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Effects of Oxalate Oxidase Transgene Expression on Mycorrhizal Colonization in American Chestnuts

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Abstract

American chestnuts have been genetically engineered to express an oxalate oxidase gene isolated from wheat, which allows the plants to degrade oxalic acid, the virulence factor produced by *Cryphonectria parasitica*, the pathogenic fungus that causes the chestnut blight. In this study, we investigated the effect of oxalate oxidase gene expression on rate of mycorrhizal colonization of American chestnut root tips. Six-month-old transgenic and wildtype plantlets were placed in a soil inoculant and cultured in a greenhouse for 7 months, then root tips were visually assessed to estimate how many were colonized (expressed as a percentage). Three genotypes were used: ELLIS 1 trees were a cloned wildtype, and Darling 54 and Darling 58 were ELLIS 1 genotypes transformed with the oxalate oxidase gene, differing only in where the gene construct was inserted into the genome. Of surviving trees, 100% of both ELLIS 1 and Darling 58 trees were >95% colonized. Ninety percent of Darling 54 were >95% colonized, and 10% were 90-95% colonized. A Fisher's exact test of independence was used to reach the conclusion that expression of the oxalate oxidase gene has no effect on mycorrhizal colonization, a result consistent with similar studies.

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Thesis essay

Introduction

American chestnuts (*Castanea dentata*) have been genetically transformed to express an oxalate oxidase gene isolated from wheat (Triticum gestivum; Lane et al. 1993). Oxalate oxidase is an enzyme that breaks down oxalic acid, the virulence factor produced by *Cryphonectria parasitica* (Chen et al. 2010), the chestnut blight fungus, to necrotize the cambium tissue of non-resistant American chestnut trees. By degrading oxalic acid, American chestnut trees become resistant to the disease (B. Zhang et al. 2013), not by killing the fungus or preventing infection, but by preventing the lethal effects and making the fungus non-virulent. Resistant trees do not harm the fungus.

The oxalate oxidase gene used to produce disease-resistant American chestnuts was isolated from wheat, but it is a gene also found in other monocots (Lane et al. 1993; Dahiya et al. 2010), so while it is a foreign gene to the chestnut, it is native to the ecosystem. However, before transformed trees are introduced to the wild for restoration, it is imperative to measure non-target environmental effects. In this study, a greenhouse bioassay was conducted, and chestnut roots were observed to measure the potential effect of the expression of oxalate oxidase on mycorrhizal colonization.

The mycorrhizal network is used by plants to enhance water and mineral absorption. Fungi are able to access these nutrients because they have a unique enzymatic capacity (Qian, Wang and Yin, 2012). They also increase the plant's root volume and can access soil pores smaller than those plant roots can reach (Dighton, 2009). Study has shown that American chestnut seedlings form ectomyccorhizal associations.

Ectomycorrhizal fungi do not penetrate the cell wall of the host plant, instead forming an intercellular interface (Dulmer, LeDuc, and Horton, 2015; Palmer, Lindner, and Volk, 2008). The effects of oxalate oxidase expression on mycorrhizal colonization of American chestnut have been previously studied and with no effect on ectomycorrhizal colonization rates (D'Amico et al. 2015). The influence of various disease resistance-enhancing transgenes in multiple plants species on soil microbe communities have also been studied, with similar results (Becker et al. 2014; Cheeke et al. 2015; Kaldorf et al. 2002; Kaur et al. 2017; Newhouse et al. 2007; Vierheilig et al. 1995; Xie et al. 2016; Zeng et al. 2014; Zhang et al. 2015). A few studies showed reduced arbuscular mycorrhizal infection in transformed Bt cotton (Beura et al. 2015; Chen et al. 2016). It is expected that we will observe no significant difference in mycorrhizal colonization between wildtype and transformed American chestnuts.

In this study, the rate of mycorrhizal colonization was compared between three American chestnut genotypes. The first was ELLIS1, a cloned wildtype. The others were Darling54 and Darling58, ELLIS1 genotypes transformed with the oxalate oxidase gene, differing only in the genome insertion sites of the oxalate oxidase (OxO) gene construct. These transgenic trees differ from ones in previous studies (D'Amico et al. 2015) by fewer genome inserts, fewer selectable marker genes present, and higher OxO gene expression levels.

Methods

Soil samples for mycorrhizal inoculum were collected from ESF's Lafayette Road Experiment Station in Syracuse, New York, in a mixed hardwood stand. 23 random soil samples were dried, then sifted through a 0.5cm mesh. Samples were dried to select for

resistant propagules such as fungal spores and sclerotia. The soil inoculant was made by combining a ratio of 1:1:2 dried soil to sand to sphagnum peat moss. The inoculant was split evenly among 45 cylindrical pots, which had been previously sterilized overnight in a 7% bleach solution. About 6-month old tissue culture-generated *C. dentata* were bare-rooted and transplanted into the pots containing the inoculant. Three types of *C. dentata* were used, 15 individuals (n=15) each of 'Ellis 1' 'Darling 54' and 'Darling 58' (**Table 1**). Trees were cultured in a greenhouse until colonization took place, about 5 months. Greenhouse temperatures ranged from 18 to 29 degrees Celsius. Plants received a 16-hour photo period under high pressure sodium lights emitting light equivalent to full sun in field conditions. Plants were watered with tap water every two or three days. Tap water had a generally high pH and low electrical conductivity, or salt/fertilizer content.

