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Ectomycorrhizae determine chestnut seedling growth and drought response

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Abstract Although agriculture focuses on row crops in the Midwestern US, chestnut (*Castanea*) would be a possible multipurpose agroforestry crop in erodible locations within this region. As ectomycorrhizal (ECM) colonization is often crucial for tree establishment and production, we 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 ECM inoculation on seedling establishment and drought response in a greenhouse. We selected 50 Chinese American hybrid chestnuts trees in a commercial orchard and assessed their level of mycorrhizal colonization in relation to distance from an adjacent forest, the source of inoculum. In the green house, we grew 80 seedlings from this orchard with and without mycorrhizal inoculation. Half were harvested to assess inoculation success and biomass; the remainder were subjected to experimental water stress which was evaluated with chlorophyll fluorescence using Fv/Fm values. In the orchard planted with uninoculated plants, ECM colonization decreased with distance from adjacent oak forest ($P = 0.015$), the putative ECM source, with an average of 29% root tips colonized. ECM inoculation increased seedling aboveground biomass by 16.4% in the greenhouse and generated 1.14 times more, but smaller stomata on leaves. Water stress in ECM inoculated seedlings also recovered faster from an experimental drought having significantly higher Fv/Fm value ($P = 0.004$) than uninoculated seedlings. Our study clearly indicates that ectomycorrhizal colonization can help chestnut trees in their early growth and stress tolerance and should be included into field plantings to minimize dependence on agricultural amendments.

Keywords Chestnut · Drought response · Ectomycorrhizae · Mycorrhizal colonization · Mycorrhizal inoculation

Introduction

Chestnuts are one of the earlier tree crops utilized in agroforestry systems (Gilman and Watson 2014), native to areas with warm temperate climates in the Northern Hemisphere. Chestnuts are useful as a multipurpose agroforestry crop. Intercropping with hay, vegetables or berries, combined with the ability to produce nuts and timber, make chestnut a profitable cash crop (Prinsley 1992). China and Korea

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are the two top producing countries for chestnut and together produce more than 40% of world's chestnuts (Gilman and Watson 2014; Hochmuth et al. 2015). The productivity and potential marketability of chestnut in North America (Hochmuth et al. 2015) has made it a prime candidate for alternative agriculture in the wake of the loss of native American chestnuts (*C. dentata* (Marshall) Borkh) to blight. After the rapid elimination of American chestnut due to blight disease caused by the ascomycete *Cryphonectria parasitica* (Murr.) Barr, a backcross breeding approach has been developed to restore the chestnut in North American forests. Blight resistant genes from Asian chestnut species, most often the Chinese chestnut (*Castanea mollisima* Blume), were bred with the American chestnut (Burnham et al. 1986; Anagnostakis 2012).

The dominant industrial crops of the central Midwest are corn and soybeans. As large portions of the region are dissected with steep slopes and extensive drainage systems, many areas are prone to erosion when plowed for row crop production (Dickey et al. 1985). This makes many areas of the region unsuitable for corn and soybean production; instead, this land is often used for hay production, grazing or left fallow. In addition, according to the research done by North Carolina State University in 2014, most of the areas in Midwest are facing intensive drought events that may result in the drop of corn yield by 15–30% in the future (North Carolina State University 2014). Agroforestry could prove an economically and environmentally valuable alternative in these regions. Agroforestry combines trees and shrubs with crops and/or livestock (USDA 2012). Chestnut appears to be an agroforestry crop well-suited to become a sustainable alternative in erodible areas or for farmers interested in exploring sustainable alternatives in other locations.

One factor which may be quite important in the establishment of alternative crops is the formation of proper mycorrhizal associations to maximize plant growth and yield (Fitzsimmons 2006). Some studies suggest that establishment of root systems colonized by mycorrhizal fungi can increase host plants' resistance to drought stress (Allen et al. 1983; Augé 2004; Rapparini and Peñuelas 2014). Mycorrhizae may increase drought tolerance of plants, a potentially critical driver as drought events have been predicted to increase in the Midwest (Mishra et al. 2010). By enhancing water uptake even at low levels of soil moisture, mycorrhizal associations can help colonized plants to avoid drought to some extent (Augé et al. 1986).

