INVESTIGATING THE ROLE OF TRANSGENIC AMERICAN CHESTNUT (*Castanea dentata)* LEAF LITTER IN DECOMPOSITION, NUTRIENT CYCLING, AND FUNGAL DIVERSITY

by

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Table of contents

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

LIST OF APPENDICES

Abstract

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.

The American chestnut (*Castanea dentata*) once played an integral part in forests within Eastern North America, providing many ecological and economic benefits. The majority of these trees were eradicated by an introduced pathogenic fungus, leaving the American chestnut functionally extinct. Disease-resistant transgenic trees containing the transgene oxalate oxidase were developed to re-establish the American chestnut to its native range. In order to introduce transgenic American chestnut trees into forests, federal policies from USDA APHIS, FDA and EPA require that the ecological functions of the transgenic trees be equivalent to those of wild-type American chestnut trees. This study is aimed at determining if the rates of decomposition, elemental concentrations and contents, and diversity of colonizing fungi are significantly different among transgenic and wild-type American chestnut leaf litter *in situ*. Litterbags containing each type of leaf litter were placed in a shelterwood plot for 12, 18, 24, and 30 months for the decomposition experiment and five months for the fungal diversity experiment. Differences among litter types in the decomposition experiment increased over time, with more differences appearing at 30 months. No clear differences among types were found in decomposition rate, C and N content, C/N ratio, or the majority of nutrients. The only statistically significant difference among types found during nutrient release was in calcium concentration and content, and phosphorus concentration, with one of the transgenic types having higher values over time than other litter types In the fungal diversity experiment, one of the transgenic events did show a slightly lower diversity of fungal species than other litter types, but the community structures were similar among litter types; after 5 months all litter types were dominated by ectomycorrizal fungi. Taken together, these studies suggest that the process of genetic engineering using the transgene oxalate oxidase does not have any measureable effect on the mineralization of plant material. Upon future approval of federal deregulation, the American chestnut will be the first genetically modified non-orchard tree to be deregulated and environmentally released, which will make the process easier for the genetic engineering of other threatened native tree species in the future.

Keywords: Transgenic trees, biogeochemistry, forest ecology, ectomycorrhizal fungi, saprotrophic fungi, biodiversity

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Chapter 1: Introduction

Chestnut blight is an aggressive diffuse canker disease, which means it spreads rapidly through the tree, immediately killing the tree tissue that it comes into contact with. The blight is caused by the fungus *Cryphonectria parasitica* (Murr.) Barr. (Jacobs, 2007). *C.parasitica* was first discovered in the United States in the early 1900's at the Bronx Zoological Park in NYC. Within 50 years of its discovery, the fungus had spread at a rapid rate, while simultaneously decimating America's native population of chestnut trees. By 1960, it had killed approximately 4 billion American chestnut (*Castanea dentata* (Marsh) Borkh.) trees, which were once dominant canopy trees in eastern North America. Because of the widespread damage caused by chestnut blight, almost all American chestnut trees that exist today are sprouts that originated from blight-infected trees, since the blight does not kill the underground portion of the tree (Jacobs, 2007).

The fungus *Cryphonectria parasitica* can be found within natural forests or planted stands containing hardwood trees within its native range. It is a necrotrophic fungus, meaning that it uses dead tissue as its energy source. *C. parasitica* can live on hardwood trees of any age and acts as a pathogen of specific hardwood tree hosts including oaks and chinquapins, but most often American chestnut in North America (Dallaville and Zambonelli, 1999). During infection, the fungus enters the tree through a wound or break in the bark and spreads rapidly through the cambium, sapwood and up to five layers of heartwood. While spreading, the fungus secretes oxalic acid, which lowers the pH of tree tissue to toxic levels (Zhang et al. 2013). The fungus enters xylem and phloem tissue, causing the tree to form a canker (a localized area of dead bark and wood on the tree) which temporarily restricts the fungus from infecting other tissue (Anagnostakis, 1987). The tree eventually girdles at the site of the canker, which results in the death of the tree due to the inability to transfer water and nutrients (OEPP/EPPO, 1982).

A successful method for producing blight-resistant chestnuts has been developed at SUNY College of Environmental Science and Forestry in Syracuse, NY. In this method, blight-resistant genes are transformed into the American chestnut. This produces blight-resistant transgenic American chestnuts with wood quality and nut production equivalent to wild-type American chestnuts. The resistance-enhancing genes that have been transformed into the American chestnuts used in this study are oxalate oxidase, which breaks down the harmful oxalic acid produced by the fungus (Polin et al., 2006; Newhouse et al., 2014) and ESF39, which codes for an antimicrobial peptide (Newhouse et al., 2014). The process of creating a transgenic chestnut event takes 18-24 months, which is far less time than what is needed to develop a hybrid through traditional breeding methods (Polin et al. 2006). Unlike hybrid trees, genetically engineered trees must go through rigorous testing in order to determine that they are ecologically equivalent to wild-type trees and that they pose no threat to other species (Sedjo, 2010).

Two transgenic events were assessed in this two-year field trial to assess any possible insertion effects and to determine if the transgenic trees are ecologically equivalent to wild-type *in situ*. The transgenic events were compared to a ¾ American-Chinese chestnut hybrid (¾ American chestnut, ¼ Chinese chestnut) and a wild-type American chestnut genotype. The site chosen for this research is a shelterwood cut of natural oak and hickory species located at Lafayette Road Field Station in Syracuse, NY. In this thesis I assess the effects of transgenic events on decomposition rates, leaf chemistry, and fungal colonization of leaf litter.

In Chapter 2, I analyze the decomposition rates, C/N ratios, and concentrations and contents of selected elements and compare values between the two transgenic events, a conventionally-bred hybrid and a wild-type American chestnut over a two-year time period. I

hypothesize that the inserted transgenes will have no effect on the decomposition rate, C/N ratio, or concentrations and contents of elements within the leaf litter.

In Chapter 3, I compare the fungal species that colonize leaf litter of the two transgenic events to those that colonized wild-type leaf litter over a six month time period. I hypothesize that there will be no difference in the diversity of species that colonize and presumably break down their litter. I also hypothesize that given the accelerated decomposition at the site, after five months the fungal decomposers would have transitioned from early-stage ascomycetes to late-stage basidiomycetes, with some ectomycorrhizal species present.

Chapter 2: Decomposition of leaf litter from transgenic American chestnut (*Castanea dentata)* and effects on nutrient cycling in a two year field trial

Abstract

After chestnut blight nearly eradicated the American chestnut (*Castanea dentata* (Marsh.) Borkh.) from its native range, disease-resistant transgenic American chestnut trees were developed for the purpose of reintroducing this economically and ecologically important species into the wild. Before releasing genetically engineered plants into the environment, federal regulations require that it be equivalent to wild type plants in ecological function. This study compares the rate of decomposition and the subsequent release of nutrients from the leaf litter of transgenic, hybrid, and wild type American chestnuts *in situ* after 12, 18, 24, and 30 months. Differences among litter types in the decomposition experiment increased over time, with more differences appearing at 30 months. No clear differences among types were found in decomposition rate, C and N content, C/N ratio, or the majority of nutrients. The only statistically significant difference among types found during nutrient release was in calcium concentration and content, and phosphorus concentration, with one of the transgenic types having higher values over time than other litter types. Overall, the transgenic trees appear to release nutrients at a similar rate to wild-type and hybrid trees during leaf litter decomposition. Upon future approval of federal deregulation, the American chestnut will be the first genetically modified non-orchard tree to be deregulated and environmentally released, which will make the process easier for the genetic engineering of other threatened native tree species in the future.

Keywords: transgenic trees, forest ecology, biogeochemistry, diversity

Introduction

Before the disappearance of the American chestnut, it was a foundation species and was widespread and dominant throughout deciduous forests of eastern North America (Ellison et al., 2005). Its range extended from Maine, through the Appalachian Mountains to Alabama, and westward to the Mississippi River (Griffin, 2000). The large, fast-growing tree played an important part in the forest ecosystem. Annually, the largest trees could produce up to 10 bushels of nuts, which were nutritious and were readily consumed by forest animals such as squirrels, wild turkeys, white-tailed deer, black bears, raccoons and grouse (Davis, 2005). Several insect species relied on the chestnut as their primary source of food, and at least seven native moth

species are believed to have become extinct with the loss of chestnut trees from the forest (Davis, 2005). Aquatic ecosystems may have also changed with the disappearance of the American chestnut. In forest streams, leaf litter inputs serve as the primary source of energy for aquatic ecosystems. American chestnut leaves falling into these systems decomposed more quickly than leaves of other species and had a higher nutritional quality, which aquatic invertebrates preferentially exploited (Ellison et al., 2005). The American chestnut was also one of the most economically valued eastern hardwood species due to its rot-resistance and its ability to grow straight and tall. It was therefore often used for lumber, shingles, fence posts, poles, furniture, pulp, paneling, and firewood (Emerson and Weed, 1918; Rice et al., 1980). During the early 1900's, American chestnut made up 25% of all timber cut in the Southern Appalachians and was listed as the most valuable tree in southern New England (Hepting, 1974).

The causal agent of chestnut blight, *Cryphonectria parasitica*, was first discovered in the northeastern United States in the early 1900's, after it had been brought over from Asia via infected chestnut nursery stock (Griffin, 2000). By 1960, chestnut blight had killed about 4 billion American chestnut trees, and so almost all American chestnut trees that are alive today are susceptible sprouts that originated from blight-killed trees (Jacobs, 2007). Due to the fact that it can now only persist as an understory sprout in its native range, American chestnut is regarded as functionally extinct (Paillet, 2002). With the disappearance of the American chestnut, other hardwood species have become the dominant species within American chestnut's native range (Wang et al., 2013). Unlike the American chestnut, some of these species only produce seed during mast years, which are unpredictable, and so animals that once relied on chestnuts may now have a more difficult time finding food (Ostfeld et al., 2008).

Due to the ecological and economical importance of the American chestnut, there have been several attempts to develop a blight-resistant American chestnut tree to introduce into its native range. Attempts have included the use of traditional breeding techniques, biological control, and genetic engineering (Wang et al., 2013). This study compares blight-resistant American chestnuts developed through two methods. A combination of litter from two trees produced through the hybridization of susceptible American chestnut and resistant Chinese chestnut were used. Two genetically engineered trees developed for blight resistance were also used.

During the genetic engineering of transgenic American chestnuts, the gene(s) of interest were inserted into the plant's genome, along with the marker genes NPT2, BAR, and GFP. Transgenic event Darling 4 contains two copies of the resistance-enhancing gene oxalate oxidase and the marker gene NPT2 and two copies of the marker genes GFP and BAR. The other transgenic event, Hinchee 1, contains three copies of the oxalate oxidase gene and the marker gene NPT2 and three copies of the marker genes GFP and BAR, along with the synthetic antimicrobial peptide ESF39 (Newhouse et al., 2014). Darling 4 and Hinchee 1 were both developed from the wild-type American chestnut genotype, WB275-27, and both showed intermediate blight resistance (higher than the WB275-27 trees but lower than Chinese chestnut trees) (Newhouse et al., 2014). While conventionally-bred hybrid trees can be planted into forests without scrutiny, genetically engineered trees must go through rigorous testing to ensure that they are ecologically equivalent to traditional species and pose no threat to the environment or other species (Sedjo, 2010). In this paper, we report on the decomposition rates and element concentrations present in the leaf litter types throughout a 2.5-year field trial.

Oftentimes, during the process of inserting genetic material, insertions of additional DNA, deletions and/or rearrangements take place. These insertional effects have the potential to produce unintended traits in plants. Unintended traits can cause phenotypic changes in the plant, which may include a new plant characteristic, the loss of a previously expressed plant characteristic, or the expression of a characteristic that is outside the range of what is normally observed in the plant species (Schnell et al., 2015). Insertional effects are not limited to genetically engineered plants; they also occur in traditional breeding and in wild-type plants. Causes of insertional effects in these plants include the movement of transposable elements, the repairing of double-stranded DNA breaks by non-homologous end-joining, and through intracellular transfer of organelle DNA (Schnell et al., 2015). During this study, we aim to determine if the transformation of American chestnut causes any differences in leaf litter decomposition when compared to conventionally bred chestnuts as a result of the insertion of our gene(s) of interest and their marker genes, or any other insertional effects that may have taken place during the genetic engineering process. Other studies on the transgenic American chestnut containing the oxalate oxidase gene have shown no differences in ecological processes when compared to wild-type American chestnuts (D'Amico et al., 2015; Tourtellot, 2013).

