitab® 17 statistical software.

3. Results and Discussion

Results - Species richness

Species richness was determined for each litter type by averaging the number of fungal types found in each individual litter bag. Once the successful morphological types had been sorted into RFLP types and sequenced to OTUs (Table 1), Estimate S was used to estimate the species richness of the population after 100 randomized runs. This interpolation found a significant difference in species richness among litter types. Zoar litter was found to have twice the species richness of Hinchee 1 ($p = 0.007$) (Table 2), and Darling 4 litter was not found to differ from Zoar or Hinchee 1. Rarefaction curves did not plateau for any of the litter types in this study (Figures 1-3), therefore expected richness estimates should be interpreted with caution.

Table 1. Operational taxonomic unit (OTU) names, match information, and ecological type of the fungi identified in this study.

RFLP	OTU name	% Identity	# BP	% Coverage	E value	Max Score	Ecological type
1	Tomentella cf. sp. 11 (Tourtellot et al.)	99%	452	100%	0.0	819	Ectomycorrhizal
2	Suillus cf. sibiricus	99%	616	100%	0.0	1123	Ectomycorrhizal
3	Tomentella cf. pilosa (Tourtellot er al.)	100%	478	100%	0.0	883	Ectomycorrhizal
4	Mycena cf. sanguinolenta	99%	558	100%	0.0	1024	Saprotrophic
6	Phanerochaete cf. laevis	99%	549	100%	0.0	998	Saprotrophic
7	Suillus sp.1	96%	616	97%	0.0	990	Ectomycorrhizal
8	Boletaceae sp.1	95%	480	90%	0.0	713	Ectomycorrhizal
9	Mycena cf. murina	99%	581	100%	0.0	1059	Saprotrophic
10	Scleroderma cf. areolatum (Tourtellot et al.)	99%	599	100%	0.0	1086	Ectomycorrhizal
11	Phanerochaete sp.1	91%	400	99%	9e-163	579	Saprotrophic
13	Tomentella cf. sp. 11 (Tourtellot et al.)	96%	476	100%	0.0	795	Ectomycorrhizal
14	Suilus cf. sibiricus	99%	546	100%	0.0	994	Ectomycorrhizal
15	Mycena sp.1	95%	328	100%	2e-149	534	Saprotrophic
18	Phanerochaete sp.2	91%	407	100%	1e-156	558	Saprotrophic
21	Tomentella cf. pilosa (Tourtellot et al.)	99%	539	95%	0.0	946	Ectomycorrhizal
23	Tomentella sp. 11 (Tourtellot et al.)	99%	582	99%	0.0	1046	Ectomycorrhizal
24	Trechispora sp.1	99%	400	100%	0.0	730	Late stage wood decomposer
25	Amphinema sp.1	98%	308	98%	1e-150	538	Ectomycorrhizal
26	Atheliaceae sp.1	95%	321	84%	6-124	449	Ectomycorrhizal

Table 2. Mean species richness, Shannon diversity index and Inverse Simpson index of Zoar, Darling 4, and Hinchee 1 leaf litter communities after interpolation using 100 replications with Estimate S. Numbers in parentheses represent the standard error of the mean.

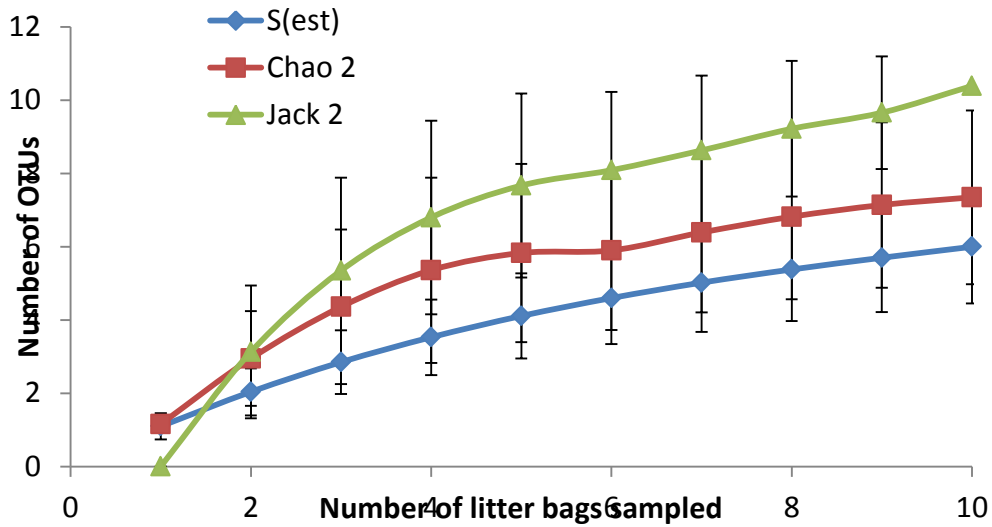|	S (est)	Shannon	Inv Simpson
Zoar	4.0 (1.1)	1.3 (0.3)	3.7 (0.8)
Darling 4	3.0 (1.0)	1.0 (0.3)	2.8 (0.8)
Hinchee 1	2.0 (0.6)	0.7 (0.3)	1.9 (0.4)