Seedling growth and establishment are highly vulnerable to many biotic and abiotic factors such as drought, flood and lack of resources (Leck et al 2008). Mycorrhizal fungi help promote seedling establishment through increasing access to soil resources (Horton and Heijden 2008). Under forest conditions, the roots of growing seedlings contact the mycelium associated with established trees, resulting in connections that may facilitate seedlings directly or indirectly (Berman and Bledsoe 1998; Dickie et al. 2002). Ectomycorrhizal (ECM) fungi are well-documented to aid the establishment of trees such as oak (*Quercus*) and chestnut by improving access to water and nutrients even in resource poor conditions, increasing a tree's ability to withstand adverse environmental conditions (Moser and Haselwandter 1983; Tinker 1984; Smith 1988; Smith and Read 2010; Kemppainen and Pardo 2010; Hiremath et al. 2014). A common ECM network can influence plant establishment, survival, physiology, growth and defensive chemistry (Wu et al. 2001; Dickie et al. 2002; Teste et al. 2009; Babikova et al. 2013). For example, seedlings colonized by ECM connected to established trees can acquire more mineral nutrients through the mycelial networks for root and shoot growth, increasing the survival of new seedlings (Teste et al. 2009; Kariman et al. 2012). While ECM may be abundant in forested systems, long-term row crop agriculture dramatically reduces their abundance, limiting regeneration of plants that depend on ECM (Teste et al. 2009). Agroforestry projects converting land from traditional agriculture to more sustainable practices may need to account for this lack of ECM.

The chestnut blight resistant hybrid 'Dunstan' (*C. mollissima* × *C. dentata*), developed by Chestnut Hill Nursery (Alachua, Florida) (Wallace 1993) is the widely planted chestnut tree in America in recent years and produces average 23–35 nuts per pound (Olsen 2000). We used progeny from this seedling line to relate ECM colonization to both plant performance and physiological responses. As ECM colonization can be crucial for tree seedling establishment and increases plant performance, this study assessed whether: (1) natural patterns of ECM colonization were sufficient to promote chestnut performance in a seed-generated commercial orchard, and (2) If ECM inoculation improves seedling establishment, growth, and drought stress response in a greenhouse setting. Together these two approaches will assess whether ECM colonization represents an important support for the establishment of chestnut as a sustainable crop for the Central Midwest.

Materials and methods

ECM colonization in a seed-generated orchard

The study site and source of seeds for the greenhouse experiment was Chestnut Ridge of Pike County, Illinois, located on the highlands between the Illinois and Mississippi Rivers. The section of the orchard studied was direct seeded with seeds from the *C. mollissima* × *C. dentata* hybrid Dunstan, (Chestnut Hill Nursery, Alachua, FL, USA) with 6 m spacing between rows in 2001 and 2002. This area was previously an upland farm used for row-crop production that had been abandoned and had become covered with invasive arbuscular mycorrhizal (AM) shrubs such as autumn olive (*Elaeagnus umbellata* Thunb.). Because of the history of tillage and extended period without ECM fungus inoculum plants, the orchard would be expected to be depauperate in ECM fungus. The surrounding sloped areas are a mixed deciduous forest including oaks (*Quercus* spp.), the potential source of ECM colonization for the orchard (Palmer et al. 2008; Dulmer et al. 2014). Currently this orchard has around 12 ha of chestnut trees in production.

