Maximizing the Symbiosis of *Tuber lyonii* with *Carya illinoinensis*

by

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ABSTRACT

Truffles are the culinary desirable ascocarps of the fungal genus *Tuber*. *Tuber lyonii* (pecan truffle) is a species of truffle endemic to North America and can also be found in pecan (*Carya illinoinensis*) orchards. Its proclivity to form ectomycorrhizae with pecan trees is the driving factor behind the research described here. The overall objective was to maximize colonization of pecan seedling roots by *T. lyonii*. Two varieties of pecan rootstock, 'Elliott' and '87Mx5-1.7' were tested and both formed ectomycorrhizae with *T. lyonii* when grown in containers and exposed to greenhouse culture conditions. Inoculation with *T. lyonii* led to taller seedlings with larger stem diameter in both varieties of rootstock. Fertilization treatment influenced colonization percentages. The foliar inorganic application treatment had the highest mean *T. lyonii* colonization compared to a foliar organic and inorganic soil drench applications. Colonization percentages were higher in root samples collected from near the substrate surface as compared to samples from the lower portions of the root system. Vernalization and fungicide application did not influence *T. lyonii* colonization in pecan roots, which remained statistically similar in treated and untreated plants. Results show that pecan seedlings can be successfully grown in containers in symbiosis with *T. lyonii* with implications for nursery production and management of inoculated seedlings. While further optimization of the production system may be required, this work is a significant first step toward developing reliable methods for producing and managing pecan truffle inoculated pecan seedlings.

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CHAPTER I

INTRODUCTION

Tuber lyonii Butters (Figure 1.1), commonly referred to as the pecan truffle, is an ascomycete fungus in the order Pezizales that forms ectomycorrhizae with the roots of several families of hardwood trees including the Juglandaceae and Fagaceae. *Carya* spp. in the Juglandaceae, especially *Carya illinoinensis* (Wangenh.) K. Kock (pecan tree), are economically significant species in the United States known and utilized for their nut crop (Figure 1.1) (Benucci et al. 2011).

Ectomycorrhizae are a type of mutualistic symbiosis between tree roots and fungi belonging to multiple phyla. The symbiosis is characterized by the formation of a sheath of fungal hyphae (Figure 1.2) (i.e., mantle) on the outside of roots and a maze-like growth of fungal hyphae between the epidermal and cortical cells (i.e. Hartig net) and a hyphal network growing into the soil (i.e., extraradical mycelium) (Smith and Read 2010). Approximately 3% of seed plants form ectomycorrhizae and almost all of the ectomycorrhizae forming plants are woody perennials (Smith and Read 2010). Considering the economic value of timber and use and distribution of other tree species in terrestrial ecosystems, ectomycorrhizae represent a significant component of global biodiversity (Meyer 1973, Smith and Read 2010).

Figure 1.1 Fresh *Tuber lyonii* ascocarps harvested in Texas (A). A typical pecan orchard in Charlie, TX showing the general appearance of a pecan orchard (B).

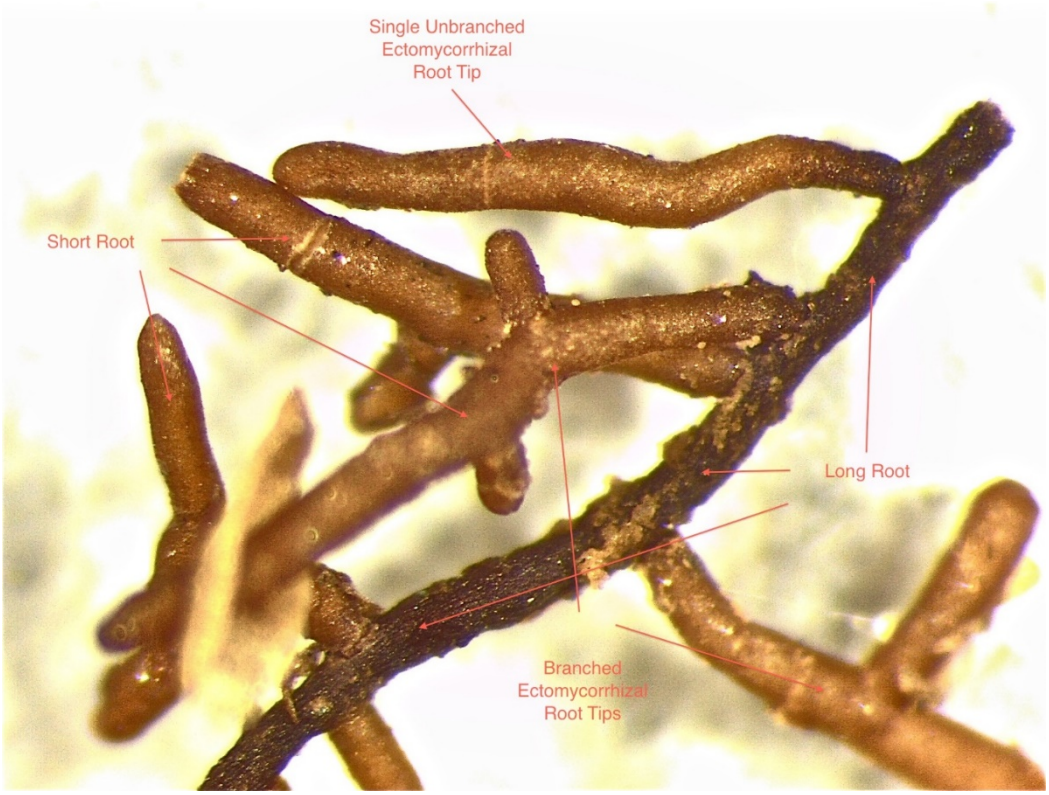

Figure 1.2 Ectomycorrhizal root tips that were formed by *Tuber lyonii* on *Carya illinoinensis*. The image distinguishes between long non-colonized and short fibrous roots that are colonized by *T. lyonii*.

The genus *Tuber* (which includes the gastronomically useful truffle producing species) has a cosmopolitan distribution and occurs in Europe, Asia, and North America. *Tuber* spp. produces a hypogeous ascocarp, some of which have aromatic characteristics that lend themselves to culinary use. These and other hypogeous fungal fruiting bodies that have aromatic characteristics are used for culinary purposes worldwide and are called 'truffles.' Besides the genus *Tuber*, edible truffles are produced by several other genera of Ascomycetes and Basidiomycetes including *Terfezia*, *Tirmania*, *Mattirilomyces*, *Leucangium*, *Kalapuya*, *Hysterangium*, and *Melanogaster* (Diez et al. 2002, Trappe et al. 2007, Trappe et al. 2010). Gastronimically prized truffles, however, occur naturally only in limited regions around the world, which primarily include the regions of Perigord, Provence, Piedmont, and Tuscany across France and Italy (Pilz et al. 2009).