Altered nutrient dynamics in the soil, resulting from the decomposition of transgenic litter, have been considered to be potential risks for planting genetically engineered plants (Trevors et al., 1994). Because there have been so few studies investigating this concern, especially regarding trees, more research is needed on the decomposition of genetically engineered plants transformed with the genes OxO and ESF39 and their role in biogeochemical cycles. During the decomposition process, organically-bound nutrients within leaf litter are released as free ions into the soil solution, where they can then be utilized by various organisms

(Berg and Laskowski, 2005). The concentrations of certain elements within decomposing leaf litter have been used to signify how quickly the mineral elements are being released into the soil, especially in the first phase of decomposition. The C/N ratio is most often used to determine the extent of decomposition, because generally decomposition rates increase with decreasing C/N ratios (Heal et al., 1997), due to preferential use of the high quality litter by soil organisms and the reduced recalcitrance of the litter.

In this study, we investigate the concentrations and contents of plant macronutrients: calcium (Ca), magnesium (Mg), potassium (K), and phosphorus (P), the plant micronutrient manganese (Mn), a plant functional nutrient sodium (Na), and the element aluminum (Al), which is known to limit plant production on acid soils (Delhaize and Ryan, 1995). Ca is an essential plant nutrient; it plays a crucial role in regulating physiological processes that influence growth and environmental stress responses such as: water and solute movement, cell division and cell wall synthesis, direct or signaling roles for plant defense and repair, rates of respiratory metabolism, and structural chemistry and function of woody support tissues (McLaughlin and Wimmer, 1999). Ca is also important in neutralizing soil acidity in temperate forests (Dijkstra, 2003). Due to the large size and age of trees and the vast need for calcium these characteristics cause, Ca supply generally places a limitation on forest structure and function, especially in older trees, late successional species, high levels of soil acidity, and when competition is high (McLaughlin and Wimmer, 1999).

P is an essential element for plants and animals, as it is required for the formation of adenosine triphosphate (ATP),which drives most biological processes, such as the uptake of nutrients and the transport of nutrients within plants (Brady and Weil, 2008). P is also the second most likely essential element to limit plant productivity. Mg is an essential plant nutrient that

plays a crucial role in photosynthesis; one fifth of Mg in plants is found as the central component of the chlorophyll molecule (Brady and Weil, 2008). Mg also plays key role in the synthesis of oils and proteins and in the activation of enzymes involved in energy metabolism (Brady and Weil, 2008). K is the third most likely essential element to limit plant productivity. It is crucial to both plant and animal nutrition, as it is known to activate over 80 different enzymes responsible for energy metabolism, starch synthesis, nitrate reduction, photosynthesis, and sugar degradation (Brady and Weil, 2008). .

The plant micronutrient Mn is essential to plants; it activates decarboxylase, dehydrogenase, and oxidase enzymes and plays an important role in photosynthesis, N metabolism, and nitrogen assimilation. Mn also catalyses peroxidase, an exo-enzyme that aids in the synthesis of lignin and monophenols (Brady and Weil, 2008). The element Na is an essential element for animals, but is not regarded as essential in plants, however many plant tissues contain high concentrations of it (Subbarao et al., 2003). Na has been described as a functional nutrient in plants, as it can substitute for potassium in non-specific functions, such as being an osmoticum during cell enlargement and a counterion during long-distance transport; is also a major contributing factor to the secondary salinization of many soil systems (Subbarao et al., 2003). Al is not a plant nutrient, but exists as a major component of most soil minerals, including clays. Al is important to study in nutrient cycling because it can play a role in soil acidity (such as in Al hydrolysis) and because Al toxicity in plants is the most common and severe problem associated with acidic soils, due to the increased solubility of Al ions in acidic soil conditions (Brady and Weil, 2008).

We hypothesize that there will be no significant difference in decomposition rates, concentrations of C and N, C/N ratio, or concentration or content of any of the elements among

the four leaf litter types. Findings from this study will contribute to the assessment of ecological equivalence between blight-resistant transgenic American chestnut and wild-type American chestnut.

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 exists in an area that was once used for agriculture. 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

Four leaf litter types were selected for this study: a "hybrid" consisting of two backcrossed, ¾ American-Chinese chestnut hybrids (GR68-B1 and K-L-BC1), Darling 4 from the transgenic event Darling 4, Hinchee 1 from transgenic event Hinchee 1, and wild-type litter

from the American chestnut genotype Zoar. Litterbags were 22 cm in length and width and made with 1mm mesh; 10 grams of leaf litter from one of the four litter types were placed into each bag. The bags were then placed on the surface of the Ap layer of soil in the lower shelterwood site at Lafayette Road Experiment Station in October 2012. A total of 120 litterbags were deployed in the field, at 30 different points, which lined the perimeter of the plot. At each point, a set of four litterbags were placed on top of the soil in order to account for effects of micro-climate and slope. After 12, 18, and 24 months, six points were randomly selected, and after 30 months the remaining 12 points were selected, and the four litterbag-set located at each point were harvested. Subsamples of senesced leaves from each litter type were retained as "initials" to assess litter chemical characteristics before decomposition.

Lab Methods

After oven drying the litter at 60°C, leaf litter was separated from the roots and grasses that had grown into the bags. Oven dry mass was determined, and the litter was ground in a Wiley Mill, and pulverized in a SPEX CertiPrep 8000 Mixer/Mill in order to homogenize the litter sample. A subsample (0.025 g) of the homogenized litter was analyzed for C and N in a Thermo Flash EA 1112 CHN/S/O Analyzer. Another 0.25 g subsample of the homogenized litter was ashed in a Sybron/Thermolyne muffle furnace at 470°C overnight, and proportion ash was determined.

An acid digest was performed on the ash as per Bickelhaupt and White (1982). 2 ml of distilled de-ionized water was added to the ash to moisten the sample, followed by 10 mL of 6 M nitric acid (HNO_3). The ash and digest mixture was heated on a hot plate. The sample was filtered through Whatman #42 filter paper, rinsed and diluted to 50 mL with de-ionized water.

The filtered digest solution was analyzed using Inductively Coupled Plasma—Optical Emission Spectrometry (ICP-OES) (Perkin Elmer Optima 3300DV). Four standards using spruce litter element data were run as a quality control.

Calculations and Statistical Methods

The rate of decomposition was modeled as an exponential decay curve using SAS ® statistical software, in order to derive the negative exponential decay constant (*k*). Olson's (1963) formula was used to model decay, which is $y = e^{-kt}$, where y is the fraction of mass remaining at some time, *t* (years). To determine differences in mass loss, C/N ratios, element contents and concentrations, means were compared among litter types with two-way ANOVA (at $\alpha = 0.05$) and Tukey's test (at $\alpha = 0.10$) using Minitab® 17 statistical software so that interaction effects could be investigated as well as main effects. All values were calculated on an ash-free basis, including decay rates (k), due to the proportion ash increasing over time in the litterbags (Figure 2.1).

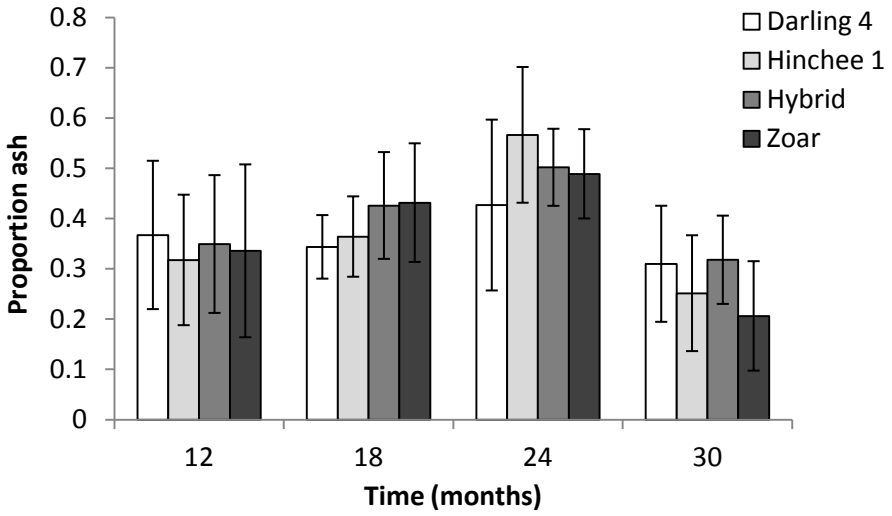


Figure 2.1. The mean proportion ash in decomposing transgenic (Darling 4, Hinchee 1), Hybrid and Zoar leaf litter over a 2.5 year period (n=6 for 12, 18, and 24 months, n=12 for 30 months). Error bars represent standard deviation of the mean.

Results

Mass remaining and decomposition rate

Throughout the 30-month decomposition period, mass remaining varied among litter types, with Darling 4 having more mass remaining throughout decomposition ($p < 0.001$). Mass remaining also varied over time between 12 and 30 months ($p < 0.001$) (Figure 2.2). There was no interaction between litter type and time for mass remaining ($p = 0.40$) The decomposition rates, expressed as k-values in this study, were similar among all litter types except Darling 4, which had a slightly lower k-value than other types, indicating a lower rate of decomposition (Table 2.1).

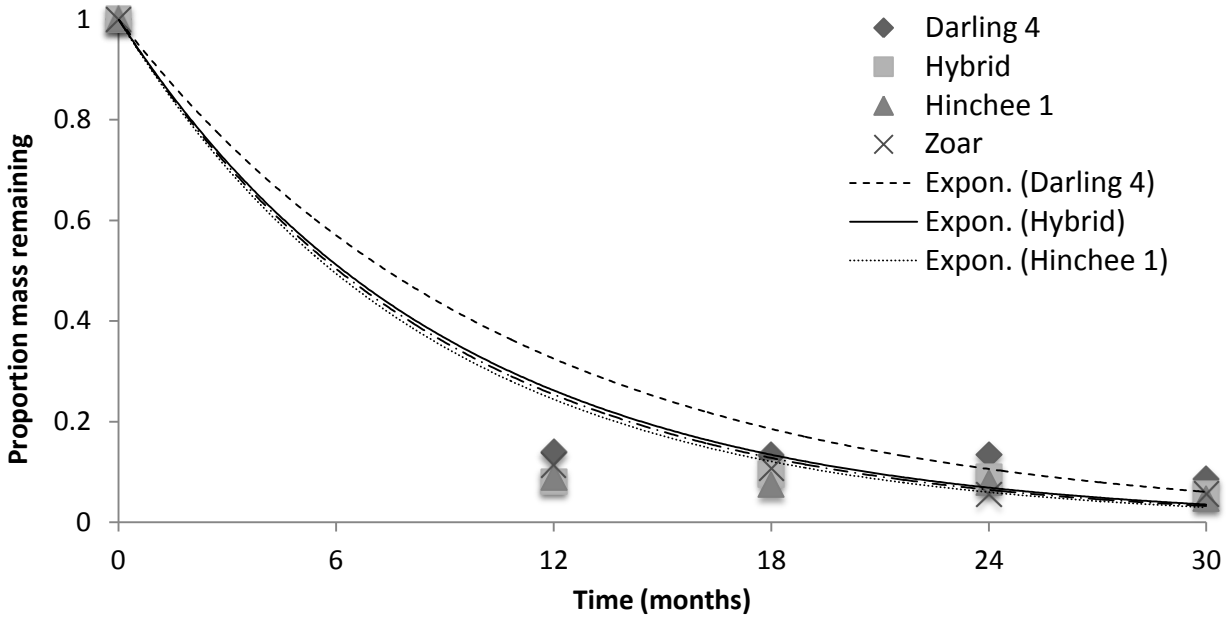


Figure 2.2. Nonlinear model ($Y = e^{-kt}$) fit to proportion of ash-free mass remaining in decomposing transgenic (Darling 4, Hinchee 1), Hybrid and Zoar leaf litter over a 2.5 year period.

