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Evaluation of Ecto-Mycorrhizae as a Determinant of Chestnut Growth and Stress Response

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Evaluation of Ecto-Mycorrhizae as a Determinant of Chestnut Growth and Stress Response

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Pabitra Aryal

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ABSTRACT

Although agriculture focuses on row crops throughout much of the Midwest, chestnut (*Castanea* spp.) appears to be an agroforestry crop well suited as a sustainable alternative to row crops in areas prone to erosion. As ectomycorrhizal (ECM) colonization is often crucial for tree establishment and production, I addressed the importance of ECM colonization on chestnut performance by assessing 1) natural ECM colonization in an established chestnut orchard and 2) the effect of experimental ECM inoculation on seedling establishment and drought response in a greenhouse. In the established orchard, I selected 50 Chinese-American hybrid chestnuts (C. *mollisima* × *C. dentata*) trees and assessed their level of mycorrhizal colonization in relation to several environmental factors, including distance from native oak forests, the source of ECM. In the orchard, ECM colonization decreased with distance from adjacent oak forest resulting in a relatively low average of 29% ECM colonization which was significantly positively correlated with leaf nutrient concentrations. In the greenhouse, I grew 80 seedlings from this orchard with and without mycorrhizal inoculation. Half were harvested to assess inoculation success and effects on biomass; the remainder were subjected to an experimental drought and their stress levels monitored with chlorophyll fluorescence. In the greenhouse, above ground biomass and number of leaves were significantly higher in ECM inoculated chestnut seedlings. Stomatal area, length and width were also smaller in

inoculated seedlings. During the experimental drought, inoculated seedlings and non-inoculated seedling were not significantly different during the drying phase, but were significantly different during the rewetting period. Stress in inoculated seedlings recovered significantly faster as assessed by chlorophyll fluorescence, indicating a role of ECM in mitigating drought recovery. My study clearly indicates that natural colonization with ectomycorrhizae can be limiting in orchards and can help chestnut trees in their early growth and stress responses.

INTRODUCTION

Chestnuts are considered one of the earlier domesticated tree crops used in agriculture (Gilman and Watson, 2014). Chestnuts (*Castanea* spp.) are native to areas with warm temperate climates in the Northern Hemisphere. Chestnuts are useful as a multipurpose agroforestry crop. Intercropping with vegetables and berries, combined with the ability to produce timber make chestnut a profitable cash crop (Prinsley, 1992). China and the Korea are the two top producing countries for chestnut and together produce more than 40 percent of world's chestnuts (Gilman and Watson, 2014; Hochmuth et al., 2015). There are few countries that have a high potential for chestnut production, but do not have a significant commercial chestnut industry; the United States is one of them. However, advancements in chestnut production, such as cultural techniques, propagation methodology and the development of new chestnut cultivars are aiding the expansion of the chestnut in many countries including in the United States (Gilman et al., 2014; Hochmuth et al., 2015).

The dominant industrial crops of Illinois are corn and soybean (NASS/USDA, 2016). As central and southern regions of the state are more dissected with steeper slopes and more extensive drainage systems, many areas of the region are prone to erosion when plowed for row crop production. This makes many areas of the region unsuitable for corn and

soybean production; instead, this land is often used for hay production or grazing. Agroforestry could prove an economically and environmentally valuable alternative in these regions. Chestnut appears to be an agroforestry crop well-suited to become a sustainable alternative in these areas or for farmers interested in exploring sustainable alternatives in other locations.

One of the major barriers to the establishment of alternative crops is the availability of sufficient culture information. Issues such as site selection, maintenance, and tree spacing have yet to be determined to encourage maximum growth and nut production for chestnut in the central Midwest. One factor which may be quite important is the formation of proper mycorrhizal associations to maximize plant growth and yield (Fitzsimmons, 2006). Mycorrhizae form a symbiotic association between a fungus and the roots of a host plant, which play an important role in soil chemistry and biology (Kirk et al., 2001). Generally, the association of fungi and host plant is thought to be mutualistic, but in particular cases these can be functionally pathogenic to the host plant (Johnson et al., 1997). Some studies suggest that establishment of root systems colonizing with the mycorrhizal fungi can increase host plants resistance to drought stress (Allen et al., 1983, Augé, 2004; Rapparini et al., 2014). Enhancing water uptake even at low level of soil moisture, plants with mycorrhizal associations can avoid drought to some extent (Auge et al., 1986). For an example, mutualistic benefits conferred by the fungus *Colletotrichum*

spp. included disease resistance, growth enhancement, and drought tolerance in tomato (Rodriguez et al., 2008). Mycorrhizal colonization within the roots of host plants is either intracellular as in arbuscular mycorrhizal fungi (AMF, called endomycorrhizal fungi) or extracellular as in ectomycorrhizal fungi (ECM). Ectomycorrhizal fungi form an intercellular interface consisting of highly branched hyphae forming a network between epidermal and cortical root cells but do not penetrate host cell walls (Parks, 2017).

Plant species forming ectomycorrhizae tend to be woody plants, including species of birch, beech, pine, willow, and dipterocarps (Smith et al., 2010). Several timber trees strongly depend on ectomycorrhizal fungi for their health and survival, even when growing in fertile soil (Hiremath et al., 2014). Ectomycorrhizal fungi have the capacity to help the establishment of these forest tree species after transplanting by increasing access to water and nutrients even in resource poor conditions and improve because these fungi have ability to withstand a variety of adverse environmental conditions (Moser and Haselwandter, 1983; Tinker, 1984; Smith, 1988; Smith et al., 2010; Kemppainen and Pardo, 2010; Hiremath et al., 2014).

Roots of chestnuts, like all species in the Fagaceae, form mutualistic ectomycorrhizal associations, such as the association between *Castanea dentata (Marshall) Borkh.* and *Scleroderma areolatum Ehrenb.* (Palmer et al., 2008). Because of their ability to

facilitate tree growth and survival, ECM are critical in the rehabilitation and regeneration of degraded forest ecosystems (Itoo and Reshi, 2013). Through an increased absorbing surface, efficient conduction through mycelial strands and enhanced hydraulic conductivity at the soil-root interface ECM symbioses help plants tolerate drought (Breda et al., 2006). For example, experiments on hybrid poplar (*Populus tremula* × *tremuloides*) seedlings inoculated with Amanita muscaria disclosed the root-fungus symbiosis improve the water transport capacity under conditions of reduced water availability (Marjanović et al., 2005). Similarly, ECM fungi *Rhizopogon vinicolor A. H. Smith* inoculated seedlings were less affected by drought than non-inoculated Douglas-fir seedlings (Parke et al., 1983). Therefore, ECM facilitates tree establishment by increasing their drought tolerance, disease tolerance, and uptake of nutrient and water.