Within a section of the orchard, five transects were set up along alternate rows of trees (every 12 m), with each transect terminating 6 m from the adjacent forest edge. Starting with the chestnut closest to the forest, alternate trees were selected (every 12 m) until a total of 10 trees were sampled for that transect. This design followed the irregular shape of the orchard perimeter and resulted in a total of 50 focal trees with known distance from the forest edge. To assess ECM colonization, root samples were collected in four, 10 cm diameter (10 cm deep, 30 cm from tree trunk) soil cores from each four cardinal points of all the focal trees on 20, May 2016. After pooling four samples, they were placed in a cooler in the field and transported to the lab. In the lab, roots were isolated from soil using #30 (0.0232 in.) soil sieve to wash of the soil and keep the clean roots. These roots were then refrigerated at 4 °C until processing. Root samples were mixed, and the first 100 tips were quantified for the presence of external ECM using the gridline intersect method (Brundrett et al. 1996). In this method, root sections were trimmed and fitted in single layer in petri dishes having horizontal and vertical grids (1 cm intervals) in water, looked at each gridline intersection for mycorrhizal roots using dissecting microscope at 40 × magnification, increasing power as necessary. Colonization proportion was expressed as number of ECM per 100 root tips. ECM colonization was assessed as a function of distance to the forest edge using a regression analysis.

Impacts of ECM inoculation in the greenhouse

To assess whether ECM colonization is important in early plant growth and establishment, 80 chestnut seedlings were started from seed acquired from the field site. These seedlings were planted in the greenhouse in a soilless potting mix (peat moss, perlite and vermiculite in a 7:2:1 ratio with powdered Dolomitic limestone to adjust pH to approximately neutral). Half of the seedlings were inoculated with ECM (Diehard ecto injectable, Horticultural Alliance Inc, Sarasota, FL, United States). This commercial inoculum contains live spores (14,537,445 spores per gram) of generalist ectomycorrhizal fungi (*Pisolithus tinctorius* (Pers.) Coker & Couch and four *Rhizopogon* species). Seeds were started on uninoculated potting mix and transplanted into a 1-gallon root pruning container (RootMaker®, Huntsville, Alabama). The use of a root pruning container helps root development, accelerates growth and has become common in the horticultural industry. Uninoculated seedlings had the same inoculant that was sterilized by autoclaving to kill the bacteria and fungi in the inoculum, but still retaining nutrients that would be released. Half of the seedlings were harvested from each treatment after 90 days to determine the success of inoculation and its effects on plant growth. ECM colonization of each seedling's root system was assessed as described above. Above ground biomass was dried for 48 h at 60 °C and then weighed. The total number of leaves was also counted for each seedling.

The remaining 40 trees were subjected to an experimental drought starting on 12 September 2016, when watering ceased. Water stress was allowed to develop over a period of 12 days (drought), when watering was resumed (recovery) upon witnessing incipient wilting. Plant stress was evaluated with chlorophyll fluorescence using Fv/Fm (ratio of variable fluorescence to maximal fluorescence and calculated as $\left(\frac{Maximal\ fluorescence\ (Fm) - Minimal\ fluorescence\ (F0)}{Maximal\ fluorescence\ (Fm)}\right)$ value. Chlorophyll fluorescence responds to a wide spectrum of plant stressors such as water stress (Lu and Zhang 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). An Fv/Fm value in the range of 0.79–0.84 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 min, as preliminary work determined this was sufficient for chestnut. Fv/Fm values were recorded on each plant across the 20 days of the experiment to capture both the rate at which stress developed and recovered following the resumption of watering. In this experiment, the root pruning containers were randomly assigned to inoculum and drought treatments and kept intermixed on the greenhouse bench. As each seedling was in a separate pot, seedlings were used as replicates in all analyses.

As gas exchange can alter plant responses to drought, we also investigated the effects of ECM inoculation on seedling stomatal characteristics. Two leaves were taken from each plant on the last day of the drought experiment (day 20). Leaf epidermal 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, left to dry completely, and the impression removed with clear tape. This tape was attached to a microscope slide and three fields of view were photographed at 400 × 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 and length was determined. All image analysis was performed in ImageJ (Schneider et al. 2012).