Figure 1. Rarefaction curve with Chao 2 and Jackknife 2 estimates for Zoar litter. Error bars represent computed standard deviation in 100 randomized runs.
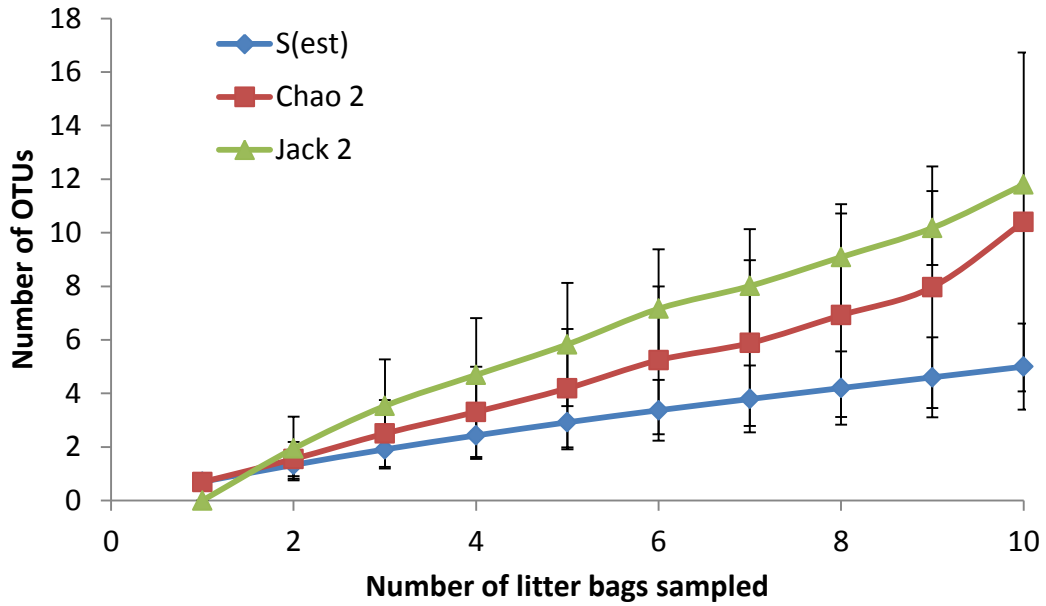
Figure 2. Rarefaction curve with Chao 2 and Jackknife 2 estimates for Darling 4 litter. Error bars represent computed standard deviation in 100 randomized runs.
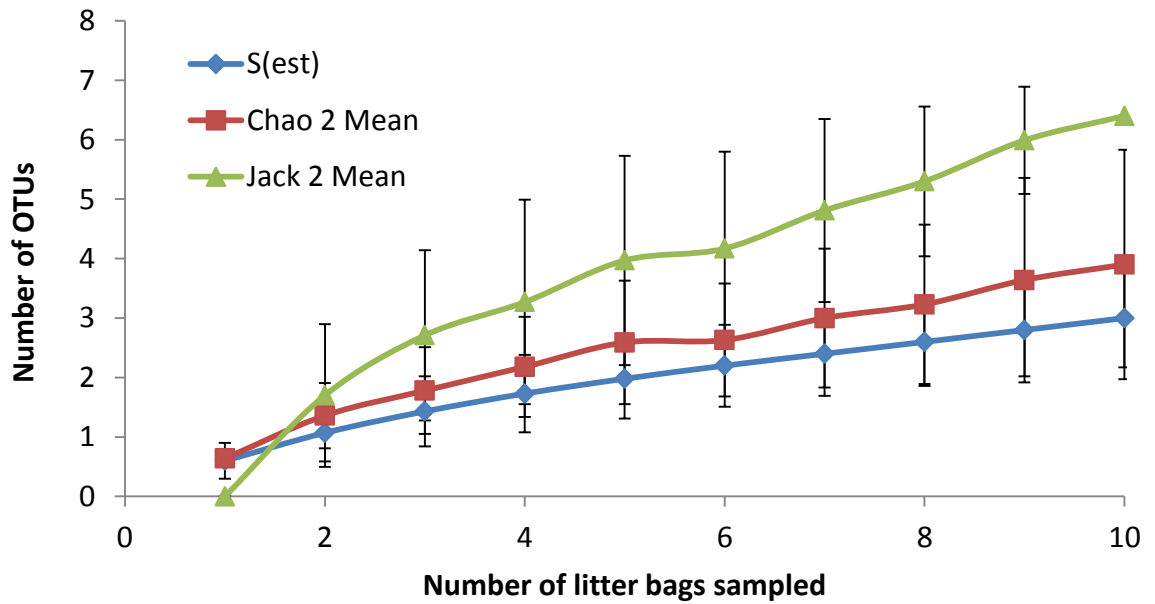

Figure 3. Rarefaction curve with Chao 2 and Jackknife 2 estimates for Hinchee 1 litter. Error bars represent computed standard deviation in 100 randomized runs.