Mycorrhizal networks can link two or more plants forming an integrated network (Gorzelak et al., 2015). Ectomycorrhizal networks can be exclusive, linking a single species (Beiler et al., 2010) or inclusive, linking multiple plant and fungal species (Molina et al., 1992). The significance of ectomycorrhizal networks to plant community establishment has been studied only in the past two decades (Simard et al., 1997). When the roots of growing seedlings contact, the mycelium connected to established trees, they get colonized) that facilitate seedlings directly or indirectly (Berman and Bledsoe 1998; Dickie et al. 2002). A common ectomycorrhizal network can influence plant

establishment, survival, physiology, growth and defensive chemistry (Horton and Bruns, 2001; Wu et al., 2001; Dickie et al., 2002; Nara, 2006; Teste et al., 2009; Teste et al., 2010; Bingham and Simard, 2011; Babikova et al., 2013). For example, seedlings colonized by ECM connected to established trees can acquire mineral nutrients from a mycorrhizal network for root and shoot growth, increasing the survival of new seedlings (Teste et al., 2009; Kariman et al., 2012). However, ECM networks are often not present in non-forested habitats or the habitat farther from forest habitats, leading to poor colonization and performance of plants that depend on ECM (Teste et al., 2009). Seedlings planted near mature ECM trees usually have higher fungal species richness than those planted in open land far from trees (Durall et al., 1999; Cline et al., 2005) where ECM colonization will depend on spores rather than established hyphae. As agroforestry activities are likely to be initiated on former agricultural land, a similar lack of ECM to colonize tree roots may exist.

As ectomycorrhizal colonization can be crucial for tree establishment and increases plant performance, this study addressed the importance of ECM colonization on chestnut performance by evaluating 1) natural patterns of ECM colonization and their relationship to growth and production in an established orchard and 2) the effects of ECM inoculation on seedling establishment, growth, and stress response in a greenhouse setting. Together

these two approaches will assess whether ECM colonization represents an important challenge for the establishment of chestnut as a sustainable crop for Illinois.

METHODS

Site description

The chestnut orchard studied was in Pike County, Illinois, USA. Pike County is in western Illinois, located on the highlands between the Illinois and Mississippi Rivers. Chestnut Ridge of Pike County is in Rockport above the Mississippi River Valley just off US-54 (Figure 1). This orchard was planted by Mr. Dale Black and his late wife Linda Black. As per the land owners, the location of the current orchard previously was agricultural land that had been abandoned and become covered with invasive shrubs such as autumn olive (*Elaeagnus umbellata* Thunb.). Currently this orchard has 30 acres of chestnut trees in production. Between 2001 and 2002, they planted more than 3,000 seedlings. These seedlings were *C. mollisima* × *C. dentata* hybrids, seedlings of Dunstan chestnut trees, that are resistant to chestnut blight (Shipman, 2011). Chestnut blight is an invasive fungal disease caused by pathogenic fungus *Cryphonectria parasitica (Murr.) Barr* that decimated the American chestnut (*C. dentata*) population, starting in the early 1900s (Anagnostakis, 1987). *Castanea mollissima* Blume is resistant to the disease.

The trees I studied were 14-15 years old and planted with 6 m between each chestnut tree. The orchard is irregularly shaped and occupies the upland portions of a dissected

landscape. The orchard is surrounded by mature oak forest that lies on the slopes surrounding the orchard.

Weather conditions

In 2016, average temperatures in nearby Pittsfield ranged from a low of 17.78 °C in January to a high of 33.88°C in July. Average monthly precipitation ranged from 3.30 mm in June to 170.43 mm in July (NCEI, 2016). The summer months prior to the field work for stress sampling of chestnut trees was good for plant growth, having average monthly temperature ranges from 25.6°C to 26.1°C and average monthly precipitation ranges from 112.52 mm to 170.43mm (NCEI, 2016). The average precipitation of the week that we work in that field for plant stress data collection was 34.0 mm in August. (The weather channel, 2016).

Linking orchard plant performance with ECM colonization

An aerial photo of the study site was used to select the sampling location within the orchard. I selected the north-west side of the orchard for this study because of variation in tree size and distances from the forest edge. Five transects were set up along alternate rows of trees (every 12 m), with each transect terminating 6 m from the oak forest edge.

Starting with the tree closest to the forest edge, alternate trees were selected (every 12 m) to be included until a total of 10 trees for that transect. This design followed the irregular shape of the orchard perimeter and resulted in 50 focal trees.

To examine the impact of surrounding forest, the likely source of ECM colonization in this orchard, on the ECM colonization, distance from forest edge was measured for each tree. To relate soil nutrients to ECM colonization the 50 selected chestnut trees were sampled on 20th of May 2016. Soil and root samples were collected by taking four, 10 cm diameter (30 cm deep, 30 cm from tree trunk) soil cores from around each of the focal trees. After pooling the samples, roots were isolated from soil, washed and refrigerated until they could be processed. Colonization rates were measured using the gridline intersect method, simultaneously measuring length of mycorrhizal roots and counting the total number of mycorrhizal tips (Brundrett et al., 1996). These tips were counted to quantify the intensity of the association, and was expressed as number of total ECM per 100 root tips. Soil samples from these same cores were dried in oven at 60°C and stored individually in tin boxes until they could be analyzed. Three macro-nutrients, nitrogen (N), phosphorus (P) and potassium (K), were analyzed for soil by using *LaMotte* soil nutrient kits (Chestertown, Maryland, USA). N and P were analyzed colorimetrically and K using titrimetric analysis following the kit's instructions.

The orchard was revisited on the 8th of August 2016. Performance of the 50 focal trees was assessed by measuring tree height (H), basal diameter (DBH), specific leaf area (SLA) and extension growth of 4 branches per individual collected along the cardinal directions. Leaves from all 4 sides were also taken from each tree (total 4 leaves per tree) and pooled for nutrient analysis. The same macro-nutrients, N, P and K, were analyzed for leaf tissues using *LaMotte* leaf nutrients kits (Chestertown, Maryland USA) following similar procedures to the soil analyses. Evaluation of nut production and total nut yield was done by counting all the fruit on each tree. Each chestnut burr can contain1-3 chestnuts, so fruit production should correlate strongly with seed yield.

During the August visit, plant stress was also determined using chlorophyll fluorescence. This method is an important tool as chlorophyll fluorescence responds to a wide spectrum of plant stressors such as water stress (Lu and Zang, 1999), plant pathogens, arthropod damage (Blanco et al., 1992) and nutrient limitation (Mee et al., 2017). Chlorophyll fluorescence is an indicator of photosynthetic energy conversion in higher plants and measured as light re-emitted by chlorophyll molecules (Maxwell and Johnson, 2000). It is essentially a measure of the maximum efficiency of PSII (the efficiency if all PSII centers were open) and is measured by calculating the ratio of Fv/Fm in dark-adapted measurements. Where Fv is the variable fluorescence (Fm-F0), Fm is maximum fluorescence and F0 is the minimum fluorescence. An Fv/Fm value in the range of 0.79

to 0.84 is the approximate optimal value for many plant species, with lower values indicating increasing plant stress (Kitajima and Butler, 1975; Maxwell and Johnson, 2000). Chlorophyll fluorescence was measured in leaves that were dark adapted for 10-20 minutes, as preliminary work determined this was sufficient for chestnut. The value of Fv/Fm was measured for 4 leaves, one on each side of the tree and averaged for a single measure of each focal tree's stress.

Linking greenhouse plant performance to ECM colonization

To assess whether ECM colonization is important in early plant growth and establishment, 80 chestnut seedlings were planted in the EIU, Thut greenhouse in sterile potting mix that were either inoculated or non-inoculated with ECM. The ECM used was the Diehard ecto injectable (Horticultural Alliance Inc, Sarasota, FL, United States). This inoculum contains live spores of generalist ectomycorrhizal fungi (*Pisolithus tinctorius* (Pers.) Coker & Couch and four *Rhizopogon* species), a water-soluble root growth stimulant with nitrogen fixing bacteria, phosphorus solubilizing bacteria and growth promoting beneficial bacteria. Seedlings were grown from seeds from Chestnut Ridge of Pike County, the same orchard used for the field study. Seeds were started on sterile potting mix, transplanted into a 1 gallon root pruning container (RootMaker®,

Huntsville, Alabama) to stimulate root growth. The use of a root pruning container helps plant root development and accelerates growth and have become common in the horticultural industry. Non-inoculated seedlings had the same inoculant that was sterilized by autoclaving to maintain equivalent nutrient additions in both inoculated and non-inoculated soils. Half (20) of the seedlings were harvested from each treatment after 90 days of growth to determine the success of inoculation and effects on plant growth. Up to one hundred root tips per seedling were randomly selected (total of 4,000 root tips) and examined with a dissecting microscope for the presence of a fungal sheath as described above. The above ground biomass was dried for 48 hours at 60 °C and then weighed. The total number of leaves was also counted for each seedling. The remaining 40 trees were used in a subsequent drought stress experiment.