The impact of mycorrhizal inoculation and experimentally imposed drought on chlorophyll fluorescence of chestnut leaves was assessed with repeated measures ANOVA using inoculation as the between-subject effect and time and the time × inoculation interaction as within-subject effects. This analysis was conducted three ways: using the entire data set, and separately for the drought (days 1–12) and recovery (days 12–20) phases. Additionally, impact of mycorrhizal inoculation on stomatal density and dimension of chestnut seedlings were assessed with ANOVA. For all the data, transformations were unnecessary as all comparisons met assumptions of normality of residuals (Shapiro–Wilk test) and homogeneity of variances (Bartlett's test). We used a standard alpha of 0.05 throughout to interpret the significance of test. All analyses were done using R version 3.1.2 (R Core Team 2014).

Results

ECM colonization in a seed-generated orchard

Although all root samples except one had some level of ECM colonization, trees ranged from no colonization (0%) to relatively high colonization (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 ($F_{1,45} = 6.38$, $P = 0.015$) with ECM colonization decreasing in trees located farther from the forest (Fig. 1).

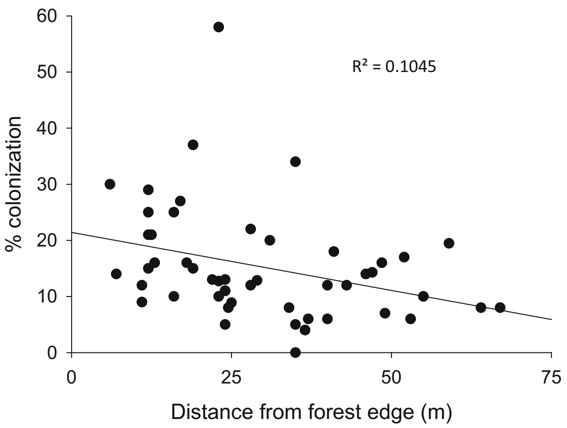

Fig. 1 Change in % ECM colonization in chestnut trees with the distance from adjacent oak forest ($P = 0.015$)

Impacts of ECM inoculation in the greenhouse

The experimental inoculation was moderately effective with inoculated seedlings having visible ECM colonization with a range from 3 to 31% ECM root tips per seedling (17% on average) at day 90. As we relied on external evidence of ECM fungi over a short time period, these rates are probably conservative estimates of colonization. Uninoculated seedlings did not have any ECM root tips. Despite having a relatively benign environment in greenhouse soil (i.e. without any competition or water stress), there was a significant response of chestnut seedlings to ECM inoculation. The number of leaves produced was 20% greater in inoculated seedlings (Fig. 2a, $F_{1,38}$ = 13.41, P = 0.0007, R^2 = 0.261). Similarly, inoculated chestnut seedlings grew around 16.4% more than uninoculated seedlings (Fig. 2b, $F_{1,38}$ = 7.13, P = 0.011, R^2 = 0.158). Greater levels of ECM colonization provided additional benefits as aboveground biomass increased with increasing mycorrhizal colonization ($F_{1,18}$ = 5.33, P = 0.033, R^2 = 0.228) among inoculated seedlings. ECM inoculation also resulted in marked changes in leaf stomata. Length of the stomatal complex were found to be shorter in inoculated seedlings ($F_{1,38}$ = 4.21, P = 0.047, R^2 = 0.099, Fig. 3a). While stomatal density was slightly higher (1.14 times) in inoculated seedlings, this was not significant between treatments ($F_{1,38}$ = 3.62, P = 0.06, R^2 = 0.087, Fig. 3b).