Table 2.1. Mean k-values, based on the exponential model $y=e^{-kt}$, for each leaf litter type as ash-free dry weight during 24 months of decomposition (n = 2 initial, n = 6 for 12, 18 and 24 months, n = 12 for 30 months). Numbers in parenthesis represent the error associated with the parameter estimate.

Zoar	Hybrid	Darling 4	Hinchee 1
1.81 (0.11)	**1.98 (0.15)**	**1.48 (0.10)**	**2.04 (0.16)**

[C], [N], and C/N ratios

Throughout the 2.5 year decomposition period, C/N ratios varied among litter types, with Zoar having the highest C/N ratio and Hinchee 1 having the lowest C/N ratio ($p < 0.001$), and decreased over time ($p < 0.001$). There was no interaction between litter type and time for C/N ratio ($p = 0.45$).

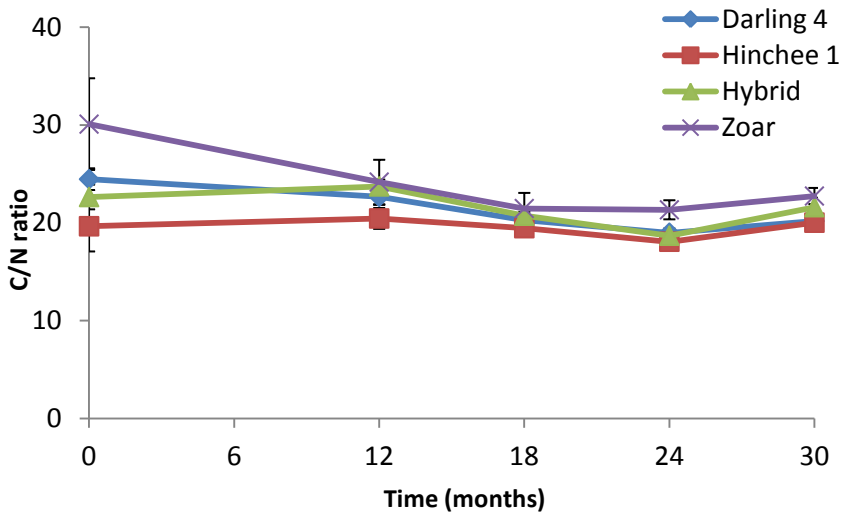


Figure 2.3. Mean C/N ratios for transgenic (Darling 4, Hinchee 1), Hybrid and Zoar leaf litter over a 2.5 year decomposition period (n =2 initial, n = 6 for 12, 18 and 24 months, n = 12 for 30 months). Error bars represent standard error of the means.

Interestingly, C concentrations did not vary among litter types throughout the 30-month decomposition period ($p = 0.68$), but did decrease over time for the first two years and increase

after two years ($p < 0.001$) (Table 2.2). There was no interaction between litter type and time for C concentration ($p = 0.51$). In contrast N concentrations did vary among litter types throughout the 30-month decomposition period ($p = 0.01$). During decomposition, N concentrations decreased for the first two years, and then increased after two years ($p < 0.001$) (Table 2.2). There was no interaction between litter type and time for N concentration ($p = 0.29$).

Table 2.2. Mean carbon and nitrogen concentrations in transgenic (Darling 4 and Hinchee 1), Hybrid, and Zoar leaf litter after 12, 18, 24, and 30 months of decomposition (n = 2 initial, n = 6 for 12, 18 and 24 months, n = 12 for 30 months). Values in parenthesis represent standard deviation from the mean

Litter Type	Time (months)	C (mg/g)	N (mg/g)
Zoar	0	489.1 (2.2)	16.7 (3.7)
	12	359.0 (77.8)	15.4 (4.6)
	18	311.7 (56.2)	15.2 (4.6)
	24	264.4 (29.2)	12.5 (2.1)
	30	404.2 (50.4)	17.9 (2.0)
Hybrid	0	490.8 134.3)	21.8 (2.3)
	12	345.2 (62.7)	14.5 (1.9)
	18	291.8 (54.7)	14.3 (3.5)
	24	259.8 (45.3)	14.0 (2.8)
	30	355.0 (40.3)	16.8 (3.5)
Darling 4	0	486.6 (23.0)	19.9 (1.4)
	12	342.9 (69.7)	15.4 (4.2)
	18	343.0 (38.3)	17.1 (2.5)
	24	297.0 (35.0)	15.7 (2.4)
	30	364.7 (58.6)	18.1 (2.6)
Hinchee 1	0	491.9 (44.7)	25.5 (4.5)
	12	368.8 (56.1)	18.3 (4.1)
	18	333.5 (41.1)	17.1 (1.7)
	24	220.2 (42.0)	12.2 (2.2)
	30	385.1 (51.0)	19.4 (3.3)

Element concentrations

Of the seven elements tested (Ca, Mg, K, P, Mn, Na, and Al) only Ca and P showed a difference among litter types in AFDW (ash-free dry weight) concentration during decomposition (Table 2.3). Throughout the 30-month decomposition period, Ca concentrations

differed with litter type ($p < 0.001$), and changed over time ($p < 0.001$). I also found an interaction between litter type and time for Ca concentration ($p < 0.001$). It is important to note that initial Ca concentrations were almost 2-fold higher in Darling 4 than Zoar and Hybrid litter ($p = 0.028$). Throughout decomposition, Ca concentration increased and then decreased after two years in all litter types except for Hinchee, in which Ca concentration increased, dropped drastically at 24 months and then increased at 30 months. Concentrations of P were found to vary among litter types ($p = 0.035$) and over time ($p < 0.01$). Initial P concentrations showed no significant difference among liter types ($p = 0.24$), however Darling 4 concentrations were 1.5 times higher than Zoar. In all litter types P concentrations decreased in the first year, then increased until 24 months, and then decreased at 30 months of decomposition. No interaction was found between type and time for P concentration ($p = 0.21$).

Concentrations of Mg, K, and Na did not differ among leaf litter types ($p > 0.001$), but did vary over time ($p < 0.05$). No interactions between litter type and time were found among these elements, with the exception of K. Interestingly, although K concentrations did not differ among the litter types at any time point, we did find an interaction between litter type and time ($p = 0.01$). Concentrations of Al and Mn increased over time for two years, and then they decreased ($p < 0.001$). No initial concentrations were taken for Al or Mn, but from 12 to 30 months of decomposition, the concentrations of these elements did not differ among litter type ($p > 0.05$). There was no interaction found between litter type and time for Al and Mn concentrations ($p = 0.70$).

Table 2.3. Mean ash-free concentrations of each element in transgenic (Darling 4 and Hinchee 1), Hybrid, and Zoar leaf litter after 12, 18, 24, and 30 months of decomposition (n = 2 initial, n = 6 for 12, 18 and 24 months, n = 12 for 30 months) . Values in parenthesis represent standard deviation from the mean.

Litter type	Time (months)	Ca (mg/g)	Al (mg/g)	Mg (mg/g)	Mn (mg/g)	P (mg/g)	K (mg/g)	Na (µg/g)
Zoar	0	11.2	-	2.8	-	1.4	7.1	222
		(1.7)		(0.4)		(0.2)	(1.0)	(18)
	12	20.4	5.4	2.5	0.9	1.3	1.8	151
		(2.3)	(4.9)	(0.9)	(0.2)	(0.4)	(0.7)	(72)
	18	22.6	7.4	2.7	1.2	1.9	1.8	285
		(3.2)	(2.6)	(6.6)	(3.0)	(3.3)	(3.9)	(76)
	24	25.2	9.5	3.2	1.4	2.0	2.6	280
		(4.9)	(3.8)	(1.0)	(0.6)	(0.5)	(0.8)	(94)
	30	18.7	4.0	2.4	1.1	1.4	1.3	286
		(3.6)	(2.8)	(0.8)	(0.3)	(1.3)	(0.4)	(106)
Hybrid	0	10.2	-	3.3	-	1.9	6.5	272
		(1.9)		(0.3)		(0.3)	(0.1)	(59)
	12	20.3	4.7	2.7	0.9	1.2	1.9	136
		(2.1)	(2.6)	(0.5)	(0.2)	(0.3)	(0.6)	(36)
	18	22.3	6.8	2.8	1.1	1.7	1.9	302
		(2.8)	(2.7)	(0.7)	(0.1)	(0.4)	(0.8)	(94)
	24	26.5	10.9	3.6	1.3	2.2	3.0	325
		(2.1)	(2.8)	(0.4)	(0.5)	(0.3)	(0.7)	(95)
	30	21.7	5.6	2.8	1.2	1.5	1.5	270
		(4.9)	(1.9)	(0.5)	(0.3)	(0.3)	(0.3)	(93)
Darling 4	0	19.0	-	3.1	-	2.2	8.2	227
		(2.4)		(0.1)		(0.5)	(0.1)	(39)
	12	33.4	4.5	2.6	1.0	1.4	1.7	135
		(6.6)	(2.4)	(0.6)	(0.3)	(0.2)	(0.6)	(49)
	18	33.3	4.6	2.8	1.2	1.7	1.2	221
		(1.4)	(1.1)	(0.5)	(0.2)	(0.4)	(0.2)	(27)
	24	32.3	9.3	3.1	1.5	1.9	2.5	363
		(4.3)	(5.1)	(0.8)	(0.3)	(0.3)	(0.7)	(159)
	30	25.8	5.3	2.8	1.2	1.5	1.4	312
		(3.4)	(2.8)	(0.7)	(0.3)	(0.2)	(0.4)	(109)
Hinchee 1	0	14.9	-	2.9	-	2.5	8.0	308
		(1.1)		(0.2)		(0.8)	(1.3)	(158)
	12	26.7	3.7	2.4	1.0	1.4	1.7	12
		(4.6)	(2.2)	(0.7)	(0.1)	(0.2)	(0.4)	(42)
	18	30.6	5.5	2.6	1.2	1.7	1.5	249
		(4.8)	(1.8)	(0.7)	(0.2)	(0.5)	(0.3)	(31)
	24	19.1	14.7	4.2	1.8	2.3	1.8	291
		(11.1)	(7.0)	(1.3)	(0.3)	(0.4)	(1.0)	(72)
	30	25.8	4.3	2.6	1.3	1.5	1.4	302
		(7.7)	(2.3)	(0.5)	(0.2)	(0.2)	(0.4)	(104)