Culinary truffles are known for their high cost and for their status as a luxury food commodity. Their natural 'rarity' and the challenges of locating and harvesting them from their hypgeous locations have led to their high economic value (Hall et al. 2007). These fungal species rely on mycophagy by mammals for spore dispersal (Frank et al. 2006) and produce aromatic compounds that indicate ripeness and attract potential dispersal agents. Traditionally, pigs were utilized to locate the underground fruiting bodies but this required ensuring that the animals did not consume the truffles (Hall et al. 2007). Over time, trained dogs have become the method of choice for locating subterranean, culinary truffles across the world (Hall et al. 2007, Pilz et al. 2009). Currently the USDA does not recognize culinary truffles as a specialty crop, although there is a generic mushroom category listed (USDA 2014).

European Truffle Industry

Research on truffle cultivation began in Europe in the mid nineteenth century and led to the discovery of ectomycorrhizae in 1885 (Lefevre and Hall 2000). European truffle production peaked in the late nineteenth century after the phyloxera epidemic nearly destroyed European viticulture (Sourzat 2009). Many vineyards were repurposed as truffieres by viticulturists who were concerned about another phyloxera outbreak. The viticulturists had had previous experience finding truffles growing in their vineyards

within proximity to downy oaks (*Quercus pubescens*), and this led to a high number of *Q. pubescens* being planted in place of grapes (Sourzat 2009). When phyloxera initially struck France there were 80,000 hectares of vineyards and as of 2009 there are only 5,000 hectares of vineyards. The two decades preceding World War I saw the highest truffle production in European history (Sourzat 2009). For example, in 1892 France estimated a harvest of 2,000 tons of Black Perigord truffles, compared to the 30-40 tons estimated in 2003 (Sourzat 2009). The post-world-war decline of the European truffle harvest has been a topic of speculation (Pruett et al. 2007, Sourzat 2009). Some believe the decline was a repercussion of each world war as two generations of European men were lost, leading to the loss of knowledge of harvesting and propagation techniques**.** Others believe that changing forest management practices, such as brush collection for fuel, may have created an environment that was no longer as conducive to truffle ecology (Sourzat 2009). Others have attributed this decrease in yield to abiotic factors like climate change as well (Sourzat 2009). Whether this was due to monotypic forest plantations or unnatural stages of succession is still undetermined (Pruett et al. 2009, Hall et al. 2003, Hall et al. 2007). As truffle yield decreased over the 20th century, their prices rose significantly. The export value of European truffles increased to $19.4 million over a ten-year period between 1992 and 2002 as a result of decreasing supply (Niskanen 2006).

Growing U.S. Market

European truffles species (*Tuber melanosporum*, *Tuber magnatum*, *Tuber aestivum*, and *Tuber borchii*, for example) have already established themselves as a culinary delicacy in the U.S. (Pilz et al. 2009). Chefs currently pay anywhere from $1000 to $1500 per pound of European truffles (Pilz et al. 2009). At the same time a North American truffle market is developing, especially in the Northwestern U.S. Native North American truffles species include *Tuber gibbosum*, *Tuber oregonense*, and *Leucangium carthusianum* (Pilz et al. 2009). *Tuber lyonii* (pecan truffle), discovered in 1903, is the latest addition to gastronomically desirable North American truffles. The species occurs naturally across the Southeastern United States and has the potential to be an economically important specialty food crop. Chefs may be particularly interested in pecan truffles because of their relatively low cost when compared to the European

truffles imported from France and Italy. Pecan truffles are priced at approximately $150-200 per pound (Sharma et al. 2012, Smith et al. 2012). Pilz et al. (2009) projected the 2030 market value of the native Oregon truffles in the range of approximately $4.5 to 600 million by considering both low and 'optimistic' demands.

Tuber lyonii **(The Pecan Truffle)**

Tuber lyonii Butters, the pecan truffle, is synonymous with *Tuber texense* Heimsch (Heimsch 1958) although there is still some speculation as to whether this reclassification is appropriate (Matt Smith, personal communication). *Tuber lyonii* has been recorded in 16 U.S. states, two northern states in Mexico, and also in two provinces of Canada (Smith et al. 2012). The known range for *T. lyonii* is distributed north to south from Quebec to Florida, and east to west from the Gulf Coast to the Rocky Mountains (Smith et al. 2012, Sharma et al. 2012). *Tuber lyonii* is currently known to form ectomycorrhizae with three genera of woody angiosperms, which include *Carya, Quercus,* and *Corylus* (Smith et al. 2012). The name *Tuber lyonii* was first applied to truffles collected in Minnesota in 1903 growing apparently with *Tilia americana,* which belongs to the Malvaceae family (Butters 1903). Some speculation remains as to whether the *T. lyonii* from Minnesota are in the same species complex as that of the *T. texense* discovered in Texas by Heimsch in 1958 (Matt Smith, personal communication).

Tuber lyonii fruiting bodies have been collected and vouchered from across the years in various parts of its range. However, it appears that fruiting is heavier during the winter months. In Texas, a methodological survey was conducted in November 2012 when pecan truffles were collected from six counties. Five of these collections were made in commercial pecan orchards while one was from an irrigated home lawn where an untrained domestic dog was locating and consuming the pecan truffles (Sharma et al. 2012). Additional pecan truffle surveys in 2013 and 2014 have indicated that there might be a strong correlation of fruiting and soil moisture. While this has not yet been quantified experimentally, pecan truffles were recently found near a damaged irrigation line at the USDA pecan research orchard in Somerville, Texas in April 2014 (Figure 1.3) (LJ Grauke, personal communication). Further, the most reliable fruiting site has so far

been in an irrigated home lawn in northwestern Texas, which only receives approximately 45 cm natural precipitation annually. Pecan truffle harvests have been larger recently in Georgia and Florida (Smith et al. 2012) where annual rainfall is significantly higher and can range from 100 to 150 cm annually.

Figure 1.3. Pecan truffles found in April 2014 at the USDA Pecan Breeding Station in Somerville, TX. These truffles were found while repairing a cracked irrigation line.