Mycorrhizal status also resulted in large differences in the drought response of greenhouse seedlings. In this study, chestnut seedlings inoculated with ECM showed faster recovery from drought than uninoculated seedlings during the rewatering phase. After the last watering on day 1, Fv/Fm values increased slightly over the first four days of the experiment (Fig. 4). Following this period, Fv/Fm values for both treatments decreased during the experimentally induced drought through day 12, when watering resumed. At day 12, stress levels in inoculated and uninoculated

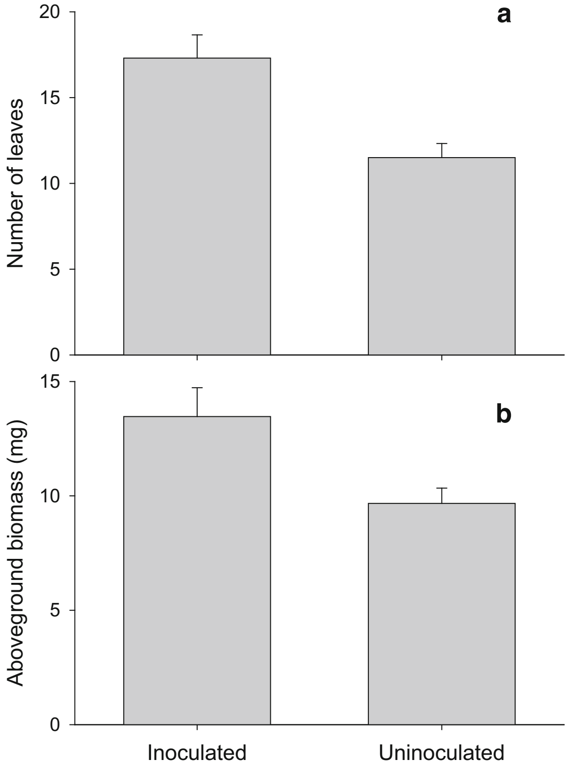

Fig. 2 Effect of mycorrhizal inoculation on the **a** leaf number (p = 0.0007) and **b** growth of chestnut seedlings (p = 0.011) grown in the greenhouse (after 90 days). Bar represents the standard errors.

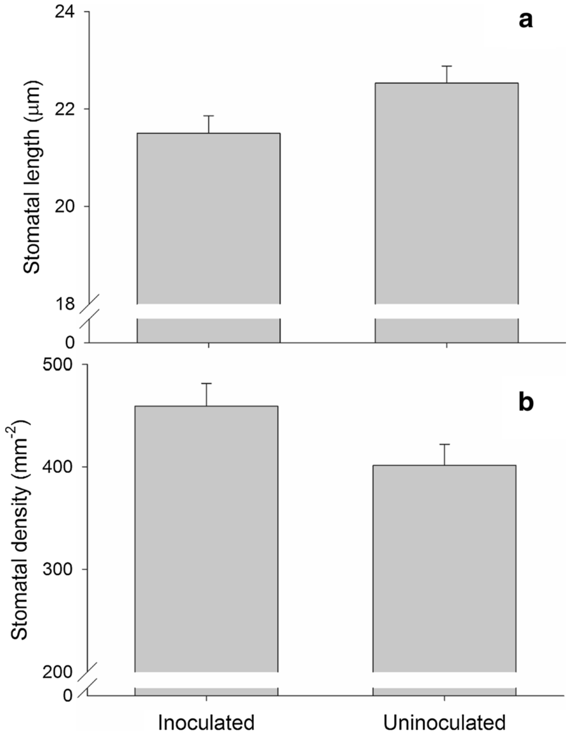

Fig. 3 Effect of ECM inoculation on average stomatal **a** length (p = 0.047) and **b** density of leaves (p = 0.06) of chestnut seedlings grown in the greenhouse (after 90 days). Bars represents the standard error.