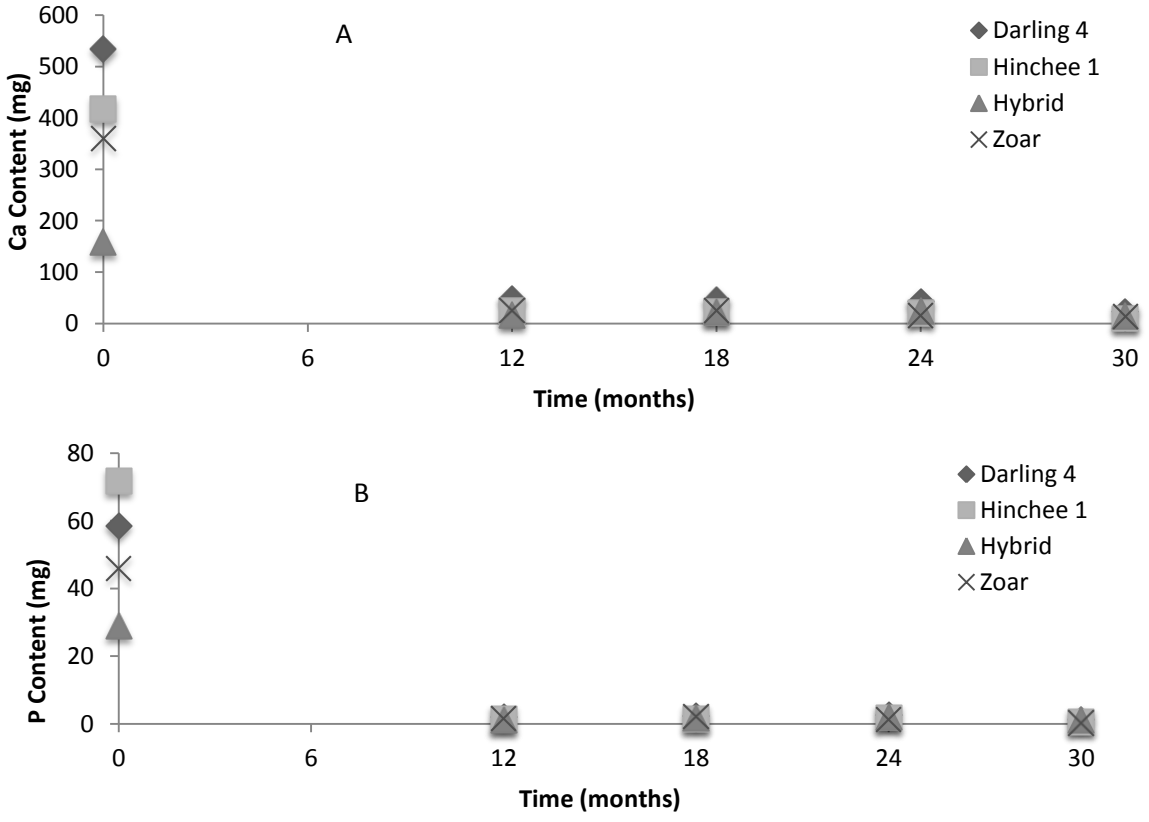
Element Contents

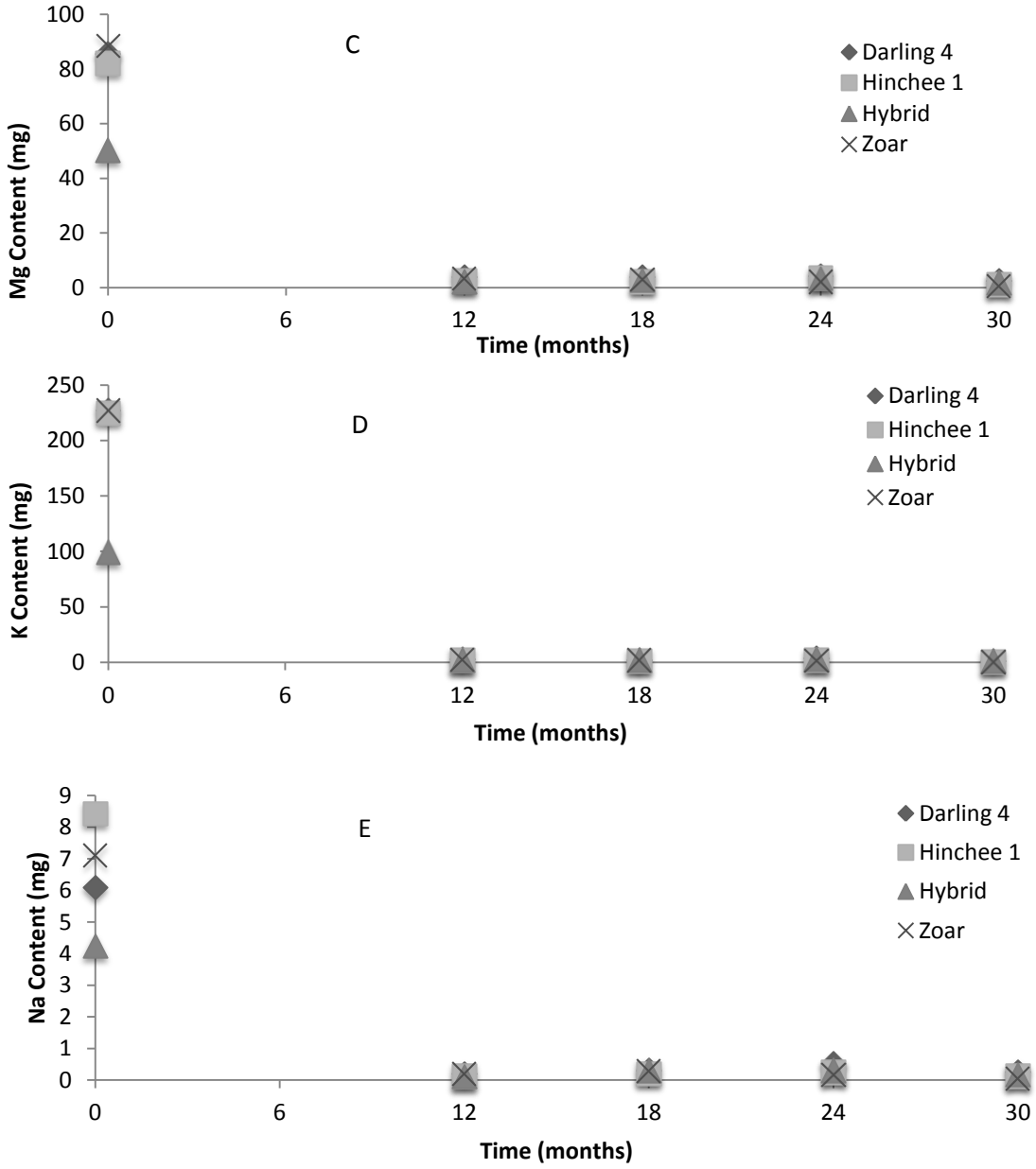
Using the AFDW concentrations discussed previously and AFDW mass remaining, we calculated the content of each element within litter at each time point. More significant differences were seen among litter types in content than in concentration, but the majority of these differences did not appear until 24 months of decomposition. The only element to show differences among litter types at more than one time point was Ca (Figure 2.4, panel A). Throughout the 30-month decomposition period, Ca contents varied among litter type ($p < 0.001$), and decreased over time ($p < 0.001$). We also found an interaction between litter type and time for Ca content ($p < 0.001$). Initially, no significant difference was found among types ($p = 0.16$), although there was an apparent 3-fold difference among types with the lowest and highest Ca content. We also found an interaction between litter type and time for Ca content ($p < 0.001$).

For other elements in which initial element concentrations (Mg, P, K, Na) were measured, contents varied by litter type ($p < 0.001$) and changed over time ($p < 0.001$) (Figure 2.4, panels B-E). There was also an interaction between litter type and time for each of these elements over the decomposition period ($p < 0.001$). Interestingly, few differences among type were found for each element when single time points were analyzed. Contents of Mg, P, K, and Na did not vary by litter type initially, at 12 months decomposition, or at 18 months decomposition ($p > 0.05$). After 2 years, several elements began showing differences in contents among types. At 24 months, the content of K was 2-fold higher in Darling 4 than Zoar and Hinchee 1 ($p = 0.042$); the content of P was 2-fold higher in Darling 4 than in Zoar ($p = 0.049$); the content of Na was 3-fold higher in Darling 4 than Zoar ($p = .033$). Unlike other elements, Mg contents did not differ among litter types at 24 months ($p = 0.15$). At 30 months, the contents of K, P, and Mg were two-fold higher in Darling 4 than Hinchee 1 and five-fold higher in Darling 4

than Zoar ($p < 0.001$); the content of Na was two-fold higher in Darling 4 than Hinchee 1 and four-fold higher in Darling 4 than Zoar ($p < 0.001$).

We did not take initial measurements for Al and Mn, so only contents measured during the 12-30 month period were evaluated. Over this time period, Mn contents increased for two years, and then decreased ($p < 0.001$), and also varied among litter types ($p < 0.001$) (Figure 2.5, panel F). There was no interaction between litter type and time for Mn content ($p = 0.18$). Al contents also increased for two years, and then decreased ($p < 0.001$), but did not vary by type ($p = 0.33$) (Figure 2.5, panel G). There was also no interaction between litter type and time for Al contents ($p= 0.09$).




C
Mg Content (mg)
100
80
60
40
20
0
0
6
12
18
24
30
Time (months)
Darling 4
Hinchee 1
Hybrid
Zoar
D
K Content (mg)
250
200
150
100
50
0
0
6
12
18
24
30
Time (months)
Darling 4
Hinchee 1
Hybrid
Zoar
E
Na Content (mg)
9
8
7
6
5
4
3
2
1
0
0
6
12
18
24
30
Time (months)
Darling 4
Hinchee 1
Hybrid
Zoar

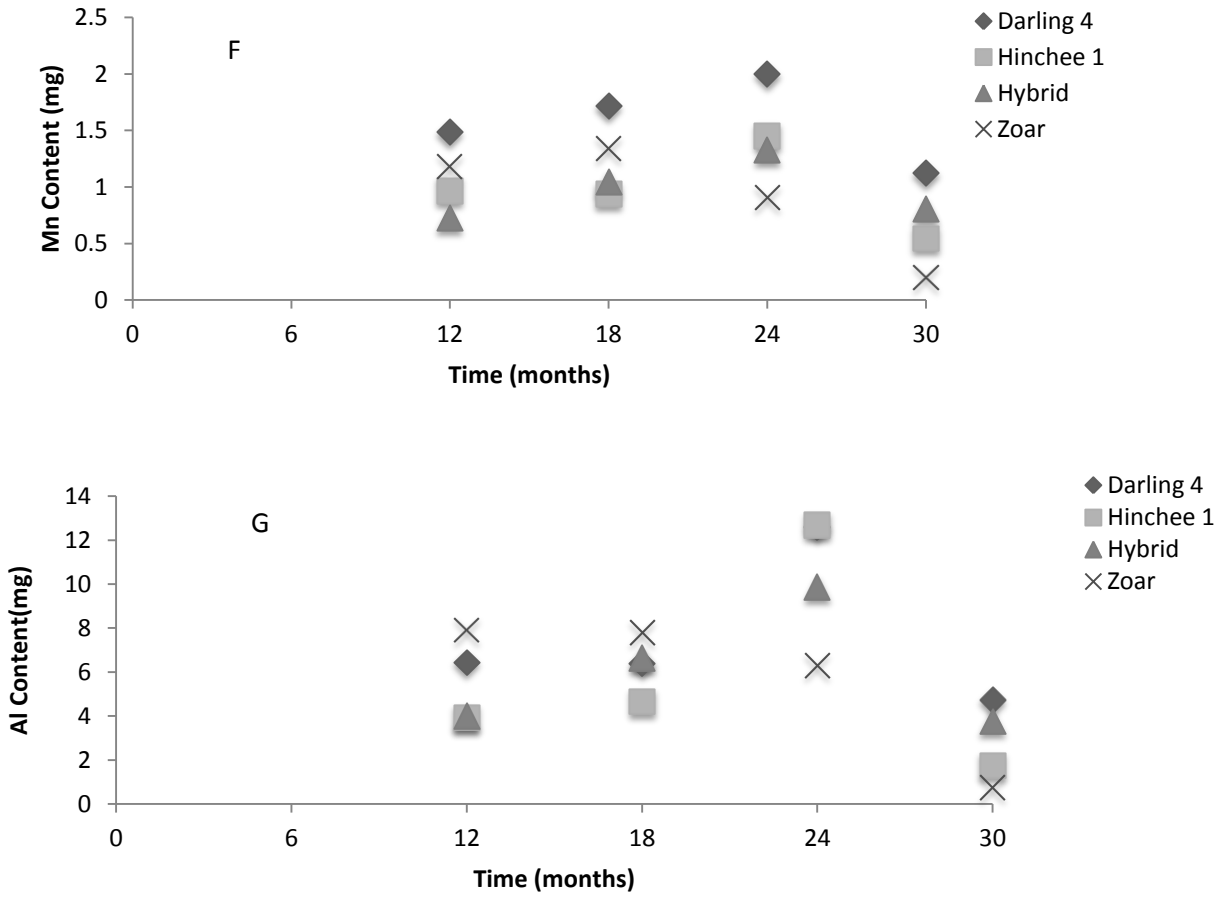


Figure 2.4. Panels A-G show mean contents of Ca, P, Mg, K, Na, Mn and Al, respectively, in decomposing transgenic (Darling 4, Hinchee 1), Hybrid and Zoar leaf litter over a 2.5 year period, excluding initial (n = 6 for 12, 18 and 24 months, n = 12 for 30 months).