Diversity characteristics

Using Estimate S software, Shannon and inverse Simpson indices were estimated for each litter type community using 100 randomized runs (Table 2). Based on the Shannon index, the diversity of the Zoar community was 16% higher than the community of Darling 4 and 50% higher than the community of Hinchee 1; the diversity of the Darling 4 community was 35% higher than the diversity of community of Hinchee 1 ($p < 0.001$). Using the inverse Simpson index, the Zoar community was 24% more diverse than the community of Darling 4 and 50% more diverse than the community of Hinchee 1; the Darling 4 community was 34% more diverse than that of Hinchee 1 ($p < 0.001$). The Hinchee 1 community overall had a lower number of OTUs found (10 for Hinchee 1 compared to 11 and 13 for Zoar and Darling 4, respectively) and had a high dominance of *Tomentella pilosa* (40% of the community), which resulted in the lower diversity for the Hinchee 1 community than Zoar and Darling 4 communities.

Community composition

Of the 73 morphological types originally found within the 30 litterbags used in this study, 39 were successfully run through PCR and RFLP analyses. The 39 morphological samples were sorted into 19 distinct RFLPs. These RFLPs were grouped into 15 distinct OTUs using a cutoff of 97% sequence similarity. OTUs were compared across litter types to identify shared OTUs and the proportion of each (Table 3). *Tomentella pilosa* and *Scleroderma aereolatum* were the only OTUs present in at least one litter bag from every litter type. When all litter types were combined, *T. pilosa* also had the highest absolute frequency at 20.6 %, and *S. aereolatum* had the second highest absolute frequency at 17.7 %. Within each litter type, dominant OTUs differed slightly from the dominant OTUs listed above. In the Zoar litter, *Tomentella* sp.11 and *Suillius*

sibiricus both have the highest absolute frequency at 23.1 % each. Within Darling 4 litter, *S. areolatum* had the highest absolute frequency at 27.3 %. Within Hinchee 1 litter, *T. pilosa* had the highest absolute frequency at 40.0 %.

The fungal OTUs were categorized into either EM or saprotrophic groups. EM groups were found to dominate all litter types after five months of decomposition. With all 10 samples of each litter type combined, Zoar leaf litter had 84.6 % EM fungal samples and 15.4 % saprotrophic samples (Figure 4). Darling 4 litterbags were found to have 72.7 % EM fungal samples and 27.3 % saprotrophic samples (Figure 5). Hinchee 1 litterbags had 70.0 % EM fungal samples and 30.0 % saprotrophic fungal samples (Figure 6).

Table 3. Absolute frequency, presented as percentage, of GenBank BLAST identified operational taxonomic units (OTUs) found in the litter of Zoar, Darling 4 and Hinchee 1 American chestnut types after five months of decomposition.