On 12th of September 2016, a drought stress experiment was started. This was the last day that the chestnut seedlings were watered. Water stress was allowed to develop over a period of 12 days, when watering was resumed. The drought stress was evaluated with chlorophyll fluorescence using the same procedure as in the orchard. Fv/Fm value was recorded for the entire 20 days of the experiment to capture the rate at which stress developed and recovered following the resumption of watering.

As gas exchange can alter plant responses to drought, I also investigated the effects of ECM inoculation on stomatal characteristics. Two leaves were taken from each plant on the last day of the drought experiment (day 20). Leaf peels were made from the abaxial leaf surface to the right side of the midvein in the middle third of each leaf. Clear nail polish was applied to this area and left to dry completely (about twenty minutes) and the impression removed with clear packaging tape. This tape was attached to a microscope slide and three fields of view were photographed at 400x for each leaf. Fields of view were chosen so that as much of the epidermal peel as possible was clearly resolved. From these images, stomatal density was determined. Size of stomata was determined by measuring the length and width of the stomate closest to the center of the image for each field of view. The area of the stomata was determined assuming that each stomate was a perfect ellipse, using length across the aperture and width across its guard cells. All image analysis was performed in ImageJ (Schneider et al. 2012).

Data Analysis

To relate soil nutrients and distance from the forest edge to ECM colonization rates, a multiple regression analyses was done. To determine the effect of ECM colonization on leaf nutrient concentrations, individual Pearson correlations were calculated between

each leaf nutrient and ECM colonization rate. To assess the relationship between soil and leaf nutrients contents, Pearson correlation tests were conducted. Similarly, to relate performance of orchard trees to mycorrhizal colonization, correlations between extension growth, number of fruit produced, tree DBH, tree height, chlorophyll fluorescence and mycorrhizal colonization were calculated. Partial correlation analysis was conducted to examine linkages between ectomycorrhizal colonization and plant performance controlling for plant size as covariate.

To determine the impact of mycorrhizal inoculation on the growth of chestnut seedlings in the greenhouse, ANOVA was done on above ground biomass and number of leaves. This analysis was followed by a regression relating aboveground biomass to ECM colonization rate within inoculated seedlings to see if the degree of colonization was also important. The impact of mycorrhizal inoculation and experimentally imposed drought on chlorophyll fluorescence of chestnut leaves was assessed with repeated measures ANOVA (RMANOVA). This analysis was conducted three ways: using the entire data set, and separately for the drought and recovery phases by splitting the data at day 12, when watering was resumed. Additionally, impact of mycorrhizal inoculation on stomatal density and dimension of chestnut seedlings were assessed with ANOVA. All analyses were done using R version 3.1.2 (R Core Team, 2014).

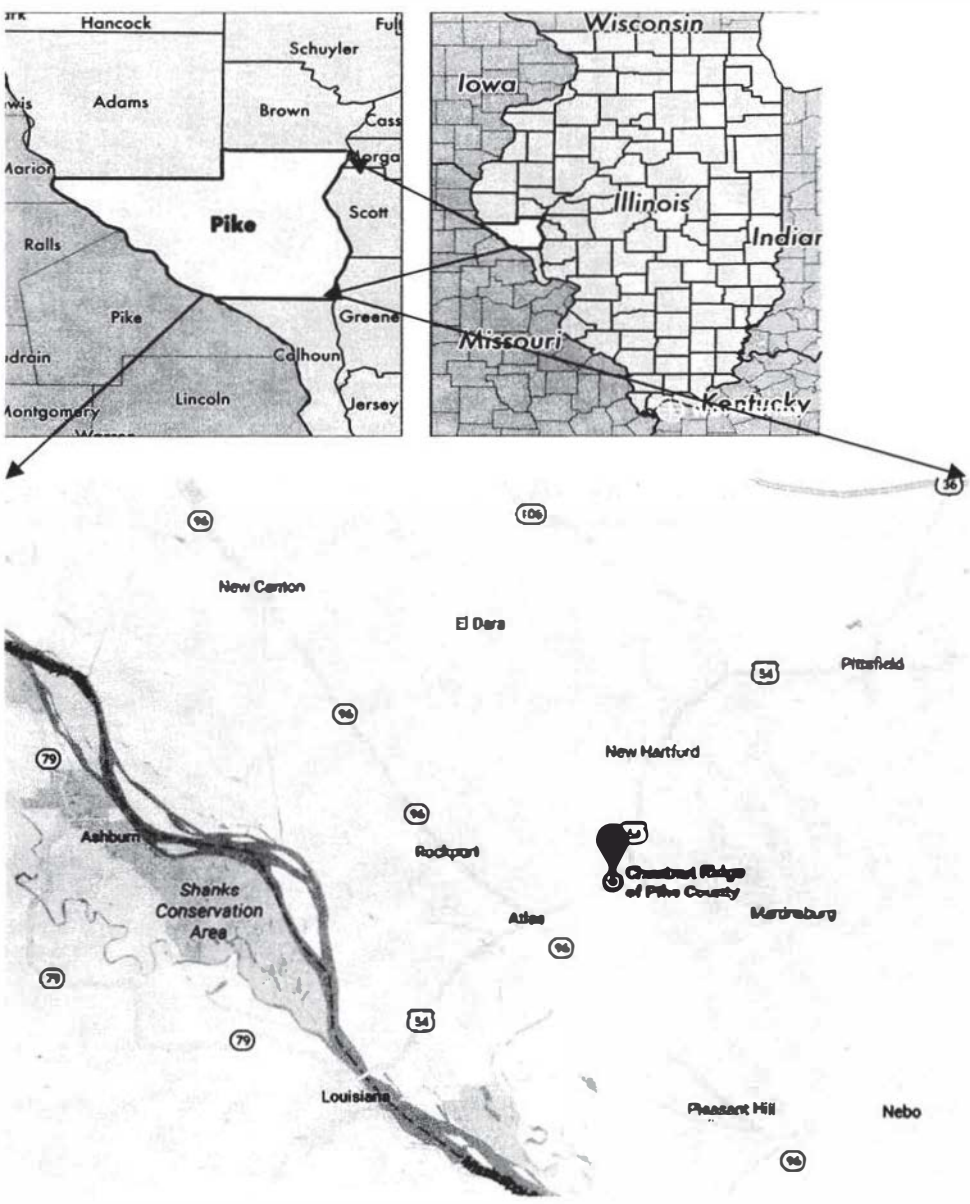

Figure 1: Location of the study site, Chestnut Ridge of Pike County. Lower panel from Google maps (39.5242° N, 90.9402° W).

