2016

Comparisons of Mycorrhizal Colonization between Transgenic and American Chestnut

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Senior Honors Project 2016

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Abstract

The American chestnut tree was once a common and important species. It has been reduced to an understory shrub by a fungal pathogen. This decimation of a once majestic tree prompted considerable restoration efforts. These have culminated in transferring genetic resistance to the chestnut by transformation with *Agrobacterium*. The past events (genetic lines from a single transformed cell) have not been shown to vary significantly in root colonization by ectomycorrhizal fungi. This experiment used three recent events to assess colonization. There was no significant variation among two of the events and the American chestnut. The significantly different event was explained by confounding experimental factors.

Introduction

American Chestnut (*Castanea dentata*) was once a keystone species of the eastern hardwood forest. It made up 30-50% of the forest before 1900 and was important to wildlife and humans (Hill 1994). The wood from American chestnut was used to make furniture, instruments, fencing, railway ties, telephone poles and a number of other products (Brooks 1937).

The fungal pathogen *Cryphonectria parasitica* was introduced on imported Asiatic chestnut trees. It has a variety of different dispersal mechanisms and spreads rapidly in the host as well as across the landscape. The pathogen can survive on other trees as a saprobe and on American chestnut stumps. It re-infects the stump sprouts, reducing the chestnut to an understory shrub (Paillet 1984).

Control of *C. parasitica* has been unsuccessful. The fungus spread throughout American chestnut's range by 1950 (Hepting 1974). A program of breeding, by backcrossing with Chinese chestnut, has been sponsored by the American Chestnut Foundation. The transgenic work took years, so it too is time consuming and difficult. It also results in an unknown combination of genes in the hybrids. Direct resistance gene transfer by *Agrobacterium*-mediated transformation has been used to create transgenic American Chestnuts that are resistant to *C. parisitica* (Newhouse et al. 2014). This method accurate but is time consuming as well.

Transgenics produce Oxalate oxidase, which degrades oxalic acid. Oxalic acid is a toxin secreted by the fungus to break down host tissue.

A number of transgenic American chestnut events have been produced. They have been shown to not vary statistically in ectomycorrhizal fungus (EMF) colonization rates compared to American chestnut (D'Amico et al. 2014). Newly produced events featured in this study are 58, 54, and 311. These were compared to American and Chinese chestnut for colonization by EMF. Event 54 has a high expression of oxalate oxidase (Oxo). Event 58 has a very high expression of Oxo. Event 311 is the same as event 58 plus a gene for GFP (green fluorescent protein). All of these events share the same promoter (Desmaris pers. comm.). While resistance to the blight is desired, resistance to mutualistic EMF in the roots is not. Governmental regulations require researchers to demonstrate that transgenic lines are no different than wild type plants for a variety of ecological features. The objective of this study was to evaluate the new lines for differences in their ability to associate with EMF compared to wild type American chestnut.

Methods

Soil was collected for inoculum just south of the shelterwood plots at SUNY ESF's Lafayette field station. The collection was done using a 5cm × 15cm (300mL) soil corer. Sixteen soil samples were randomly chosen and combined and sifted through 2cm × 2cm wire mesh. 100mL of this soil was used to inoculate each chestnut, which

was then repotted with Fafard's Nursery Mix (80% composted bark mulch and 20% fine peat). The transgenic events consisted of five 311's, three 58's, and five 54's. These plantlets were inoculated. Five American and Chinese were also inoculated. Four plants were not inoculated (54, 58, Chinese and American). These were repotted without inoculum to serve as controls. While the sample size of these treatments was admittedly low, I was limited by the availability of plants for this experiment.

The plantlets were leached for one month prior to inoculation. The American plants were not fertilized but the Chinese were fertilized until the day of inoculation. Leaching of fertilizer primary to planting the seedlings was done to encourage the plants to associate with EMF. It was not done with the Chinese because they were being fertilized at the beginning of the experiment. The chestnuts were grown for five and a half months in the greenhouse under a temperature maintained between 21-26 °C, 18 hours of light a day, and watered as needed. The plants did not receive fertilizer or pH amendments during the experiment to encourage the plants to associate with mycorrhizal fungi. Some did not survive and were replaced, they were inoculated with the soil from the dead plants. This changed the counts to six 54's, six 58's, six 311's, seven Chinese, and six American.