| OTU	Zoar	Darling 4	Hinchee 1	ALL
Tomentella sp. 11	23.1%	9.1%		11.8%
Tomentella pilosa	15.4%	9.1%	40.0%	20.6%
Suillis sibiricus	23.1%	9.1%		11.8%
Suillus sp. 1	7.7%			2.9%
Trechispora sp. 1	7.7%		10.0%	5.9%
Mycena sanguinolenta			10.0%	2.9%
Mycena murina		9.1%		2.9%
Mycena sp. 1	7.7%			2.9%
Scleroderma aereolatum	7.7%	27.3%	20.0%	17.7%
Boleataceae sp.1		9.1%	10.0%	5.9%
Phanaerochaete laevis		9.1%		2.9%
Phanaerochaete sp. 1			10.0%	2.9%
Phanaerochaete sp. 2		9.1%		2.9%
Amphinema sp. 1	7.7%			2.9%
Atheliaceae sp. 1		9.1%		2.9%
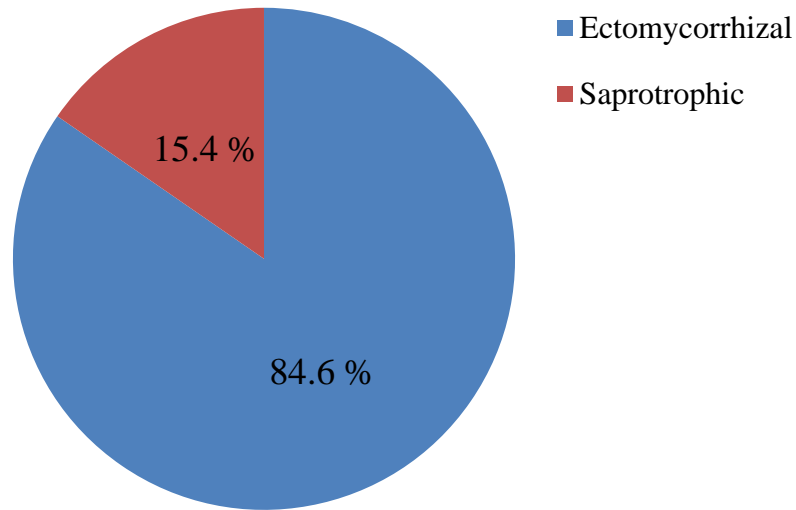
Figure 4. Percentage of EM and saprotrophic fungal samples in Zoar litterbags after five months of decomposition (n=10).
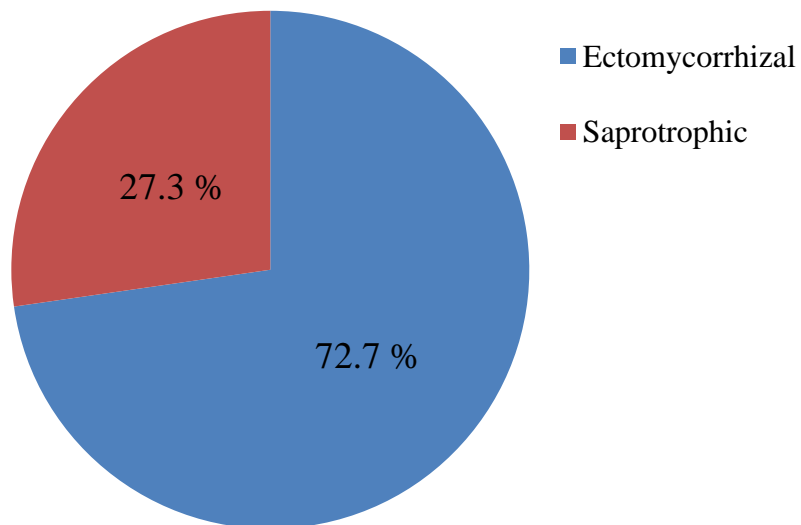

Figure 5. Percentage of EM and saprotrophic fungal samples in Darling 4 litterbags after five months of decomposition (n=10).
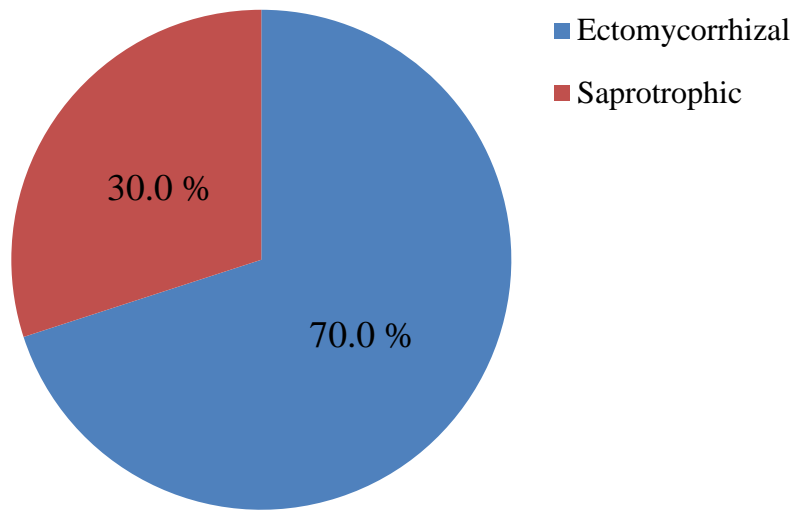
Figure 6. Percentage of EM and saprotrophic fungal samples in Hinchee 1 litterbags after five months of decomposition (n=10).

Jaccard indices were calculated in Excel for each pair of communities. The Zoar community was found to be equally similar to both Darling 4 and Hinchee 1 communities in the number of OTUs shared with a Jaccard index of 0.33 for both sets of communities. When the two transgenic communities were compared to one another, the Jaccard index was found to be 0.25, apparently lower than those of the sets of communities listed above. These data suggest that the transgenic communities are slightly more similar to the wild-type community than they are to each other, which is likely an artifact of low sample size.

There have been relatively few studies on the colonization and decomposition of transgenic leaf litter by fungi (Vauramo et al., 2006; Escher et al., 2000). Since fungi are considered to be key drivers of litter decomposition (Vořísková and Baldrian, 2013), it is important to study the potential effects that each genetically engineered plant may have on fungal decomposition. Previous studies have not shown a difference in the activity of fungal decomposers between transgenic and wild-type plants. For example, a study using transgenic maize engineered to express cry genes (genes that code for toxic crystals) from *Bacillus thuringiensis* found no difference in the growth of fungi in transgenic litter under varying nutritional conditions when compared to wild-type litter (Escher et al., 2000). For transgenic *Betula pendula* leaf litter engineered to express a chitinase gene, no difference was found in the ergosterol (a ubiquitous fungal sterol) content in the transgenic and wild-type litter (Vauramo et al., 2006).