RESULTS

Linking orchard plant performance with ECM colonization

Although all the root samples except one had some level of ECM colonization, trees ranged from no colonization (0%) to relatively high (58%), with an average of 29% of root tips colonized. Distance of individual trees from adjacent oak forest, the putative source of ECM colonization, was significantly related to ECM colonization of chestnut within the orchard ($F_{1,45} = 6.38$, P= 0.015; Table1) with ECM colonization decreasing in trees located farther from the forest (Figure 2). Of the major soil nutrients, only K content was significantly positively associated with the ECM colonization ($F_{1,45} = 5.76$, P= 0.020; Table 1). Soil N and P were not significantly related to the ECM colonization rate of orchard trees (Table 1).

Mycorrhizal colonization rates were associated with improved nutrient status of chestnut leaves with all leaf nutrients positively correlated with ECM colonization N (R= 0.38, P= 0.005), P (R= 0.55, P= 0.001), and K (R= 0.66, P= 0.001). Any correlation between soil nutrient content and leaf nutrient levels could account for the patterns with ECM colonization. However, only K content of leaves had a significant positive correlation with the K content of the soil (R = 0.43, P= 0.001). In contrast, neither leaf N (R= 0.04,

and P= 0.792) nor leaf P (R= 0.23 and P= 0.092) had any association with their levels in the soil.

In the orchard, ECM colonization was unrelated to any of the measure of plant size or performance (DBH, height, specific leaf area (SLA), or extension growth, fruit production, or stress levels (Table 2). However, some of the performance variables were correlated with each other. For example, DBH and height showed strong and significant negative correlations with extension growth (R= -0.51, P= 0.001, R= -0.56, P< 0.001 respectively; Table 2) and significant positive correlations with plant stress (Fv/Fm) and specific leaf area (Table 2). Similarly, total fruit production was positively correlated with DBH (R = 0.36, P= 0.009) and Fv/Fm (R= 0.29, P= 0.036) (Table 4). The only variable that was positively correlated with ECM colonization was extension growth, when plant stress (Fv/Fm) and plant height were accounted for using as a co-variate in partial correlation analysis at different time (R= 0.28, P= 0.048 and R= 0.29, P= 0.039 respectively).

Effect of ECM colonization on greenhouse plant performance

The experimental inoculation was effective with inoculated seedlings having ECM colonization with a range from 3%-31% ECM root tips per seedling (17% on average) at day 90. Non-inoculated seedlings did not have any seedlings with ECM root tips. The inoculation treatment generated several changes in chestnut seedlings in the greenhouse. The number of leaves produced was greater in inoculated seedlings (Figure 3 A, $F_{1,38}$ = 13.41, P= 0.0007, R^2= 0.261). Similarly, inoculated chestnut seedlings grew more than non-inoculated (Figure 3 B, $F_{1,38}$ = 7.13, P= 0.011, R^2=0.158). Greater levels of ECM provided additional benefits as within inoculated greenhouse seedlings, aboveground biomass increased with increasing mycorrhizal colonization (Figure 4, $F_{1,18}$ = 5.33, P= 0.033, R^2= 0.228).

ECM inoculation also resulted in marked changes in leaf stomata. Length and area of the stomatal complex were found to be smaller ($F_{1,38}$ = 4.21, P= 0.047, R^2= 0.099 and $F_{1,38}$ = 4.11, P=0.049, R^2= 0.097, respectively) in inoculated seedlings (Figure 5 A and C, respectively). Stomatal complex width was also found to be smaller, but not significantly so in inoculated seedlings ($F_{1,38}$ = 2.25, P= 0.14, R^2= 0.055, Figure 5 B). While stomatal

density was slightly higher in inoculated seedlings, there was no significant difference between treatments ($F_{1,38} = 3.62$, $P = 0.06$, $R^2 = 0.087$, Figure 5 D).

Mycorrhizal status also resulted in large differences in the drought response of greenhouse seedlings. After the last watering on day 1, Fv/Fm values increased over the first four days of the experiment (Figure 6). Following this period, Fv/Fm values drastically decreased during the experimentally induced drought through day 12, when watering resumed. At day 12, stress levels in inoculated and non-inoculated plants was nearly identical. However, following the resumption of watering, inoculated seedlings recovered much faster than non-inoculated ones. Across the entire drought experiment, Fv/Fm values significantly varied with inoculation and time ($F_{1,38} = 8.67$, $P = 0.018$ and $F_{11,28} = 0.05$, $P < 0.001$ respectively). Similarly, the time × inoculation interaction was also significant ($F_{11,28} = 2.14$, $P = 0.013$; Table 3). When the experiment is separated into drying and recovery phases, differences occur. During the drying phase, Fv/Fm responses for inoculated and non-inoculated chestnut seedlings were not significantly different ($F_{11,28} = 0.95$, $P = 0.333$; Table 3) but did increase with time ($F_{7,32} = 10.72$, $P = 0.001$; Table 3) with no interaction ($F_{7,32} = 0.90$, $P = 0.510$). In contrast, Fv/Fm values of chestnut seedlings during the recovery phase showed significant difference between treatments ($F_{1,38} = 8.67$, $P = 0.004$; Table 3) and time ($F_{3,36} = 0.047$, $P = 0.902$; Table 3) with no interaction ($F_{3,36} = 2.14$, $P = 0.153$; Table 3).

As transpiration can be associated with stomatal structure, stomatal characteristics could be expected to have some impact on drought tolerance. As both stomatal complex length and area significant differed with inoculation, both were examined if they could be responsible for drought responses. However, neither were significantly correlated with value of Fv/Fm (Table 4).

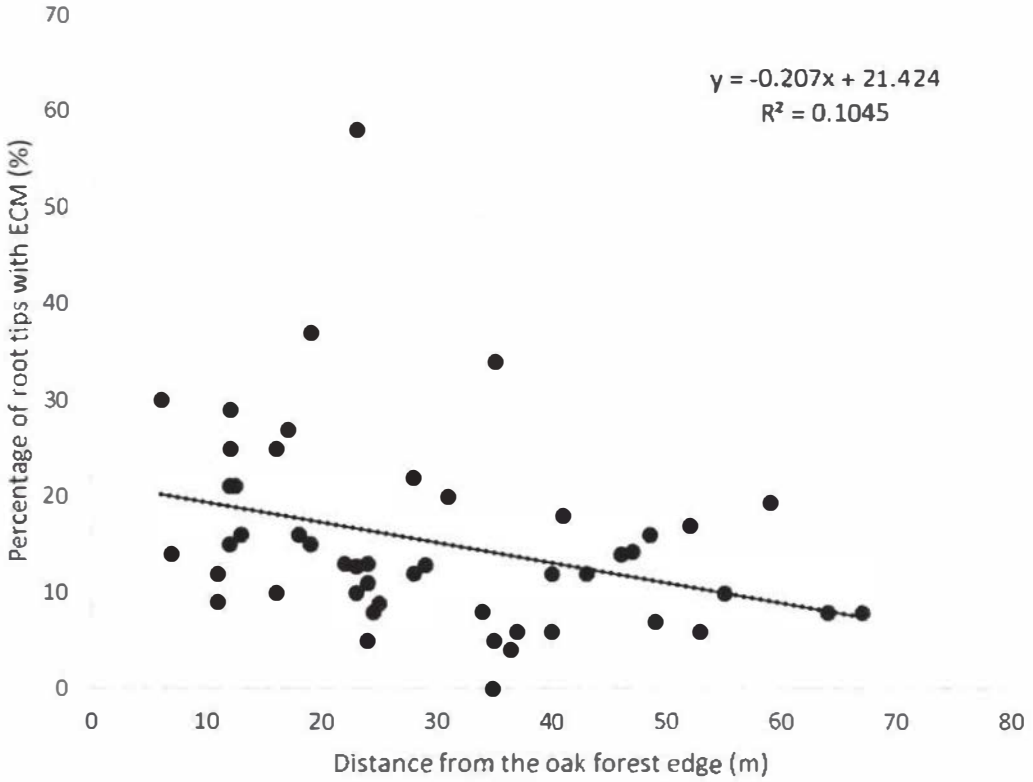

Figure 2. Change in the number of ECM root tips in chestnut trees with the distance from adjacent oak forest (P = 0.015)