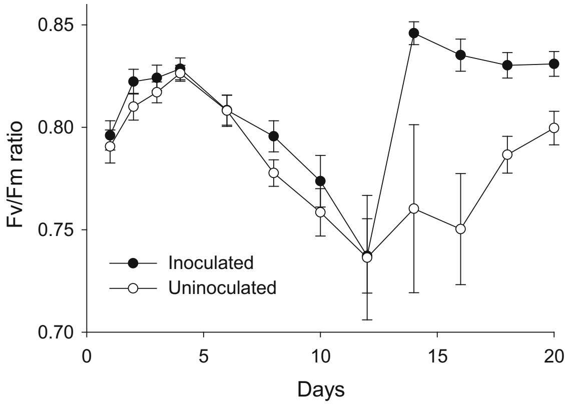

Fig. 4 Effect of experimentally imposed drought on the chlorophyll fluorescence (Fv/Fm, as an indicator of plant drought stress) of inoculated and uninoculated chestnut seedlings. Watering last occurred at day 1 and resumed on day 12 only. Bars represents standard error

plants was nearly identical. However, following the resumption of watering, inoculated seedlings recovered much faster than uninoculated ones. Across the entire drought experiment, Fv/Fm values significantly varied with inoculation and time ($F_{1,38}$ = 6.03, P = 0.018 and $F_{11,28}$ = 13.39, $P < 0.001$ respectively). Similarly, the time × inoculation interaction was also significant ($F_{11,28}$ = 2.82, P = 0.013; Table 1). When the experiment was separated into drying and recovery phases, differences were clarified. During the drying phase, Fv/Fm responses for inoculated and uninoculated chestnut seedlings were not significantly different ($F_{11,28}$ = 0.96, P = 0.333; Table 1) but did change with time ($F_{7,32}$ = 10.72, P = 0.001; Table 1) 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 differences between treatments ($F_{1,38}$ = 9.14, P = 0.004; Table 1) and time ($F_{3,36}$ = 0.19, P = 0.900; Table 1) with no interaction ($F_{3,36}$= 1.86, P = 0.153; Table 1).

Table 1 Repeated measures ANOVA 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	13.39	**0.001**
Day × inoculation	11,28	2.82	**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	9.14	**0.004**
Day	3,36	0.19	0.900
Day × inoculation	3,36	1.86	0.153

Values in bold are significant values

As transpiration can be associated with stomatal structure (Jarvis and McNaughton 1986), and stomatal length differed with inoculation, it was examined if it could be responsible for drought responses in both treatments. However, stomatal length was not correlated with value of Fv/Fm in either treatment (inoculated: Pearson R = – 0.03, P = 0.8 and uninoculated: Pearson R = 0.06, P = 0.7).

Discussion

ECM colonization in a seed-generated orchard

Growing trees that have a high degree of specificity for their mycobiont in a new locality, such as *Pinus* and *Eucalyptus* species, often requires a supporting ectomycorrhizal partner (Díez 2005). Seedling establishment in case of primary successional setting could be critical to due to lack of contact to ECM networks (Nara 2015). 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 and spores into nearby vegetation (Dickie et al. 2004). The higher ECM colonization of chestnut root tips near the forest edge could have been generated by mycorrhizal fungi present on the existing forest vegetation (Dickie et al. 2002; Palmer et al. 2008; Dulmer et al 2014). The lower colonization with greater distance from forest edge could be due lack of contact to other trees (Kranabetter and Friesen 2002) and dependence on decreasing ECM spore dispersal. For an example, Nara (2015) studied the role of ECM in seedling establishment in volcanic desert and found that the seedlings transplanted near the pre-established willow shrubs, where ECM networks are available, readily formed ECM associations and grew well.

The declining rate of ECM colonization with distance from surrounding forest in this study is consistent with the results of Bauman et al. (2012) which found the highest rate of ECM colonization (58%) of American chestnut (*Castanea dentata*) along a forest edge, followed by 14% in seedlings growing along an established *Pinus virginiana* stand. Similarly, D'Amico et al. (2015) found colonization rates of *Castanea* from *Quercus forest* inoculum of 45–55% on average, though their seedlings were grown for much longer. The average rate of ECM colonization (29%) in our study appears to be relatively low compared to other studies such as in *Abies alba* growing outside of its native range, nearly 100% ECM colonization was found (Rudawska et al. 2016) and around 75% of ECM colonization was found in *Quercus garryana* studied in southern Oregon (Valentine et al. 2004). A study by Nara and Hogetsu (2004) showed that the growth and nitrogen content of the seedlings increased significantly with the numbers of ECM root tips. The low level of ECM colonization in our study may inhibit chestnut performance in this orchard. As the orchard has recently adopted a fertilization schedule, we were not able to relate ECM colonization to plant performance.