Discussion

Mass loss and decomposition rate

During the 30-month decomposition period, leaf litter type had a statistically significant effect on mass remaining, with the difference becoming more pronounced as mass remaining decreased over time. However, there was a greater range in mass remaining between the two transgenic types (Darling 4 and Hinchee 1) than there was between either of the transgenic types and the wild-type, Zoar or the conventionally-bred, Hybrid. The results show more variation

within the two very similar transgenic events than between genetically engineered lines and conventionally bred lines. This is interesting because the transgenic events are very similar in terms of genes inserted. Darling 4 has two copies of the resistance-enhancing gene oxalate oxidase, as well as the gene NPT2, a selectable marker; Hinchee 1 has three copies of the resistance-enhancing gene oxalate oxidase, as well as another resistance-enhancing gene, ESF39 which codes for an antimicrobial peptide, and the gene NPT2. 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.

All litter types in this study had fallen below 20% mass remaining after 12 months of decomposition, indicating that at this time point all types had probably transitioned into late-stage decomposition. Unlike early-stage decomposition, late-stage decomposition is not driven by nutrient concentration, but instead is driven primarily by lignin degradation (Berg and Staaf, 1980). Late-stage decomposition also proceeds very slowly; decomposition rates during late-stage have been shown to approach 0 (Howard and Howard, 1974; Berg and Ekbohm, 1991). At these states of decomposition, white-rot fungi dominate the litter; these fungi use enzymes to break down lignin, as well as tannins, melanins, humic substances, and cutin (Osono, 2007).

Over the 30-month study, the decomposition rates differed slightly among types, with Darling 4 having a lower k-value, suggesting that this transgenic type may decompose more slowly over time than Zoar, Hybrid, and Hinchee 1 litter. There is no obvious reason as to why Darling 4 would decompose more slowly than any other type, especially given that Darling 4 and Hinchee 1 are very similar events, with the exception of Hinchee 1 having the extra resistance-enhancing gene ESF39. Admittedly, the negative exponential model is a relatively poor fit for

these data, overestimating mass remaining at 12 months and underestimating mass remaining at 30 months. Nevertheless, k-values are commonly reported and serve as a means to readily compare mass loss rates among species and systems. The small differences and high variation found in k-values in the current study may not be very informative for comparing mass loss among the four litter types.

There have been several studies of decomposition in transgenic plants under field conditions (Donegan et al., 1997; Hay et al., 2002; Lachnicht et al., 2004; Tilston et al., 2004; Vauramo et al., 2006) but the majority of these studies have focused on fast-growing plant species. For trees, only a few products of genetic engineering have been studied, and results have varied depending on the inserted gene. Decomposition field studies have not yet been done for oxalate oxidase in trees. In a field trial using woody trunk material from poplar (*Populus tremula* and *P. alba*) trees that had modifications in lignin biosynthesis, no difference was found in the rate of decomposition between genetically modified trees and wild-type trees (Tilston et al., 2004). A study of transgenic chitinase silver birch (*Betula pendula*) trees under field conditions similarly found no difference in decomposition rate between the transgenic types and the wild-type (Vauramo et al., 2006). However, a study using similar chitinase silver birch trees in a microcosm experiment found transgenic leaves to have a significantly higher decomposition rate than the non-transgenic controls (Kotilainen et al., 2005). Based on the available literature dealing with genetic engineering using lignin-and chitin-altering genes, the effects on leaf litter decomposition appear to differ even among the same tree types, indicating that there are other factors influencing the decomposition rate. It is also important to note that the effects of genetically engineered plants will vary depending on the genes that are altered or inserted and how they function in the plant. Finally, it is also possible that the differences seen in the

microcosm experiment and our experiment could be attributed to natural variation in leaf litter quality and chemical composition within the species. Differences found among genotypes of a single species in leaf litter chemistry and decomposition have increasingly been reported (Schweitzer et al., 2005; Madrich et al., 2006; Lecerf and Chauvet, 2008), indicating that genetic variability may play a significant role in decomposition and nutrient cycling.

Little work has been done on the decomposition of leaf litter from American chestnut trees, which have been absent as a dominant canopy species in their native forests for decades, making it difficult to investigate natural variation in the species. A field study comparing the mass loss of wild-type American chestnut and a hybrid chestnut line to other hardwood species found mass remaining to be around 50% for wild-type and hybrid chestnuts after one year of decomposition (Rosenberg, 2010). All litter types used in our study had a much lower mass remaining after one year, due to unexpectedly rapid decomposition at our field site. There are many possible reasons for altered litter decomposition rates. Like many forests in North America, our site is predicted to be inhabited by non-native earthworms (though none were found in the bags), which have been found to significantly increase decomposition rates as measured in a temperate mixed forest in NY (Ashton et al., 2004). Environmental conditions in the soil such as moisture, temperature, and organic matter content can also influence decomposition rates (Tilston el al., 2004). Finally, the relatively high light environment at our site, a shelterwood, could be influencing decomposition; leaf litter decomposition rates have been found to be significantly higher in *Jatropha curcas* leaves in an open canopy environment compared to a closed canopy environment (Abugre et al., 2011).

C/N ratio

Initial C/N ratios did not differ among litter types but ranged from 20:1 in Hinchee 1 to 30:1 in Zoar. After 12 months, no difference was found among litter types and the C/N ratios ranged from 20:1 in Hinchee 1 to 24:1 in Zoar. At 18 months, no difference was found among litter types and the C/N ratios ranged from 19:1 in Hinchee 1 to 21:1 in Zoar. After 24 months, Zoar litter had a significantly higher C/N ratio at 21:1 compared to litter from hybrid and transgenic trees, with C/N ratios between 18:1 and 19:1, and after 30 months, Zoar litter had a significantly higher C/N ratio at 23:1 compared to litter from hybrid and transgenic trees, with C/N ratios between 20:1 and 22:1. The initial C/N ratios recorded for wild-type American chestnut and a 7/8 hybrid chestnut in a previous study were found to be 37:1 and 39:1, respectively (Rosenberg, 2010). Given the large range in ratios seen between our initial wild-type (30:1) and another wild-type (37:1), it can be argued that the American chestnut species may have a wide range of litter C/N ratios due to intraspecific variation.

The recent literature regarding C/N ratios of transgenic trees shows similar results to our first two time points, in that there is no difference among transgenic and wild-type litter varieties. For example, transgenic chitinase silver birch leaf litter showed no difference in C/N ratio compared to wild-type silver birch after 11 months of decomposition (Vauramo et al., 2005), which implies that the chemical composition of the litter is unchanged in transgenic varieties expressing these genes. More research on litter C/N ratios is needed for transgenic trees, especially if they are expressing genes that could alter the C or N composition of leaves.

Generally, C/N ratios in leaf litter have been found to be negatively correlated with N mineralization and nitrification (Wedin and Tilman, 1990; Hobbie, 1992; Finzi et al.,1998),

which are important in making N more available to the ecosystem. Microbial decomposers release N from litter when their own N requirements have been met. When the C/N ratio is low (high N concentration), decomposers will have their N requirements met directly from the litter, and will thus begin to release N from the litter. During the decomposition process, C/N ratios typically decrease even throughout late-stage decomposition (Gosz et al., 1973; Fioretto et al., 2005). As expected from these previous studies, the C/N ratio in all litter types in our experiment decreased at each sampling time point.

Within the forest floor and topsoil layers, tree species is thought to be the primary driver of C/N ratios. On average, the majority of C/N ratios in European forest soils were found to be between 16:1 and 44:1 in forest floor layers; the lowest C/N ratios were found under N-fixing tree species and the highest C/N ratios were found under evergreen species (Cools et al., 2013). Since transgenic and hybrid chestnuts will be planted into mixed forests, slight differences in C/N ratios may not have any more impact on the ecosystem than planting pure wild-type American chestnuts. Given the large range in C/N ratios across tree species and the range in initial C/N ratios seen between our wild-type chestnut litter and reported by Rhoades (2007), the small differences between wild-type litter and the other 3 types may not be biologically meaningful; these small differences are not likely to alter ecosystem dynamics.

Element concentrations and contents

Out of the 7 elements analyzed (Ca, P, K, Mg, Mn, Na and Al), only Ca and P concentrations differed among litter types throughout the 2.5 year field trial, with Ca concentration increasing and then decreasing after two years in all litter types except for Hinchee 1, in which Ca concentration increased, dropped drastically at 24 months, and then increased

again at 30 months. With regards to contents, Ca was the only element to show differences among litter types at more than one time point, with Darling 4 having about a 2-fold greater Ca content compared to other litter types. The decomposition results reflect the initial concentrations (Darling 4: 19.0 mg/g Hinchee 1: 14.9 mg/g, Hybrid: 10.2 mg/g, Zoar: 11.2 mg/g) with Darling 4 having higher Ca concentrations. Thus, it can be argued that the initial litter concentration of calcium in the leaves, not differences in Ca mineralization, was the main driver for the differences in Ca over time. While Ca is more abundant in transgenic leaves, the oxalate oxidase transgene is not the likely cause. Oxalate oxidase breaks down oxalic acid, which is naturally produced by plants to bind Ca and form Ca-oxalate crystals. If there were to be any effect on Ca caused by the transgene, it would be a reduction in Ca, due to the rapid loss of Ca ions from litter. It is possible that insertional effects could account for a change in Ca uptake, but it is very unlikely that the same insertional effect would occur in two separate transgenic events (Darling 4 and Hinchee 1), as insertional effects that lead to phenotypic changes do not occur often (Schnell et al., 2015).

In a study on decomposing leaf and branch litter, Ca was found to be less susceptible to leaching than other elements, and to be correlated with mass loss, consistent with its importance as a structural component of leaves (Gosz et al., 1973; Dijkstra, 2003). Different tree species have been found to have different Ca mineralization rates, which may affect nutrient balances in the soil. For example, calcium mineralization rates in the forest floor per amount of decomposed litter have been found to be significantly higher in sugar maple (*Acer saccharum*) and white ash (*Fraxinus americana*) than in American beech (*Fagus grandifolia*), red maple (*Acer rubrum*), red oak (*Quercus rubra*), or Canadian hemlock (*Tsuga canadensis*) (Dijkstra, 2003).

Mineralization rates, however, may also be influenced by soil type. A study focusing on foliar analysis of American chestnut and mixed deciduous tree plots found Ca concentrations in the O layer below American chestnut trees to be 15.9 mg/g on Greenridge silt loam, lower than concentrations found under mixed canopies on the same soil (18.2 mg/g). However on Council sandy loam, concentrations of Ca in the O layer were found to be 15.2 mg/g under American chestnut, higher than concentrations found under mixed species plots on the same soil type (14.8 mg/g) (Rhoades, 2007), which implies that soil type may play a significant role in Ca mineralization for mixed species plots, while seemingly not having much of an effect on pure American chestnut litter.

The elements Mg, K, P, and Na decreased significantly in content throughout the 30-month decomposition period. This agrees with the current literature. For example, K and Mg have been observed to largely decrease in content within the first few months of decomposition due to rapid leaching (Gosz et al., 1973). Our measurements only account for contents after one year of decomposition, so we cannot determine how quickly K and Mg were lost, but by 12 months, the contents of K, Mg, P, and Na are approaching 0, and do not change much between 12 and 30 months. Therefore, we deduce the genetic engineering of American chestnut did not have a meaningful effect on the mineralization of plant materials, at least during late-stage decomposition.