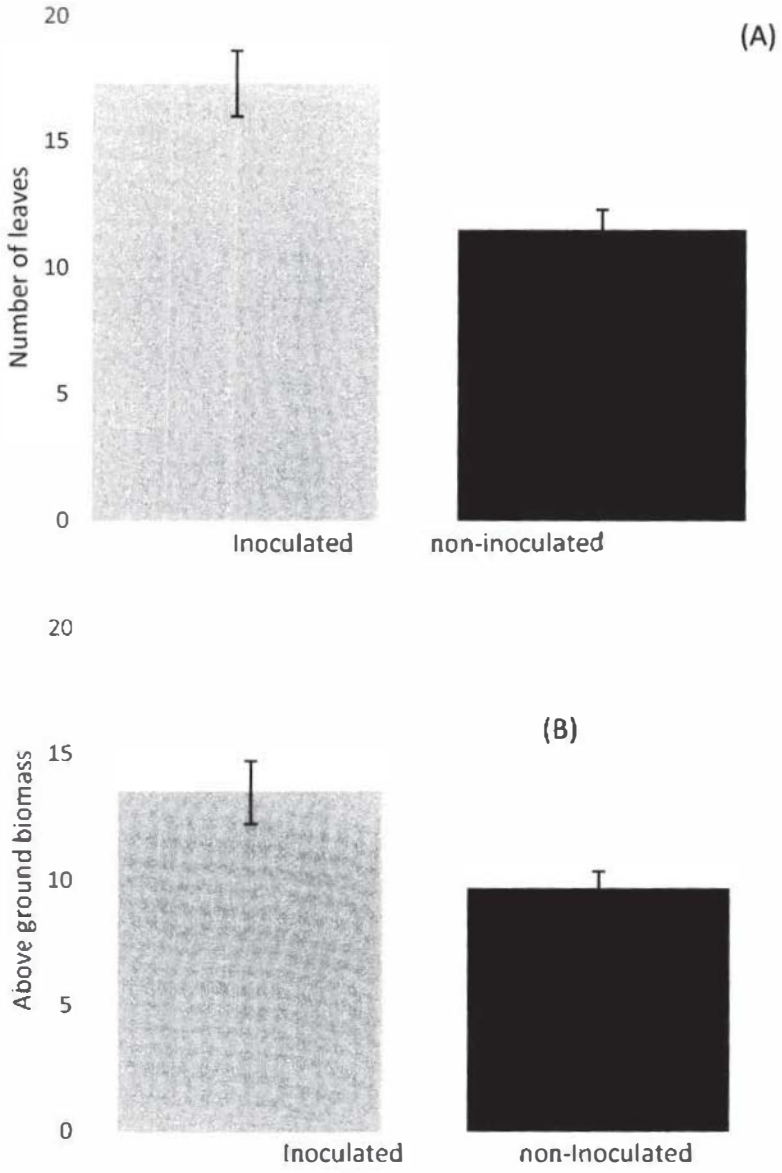

Figure 3. Effect of mycorrhizal inoculation on the (A) Leaf number and (B) Growth of chestnut seedlings grown in the greenhouse. Bars represent the standard error.

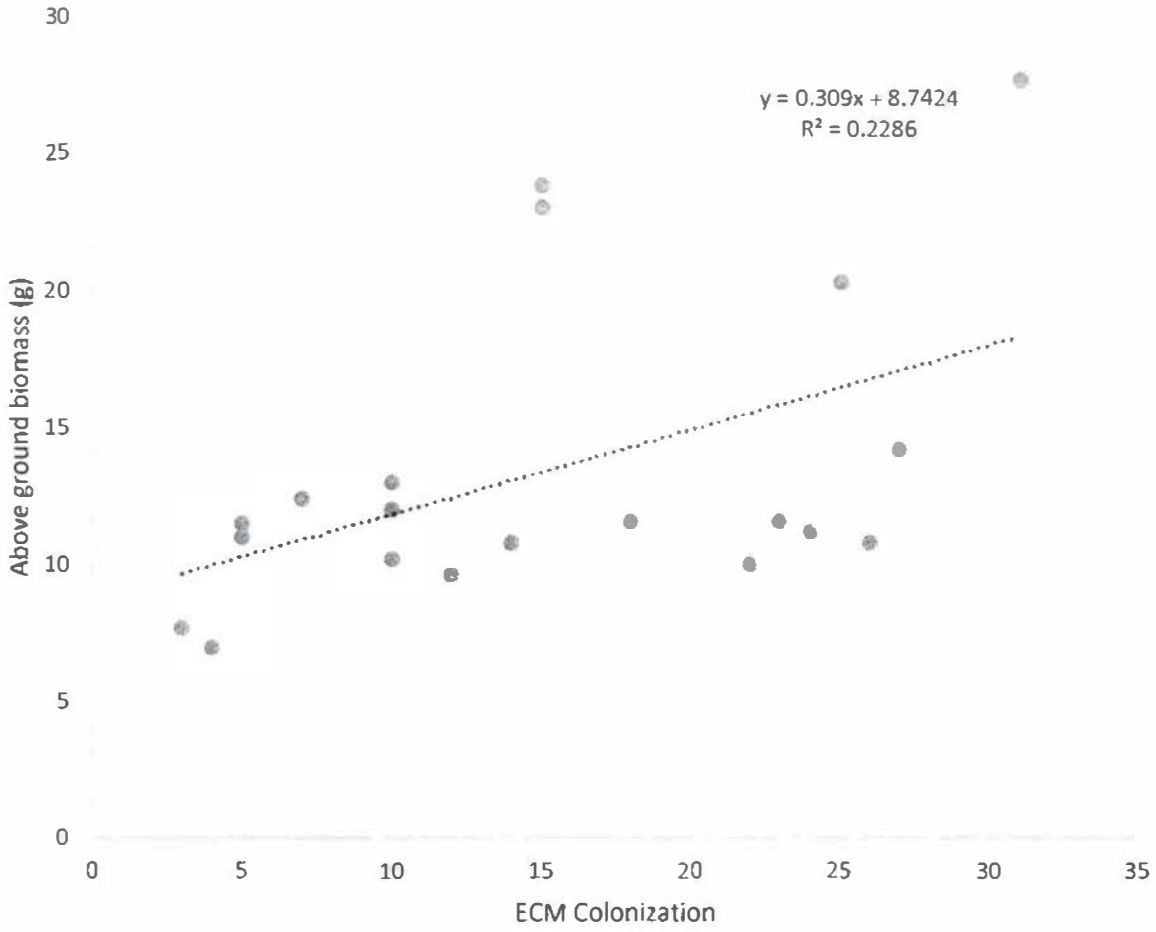

Figure 4. Growth response of chestnut trees in the greenhouse to increasing levels of ECM colonization. Data presented are for inoculated seedlings only (P = 0.033).