S*pecies richness and OTU diversity*

None of the three litter types used in this study showed a plateau on the species rarefaction curves. Another study in this same site sampled over 100 root tips of wild-type and

transgenic American chestnuts and found that the rarefaction curve did not plateau, and was still increasing after 126 root tips were sampled (Tourtello, 2013). Because I was also not able to fully sample the fungal community present at the site, my estimate of diversity for the site is likely conservative, and inferences about differences in diversity are most likely premature.

The estimated OTU richness via Estimate S interpolation for 100 randomized runs trended higher in Zoar than in Hinchee 1 litter. Species richness of Darling 4 was not significantly different from Zoar or Hinchee 1. This is interesting because the transgenic events Darling 4 and Hinchee 1 are very similar in terms of genes inserted, with the exception of one gene. Darling 4 has two copies of the resistance-enhancing gene oxalate oxidase, as well as NPT2, which is a selectable marker; Hinchee 1 has three copies of the resistance-enhancing gene oxalate oxidase and the selectable marker NPT2, as well as an additional resistance-enhancing gene, ESF39 which codes for an antimicrobial peptide. Both transgenic events were co-transformed with a plasmid containing the selection markers GFP and BAR (Newhouse et al., 2014), and both transgenic events were developed using the wild-type American chestnut genotype, WB275-27. Therefore, one possible explanation is that the antimicrobial gene ESF 39 is having a negative impact on the diversity of fungal colonizers. However, possible insertional effects that could have occurred during the development of Hinchee 1 cannot be ruled out.

It is also interesting that we see a difference in species richness at all; in our study the transgenic event Hinchee 1, which has an extra resistance-enhancing gene and an extra copy of OxO, differed statistically from Zoar (a wild-type), whereas the other transgenic event, Darling 4, did not differ statistically from Zoar or Hinchee 1. This result is possibly due to small sample size. In a recent study on EM fungi and saprotrophic fungi, no difference was found in species richness across soil plots and horizons with different chemical compositions for either group of

fungi (Talbot et al., 2013). In fact, the species richness of EM fungi and saprotrophic fungi was not correlated with any environmental parameter examined and spatial patterns across plots were not detected (Talbot et al., 2013). However, species richness in another study using methods of morphological typing and sequencing similar to those used in our study found saprotrophic species richness ranging from five to 20 per sample and that there were significant differences in richness across stands of different tree species throughout the course of litter decomposition (Kubartová et al., 2008).

Diversity characteristics and community compositions

Zoar litter was found to be slightly more diverse in fungal OTUs than Darling 4 and notably more diverse than Hinchee 1 using both Shannon's index and Inverse Simpson's index. Hinchee 1 litter actually had the fewest OTUs after successful DNA sequencing and the litter was also highly dominated by one OTU, *T. pilosa*, which made up 40 % of the total fungal samples collected from the 10 Hinchee 1 litterbags. This high dominance by a single OTU, together with the lower number of OTUs found in Hinchee 1 litter bags could be impacting the diversity results in this study.

In our study, two OTUs were found in all litter types, *T. pilosa*, and *S. aereolatum*, both of which are EM fungi. Even though only two OTUs were shared among all litter types out of the 15 OTUs found, this is actually a commonly observed representation. Other studies have found as few as five of the same EM species to be present in more than one soil sample collected out of 23 species found (Talbot et al., 2013), or as few as 13 saprotrophic species present on two tree species out of 69 total species found (Kubartová et al., 2008).

Community composition has been shown to vary in EM fungi and saprotrophic fungi as a function of soil chemistry (Talbot et al., 2013). Results from chapter 2 show that the chemical compositions of Zoar, Darling 4 and Hinchee 1 litter are very similar. Darling 4 and Hinchee 1 litter mainly differed from Zoar litter by having higher calcium concentrations before and during decomposition. Leaf litter chemistry has also been shown to be more of a driver of decomposition during early stages of decomposition, while late stage decomposition has been associated with lignin concentrations within the litter (Berg, 2000). Previous work at the site used in this study showed rapid decomposition rates, with late stage decomposition being reached by six months, so it is likely that the litter collected in this study had transitioned into late stage decomposition, or was very close to transitioning into late stage decomposition. Species richness of saprotrophic fungi has been found to decrease when leaf litter transitions into late stage decomposition (Kubartová et al., 2008), which is due to the recalcitrance of the litter at this stage.