Tuber lyonii is an ideal candidate for co-cropping in commercial pecan orchards potentially across all regions where pecan trees are cultivated for nut production because: (1) pecan truffle associates naturally with pecan trees, (2) pecan orchards have a concentration of the host tree, and (3) the orchards provide easier access than natural forests. Additionally, the culinary market in the United States is beginning to recognize the pecan truffle for its gastronomic potential, with it commanding between $150-200 per pound (Sharma et al. 2012, Smith et al. 2012). Co-cropping pecan truffles along with the pecan trees could be a source of alternative income for pecan growers. Pecan truffles are prevalent enough in some orchards that workers have found truffles on the sorting table that had been picked up by the mechanical sweepers at harvest (Tim Brenneman, personal communication). Further, small farms may benefit more because of lower vehicular traffic in the orchard, which may limit the amount of compaction caused by machinery and harvest equipment (Hall et al. 2007). However, further research is needed to optimize the production methods for producing inoculated pecan seedlings, orchard management practices, and harvest, storage, and marketing practices before pecan truffle and other North American truffles can become better known across the continent and across the world. In the past, unreliable harvesting techniques have damaged the culinary reputations of North America's finest truffle species. Animal assisted harvesting is routine in Europe and helps maximize the chances of harvesting ripened truffles, but in the U.S. and China, most truffle hunters have traditionally used a rake to search through the leaf litter and topsoil (Pilz et al. 2009). Raking indiscriminately harvests truffles without regard to the ripeness of the specimen, which results in the harvesting of many unripe, and undesirable truffles. These immature truffles negatively impact the reputation and marketability of native North American truffles. The use of dogs to locate truffles is ideal because the dogs are trained to locate ripe truffles. The benefit of this is two-fold: (1) unripe truffles are left to be harvested later when ripe, and (2) dog located truffles are of higher and more uniform quality.

Optimal Culturing Conditions

Ecological and cultural data for *Tuber lyonii* are sparse and just now becoming available (Beuchat et al. 1993, Bonito et al. 2011, Sharma et al. 2012, Smith et al. 2012).

Inferences about *T. lyonii* are thus based on the information available for the European truffles, particularly *T. melanosporum* and *T. aestivum*. However, it is worth noting that culture requirements and growing conditions for *T. lyonii* may be significantly different and need to be established for the species by conducting manipulative experiments. In U.S. orchard surveys, *T. lyonii* was found more often in orchards with higher pH soils averaging 6.2 compared to an average of 5.7 for non-*T. lyonii* orchards (Matt Smith, personal communication), whereas *Tuber melanosporum* thrives in the calcareous soil of the Piedmont region, preferring a pH between 7.6 and 8.3 (Hall et al. 2003, Pilz et al. 2009). In comparison, *Tuber aestivum* seems to perform best in a pH range between 6.7 and 7.5 (Pruett et al. 2009). *Tuber* spp. generally prefers well-drained calcareous soils with warm summer temperatures and relatively low, but well partitioned summer rainfall (Bonet et al. 2009, Hall et al. 2007). Due to their low nutrient requirements, European truffle cultivation is being encouraged in rural areas with poor soil quality as an alternate means of agroforestry income (Hall et al. 2007).

Anthropogenic deposition of nitrogen and phosphorous has been shown to have an effect on ectomycorrhizal colonization density, as well as ectomycorrhizal community structure (Lilleskov et al. 2002). Some genera of mycorrhizal fungi benefit from additional amounts of nitrogen, and phosphorous while others are adversely affected by it (Lilleskov et al. 2002, Liu et al. 2012). To my knowledge, literature on effects of fertilizers on the *Tuber* genus is limited. Considering that pecan orchards are agricultural systems that receive external fertilizer inputs, knowledge of how additional mineral nutrients impact the mycorrhization, growth, and fruiting of pecan truffle would be of importance.

Pecan growers also utilize a variety of fungicides to protect the pecan tree's leaves and nuts from fungal diseases (scab, powdery mildew, downy spot, leaf scorch, brown leaf spot, vein spot, hypoxylon canker, cotton root rot and kernel decay) to which many commercial varieties are susceptible (Stein and McEachern 2007). Fungicides are primarily applied as a foliar spray to prevent or destroy plant pathogenic fungi (Stein and McEachern 2007). Despite the use of fungicides in commercial pecan orchards, *T. lyonii*

has been located in orchards where such practices are a routine part of the pecan orchard operations (Sharma et al. 2012). This observation raises the question whether fungicides used for pecan pathogens negatively impact the development of ectomycorrhizae, including *Tuber lyonii*. Zambonelli and Iotti (2001) showed that different fungicides had differing effects on the interactions between *Tuber borchii*, the bianchetto truffle, and *Hebeloma sinapizans* an EcM basidiomycete that competes with *Tuber* for host root space on *Quercus*. Some nematocides (1,2-dibromo-3-chloropropane) as well as fungicides (Captan®, Demosan®, Dexon®, Terrazole®) have been shown to affect the community structure, affecting colonization levels for *Tuber* as well as *Schleroderma* (Zambonelli and Iotti 2001, Bonito et al. 2011).

Finally, vernalization and dormancy is important for the development of many temperate hardwood tree species. Most research regarding *C. illinoinensis* and vernalization focuses on its effect on flower production, particularly the differentiation and development of pistillate catkins (Smith 2012, Wood et al. 2003). Dormancy has been shown to have a positive effect on the uniformity of bud-break in the spring (Sparks 1993). There is insufficient research on the direct effects of vernalization and dormancy on *C. illinoinensis* during its juvenile, or vegetative, growth stage. At the same time, there is no information on vernalization and temporary dormancy and its effect on ectomycorrhizal colonization of the host plant's root system.

Overall, there is little information on the culturing techniques used to propagate *Tuber lyonii* inoculated pecan seedlings. There is currently no literature concerning the management of adult *T. lyonii* inoculated trees and enhancement of EcM root colonization with regard to increasing the subsequent production of *T. lyonii* fruiting bodies. A set of research experiments was conducted to fill some of the gaps in knowledge with respect to pecan seedling inoculation and culture conditions to obtain plants that can be transplanted into pecan orchards for truffle culture. Specific aims and relevant hypotheses for this project are listed below.