At the end of five and a half months, two 2cm (internal) diameter soil cores were take from each pot. The pot was cored 2 cm from the rim on opposite sides and down to the bottom of the pot. The roots and soil were washed with water in a #35

USA Standard soil sieve (0.5mm mesh) to release the roots from the soil. A subsample or all the roots in the core were placed in 1.5 mL Eppendorf ® centrifuge tubes and filled with sufficient 2% CTAB solution (100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% CTAB; after Gardes and Bruns 1993) to cover, then refrigerated at 4°C. Mycorrhizal colonization was quantified following Brundrett et al. 1994. The roots in the ependorf tubes were arranged on a 0.5 cm grid and viewed under a dissecting microscope (Nikon SMZ645 and a Fostec Ace I light fiber optic light source. The viewing window was 6 cm in diameter. Root tips were counted that crossed the gird lines. Root tips were counted as either mycorrhizal or non-mycorrhizal. All intersections were counted and a percent colonization was obtained from this following Brundrett et al. (1996).

The three events and the two species averages and standard deviations were calculated. These were used for a one-way ANOVA to determine variance under the assumption that the species were normally distributed. A 95% confidence interval was used. A Tukey pairwise comparison was used to group the different treatments. A Kruskal-Wallis test was also used to compare the differences assuming non-normal distribution plots. All data were analyzed in Minitab.

Results

There was considerable variation in the number of roots in the cores taken from the various plants. Some had an abundance of roots, and some had none. The Chinese and American had considerably more root mass than the transgenic events.

A one-way ANOVA showed at least one mean was statistically different (Table 1). Tukey Pairwise comparison showed the difference to be between the American chestnut and event 58 (Table 2). Wild type American had a significantly higher percent colonization than event 58 (Tukey test, $p<0.02$). A Kruskal-Wallis test also showed significant differences at $p=0.002$, presumably the result of differences between wild type American and event 58 (Table 3).

Table 1. ANOVA results comparing EMF colonization by plant treatments.

Source	DF	Adj SS	Adj MS	F -value	P-value
Factor	4	0.7776	0.19440	3.07	0.023
Error	56	3.5482	0.06336		
Total	60	4.3258			

Table 2. Tukey Pairwise Comparisons
Means that do not share a letter are statistically significant different ($p < 0.02$)

Plant	Grouping
American	A
311	A B
Chinese	A B
54	A B
58	B

Table 3. Comparison of Colonization across plants tested. Based on the Tukey test (Table 2) only event 58 and the American were statistically different from one another.

Table 3. Kruskal-Wallis Test on EMF percent colonization by EMF on root tips

Plants	Median	Rank
American	0.9800	48.2
311	0.8850	33.9
54	0.8610	26.9
58	0.7961	25.4
Chinese	0.8105	21.5

Discussion/Conclusion

There were 31 samples used in this experiment. This was due to seedling and plantlet availability during the time of the experiment. Even though some plantlets and seedlings did not receive field soil inoculum directly or at the same time, they all developed mycorrhizal colonization. Two possible factors contributing to this were: spores in the soil likely became airborne during the inoculation process, and the potting mix used was not sterilized. While EMF colonization levels did not vary for most of the treatments, event 58 was significantly different from the wild type American, with lower EMF colonization levels than wild type American.

It did not vary significantly from the Chinese or the other events.
There may be a few plausible explanations for these results. Some of the plantlets died and had to be replaced. The last experimental explanation for the difference is that three 58's were inoculated, but due to death and replacement of some of the other treatments, six 58's were assessed for colonization. This was because they were what was available for replacement.

There is the possibility that the higher expression of Oxo in event 58 caused the colonization to be lower. This was rejected because event 311 is basically the same as event 58 with the addition of GFP (Desmarais pers. comm.). Another explanation is that the insertion of genetic material into the genome in events is unpredictable in

terms of where the material is inserted. It could be that the genetic material in Event 58 was inserted into a region of the genome that negatively impacted the ability of the fungus to colonize roots (form mycorrhizal associations) while the insertion of event 311, even with the extra GFP reporter gene, occurred in a region that did not impact root colonization. Sorting out these possibilities was beyond the scope of this project.

In conclusion this experiment finds that the new transgenic American chestnuts Events 311 and 54 did not vary significantly from wild type American chestnut in their percent colonization by ectomycorrhizal fungi. Event 58 did differ from wild type, but additional work is necessary to identify if this is related to the antimicrobial polypeptide inserted or a result of some other factor including the experimental design (low sample size).

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