In a recent experiment on fungal succession during leaf litter decomposition, three distinct groups of fungal functional groups were found to dominate during different stages of decomposition based on the mass loss of hemicelluloses, celluloses, and lignin from the litter (Vořísková and Baldrian, 2013). In the same study, the first year of decomposition was associated with a loss in litter mass, a rapid degradation of cellulose, and the dominance of Ascomycetes. Saprotrophic Basidiomycete fungi and EM fungi began appearing on the litter at one year of decomposition, and at two years, cellulolytic activity decreased and lignolytic enzymes began to increase. The increase in lignolytic enzymes in year two was characterized by the dominance of Basidiomycetes in the litter, which have the ability to degrade lignin

(Vořísková and Baldrian, 2013). Interestingly, EM fungi have also been found to dominate leaf litter during late stages of decomposition (Lindahl et al., 2007; Vořísková and Baldrian, 2013).

In our study, EM fungi were found to dominate all litter types after five months of decomposition, and were actually more dominant in Zoar litter bags than in Darling 4 and Hinchee1 litterbags. The proportion of EM fungi and saprotrophic fungi found in the litter is surprising when compared to other studies, which is probably related to the different environmental conditions found at our site, such as soil type, temperature, and precipitation. In a 24-month study of oak leaf decomposition in the Czech Republic, EM fungi began appearing on the litter at 12 months, and the percentage of EM fungi was 50 % at 24 months (Vořísková and Baldrian, 2013). A study using soil from a *Pinus sylvestris* forest in Sweden found that after three to five years, the fungal community was most likely to shift from being dominated by Ascomycetes to Basidiomycetes, and that during late-stage litter decomposition, the majority of fungal species were actually ectomycorrhizal (Lindahl et al., 2007). It is difficult to explain the high dominance of EM fungi in this study after just five months of decomposition, since this time frame is much shorter than what has been documented for shifting fungal communities. Because only 39 of the 73 morphotypes collected for this study were successfully sequenced, it is possible that the high dominance by EM fungi is an artifact of this success rate. However, since another experiment in this same location has shown an incredible diversity of EM fungi at this site (Tourtellot, 2013), and previous work has shown that our site is prone to rapid decomposition, it is also entirely possible that these proportions represent the true composition of fungal species in the litter at this site.

In a previous experiment, we found that calcium (Ca) concentration was higher in senesced litter from Darling 4 than in Zoar and Hinchee 1, and that it remained higher during 2.5

years of decomposition. Litter Ca has been found to have a strong relationship with forest floor biogeochemical dynamics, including soil acidity, decomposition rates, and heterotrophic community composition (Reich, 2005; Hobbie et al., 2006). Ca-induced changes in soil acidity have been found to play a key role in determining differences in observed root tip EM fungal communities among sites (Aponte et al., 2010). The most significant change in EM fungal community composition seen when Ca concentrations were high in litter, was a shift in the dominant taxa from russuloids to tomentelloids (Aponte et al., 2010). In the current study, no russuloids were found in any litter types, perhaps because russuloids are short-distance exploration types and would therefore only be found near the root tips of plants. However, Darling 4 litter, which had higher Ca contents, actually showed a shift in dominant taxa away from tomentelloids and towards a single *Scleroderma* species.

The most common EM fungal species found in this study were tomentelloids, suilloids, and *Scleroderma* species. While certain EM fungi have been shown to have retained certain enzymes that could facilitate leaf litter decomposition, previous studies have stated that the EM Boletales, which include Suillineae and Sclerodermatineae, have lost the ability to produce the brown-rot decay enzymes necessary to decompose litter (Kohler et al., 2015). However, one of the fungi within this group, *Paxillus involutus*, has the capacity to oxidize organic matter in a way that is similar to brown-rot fungi (Rineau et al., 2013). Because not much research has been done on brown-rot abilities of EM fungi, it could be possible that other members of the EM Boletales also have this ability, including some of the species found in this study.

Chen et al. (2001) focused on the ability of EM fungi to produce white-rot-like enzymes found that several ectomycorrhizal species possess certain genes that could potentially allow them to oxidize organic matter using peroxidases, a white-rot mechanism. Several Boetales and

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Thelephorales possessed at least one of these peroxidase genes, and many EM fungi possessed one or more of these genes. This lends further support for the possibility that certain EM fungi have retained some ability to decompose leaf litter as mycorrhizal fungi.

The fungal species observed in this study have been associated with different environment types. Fungi in the genus *Scleroderma* have been found to increase in relative abundance after logging (Ingleby et al.,1998), to be common on a primary succession site on Mount Fuji (Nara et al., 2003), and to be common in abandoned farmlands (Karpati et al., 2011). *Scleroderma* species have also commonly been found in nursery stocks (Martin et al., 2003), and were found to be the dominant fungal type colonizing American chestnut seedlings on an abandoned strip mine site in Ohio (Bauman, 2010). The most common genus found in this study was *Tomentella* at 32.3 % relative abundance among all litter types combined. Members of Thelephoraceae such as *Tomentella* spp. are often dominant in mature conifer forests (Horton and Bruns, 2001), and while this study aimed at investigating American chestnut litter, there were pine trees within the experimental plot. In another study investigating the EM fungi on the roots of American chestnut in the same experimental plot, *S. areolatum* was found on 2.4 % of roots, *T. pilosa* was found on 2.4 % of roots, and *Tomentella* sp. 11 was found on 1.6 % of roots (Tourtellot, 2013), showing that these three species are present on both the roots and the decomposing leaf litter of American chestnut.