Specific Aims and Hypotheses

Specific Aims

1. Assess ectomycorrhizal (EcM) colonization in two varieties of *Carya illinoinensis* after inoculation with *Tuber lyonii.*
2. Determine the effect on plant growth and EcM colonization of vernalization on plants of 'Elliott' variety that were either un-inoculated or inoculated with *Tuber lyonii*.
3. Evaluate plant growth and EcM colonization in response to interactions of plant variety and fungicide treatments.
4. Evaluate plant growth and EcM colonization in response to interactions of plant variety and fertilizer treatments.

Hypotheses for Specific Aim 1

1a. Plants inoculated with *T. lyonii* will have higher colonization by EcM than un-inoculated plants.

1b. There will be no difference in EcM colonization levels between the two rootstock varieties.

Hypotheses for Specific Aim 2

2a. Vernalized seedlings will show an increase in spring growth in comparison to non-vernalized seedlings.

2b. Colonization of *T. lyonii* will be higher in vernalized plants in comparison to non-vernalized seedlings.

Hypotheses for Specific Aim 3

3a. Fungicide application will cause a reduction in EcM colonization compared to plants untreated with fungicide.

3b. Plant growth will remain may improve when plants are treated with fungicide.

Hypotheses for Specific Aim 4

4a. Pecan seedlings fertilized with a soil drench of inorganic fertilizer (SI) will show a decrease in EcM colonization and a decrease in overall root production due to the prevalence of nutrients in the rhizosphere.

4b. Soil inorganic fertilizer (SI) treated plants will also show more vigorous seedling growth.

4c. Colonization by EcM will be greater in plants with foliar fertilization, albeit with lower growth rates.

CHAPTER II

MATERIALS AND METHODS

Materials and methods that were common to all experiments are presented first. Experimental design for each specific aim is discussed subsequently.

Study Species

Seeds of *Carya illinoinensis* variety 'Elliott' were purchased from a pecan grower in Texas. Seeds were harvested in October of 2012 and used subsequently in year 2013 for various experiments. Additionally, seeds of another variety were included in some experiments. For this purpose, Dr. LJ Grauke (USDA Pecan Breeding Station, Somerville, Texas) collected and provided the seeds of a variety of Mexican origin called '87MX5-1.7'. Seeds were stored dry at room temperature until they were used for experiments.

Fruiting bodies of *Tuber lyonii* were obtained or from our collections made in 2012 within Texas or from Florida (Dr. Matt Smith, University of Florida). Fruiting bodies were kept frozen at -20°C until they were used for experiments.

Seed Pre-germination Treatments, Germination, and Seedling Growth

Seeds of the 'Elliott' variety were first rinsed under tap water to remove loose debris. Subsequently, they were soaked for 5 min in a 10% bleach (6% NaOCl) solution. After rinsing with DI water, seeds were placed in a plastic bag containing moistened, autoclaved (121°C, 15 psi, 2x for 1 hour), coarse vermiculite. The bag was stored at 4C for 35 days to cold-stratify the seeds. We used a 40 days stratification period for 'Elliott' seeds that were used for the fertilizer experiment (Specific Aim #4). After the stratification period was completed, seeds were sown in a 23 cm x 41 cm x 8 cm plastic flat by placing them in autoclaved coarse vermiculite at the depth of approximately 2.54 cm. Sown seeds were watered as needed.

Seeds of the Mexican variety, '87Mx5-1.7', were processed similarly except that

these were not stratified because the variety is native to warm climates where they do not undergo a cold-dormancy. Instead of stratification, seeds were soaked in DI water for 10 days and water was changed daily. Sowing of the seeds was carried out as described for seeds of Elliott.

To ensure that the seeds of both varieties were sown on the same day for a given experiment, we first started the stratification of seeds of 'Elliott', and then 10 days prior to the sowing date, we started the soaking treatment of the Mexican variety. Seeds of both varieties germinated within approximately two weeks from sowing. Seedlings were then allowed to grow and develop expanded leaves over another two weeks prior to further use.

Inoculation

Each variety was handled separately to ensure that mixing of the seedlings from the two varieties did not occur. All plants that were assigned randomly to non-inoculation treatment were planted in their individual containers first to avoid the presence of any truffle inoculum in the area while the un-inoculated plants were being prepared and planted. Seedling roots were gently washed free of vermiculite by placing the roots of the seedlings in a tub filled with autoclaved DI water. Once vermiculite was loosened from the roots, seedlings were individually planted in appropriately labeled 7.5cm diameter containers (Figure 2.1). Substrate for these seedlings was composed of an autoclaved (121°C, 15 psi, 2x for 1 hour) mix of 2:1:1:1, v:v:v:v, peat:perlite:sand:vermiculite. Un-inoculated seedlings were manually handled as they would be for inoculation procedures to ensure that we did not create handling artifacts for the inoculated plants. Planting of un-inoculated plants was completed for each variety prior to handling the inoculum.