Between 12 and 24 months Mn and Al contents increased. Leaching has been hypothesized to be an important removal process for Mn in leaf litter, due to the water-soluble form that is present in leaf litter (Tukey, 1970). Soil organisms may release compounds that convert Mn in leaf litter to an insoluble form (Alexander, 1967) which could explain an increase in concentration, but not content. Increasing contents of Mn and Al in our litter bags could be

coming from soil particles within the litterbags. As the tenth most abundant element in the earth's upper continental crust, the average concentration of Mn found in the surface layer (0-20cm) of soils in the U.S. is 560 mg/kg (Shacklette et al., 1971). As the third most abundant element in the earth's crust, the typical range of Al abundance in U.S. soils is between 10,000 and 300,000 mg/kg (Dragun, 1988). Therefore, increasing contents of Al and Mn in our litter and high ash proportions can be attributed to mineral soil particles.

Conclusion

The results of this study show only a small variation in the rate of litter mass loss over the 2.5- year *in situ* incubation period between transgenic American chestnuts and Zoar and hybrid chestnuts, at least during late stage decomposition, which is consistent with other studies reporting minimal variation (Vauramo et al., 2006; Tilston et al., 2004). Taken together, these studies suggest that the process of genetic engineering using a selected transgene does not have any measurable effect on the mineralization of elements examined. However, depending on the transgene itself and what it expresses in the transformed plant, as well as where it is inserted in the genome, potential effects on decomposition cannot be ruled out.

Intraspecific variation within a tree species may have greater effects on decomposition rate than genetic engineering within a single genotype (Tilston et al., 2004). The natural intraspecific variation among American chestnut genotypes in traits such as decomposition rate and nutrient concentration has yet to be explored, but could account for the differences seen in this study. To accurately assess natural variation, future studies should compare decomposition and nutrient mineralization rates of multiple wild-type genotypes and transgenic events. Since the first sampling point of the study (12 months) proved to be too late to observe characteristics

of early-stage decomposition, future studies should begin sampling earlier. To observe the initial leaching of nutrients from the litter, future sampling should be initiated after one month of incubation.

If granted federal approval, the American chestnut will be the first genetically modified 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 fungal diversity, nematode diversity, and the release of other important nutrients, so that informed decisions can be made on their deregulation status. 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*).

Chapter 3: The impact of transgenic American chestnut (*Castanea dentata*) leaf litter on fungal diversity

Abstract

Once chestnut blight had nearly eradicated the American chestnut (*Castanea dentata* (Marsh.) Borkh.) from its native range, disease-resistant transgenic American chestnut trees were developed for the purpose of reintroducing this economically and ecologically important species into its native forests. Before releasing genetically engineered plants into the environment, federal regulations require that it be equivalent to wild type plants in ecological function. This study aims to determine if there is a significant difference in the diversity of fungi colonizing leaf litter of transgenic American chestnuts and wild-type American chestnuts. Litterbags were filled with litter from two transgenic events and a wild-type American chestnut line, and were placed on top of the mineral layer of soil for 5 months. Results indicate that one of the transgenic events had a slightly lower diversity of fungal species than other litter types, but that the community structures were similar among litter types; after 5 months all litter types were dominated by ectomycorrizal fungi. Overall, the process of genetic engineering using the transgene oxalate oxidase does not appear to have any measureable effect on the diversity of fungi that colonize leaf litter. Upon future approval of federal deregulation, the American chestnut will be the first genetically modified non-orchard tree to be deregulated and environmentally released, which will make the process easier for the genetic engineering of other threatened native tree species in the future

Keywords: saprotrophic fungi, ectomycorrhizal fungi, transgenic trees, nutrient cycling

Introduction

American chestnut was once a widespread, dominant tree species throughout the deciduous forests of eastern North America, but is currently described as functionally extinct (Griffin, 2000). Almost all American chestnut trees that exist today are susceptible sprouts that originated from blight-infected trees (Jacobs, 2007). There have been several attempts to create a blight-resistant American chestnut tree to introduce into its native range. One method is the development of genetically engineered American chestnut trees that contain a blight-resistance

gene. In order to plant a genetically engineered tree in the wild, federal regulations from USDA APHIS, EPA, and FDA require that it be ecologically equivalent to traditional species and pose no threat to the environment or other species (Sedjo, 2010). American chestnut, like other members of the Fagaceae family, forms symbiotic associations with ectomycorrhizal (EM) fungi of both Basidiomycota and Ascomycota (Bauman, 2010). The fungal symbionts of the American chestnut are similar to those of red oak (Dulmer et al., 2014) which means that these symbionts are still abundantly present in the soil ecosystem While some research has been done on the colonization of American chestnut roots by mycorrhizal fungi (Tourtellot, 2013; Dulmer et al., 2014; D'Amico et al., 2015), no work has been done on the fungi that colonize and break down its leaf litter.

Leaf litter decomposition plays a crucial role in maintaining site fertility and productivity in forest ecosystems (Prescott, 2005). The change in leaf litter quality during decomposition triggers a general successional pattern of fungal functional groups. Functional groups that are involved in decomposition consist of litter-decomposing fungi, wood-decomposing fungi, and mycorrhizal fungi. After fresh leaves have fallen onto the soil, bacteria and saprotrophic Ascomycetes are generally first to colonize the litter, and continue to dominate the litter throughout the early decomposition stage. The Ascomycetes are able to break down the less recalcitrant components of litter, such as sugars and sometimes cellulose, in order to obtain carbon for energy. The late stage of decomposition is characterized by litter that is high in lignin (a recalcitrant substance), and low in nitrogen. The bacteria and Ascomycetes cannot breakdown lignin, and so saprotrophic Basidiomycetes become dominant during the later stage, followed by EM Basidiomycetes (Voříšková and Baldrian, 2013). Litter-decomposing lignolytic Basidiomycetes use enzymes to break down lignin, as well as tannins, melanins, humic

substances, and cutin (Osono, 2007). The decomposition of lignin is correlated with accumulating C and N in soil, which has an impact on long-term ecosystem functioning (Amundson, 2001; Berg and McClaughtery, 2003; Lal, 2005).

Saprotrophic fungi are generally placed into one of two groups: white-rot fungi and brown-rot fungi. This study focuses solely on white-rot fungi, which produce lignin-degrading enzyme, typically found in leaf litter. Brown-rot fungi do not produce any lignin-degrading enzymes and therefore are seldom found in leaf litter, but are most commonly found as decomposers of decaying wood (Goodell, 2003). To date, 31 genera of white-rot fungi have been determined to be decomposers of leaf litter and forest floor materials; this includes 23 Basidiomycetes and 8 Ascomycetes. Basidiomycetes are accepted to have greater lignolytic properties than Ascomycetes in general (Osono and Takeda 2002; Osono and Takeda, 2006). The most common Basidiomycete lignolytic genera consist of *Clitocybe*, *Collybia*, *Marasmius*, and *Mycena* (Osono, 2007). It is generally thought that phenol oxidase and manganese peroxidase (MnP) are the primary enzymes responsible for lignin degradation by litter-decomposing Basidiomycetes (Steffen, 2003). The activities of these two enzymes have been found to vary with leaf litter species (Kourtev et al. 2002; Sinsabaugh et al., 2002; Allison and Vitousek, 2004).

While the role of saprotrophic fungi in leaf litter decomposition has been relatively well-studied, the role of ectomycorrhizal fungi (EM) in litter decomposition has received relatively little attention. Studying EM fungi as a distinct group can be difficult because EM fungi do not share a single common ancestor, but instead have evolved independently from lineages of saprotrophic fungi at least eight times with angiosperms and between six and eight times with conifers (Hibbett and Matheny, 2009). Few mycorrhizal genomes have been sequenced to date,

however, the first two EM genomes sequenced (*Laccaria bicolor* and *Tuber melanosporum*), show a major loss of genes coding for plant cell wall-degrading enzymes (PCWDE), which include enzymes needed to break down lignin during decomposition (Kohler et al., 2015). The loss of PCWDE genes is thought to have been a critical driver for symbiosis, as the fungi would appear to be less of a threat to the plant if these genes were no longer present (Plett and Martin, 2011). Although many PCWDE genes were lost during the transition to symbiosis, EM fungi have been found to have retained unique sets of PCWDE genes (Kohler et al., 2015).

It has been hypothesized that the retention of unique PCWDE genes allow EM fungi to "mine" the material that is released during litter decomposition in order to obtain nutrients for the host plant (Plett and Martin, 2011). It is also thought that by retaining some PCWDE genes, EM fungi assure their survival on leaf litter in cases where they are not able to find plant hosts (Martin et al., 2008). A preliminary search through 28 EM gene families found that on average, EM fungi retain about 62 PCWDE genes, while white-rot saprotrophs have an average of 133 and brown-rot saprotrophs (which have the ability to degrade cellulose, but not lignin) have an average of 81. The evolution of EM fungi and brown-rot fungi are similar, with both lineages losing much of the white-rot capability of ancestral fungi. Interestingly, not all EM species have lost the same sets of genes, and some EM fungi have been found to possess certain unique lignocelluloses-degrading genes (Kohler et al., 2015). Therefore, current evidence does not point to a clear decomposition mechanism for all EM fungi, but rather to unique mechanisms possibly depending on ancestral lineages.

During the succession of functional groups during leaf litter decomposition, EM species have been found to dominate during late-stage decomposition, three to five years after litter fall (Lindahl et al., 2007). One possible way to explain the late-stage dominance is that EM fungi are

weaker competitors than saprotrophic fungi for organic substrate, and there is therefore less competition with saprotrophic fungi for substrate at that time. Another scenario is that saprotrophic fungi consume most of the labile carbon in litter and are less competitive than EM fungi for the remaining nitrogen held up in more recalcitrant substances in the litter. Both scenarios may be occurring to influence the fungal groups on the litter over time. Some EM fungi have actually been found to be able to successfully compete with saprotrophic fungi for organic nitrogen (Schimel and Bennet, 2004; Hobbie and Hobbie, 2008), and the competition is aided by direct access to carbon from a plant host. For example, the EM genera *Cortinarius*, *Piloderma*, and *Tricholoma* are thought to be able to obtain organic nitrogen particularly well, due in part to their ability to use proteins as their sole nitrogen source, and possibly through the production of unique enzymes that allow for the harvest of organic N (Lilleskov et al., 2011).

With evidence for EM degradation of litter by brown-rot and white-rot mechanisms, but genomic studies showing major losses in genes involved in these processes, more information is needed in order to elucidate the ecological function of EM fungi during leaf litter decomposition. With unique sets of genes coding for decomposition conserved in different EM species, it is best not to group all EM species together, but rather to treat each species as a unique decomposer.

In this study, 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 (chapter 2) has demonstrated that the litter quality of each type is similar during 2.5 years of *in situ* decomposition in almost every aspect. We harvest hyphae from the litter bags and identify the fungi using PCR-based methods (Gardes and Bruns, 1993; Horton and Bruns, 2001; Schoch et

al., 2012). We aim to discover more information about the fungal species that decompose American chestnut leaf litter and to determine if transgenic litter is ecologically equivalent to wild-type in the diversity of fungal colonizers via diversity indices and species richness.

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.

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 3.1), 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 3.2), 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 3.1, 3.2 and 3.3), therefore expected richness estimates should be interpreted with caution.

Table 3.1. 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 et 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 (Tourtello tet 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 3.2. 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)

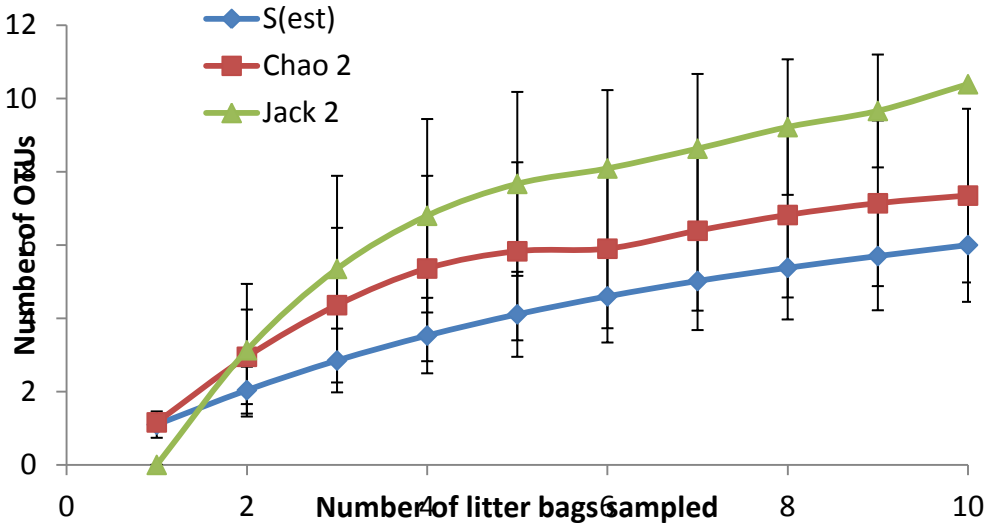


Figure 3.1. Rarefaction curve with Chao 2 and Jackknife 2 estimates for Zoar litter. Error bars represent computed standard deviation in 100 randomized runs.