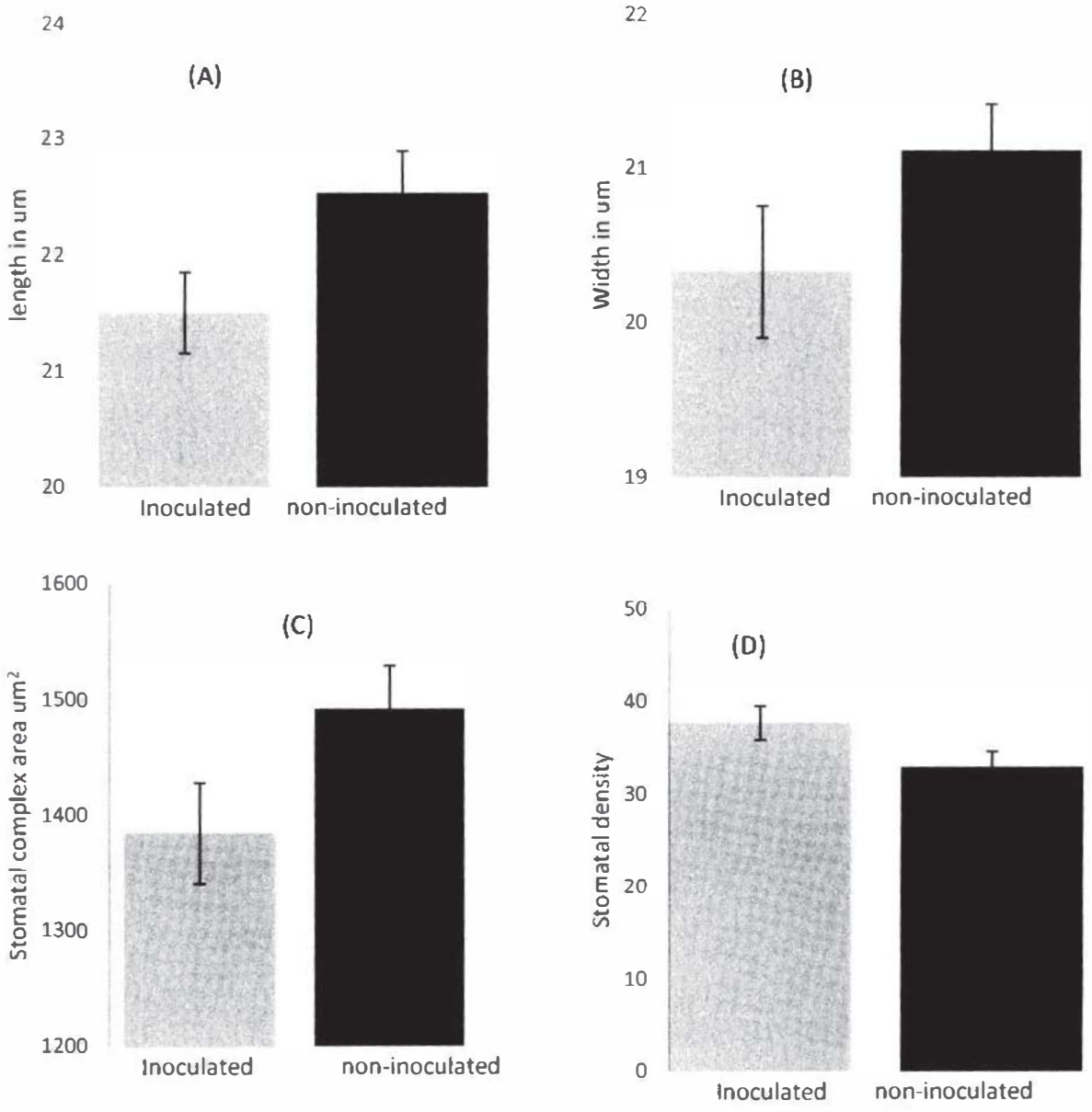

Figure 5. Average stomatal (A) length (B) width (C) Area and (D) Density of leaves of inoculated and non-inoculated chestnut seedlings grown in the greenhouse. Bars represents the standard error.

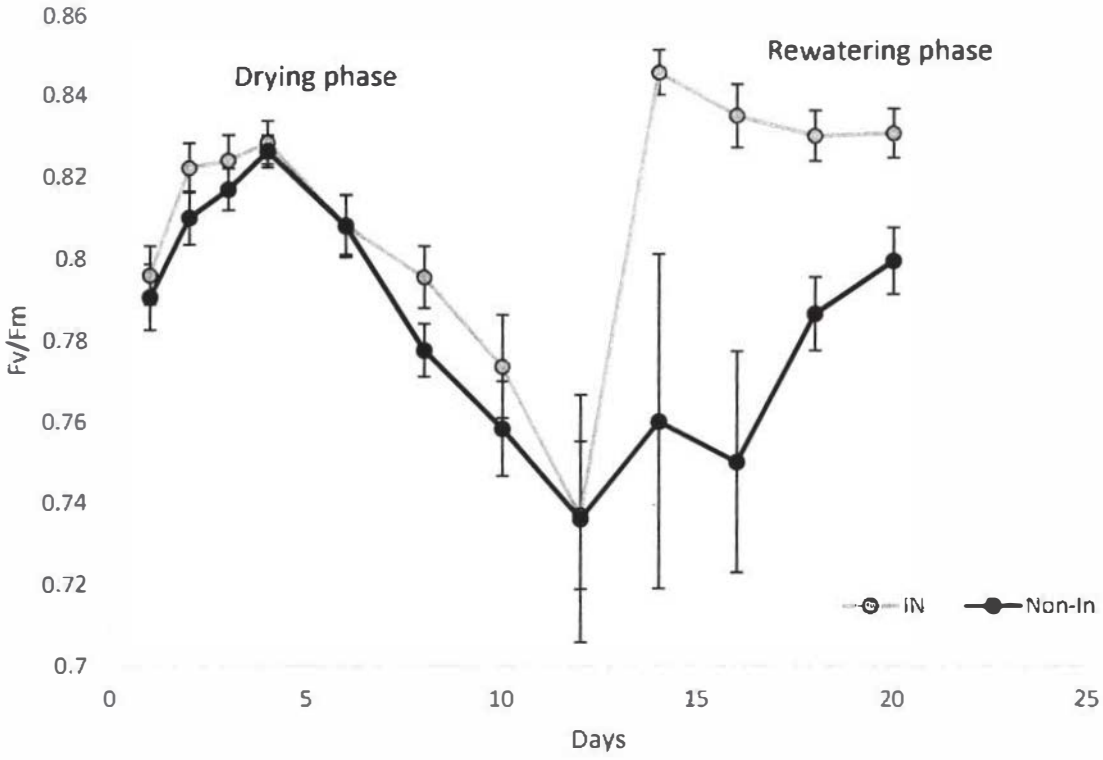

Figure 6. Effect of experimentally imposed drought on the chlorophyll fluorescence of inoculated (IN) and Non-inoculated (Non-In) chestnut seedlings. Watering last occurred at day 1 and resumed on day 12 only. Bars represents standard error.