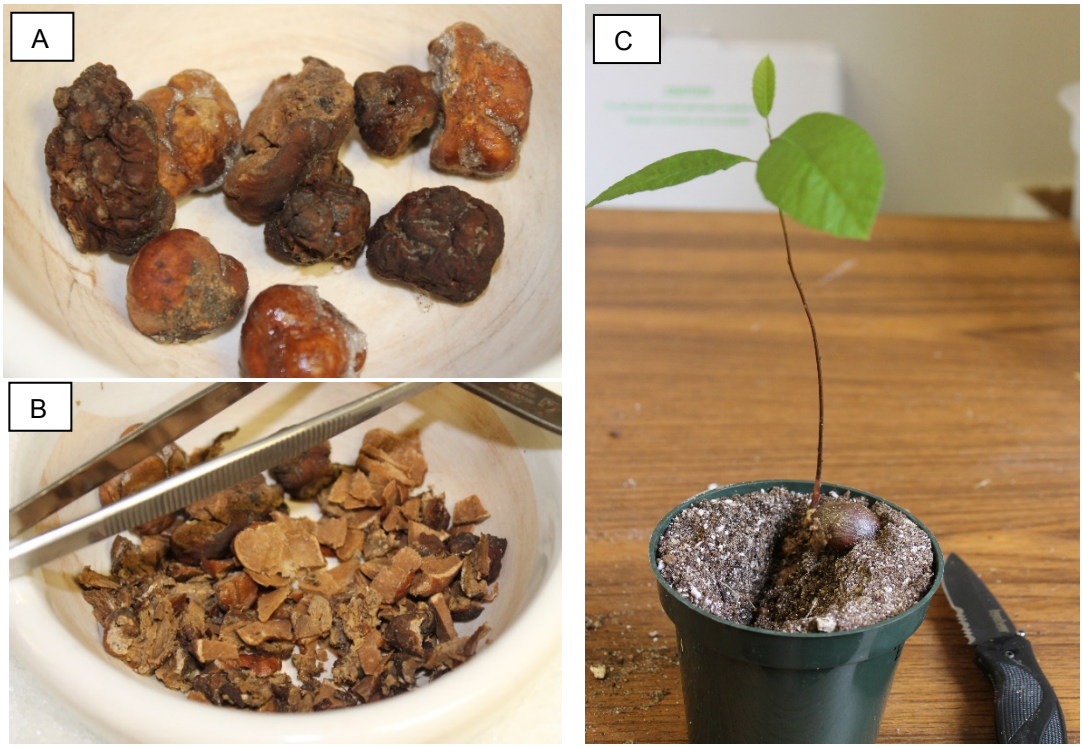

Figure 2.1 Whole *Tuber lyonii* fruiting bodies in mortar **(A)**. Sliced *T. lyonii* ready to be crushed into a paste **(B)**. A freshly inoculated seedling **(C)**.

Next, plants selected randomly for the inoculation treatment were processed for each variety separately. We followed the same planting methods for these plants as we did for the un-inoculated plants except that we applied truffle inoculum to the roots. Two grams (2 g) of truffle ascocarp for each inoculated seedling was ground with mortar and pestle and DI water was added to make a thick paste (Figure 2.1). The truffle paste was applied to a single root system with a spatula by smearing onto the fibrous roots. Seedlings were planted into the medium as described in the paragraph above without disturbing the roots and the inoculum that was applied to them (Figure 2.1). Finally, we added a few milliliters of DI water to the weigh boat used to measure and hold the inoculum for each plant to rinse the leftover spores into the medium around the seedling. All containers were watered with autoclaved DI water without allowing drainage of excess water.

Growth after Inoculation

All inoculated and un-inoculated seedlings were placed on a culture rack in the laboratory under a 16:8 light: dark photoperiod provided by four Sylvania 40W 'Cool white' fluorescent bulbs on each culture rack (Figure 2.2). Plants were watered as needed using autoclaved DI water. Leaves were sprayed several times a day to prevent transplant shock and dehydration. Plants were fertilized with Miracle-Gro Water Soluble All-purpose Plant Food (24-8-16) by using a foliar spray application every three days while preventing drip into the medium. This was done by placing a plastic sheet over the medium and around the plant stem to cover the surface of the substrate. Plants were grown in the laboratory for approximately four to six weeks depending upon the experiment. It was observed that a four-week growing period was preferable to a six-week period. Six weeks of growth in small containers led to root-circling which may stunt the subsequent growth of seedlings. Pest control was not necessary for plants while growing in the laboratory.

Relocation to Greenhouse

Plants were relocated into the greenhouse at Texas Tech University where the seedlings were transplanted into 30 cm deep tree-pot containers and experimental treatments were applied (Figure 2.2). The plants were grown under 15**%** shade cloth throughout the experimental period. Plants were irrigated as needed with reverse osmosis treated but unsterile water. Every three days, a foliar spray of Miracle-Gro (Water Soluble All-purpose Plant Food (24-8-16)) was applied to all plants unless plants were under the fertilization experimental treatments. Once every four weeks, NickelPlus (5ml/ 2L RO water. Nipan LLC. Valdosta GA) was applied as a foliar spray to all plants throughout the experimental period. We sometimes observed black aphids (*Melanocalis caryaefoliae*) and mites on the pecan seedlings growing in greenhouse conditions. Pyrethrin-based formulation (Pyrethrin Garden Insect Spray by Bonide Products Inc. Oriskany, NY) was used as needed when black aphids were visible, whereas Abamectin (Abamectin E-Pro 0.15 EC by Etigra. Cary NC) was used for controlling mites. When foliar thrips were observed Conserve (Conserve SC by Dow AgroSciences LLC. Indianapolis, IN) was used to control them.

Figure 2.2 Lab incubation rack with freshly inoculated pecan seedlings **(A)**. Pecan seedlings after being transplanted into tree-pot containers in the greenhouse **(B)**. Inoculated pecan seedling being transplanted into a 30.5 cm deep tree container **(C)**

Experimental Treatments

Specific Aim 1: Assess ectomycorrhizal (EcM) colonization in two varieties of *Carya illinoinensis* after inoculation with *Tuber lyonii*

Only inoculated plants of 'Elliott' and '87Mx5-1.7' were used for this experiment. We used 21 experimental units of 'Elliott' and 6 experimental units of '87Mx5-1.7' for this purpose.

The experimental units were grown in the greenhouse for approximately 1.5 years before their roots were sampled for *T. lyonii* colonization. Three inoculated experimental units of each variety were selected; three root samples from each of the three selected plants were obtained (Figure 2.3). The three root samples were randomly selected proportionally throughout the fibrous root zone in three equidistant locations, and organized from proximal to distil sample locations. Samples were stored at 4°C after wrapping in moist paper towels in individual plastic bags until they were processed further for colonization estimation.

Figure 2.3 *Carya Illinoinensis* root system showing four sample stratification levels throughout the fibrous root system. The arrow at the top of the picture indicates Level 1 with four respective levels marked by the arrows continuing down the fibrous root system. This was adjusted to three collection points throughout the fibrous root system.

In the laboratory, root samples were cleaned and freed of all soil medium debris. Each sample was placed in its own cassette (Tissue Path IV, Thermo Fisher Scientific Inc.) and individually labeled with pencil. The cassettes were submerged in DI water to prevent desiccation of the roots prior to staining. Staining with Trypan Blue (Trypan Blue, dye content > 60% by Alfa Aesar. Ward Hills, MA) was conducted as follows. Roots samples enclosed within the cassettes were submerged in 10% potassium hydroxide (KOH). Cassettes were then microwaved in KOH for approximately two to four minutes depending upon the batch by covering the beaker to prevent fume inhalation. After draining the KOH solution, cassettes were washed under running tap water to remove potassium hydroxide. A 25% solution of bleach (6% NaOCl) was then used to submerge the samples to remove phenolic and tannic compounds from the roots. This treatment lasted for approximately one minute. Samples were then washed under running tap water to remove traces of the bleach solution. Subsequently, a 3.33% 1N HCl solution was used to acidify roots for five minutes. Finally, a 0.2% solution of Trypan Blue in 1:1:1, v:v:v, glycerin: lactic acid: DI water was used to stain the roots after the HCl treatment by microwaving the submerged cassettes for approximately two minutes. Samples were then allowed to soak in the staining solution for two hours at room temperature. After draining the staining solution, samples enclosed within the cassettes were placed in DI water and stored at 4C to remove the excessive stain. Water was changed when it looked blue in color.