Impacts of ECM inoculation in the greenhouse

ECM colonization of plant roots often improves the water and nutrient uptake of hosts (Smith and Read 2010). Chestnut seedlings that were inoculated with ectomycorrhizal fungi were larger than uninoculated seedlings. This study clearly indicates that colonization with ectomycorrhizal fungi helps chestnut seedlings in their early growth and development, even under relatively benign culture conditions. The increase in above ground biomass with the increasing ECM colonization in this study is consistent with a study showing that an increased shoot height and biomass with ECM colonization in *Shorea seminis* (Turjaman et al. 2006). Although it is well documented that ECM fungi have beneficial effects on plant growth and production, it is unclear whether ECM activity was the driver of plant growth, or if plant fitness contributed to ECM colonization (Bauman et al. 2013). As ECM on *Castanea* produce smaller external structures (Palmer et al. 2008), estimates from inoculated seedlings are likely quite conservative.

ECM inoculation also resulted in marked changes in leaf stomata with significantly shorter stomatal complex lengths in the inoculated chestnut seedlings. As plants with smaller stomata may have better drought tolerance (Mehri et al. 2009), inoculated chestnut seedlings should also tolerate drought better than uninoculated chestnut seedlings. However, changes in stomatal characters were not related to drought tolerance, as Fv/Fm was similar between inoculated and uninoculated plants throughout the drought phase of the experiment. However, the larger size of inoculated seedlings should have resulted in more rapid depletion of water during the experiment, so equivalent stress between treatments is still somewhat surprising in this study. As ECM fungi in oaks and other trees of Fagaceae increase water absorption (Egerton-Warburton and Allen 2001), mycorrhizal inoculation may have altered the physiological constraints on the plant sufficiently to alter leaf properties, and therefore water balance. As there was no significant correlation between stomatal length and Fv/Fm, this argues that the effect of mycorrhizal colonization on drought response was not primarily generated by the alteration of stomatal characteristics. But, as fungal hyphae can access smaller pore spaces in soils through an increased absorbing surface, efficient conduction through mycelial strands and enhanced hydraulic conductivity at the soil–root interface accessing water as soils dry, ECM symbioses help plants tolerate drought (Breda et al. 2006).

Water scarcity is one of the key limiting factors for the development of trees (Clark and Kjelgren 1990), leading to reductions in seedling recruitment, susceptibility to pathogen or insect attack, reductions in photosynthesis, and mortality with exposure to drought (Zhao and Running 2010; Reichstein et al. 2013). At day 12, stress levels in inoculated and non-inoculated plants were nearly identical, suggesting similar impacts on inoculated and uninoculated plants. Following the resumption of watering, inoculated seedlings recovered much faster, 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). Sánchez-Blanco et al. (2004) also found similar results; under drought conditions leaf water potential decreased in both non-mycorrhizal and mycorrhizal plants, but this decrease were lower in mycorrhizal plants. During drought, mycorrhizae could increase water uptake through lowered resistance to water flow from soil to roots and increased absorptive surface to reduce plant moisture stress (Reid 1979). While it is not clear why we 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 natural source of colonization but was still overall quite low and potentially problematic. To reduce the future risk of drought stress, direct ECM inoculation of existing orchard trees may be useful. Our results also suggest that ECM inoculation should be included to improve seedling growth and survival, and to establish ECM at orchard initiation. Managing mycorrhizal associations may prove to be an important component of chestnut culture as a part of a regional sustainable agriculture program, particularly when converting land from contemporary row crop agriculture practices. Future research should look at the long-term benefits of ECM inoculation in field settings to quantify value of managing ECM in sustainable agriculture.

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