Mycorrhizal colonization rate was assessed by collecting a continuous root length of at least 15cm after six months in the greenhouse. All root tips along the sample were observed, and the percentage of root tips that were colonized (categorized as mycorrhizal), and uncolonized (categorized as nonmycorrhizal) were visually estimated and assigned to categorical percentage ranks (less than 5%, more than 95%, or within ranges of 10%). A root tip was considered mycorrhizal if it was actively colonized or senescent with indications of previous colonization. The frequencies of each category in each treatment were calculated. A Fisher's exact test of independence, with a significance of 0.05, was used to test the null hypothesis that there was no significant difference in colonization between any two treatments.

Results

Mortality rate was 0% among ELLIS1 genotypes, 33% among Darling54 genotypes, and 20% among Darling58 genotypes. All surviving trees had greater than 90% mycorrhizal colonization. 100% of root tips were colonized in all Darling 58 and Ellis1 trees, and 90% of Darling54 trees. 90-95% of root tips were colonized in 10% of Darling54 (**Table 1**).

Discussion

There were no statistical differences between the rates of mycorrhizal colonization of the three genotypes of American chestnut, two of which were engineered to express oxalate oxidase. As previously stated, American chestnut associates with ectomycorrhizal fungi, which take part in chemical exchange with the plant host without breaking the plant cell wall or plasma membrane. Oxalate oxidase produced as a result of the expression of the transgene may not come into contact with the mycorrhizal fungi if it is present in the cytoplasm, apoplast, or some other intercellular structure and is not exported past the cell wall, or if it is differentially expressed in tissues and not root tissues.

If the gene is expressed in the roots, it may have no effect on mycorrhizae because oxalic acid production is associated with pathogens and saprobes, specifically brown-rot fungi that rely on the breakdown of plant tissues for survival (Green and Clausen, 2003; Hastrup et al. 2012; Na et al. 2017). Mycorrhizae depend on a symbiosis with their plant hosts, an exchange of water and minerals for the carbohydrate products of

photosynthesis, and do not rely on the action of oxalic acid, so its absence would likely have little effect on mycorrhizal colonization.

Hydrogen peroxide and carbon dioxide are the byproducts of oxalic acid decomposition. Hydrogen peroxide is an oxidative agent that is sometimes toxic to some mycelial fungi in high concentrations (El-Gazzar and Marth, 1988; Zharare, Kabanda and Poku, 2010), but it had no negative effects on the arbuscular mycorrhizae that colonized our chestnuts. This may be because hydrogen peroxide is associated with the temporal and spatial control of arbuscular mycorrhizae colonization; hydrogen peroxide was observed to accumulate in cortical root cells occupied by arbuscules, the haustorial organs of arbuscular mycorrhizae, and around hyphal tips attempting to penetrate host tissues (Salzer, Corbière and Boller, 1999). It has also been found that arbuscular mycorrhizae use hydrogen peroxide as a signal molecule in a pathway that enhances plant host resistance to pathogens (R. Zhang et al. 2013). Increasing the concentration of hydrogen peroxide, to a certain degree, would not harm the mycorrhizae, as they create and use this compound themselves. However, it may be harmful in very high amounts, and future study should look at hydrogen peroxide accumulation in transgenic American chestnut tissues over time, as oxalic acid is continuously broken down.

The results of this experiment are comparable to the results of similar studies (Becker et al. 2014; Cheeke et al. 2015; Kaldorf et al. 2002; Kaur et al. 2017; Newhouse et al. 2007; Vierheilig et al. 1995; Xie et al. 2016; Zeng et al. 2014; Zhang et al. 2015). One such study also investigated the mycorrhizal colonization of wildtype and blight-resistant genetically engineered American chestnut, as well as related species including red oak, American beech, and Chinese chestnut, and found that mycorrhizal colonization

rates differed between soil types, but not between tree genotypes (D'Amico et al. 2015). In another study, wheat was transformed with an apoplast-targeted defensin gene which granted the wheat significant resistance to infection by fungal rust. However, expression of the foreign gene had no effect on the plant's symbiotic relationship with mycorrhizal fungi (Kaur et al., 2016). A third study found that vesicular-arbuscular mycorrhizal colonization was not affected by various constitutively-expressed pathogenesis-related proteins, including chitinases, in tobacco plants (Vierheilig, 1995). In a final study, fungal symbionts were not affected by transgenic cotton expressing a foreign insecticidal gene that targeted lepidopteran pests. There were differences between the population size and structure of rhizosphere soil fungi between the transformed and conventional cotton only at some stages of plant growth. But fungal population size and structure were significantly influenced by variation in years and the stage of plant growth, and there was no difference in dominant fungi between cotton genotypes at any stage, indicating the transgenic, pest-resistant cotton had no significant effect on fungal community size or structure (Xie et al. 2016). These studies corroborate our results, demonstrating that expression of transgenes which enhance disease resistance have little or no effect mycorrhizal communities.

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Appendices

Table 1. Tree mortality, as a percentage of the sample size (n=15) and colonization of ectomycorrhizal fungi of wildtype and transgenic American chestnut (*C. dentata*) plantlets, measured in % root tips colonized.

Tree Type	**Number of surviving trees observed**	**Tree mortality**	**Surviving trees with >95% mycorrhizal colonization**	**Percent of surviving trees with 90-95% mycorrhizal colonization**	**Percentage of surviving trees trees with <90% mycorrhizal colonization**
'Ellis 1'	15	None	15 (100%)	None	None
'Darling 54'	10	5 (33%)	9 (90%)	1 (10%)	None
'Darling 58'	12	3 (20%)	12 (100%)	None	None