Once the roots had de-stained (over approximately one week), each root sample was individually inspected under a stereoscope to record the number of *T. lyonii* EcM and non-EcM root-tips (Figure 2.4). First, the total root length of the sampled long root was determined. Visibility of the mantle and whether the mantle was fully developed or immature were both used as an indication of mycorrhization (Figure 2.4). When we encountered atypical, non- *T. lyonii* EcM root tips, their occurrence was also noted.

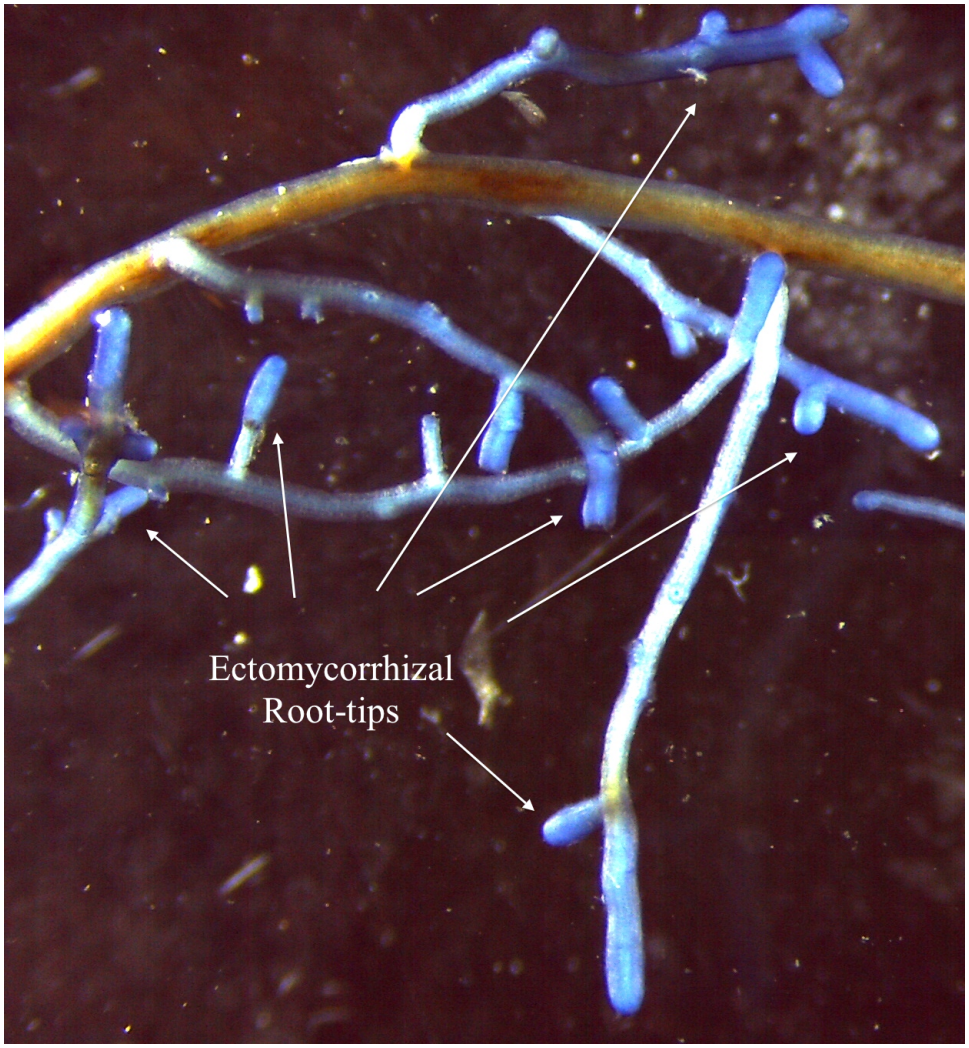

Figure 2.4 Ectomycorrhizal root tips formed by *Tuber lyonii* colonizing *Carya illinoinensis* roots. Trypan Blue stain was used to visualize ectomycorrhizal root tips.

Specific Aim 2: Determine the effect on plant growth and EcM colonization of vernalization on plants of Elliott variety that were either un-inoculated or inoculated with *Tuber lyonii*

For this experiment, only the 'Elliott' variety was utilized. A factorial arrangement of treatments was used with two inoculation treatments and two vernalization treatments. Six replicates of each treatment combination were utilized. Twenty-four plants were grown as described earlier. Twelve plants were inoculated with *T. lyonii* and the other twelve remained un-inoculated. Half of the plants (i.e., six experimental units) within each of the inoculation treatments were randomly assigned to vernalization treatments. Consequently, six inoculated plants and six un-inoculated plants received the vernalization treatment (Figure 2.5) that included placement of the plants in a poly-covered cold-frame. There was no additional climate control in the cold frame. However, if the nighttime minima dropped below -7°C, a small space heater was used by timing it to turn on and off in two hour intervals between 12 AM and 5 AM. Plants were grown in the greenhouse for 36 weeks prior to the vernalization treatment. On November 20, 2013 the vernalization treatment was initiated for the respective experimental units. These plants remained in the vernalization treatment until March 21, 2014. The vernalization period amounted to a duration of seventeen weeks. At the end of this period, treated plants were relocated to the greenhouse and placed beside their non-vernalized counterparts (Figure 2.6).