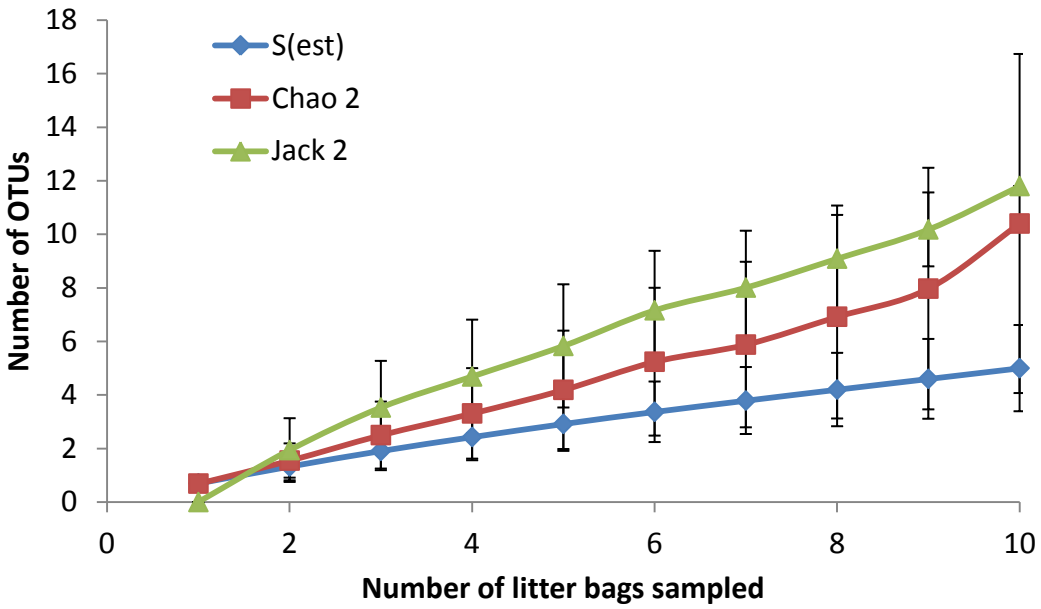


Figure 3.2. Rarefaction curve with Chao 2 and Jackknife 2 estimates for Darling 4 litter. Error bars represent computed standard deviation in 100 randomized runs.

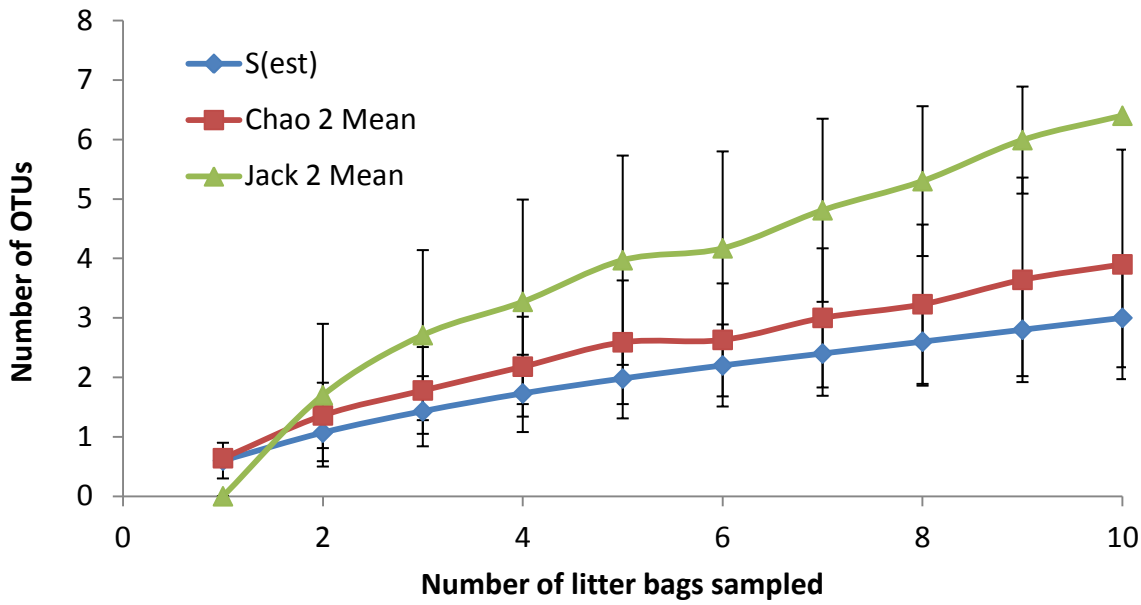


Figure 3.3. Rarefaction curve with Chao 2 and Jackknife 2 estimates for Hinchee 1 litter. Error bars represent computed standard deviation in 100 randomized runs.

Diversity characteristics

Using Estimate S software, Shannon and inverse Simpson indices were estimated for each litter type community using 100 randomized runs (Table 3.2). Based on the Shannon index, I found that the diversity of the Zoar community was 16% higher than the community of Darling 4 and 50% higher than the community of Hinchee 1, and 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, I found that the Zoar community was 24% more diverse than the community of Darling 4 and 50% more diverse than the community of Hinchee 1, and that 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 3.3). *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 %.

Table 3.3. 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%

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 3.4). Darling 4 litterbags were found to have 72.7 % EM fungal samples and 27.3 % saprotrophic samples (Figure 3.5). Hinchee 1 litterbags had 70.0 % EM fungal samples and 30.0 % saprotrophic fungal samples (Figure 3.6).

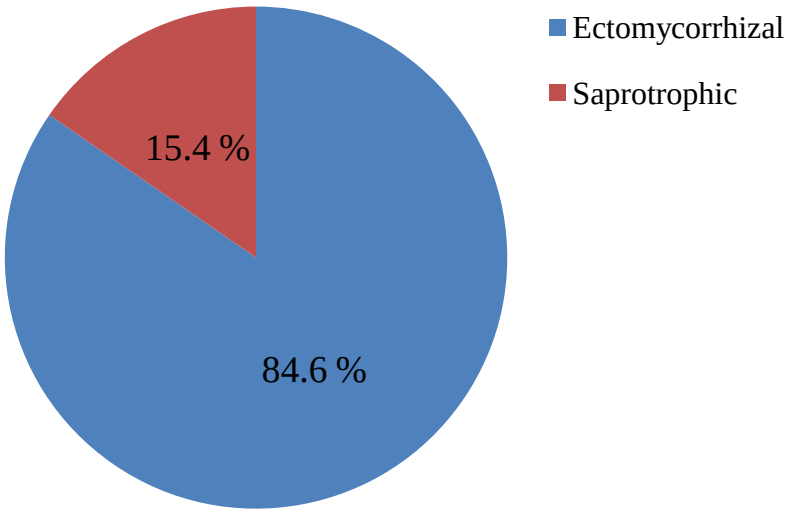


Figure 3.4. Percentage of EM and saprotrophic fungal samples in Zoar litterbags after five months of decomposition (n=10).

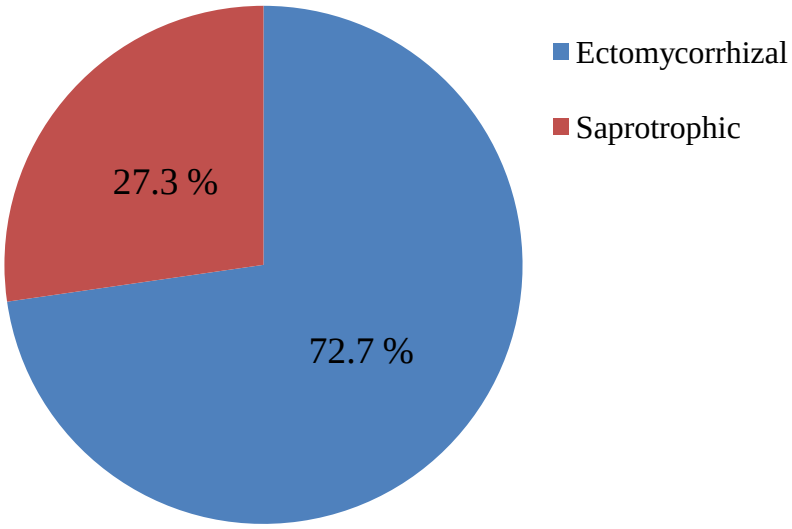


Figure 3.5. Percentage of EM and saprotrophic fungal samples in Darling 4 litterbags after five months of decomposition (n=10).

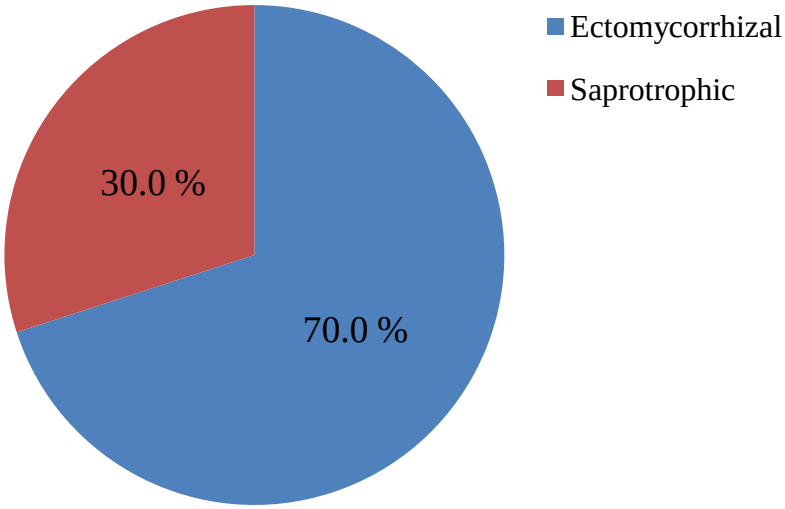


Figure 3.6. Percentage of EM and saprotrophic fungal samples in Hinchee 1 litterbags after five months of decomposition (n=10).

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.

Discussion

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 (Voříšková 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 et al., 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 (chapter 2), 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 (Voříšková 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 (Voříšková and Baldrian, 2013). Interestingly, EM fungi have also been found to dominate leaf litter during late stages of decomposition (Voříšková and Baldrian, 2013; Lindahl et al., 2007).

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 (Voříšková 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 (chapter 2), 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 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*).

CHAPTER 4: SUMMARY

The results of the experiments presented in this thesis provide information on the ecology of transgenic American chestnut (*Castanea dentata*) trees that may be deregulated and introduced into forests in the future. No major consistent differences were found among leaf litter types in the decomposition rate or chemical composition of the litter, except for the nutrient calcium, with the transgenic litter generally having more calcium than Zoar and Hybrid litter. Because the transgenic trees were developed from a different wild-type genotype than the one used in this study, it is possible that the difference in calcium concentration reflects natural variation within the species. More experiments are needed in order to determine the natural variation in chemical composition within American chestnut leaf litter.

When investigating the diversity of fungi colonization, differences were found among leaf litter types when using interpolation for 100 replications in Estimate S software. Hinchee 1 leaf litter was less diverse than Darling 4 and Zoar litter. A previous study using this transgenic event also found lower fungal colonization rates on roots of seedlings in the first year of planting when compared to other transgenic events and wild-type trees. It is possible that the ESF39 antimicrobial peptide is having a negative effect on the fungal community. More research is needed in order to determine if this gene could also have a negative effect on other communities in the forest.