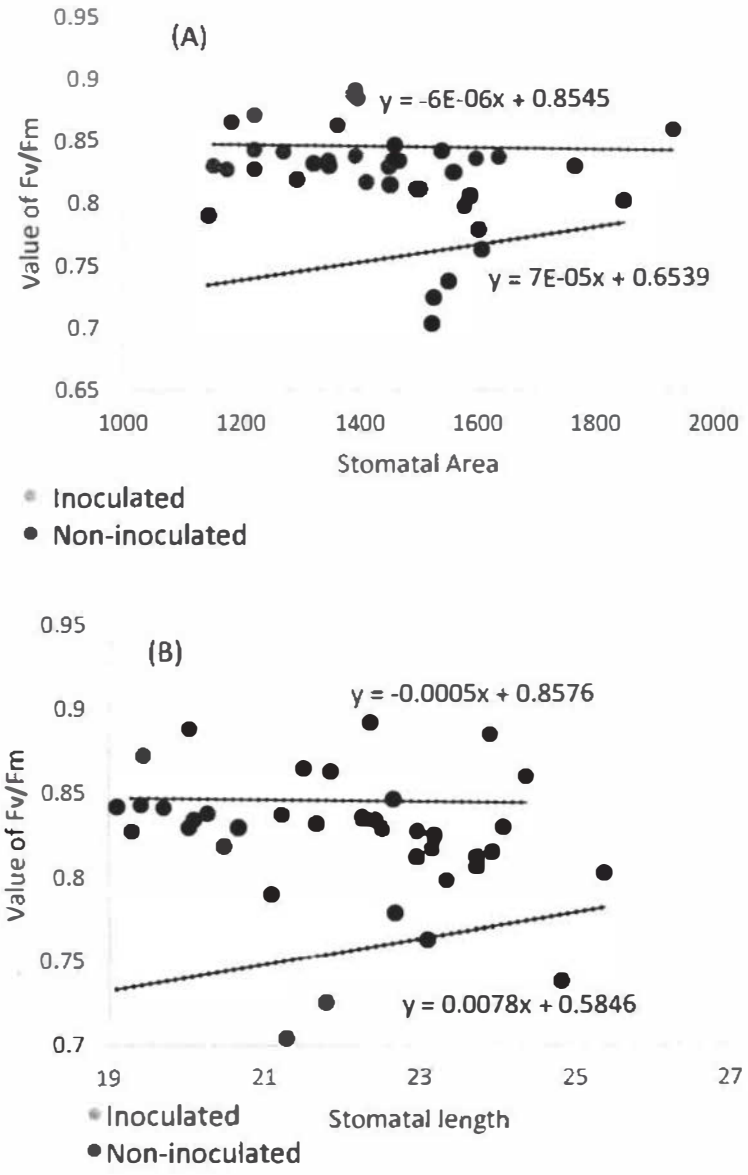

Figure 7. Plant stress in response to A) Stomatal complex area, B) Stomatal length, during drought of inoculated and non-inoculated chestnut. Grey dots=inoculated, Black dots=non-inoculated).

Table 1. Multiple regression on impact of soil nutrients and distance from edge on ECM colonization of orchard chestnut trees.

	DF	MS	F-Value	P value	R^2
Distance	1	514.22	6.38	**0.015**	0.264
Nitrogen	1	6.95	0.08	0.770	
Phosphorus	1	313.55	3.89	0.054·	
Potassium	1	464.08	5.76	**0.020**	
Error	45	20.25			
total	46	80.53			

DF = degree of freedom

MS = Mean sum of squares

Table 2. Correlations among plant mycorrhizal colonization and plant performance values in orchard chestnuts.

Variables	*ECM colonization*	*Extension growth*	*Fruit production*	*DBH*	*Height*	*Fv/Fm*
Extension growth	0.196					
Fruit production	0.154	0.079				
DBH	0.076	**-0.516**	**0.364**			
Height	0.082	**-0.569**	0.182	**0.818**		
Fv/Fm	0.144	**-0.414**	**0.296**	**0.472**	**0.575**	
SLA	-0.113	**-0.579**	-0.046	**0.358**	**0.524**	**0.338**

Bold values indicate a significant correlation

Table 3. RMANOVA of the impact of mycorrhizal inoculation and experimentally imposed drought days on chlorophyll fluorescence.

Model term	DF	F	P
Entire experiment			
Inoculation	1,38	8.67	**0.018**
Day	11,28	0.05	**0.001**
Day× Inoculation	11,28	2.14	**0.013**
Drought phase			
Inoculation	1,38	0.96	0.333
Day	7,32	10.72	**0.001**
Day × Inoculation	7,32	0.90	0.51
Recovery phase			
Inoculation	1,38	8.67	**0.004**
Day	3,36	0.05	0.900
Day × Inoculation	3,36	2.14	0.153

Values in bold are significant values

Table 4. Correlations between stomatal characteristics with value of Fv/Fm of inoculated and non-inoculated chestnut seedlings.

Variables	DF	Pearson R	P-value
Inoculated			
Area	18	-0.046	0.845
Length	18	-0.035	0.880
Width	18	-0.243	0.300
Density	18	0.409	0.072
Non-Inoculated			
Area	18	0.060	0.800
Length	18	0.066	0.781
Width	48	-0.019	0.930
Density	18	-0.184	0.430

DISCUSSION

Orchard plant performance

Source of ECM colonization and effects of soil nutrients

Growing trees that have a high degree of specificity for their mycobiont in new locality, such as *Pinus* and *Eucalyptus* species, often requires a supporting ectomycorrhizal partner (Díez, 2005). The distance from nearby host plants can be very important in ECM colonization in newly planted trees (Fujiyoshi et al., 2011) as existing vegetation and their common mycorrhizal networks can spread ECM hyphae to nearby vegetation (Dickie et al., 2004). New trees that established along forest edges can be supported by ECM colonization from those existing forest trees (Jonsson et al. 2001) and allow connection to the hyphal network. The higher ECM colonization of root tips (58%) near the forest edge in my study could have been generated by mycorrhizae on the existing vegetation (Dickie et al. 2002). The lower colonization (0-4%) with greater distance from forest edge could be due lack of contact from other trees (Kranabetter and Friesen, 2002) and dependence on decreasing ECM spore dispersal. Another important factor related to decreasing rate of ECM colonization with the distance from forest edge could be the effect of change

in micro-climatic condition like soil moisture and soil temperature (Swaty et al. 1998; Kennedy and Peay, 2007)

The declining rate of ECM colonization with distance from surrounding forest in my study is in consistent with the results of Bauman et al. (2012) which found the highest rate of ECM colonization (58%) along a forest edge, followed by 14% in the plot of American chestnut (*Castanea dentata*) seedling growing along an established *Pinus virginiana* stand. The average rate of ECM colonization (29%) in my study appears to be limited compared to other studies such as in Black Spruce (*Picea mariana*), a range of colonization 49% to 95% (Reithmeier and Kernaghan, 2013). Similarly, nearly 100% ECM colonization was found in *Abies alba* growing outside of its native range (Rudawska et al., 2016) and around than 75% of ECM colonization was found in *Quercus garryana* studied in southern Oregon (Valentine et al., 2004).

One important factor that can influence ECM colonization is soil nutrients (Avis et al., 2003), though the relationships appear to vary dramatically (Twieg et al., 2009). For example, studies on *Pinus* and boreal mixed forests found that the ECM communities were not correlated with the mineral soil nutrients, including available N and P, demonstrating that soil chemistry was not driving ECM community

structure (Douglas et al., 2005, Kemaghan et al., 2003). Whereas Kernaghan et al. (2003) found good correlations between soil exchangeable cations and ECM colonization but no direct relationship with other soil factors such as N and P. Similarly, in backcrossed hybrid chestnut (*C. dentata* × *C. mollissima*) Bauman et al. (2012) also found that colonization by ECM fungi, though limited, was significantly associated with cation exchange capacity. These results match my study where I found soil N and P were not correlated with ECM colonization, but strongly positively correlated with one of primary cations, K.