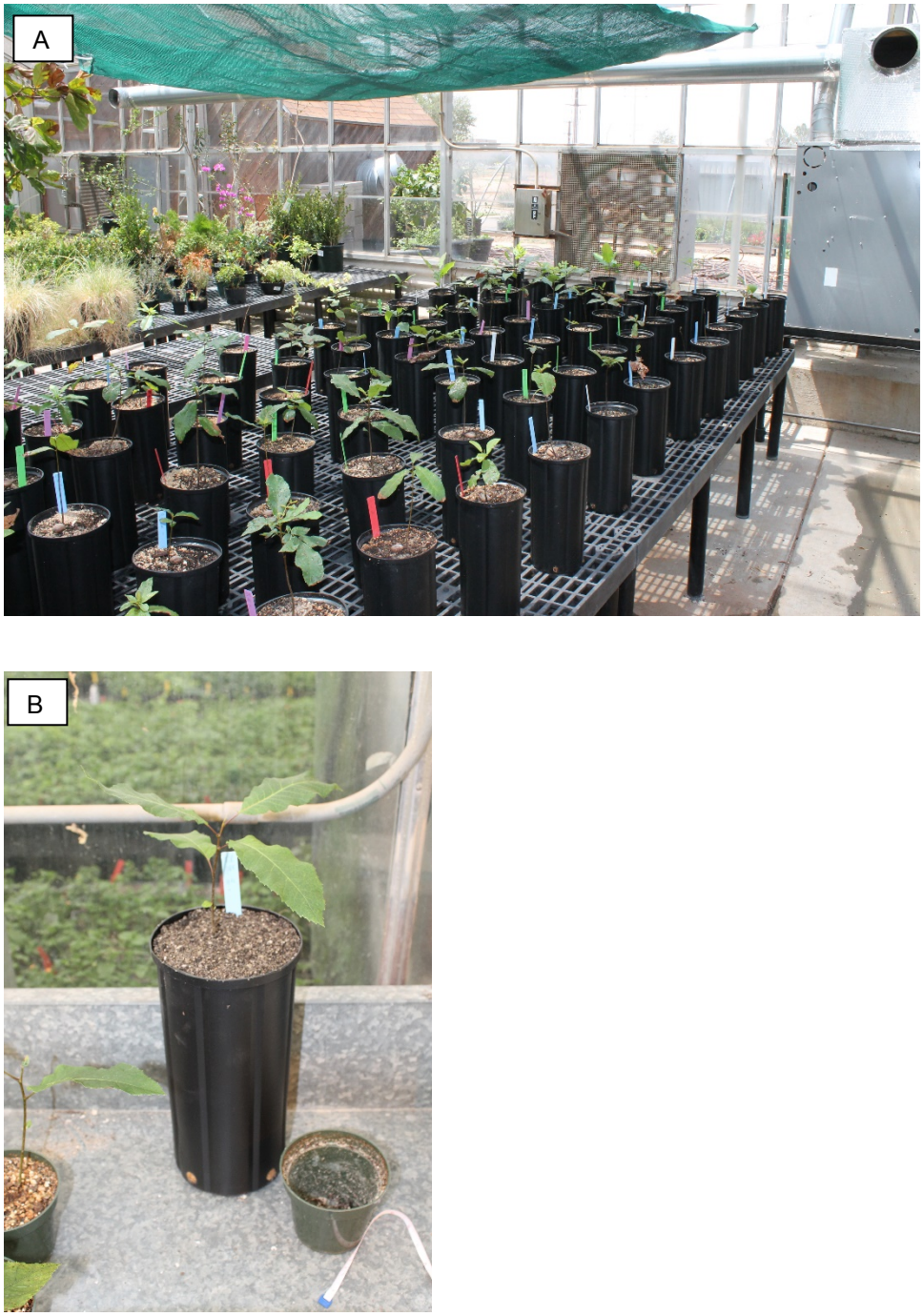

Figure 2.5 Multiple pecan plants at the Texas Tech University Plant and Soil Science greenhouse. A 15**%** shade cloth was used to reduce temperature and light intensity **(A)**. A fresh transplant from a 7.6 cm cup to a 30.5 cm deep tree container **(B)**.

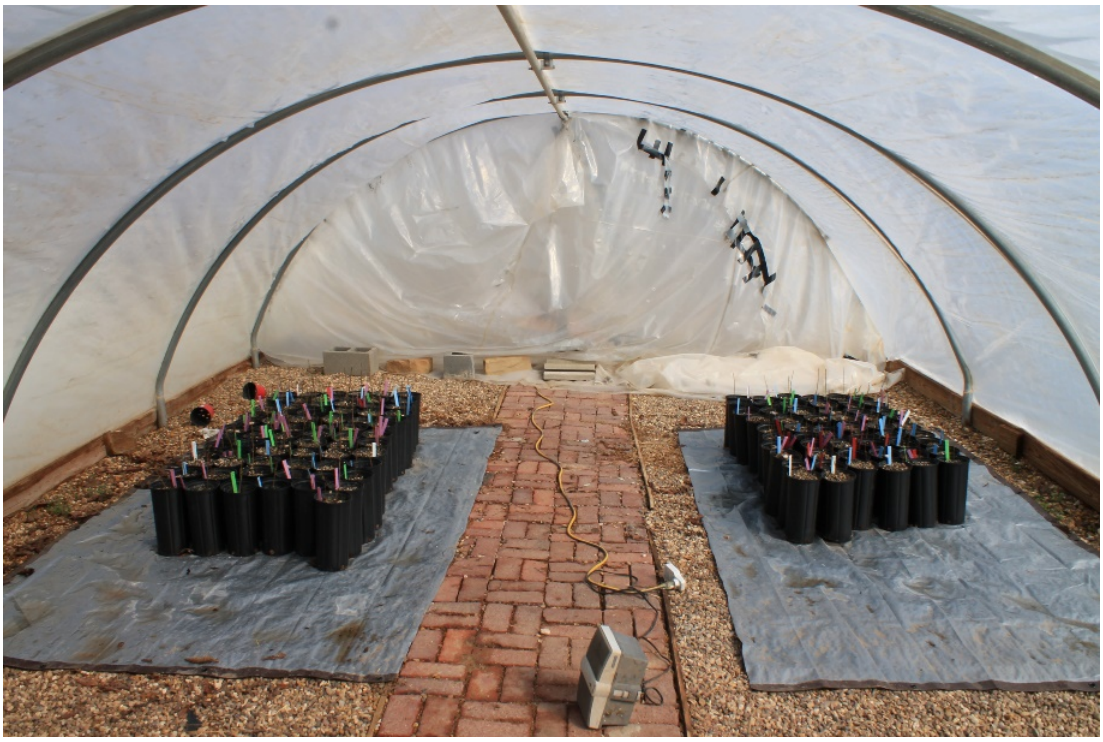

Figure 2.6 Pecan seedlings were vernalized in an un-heated quonset greenhouse with a poly-vinyl covering. A portable space heater was used on nights when the minimum temperature dropped below **-7 °C.**

While the plants were in the greenhouse and actively growing (pre- and post-vernalization), growth data were collected once every three weeks. Stem height was measured by measuring the height of the main stem from root collar to the leaf bud located at the apical meristem region. Juvenile leaves were counted by counting the number of leaves with a mid-rib exceeding 2.54 cm. Number of all compound leaves with at least 5 leaflets, each exceeding 2.54 cm mid-rib length, was recorded. Diameter of the main stem was recorded to the tenth decimal place by using a pair of digital calipers. The stem diameter was measured for each plant 2.54 cm above root collar. Substrate pH was recorded at the depth of approximately 2 cm by using a portable pH meter designed for measuring soil pH (FieldScout SoilStik pH Meter by Spectrum Technologies, Inc. Aurora, IL). Colonization of roots was quantified as described in Specific Aim 1.