Conclusion

This study reports fungi that colonize leaf litter of two transgenic and a wild-type American chestnut. Few studies have described fungal communities, especially on those that inhabit or degrade plant litter associated with genetically engineered plant species. All three fungal communities investigated in this study were fairly diverse and even, however the data

show a significant difference in the diversity and dominance of fungi among those that colonize Zoar and Darling 4 litter and those that colonize Hinchee 1 litter. Hinchee 1 litter contains a gene that codes for the microbial peptide ESF39, which has been shown to possibly have an effect on the ectomycorrhizal colonization of roots, especially within the first year (Tourtellot, 2013). The gene ESF 39 could be influencing the litter itself or the transformation of Hinchee 1 could have caused an insertional effect that is influencing the colonization of fungi

The method used in this study for collecting hyphae visually was biased towards Basidiomycetes because their hyphae are generally larger and easier to see under a dissecting microscope. Since it was probable that I was mainly collecting Basidiomycetes, the Basidiomycete-specific PCR primer ITS-4B was used during PCR amplification, unless ITS-4B did not yield successful PCR products, in which case the more general ITS-4 was used (with the fungal specific primer ITS-1f in all cases (White et al., 1990). Because Ascomycetes were not found at all in this study, placing the functional groups on a timeline of decomposition is nearly impossible. The quantity and/or quality of hyphae removed from the leaf litter was insufficient in some cases for successful DNA extraction, so not every morphological type found could be sequenced. In future research, if possible, Next Gen sequencing should be used in order to assess all fungal OTUs present on the litter, including Ascomycetes and Zygomycetes, and to assure that a greater number of species is sequenced for assessment of species richness.

If granted federal approval, the transgenic American chestnut will be the first genetically engineered non-orchard tree to be de-regulated and environmentally released. More information is needed on the potential ecological effects of transgenic American chestnuts on forest ecosystems, including herbivory patterns, nutritional content of chestnuts, and the release of nutrients, so that informed decisions can be made during their deregulation. Research

surrounding the deregulation process for American chestnut could prove useful for other

threatened native tree species that may undergo genetic engineering in the future, such as

American butternut (*Juglans cinerea*), white ash (*Fraxinus americana*), and eastern hemlock

(*Tsuga canadensis*).

Literature Cited

Altschul, S.F., Madden, T.L, Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research 25 (17), 3389-3402.

Aponte, C., García, L.V., Marañón, T., Gardes, M., 2010. Indirect host effect on ectomycorrhizal fungi; Leaf fall and litter quality explain changes in fungal communities on the roots of co-occurring Mediterranean oaks. Soil Biology and Biochemistry 42, 788-796.

Bauman, J.M., 2010 Ectomycorrhizal communities associated with restoration plantings of American chesntut (*Castanea dentata*) seedlings on Ohio mine lands: Planting methodologies to promote root colonization. Doctoral thesis: Miami University.

Berg, B., 2000. Litter decomposition and organic matter turnover in northern forest soils. Forest Ecology and Management 133, 13-22.

Colwell, R.K., 2013. Estimate S: Statistical estimation of species richness and shared species from samples. Version 9 and earlier. User's guide and application

Chen, D.M., Taylor, A.F.S., Burke, R.M., Cairney, J.W.G., 2001. Identification of genes for lignin peroxidases and manganese peroxidases in ectomycorrhizal fungi. New Phytologist 152, 151-158.

Escher, N., Kach, B., Nentwig, W., 2000. Decomposition of transgenic *Bacillus thuringiensis* maize by micro-organisms and woodlice *Porcello scaber* (Crustacea: Isopoda). Basic Applied Entomology 1, 161–169.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2, 113-118.

Hobbie, S.E., Reich, P.B., Oleskyn, J., Ogdahl, M., Zytkowiak, R., Hale, C., Karolewski, P., 2006. Tree species effects on decomposition and forest floor dynamics ni a common garden. Ecology 87, 2288-2297.

Horton, T.R., Bruns, T.D., 2001. The molecular revolution in mycorrhizal ecology: Peeking into the black-box. Molecular Ecology 10 (8), 1855-1871.

Hutton, F.Z., Rice, E., 1977. Soil Survey of Onondaga County, New York.

Ingleby, K., Munro, R.C., Noor, B., Mason, P.A., Clearwater, M.J., 1998. Ectomycorrhizal populations and growth of *Shorea* parvifolia (Dipterocarpaceae) seedlings regenerating under three different forest canopies following logging. Forest Ecology and Management 111 (2-3), 171-179.

Karpati, A., Handel, S., Dighton, J., Horton, T., 2011. *Quercus rubra*- associated Ectomycorrhizal fungal communities of disturbed urban sites and mature forests. Mycorrhiza 21 (6), 537-547.