If granted federal approval, the transgenic American chestnut will be the first genetically engineered non-orchard tree to be de-regulated and environmentally released. I believe that more information is needed on the potential ecological effects of transgenic American chestnuts on forest ecosystems, especially on transgenic events containing the antimicrobial gene ESF39.

Future studies should examine herbivory patterns, nutritional content of chestnuts, and the diversity of earthworms and bacteria on the litter, so that informed decisions can be made on the deregulation status.

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Appendix 1: Policies leading to commercializing transgenic trees

Introduction

The demand for wood products has traditionally been met through harvesting trees from natural forests, but this approach is being challenged by the novel idea of planting trees like agricultural crops. Many orchard and ornamental trees have been manipulated by humans for hundreds to thousands of years, but planting trees for timber has only been around for the last few decades. Therefore, unlike many agricultural crops, trees have only been partly domesticated in the last 50 years (Sedjo, 2004). Forest biotechnology, which includes genetic engineering, provides many incentives for the improvement of planting stock through the addition of desired traits. The genetic engineering of forest trees has the possibility to increase productivity, increase product quality, expand the range and types of land and climatic conditions under which planted forests can survive (Sedjo, 2004), and to bring back lost tree species, like the American chestnut, which exists as functionally extinct because of an invasive fungus.

The United States Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS), US Food and Drug Administration (FDA), and US Environmental Protection Agency (EPA) require that a series of steps to be followed in order for transgenic American chestnut trees to be introduced into forests within its native range. This chapter will discuss the rise of transgenic crops and trees in the Unites States and the policies surrounding their release. It explains the entire process from field trials to commercialization. This information may prove useful to the American Chestnut Restoration Project after the field trials for the transgenic American chestnut trees have been completed and de-regulation becomes the next objective. It should also be useful to other researchers who want to develop genetically modified trees of other species in the future.

History of transgenic regulation

A transgenic plant is defined as a genetically modified plant, whose genome has been altered by the insertion of a gene using a nonsexual approach. In the United States, transgenic plants are technically subject to regulation if the plant has the potential to be a plant pest. In practice, however, all transgenic plants are automatically subject to regulation in the United States (Sedjo, 2004). Transgenic crops have become increasingly important in U.S. agriculture. As of 2003, transgenic crops were grown on more than 100 million acres in the U.S. and the vast majority of soybeans and cotton grown in the U.S. were genetically modified (Pew, 2003). Before these crops gained such a large foothold in American agriculture, they were approved by the various federal agencies that oversee transgenic plants. The regulatory process that authorizes the planting of transgenic trees has only existed for 40 years, and will most likely evolve over time.

The beginning of transgenic plant policy occurred in October of 1974, when the National Institute of Health (NIH) established the Recombinant DNA Advisory Committee (RAC) in response to public concerns surrounding the safety of DNA manipulation techniques. The RAC developed a set of guidelines, published in 1976, which are periodically revised. The purpose of the guidelines is to describe practices that will prevent accidental release or exposure to either genetically modified organisms (GMOs) or recombinant DNA. These guidelines are voluntarily followed by industrial scientists worldwide, but are mandatory for any research that receives support from the NIH (Mackenzie, 2000).

The first environmental release of a GMO in the United States occurred in 1983 after approval by the National Institutes of Health (NIH). The release was a field test of “ice-minus”

bacteria that could prevent frost damage to strawberries (Mackenzie, 2000). The NIH approval fueled controversy and prompted several court cases. The attention surrounding the genetically modified bacteria encouraged a White House committee formed under the Office of Science and Technology Policy (OSTP) to develop a plan to federally regulate biotechnology. The Coordinated Framework for the Regulation of Biotechnology (CFRB) was published by the OSTP in 1986 to serve as this plan. The framework is based on the principle that the techniques involved in biotechnology are not risky, and so biotechnology should not be regulated as a process, but that the products of biotechnology should be regulated in the same way as products of other technologies (OSTP, 1992).

The 1986 CFRB created a complex set of rules using the regulatory authority of three agencies: EPA, FDA, and USDA APHIS. The EPA regulates plant-incorporated protectants (PIPs) which include pesticides produced by plants, and the genetic material needed for the plant to produce the substance (Sedjo, 2010), and also assesses the general environmental safety of GMOs through the National Environmental Protection Act (NEPA; Public Law 91-190, with several amendments). The FDA is responsible for determining human and animal food safety and wholesomeness of all plant products including genetically engineered crops, and it bases its approach on the concept of 'substantial equivalence' between the genetically engineered plant and conventionally bred or wild-type plants (FDA, 1992).

USDA APHIS is responsible for any genetically engineered organism or product that could pose a risk to an agricultural plant. Annual and perennial plants, including crops and trees, are regulated largely by APHIS, except where food, drugs, or PIPs are involved (Sedjo, 2010). USDA APHIS gained control over GMOs through The Plant Protection Act of 2000, which superseded the agency's authority under previous acts, such as the Federal Plant Pest Act (Sedjo,

2004). The Plant Protection Act extended the authority of APHIS to regulate noxious weeds and made the agency responsible for determining direct or indirect injury or damage (Sedjo, 2010). In addition, the Biotechnology, Biologics and Environmental Protection (BBEP) unit of the USDA APHIS is charged with investigating any environmental impacts of genetically engineered plants under revised regulation 7 CFR Part 340.

The USDA APHIS/EPA regulation of the environmental release is based on the concept of 'familiarity' (OECD, 1993) which can be seen as the counterpart to 'substantial equivalence.' Familiarity considers whether the modified plant is comparable to its traditionally bred counterpart in environmental safety. It includes the biology of the plant species, the trait introduced, and the agricultural practices and environment used for crop production. A related concept is 'antecedent organism', which is when an organism has already been evaluated and is familiar, future assessments of that organism can be more lenient.

The regulatory process

For a transgenic plant in the United States to be grown in large, commercial operations, the plant must first be federally deregulated (Sedjo 2004). The responsibility for deregulating transgenic plants belongs to the USDA APHIS and other government agencies. In order to receive a permit from APHIS for the deregulation of a genetically engineered plant, APHIS must first come to a Finding of No Significant Impact (FONSI) (Mackenzie, 2000). BBEP reviews about 1000 applications a year for field testing and deregulation (National Academy of Science, 2002). A review takes an average of 10 months if it does not include the notification process. If the EPA needs to approve the genetically engineered plant, their reviews take about 18 months (CAST, 2000).

Once a developer of a transgenic plant obtains a permit from APHIS for the importation, interstate movement, or release of the genetically engineered plant into the environment, the developer must notify APHIS that field-testing is about to begin. The developer must submit a petition for deregulation to APHIS detailing the results of the field test and a literature review, along with any other relevant information (Sedjo, 2004). The developer of a transgenic plant uses field-testing to assess the safety and environmental implications of the modified plant. As of 2004, APHIS has authorized thousands of field tests for more than 50 plant species, most of which are agricultural crops, and many of which have since been deregulated. After the developer receives a permit from APHIS, APHIS will authorize the developer to gather information through field-testing including statistical analysis, laboratory tests, and literature reviews to confirm that the transgenic plant is as safe to the environment as traditional varieties of that plant. Transgenic perennials such as trees must be field-tested for more than a year to ensure that the testing reflects the behavior of the plants over a multiyear time span, and to evaluate the potential for genetic escape into the environment and the ability for the trees to interbreed with related natural plants (Sedjo, 2010).

After the required information is collected from field trials, the developer can petition APHIS to make a "Determination of Non-regulated Status". Upon receiving the petition, a team of APHIS scientists reviews the information and APHIS will then make a public announcement that the petition has been received. Upon completion of the review, APHIS publishes the "Determination of Non-regulated Status" petition in the *Federal Registrar*. When reviewing the petition and public comments, APHIS must find that the organism does not directly or indirectly cause disease or damage to plants, plant parts, or processed products of plants and that the risks involved are not greater than those for traditional plants. If APHIS determines that a plant variety

or line has met all of the requirements for deregulation, it makes a "Determination of Non-regulated Status" and the product of the genetic engineering and its offspring will no longer require APHIS authorization for transport, release, or communication in the United States. However, frequently, APHIS neither accepts nor denies the developer's petition, but instead returns it to the developer with requests for additional information (Sedjo, 2004).

Analysis of Transgene Escapes and Relevance to Regulatory Process

Ever since the first transgenic crops were commercialized in the mid-1990s, there has been a continuous increase in reports of transgenes occurring outside of their intended situations (Greenpeace, 2007). These transgenes that Greenpeace and other environmental organizations are concerned about have been able to serve an unintended scientific purpose by allowing scientists to observe the method of crop gene flow. Researchers have found that there a variety of cases in which transgenes have been found outside of the intended organism (Ellstrand, 2012). Some cases involved transgenes that have not been deregulated being found in seed lots, in plants under protection, or in plants growing in the wild. In other cases, transgenes that have attained deregulation status in one country were found in plants or seeds in another country where they transgenic plants are not deregulated.

Throughout 30 years of planting transgenic crops, 22 incidents have been discovered in which the transgenes were detected in either seeds or plants that they were not supposed to be in (Ellstrand, 2012). These transgenes rarely flowed into wild populations, and were mainly found in a different variety of the same species (Ellstrand, 2012). . One of the main concerns people have in planting transgenic crops is the theory that the crops will spontaneously hybridize with a wild relative growing nearby, delivering a new trait that could, if transferred into a weedy

species, create a more persistent weed (Colwell et al., 1985; National Research Council, 1989). The findings of Ellstrand (2012) show that the fear that many people have of the hybridization between transgenic and wild-type plants is scientifically unwarranted

The transgenes that have escaped tell a very interesting story. Wild transgenic plants and their progeny have been found as free-living plants in ruderal habitats. Only two instances of transgenic escape show transgenes moving into the wild: a deregulated event for herbicide tolerance in canola flowing into wild populations and a regulated event for herbicide tolerance in a creeping bentgrass cultivar into wild canola populations. In wild canola in Canada and Japan, different transgenic lineages have crossed, giving rise to descendants with multiple transgenic deregulated events, each belonging to a different company. The canola crops with multiple transgenic events for herbicide tolerance have begun to pose a threat to farmers who must use less desirable herbicides to control them. The frequency of the bentgrass transgene event in wild populations has increased but in thought to pose no environmental threats. Interestingly, soybean and cotton are two of the world's most important transgenic crops, but there are no reports of out of place transgenes for either of them, most likely due to the fact that they are mostly self-pollinated (Ellstrand, 2012).

Implications for transgenic American chestnut

As of 2000, only 124 field tests of genetically modified trees had been authorized (McLean and Charest, 2000), such as transgenic spruce, pine, poplar, walnut, citrus, cherry, apple, pear, plum, papaya, and persimmon (Sedjo, 2004). Out of these trees, only the papaya and the plum have been deregulated thus far, and they are both domesticated orchard trees (Sedjo, 2010). The biotechnology that has been developed for agriculture can be directly applied to

forestry, but there are a few differences in trees that give some people pause. Trees are long-lived, outcrossing plants and can disperse pollen and seed long distances. Trees are also more likely to be planted within potential mating proximity of wild, compatible populations of related species because they were so recently domesticated (DiFazio et al., 1999). Orchard trees, such as papaya and plum have not posed any environmental concerns because they are highly domesticated and are therefore unlikely to cross-pollinate in the wild (Sedjo, 2010). Deregulating non-orchard trees like the American chestnut may take more time and may face more controversy than the deregulated orchard trees.

In order for the genetically engineered American chestnut to be planted throughout forests in its native range, it must pass through three government agencies. Because it is a genetically modified organism, the USDA APHIS must approve its deregulation. Because some transgenic lines contain an anti-microbial peptide, the trees must also be approved by the EPA which regulates plant-incorporated protectants (PIPs). Finally, the transgenic American chestnut must also pass through the FDA since animals and people will be consuming nuts from the transgenic American chestnut trees.

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