Together with soil chemistry, mycorrhizae play vital roles in soil biology and plant nutrition. ECM mycelium can increase access to many soil nutrient sources (Li et al., 2006) and can mobilize nutrients by acting as a decay organisms (Bücking et al., 2012). In many terrestrial ecosystems in which soil N availability is limiting to plant growth, mycorrhizal fungi can mobilize amino acids and amides which can represent a significant N source (Smith et al., 2010). Similarly, some ectomycorrhizal fungi are able to release organic compounds (organic anions or phosphatases) to mobilize unavailable P in the soil (Becquer et al., 2014). Some ECM fungi, such as *Hebeloma cylindrosporum,* express two P transporters, for high and low P conditions to efficiently take up P locally varying concentrations (Bücking et al, 2012). Evidence for improved nutrient uptake in my study was found in the significant positive

correlation of chestnut leaf N and P with the rate of ECM colonization, while there was no correlation with levels in the soil. As soil K was strongly correlated with leaf K as well as ECM colonization, ECM function for K transport could not be isolated. My results indicate that ECM colonization facilitated N and P uptake in orchard chestnuts.

Growth factors and production

Mycorrhizal colonization increases plant growth and performance (Aggangan et al., 2010). When either Fv/Fm or plant height was used as a covariate (both were correlated), extension growth showed a significant positive relation with mycorrhizal colonization. Extension growth had a strong negative correlation with DBH, height and specific leaf area (SLA), which indicate that these growth variables have strong influence on each other. SLA was strongly correlated with growth rate, and therefore with tree size. Larger trees would proportionately slow down individual stem growth. On the other hand, the significant positive correlations of Fv/Fm with SLA and with chestnut production clearly indicates that specific leaf area and the nut production increases in chestnut tree that are experiencing less stress. Nevertheless, as phenotypic plasticity in allocation occurs in plants grown in contrasting nutrient

regimes (King, 2003), we can expect variation in plant traits with changing soil resources and ECM levels. As some studies have shown mycorrhizal colonization significantly increases plant height and above ground biomass (Sim and Eom, 2006), basal diameter and leaf area (Bauman et al., 2011), plant total biomass and photosynthetic rate (Kayama and Yamanaka, 2016), I expected the same relationship in the orchard. Nonetheless, due to relative limitation of ECM colonization in this orchard, I did not find a significant relationship of ECM colonization to plant other performance measures.

Greenhouse plant performance

Growth

Despite having a relatively fertile environment in greenhouse soil, there was a significant response of chestnut seedlings to ECM inoculation. ECM colonization of plant roots improves the water and nutrient uptake of host plants. Thus, ECM fungi probably play critical roles in plant growth and survival in a greenhouse environment (Smith and Read 2010). Chestnut seedlings inoculated with ectomycorrhizae had greater above ground biomass and number of leaves than non-inoculated seedlings.

This study clearly indicates that the colonization with ectomycorrhizae helps chestnut seedlings in their early growth and development. The increase in above ground biomass with the number of ECM root tips colonized in the inoculated seedlings in my study relates to other studies which showed that the growth of the seedlings increased significantly with the numbers of associated ECM fungal species and ECM root tips (Nara et al., 2004). Although it is well documented in the literature that ECM fungi have beneficial effects on plant growth and production, sometimes it is unclear whether ECM activity was the driver of plant growth, or if, plant fitness contributed to ECM colonization (Bauman et al., 2013). Chestnut seedlings that performed poorly in non-inoculated soils and performed better in inoculated soils clarifies this doubt in my study and supports the hypothesis that ECM inoculation does have beneficial effects on plant growth.

Stomatal characteristics and drought tolerance

ECM inoculation brought marked changes in leaf stomata. Significantly smaller stomatal complex area and stomatal length and smaller stomatal width (although not

significant) in inoculated chestnut seedlings relates to studies that showed crops with smaller stomata had better drought tolerance (Mehri et al., 2009). This suggests that inoculated chestnut seedling would tolerate drought better than non-inoculated chestnut seedlings. However, changes in stomatal characters were not related to drought tolerance as Fv/Fm was consistent between inoculated and non-inoculated plants throughout the drought phase of the experiment. Nevertheless, the larger size of inoculated seedlings may have resulted in more rapid depletion of water, so equivalent stress between treatments is still somewhat surprising in this study.

Mehri et al. (2009) suggested that drought tolerant wheat cultivars and other crops have lower stomatal densities. Similarly, drought tolerance of black poplar (*Populus nigra* L.) clones was associated with lower stomatal density (Regier et al., 2009). This pattern of stomatal frequency did not match the seedlings studied here as the inoculated seedling had slightly greater stomatal density than non-inoculated ones. As ECM fungi in oaks and other trees of Fagaceae increase water absorption (Egerton-Warburton and Allen 2001), this study does show that the mycorrhizal treatment altered the physiology of the plant sufficiently to alter leaf properties, which may also alter drought tolerance in chestnut. The mycorrhizae also should alter water availability to the plant which we did not measure and would more directly affect drought tolerance. On the other hand, no significant correlations

between stomatal characteristics and Fv/Fm clearly argues that the effect of mycorrhizal colonization did not alter drought response by altering stomatal characteristics. It suggests that the driver was something else; probably water access.

Drought tolerance

Water scarcity is one of the key limiting factors for plant growth and production (Haferkamp, 1988). Negative impacts on seedling recruitment, susceptibility to pathogen or insect attack, vulnerability to fire, reductions in photosynthetic capacity or productivity, and mortality are some of the documented consequences of exposure to drought in trees (Zhao and Running, 2010, Reichstein et al., 2013). In this study, chestnut seedlings inoculated with ECM showed faster recovery from drought than non-inoculated seedlings during the rewatering phase, similar to the increases in net photosynthesis and water use efficiency in ECM-inoculated plants in a drought/rewatering experiment by Peter et al. (2016). Although the average of Fv/Fm was slightly higher in inoculated seedlings during the drought period, they were not significantly different. Sánchez-Blanco et al. (2004) found a parallel result; under drought conditions leaf water potential decreased in both non-mycorrhizal and mycorrhizal plants, but this decrease was lower in mycorrhizal plants. During

drought, mycorrhizae could aid to increased water uptake through lowered resistance to water flow from soil to roots and increased absorptive surface to reduce plant moisture stress (Reid, 1979). Rates of net CO_2 fixation during drought were reduced less and recovered more rapidly in a study of Douglas-fir by Parke et al. (1983). While it is not clear why my study found minimal effects of ECM on their drought response, ECM clearly improved drought recovery rates, which can be quite important in a field setting.

Conclusions

Natural ECM colonization in the chestnut orchard was influenced by the distance from the source of colonization but was overall quite low. ECM inoculation occurred naturally in the field via spore and/or mycelium propagules from the roots of the adjacent forest trees, and was influenced by K in the soil. To reduce the future need for fertilizer application, direct ECM inoculation of existing trees in orchard may be beneficial. My results also suggest that while producing plants in greenhouse, ECM inoculation should be included to improve seedling growth and survival, and to establish ECM at orchard initiation. Inoculation with ECM also altered leaf properties which could change water usage. My greenhouse results showed the

importance of ECM in mediating drought recovery in chestnut seedlings, though this was not related to stomatal changes. Managing mycorrhizal associations may prove to be an important component of chestnut culture as a part of a regional sustainable agriculture program.

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