Kohler, A., Kuo. A., Nagy, L.G., Morin, E., Barry, K.W., Buscot, F., Canbäck, B., Choi, C., Cichocki, N., Clum, A, Colpaert, J., Copeland, A., Costa, M.D., Doré, J., Floudas, D., Gay, G., Girlanda, M., Henrissat, B., Herrmann, S., Hess, J., Högberg, N., Johansson, T, Hassine-Radhouane, K., LaButti, K, Lahrmann, U., Levasseru, A., Lindquist, E.A., Lipzen, A., Marmeisse, R., Martino, E., Murat, C., Ngan, C.Y., Nehls, U., Plett, J.M., Pringle, A., Ohm, R.A., Perotto, S., Peter, M., Riley, R., Rineau, F., Ruytinx, J., Salamov, A., Shah, F., Sun, H., Tarkka, M., Tritt, A., Veneault-Fourrey, C., Zuccaro, AL., MGICA, Tunlid, A., Grigoriev, I.V., Hibbett, D.S., Martin, F., 2015. Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. Nature Genetics 47, 410-415.

Kubartová, A., Ranger, J., Berthelin, J., Beguiristain, T., 2008. Diversity and decomposing ability of saprotrophic fungi from temperate forest litter. Microbial Ecology 58, 98-107.

Lindahl, B.D., Ihrmark, K., Boberg, J., Trumbore, S.E., Högberg, P., Stenlid, J., Finlay, R.D., 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytologist 173, 611-620.

Martin, T.P., Harris, J.R., Eaton, G.K., Miller, O.K., 2003. The efficacy of Ectomycorrhizal colonization of pin and scarlet oak in nursery production. Journal of Environmental Horticulture 21 (1), 45-50.

Nara, K.,Nyaka, H., Wu, B., Zhou, Z., Hogetsu, T., 2003. Underground primary succession of Ectomycorrhizal morphotype *Pinirhiza sclerotia* is formed by *Acephala macrosclerotiorum* sp. nov., a close relative of *Phialocephala fortinii.* Mycorrhiza 19 (7), 481-492.

Newhouse, A.E., Polin-McGuigan, L.D., Baier, K.A., Valetta, K.E.R., Rottman, W.H. Tschaplinski, T.J., Maynard, C.A., Powell, W.A., 2014. Transgenic American chestnuts show enhanced blight resistance and transmit the trait to T1 progeny. Plant Science 228 88-97.

NOAA, 2013. Northeast Regional Climate Center. 2013. Annual Temperature, Precipitation, and Snowfall Data. Syracuse, New York. http://www.weather.gov/bgm/climateSYRAnnualTotals.

Nowak, C., 2015. Unpublished raw data.

Reich, P.B., 2005. Linking litter calcium, earthworms and soil properties: A common garden test with 14 tree species. Ecology Letters 8, 811-818.

Rineau, F.,Shah, F.,Smits, M. M., Persson, P., Johansson, T., Carleer, R., Troein, C., Tunlid, A., 2013. Carbon availability triggers the decomposition of plant litter and assimilation of nitrogen by an ectomycorrhizal fungus. The ISME Journal 7, 2010-2022.

Talbot, J.M., Bruns, T.D., Smith, D.P., Branco, S., Glassman,S.I., Erlandson, S., Vilgalys, R., Peay, K.G. 2013. Independent roles of Ectomycorrhizal and saprotrophic communities in soil organc mater decomposition. Soil Biology and Biochemistry 57, 282-291.

Tourtellot, S.G., 2013. The impact of transgenic American chestnuts (*Castanea dentata*) on 0ectomycorrhizal fungi in open-field and mature forest sites. M.S. Thesis. SUNY College of Environmental Science and Forestry.

Vauramo, S., Pasonen, H.-L., Pappinen, A., Setälä, H., 2006. Decomposition of leaf litter from chitinase transgenic silver birch (*Betula pendula*) and effects on decomposer populations in a field trial. Applied Soil Ecology 32,338-349.

Vořísková, J., Baldrian, P., 2013. Fungal community on decomposing leaf litter undergoes rapid successional changes. The ISME Journal 7: 477-486.

White, T. J., Bruns,T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications, eds. Academic Press, Inc., New York, pp. 315-322

4. Published works and presentations:

A.G. Gray. Investigating the role of transgenic American chestnut (*Castanea dentata*) leaf litter in decomposition, nutrient cycling, and fungal diversity, 84 pages, 6 tables, 11 figures. 2015. MS Thesis, SUNY College of Environmental Science and Forestry

5. **Press coverage (publication title, date, article title) and any website links in which the project appears: None as of this date**

FINANCIAL REPORT:

Expenditures totaling $1,900 covered costs associated with supplies (e.g., mesh for litterbag fabrication, RFLP primers), DNA sequencing at the Cornell lab, and tissue analysis at our laboratory here at ESF.