Specific Aim 3: Evaluate plant growth and EcM colonization in response to interactions of plant variety, inoculation, and fungicide treatments

Twenty plants each of 'Elliott' and '87Mx5-1.7' varieties were grown as described earlier. A factorial arrangement of treatments was used with two rootstock varieties, two inoculation treatments, and two fungicide treatments. Five replicates of each treatment combination were utilized. Plants were grown in the greenhouse for 28 weeks before a factorial arrangement of the two pecan varieties, two inoculation treatments (inoculated and un-inoculated), and two fungicide treatments (sprayed and non-sprayed) was applied. Five replicate experimental units were assigned to each of the eight treatment combinations. The plants slated for fungicide treatments received a foliar spray of the fungicide at the recommended rate (134 uL/1 L RO water for a single annual application) with a hand-held spray bottle without preventing drip into the soil. Additionally, two sprays were applied to the soil surface to ensure some contact of the fungicide with the media.

We selected the fungicide Stratego 250 EC (Bayer CropScience LP. Research Triangle Park, NC) for this experiment, which is used in commercial pecan orchards. The reasons for selecting this particular formulation over many other fungicides used in pecan

orchards were two-fold: first, it includes two active ingredients propicanozole and strobilurin, conveying a broader spectrum of fungicides, and second, propicanozole has been noted for its efficacy in treating fungal pathogens from the Ascomycota.

Plants were irrigated, fertilized, and managed for insect pests as described in 'translocation to greenhouse' section. Plant growth was monitored and recorded as described in Specific Aim 2. Colonization of roots was quantified as described in Specific Aim 1.

Specific Aim 4: Evaluate plant growth and EcM colonization in response to interactions of plant variety, inoculation, and fertilizer treatments

Thirty seedlings each of 'Elliott' and '87Mx5-1.7' varieties were grown as described earlier in the laboratory. A factorial arrangement of treatments was used with two rootstock varieties, two inoculation treatments, and three fertilizer treatments. Five replicates of each treatment combination were utilized. Plants were then relocated to the greenhouse and a factorial arrangement of the two pecan varieties, two inoculation treatments (inoculated and un-inoculated), and three fertilizer treatments (listed below) was utilized to apply the experimental treatments immediately. Five replicate experimental units were assigned to each of the 12 treatment combinations. The three nutrient formulations and their application methods were as follows:

1. Foliar application of an inorganic fertilizer (Miracle-Gro Water Soluble All-purpose Plant Food (24-8-16) N - 24%, P_2O_5 - 8%, K_2O - 16%, B - 0.02%, Cu - 0.07%, Fe - 0.15%, Mn - 0.05%, Mo - 0.0005%, Zn- 0.06%). Application rate = 6.7g/L RO water. Plants were sprayed with a handheld spray bottle until foliage was evenly covered.

2. Foliar application of organic fertilizer (Organo-Choice. Royal-grow Products, Tulsa, OK. N -24%, P_2O_5 - 8%, K_2O - 16%, Fe - 0.10%, Zn -0.05%). Application rate = 15ml/L RO water. Plants were sprayed with a handheld spray bottle until foliage was evenly covered.

3. Soil application of an inorganic fertilizer [Miracle-Gro Water Soluble All-purpose Plant Food (24-8-16)] N - 24%, P_2O_5 - 8%, K_2O - 16%, B - 0.02%, Cu - 0.07%, Fe - 0.15%, Mn - 0.05%, Mo - 0.0005%, Zn - 0.06%). Application rate = 6.7g/L RO water. Approximately 200ml of the fertilizer solution was applied evenly to the surface of the medium.

Plants were irrigated and managed for insect pests as described in 'translocation to greenhouse' section. Plant growth was monitored and recorded as described in Specific Aim 2. Colonization of roots was quantified as described in Specific Aim 1.

Data Analyses

Analysis of Variance (ANOVA) was performed to test treatment differences and to conduct mean separation. Either a one-way, two-way, or a three-way ANOVA was utilized depending on the experimental design. The General Linear Model (GLM) procedure was used in SAS version 9.3 to conduct the analysis of variance for each experiment and Least Significant Difference (LSD) was used to separate the means. Dependent and independent variables used in each experiment are described below.

Specific Aim 1: Assess ectomycorrhizal (EcM) colonization in two varieties of *Carya illinoinensis* after inoculation with *Tuber lyonii*

A one-way ANOVA was utilized for the data obtained from Experiment 1 to test the effect of rootstock variety of *Carya illinoinensis* on EcM colonization (i.e., the response variable). A separate one-way ANOVA was used to test sample location on EcM colonization as well.

Specific Aim 2: Determine the effect on plant growth and EcM colonization of vernalization on plants of Elliott variety that were either un-inoculated or inoculated with *Tuber lyonii*

Experiment 2 had two independent variables: inoculation treatment and vernalization treatment. Five dependent variables were analyzed, two growth variables and two colonization variables: seedling height, seedling stem diameter, number of EcM root-tips, EcM colonization percentage, and root-tip density (RTD). A separate one-way ANOVA was used to test sample location effect on EcM colonization.

Specific Aim 3: Evaluate plant growth and EcM colonization in response to interactions of plant variety, inoculation, and fungicide treatments

Experiment 3 had three independent variables: rootstock variety, inoculation treatment, and fungicide treatment. Five dependent variables were analyzed in this study: seedling height, seedling stem diameter, number of EcM root-tips, EcM colonization percentage, and root-tip density (RTD). A separate one-way ANOVA was used to test sample location effect on EcM colonization.

Specific Aim 4: Evaluate plant growth and EcM colonization in response to interactions of plant variety, inoculation, and fertilizer treatments

Experiment 4 had three independent variables: rootstock variety, inoculation treatment, and fertilizer treatment. Five dependent variables were analyzed in the study: seedling height, seedling stem diameter, number of EcM root-tips, EcM colonization percentage, and root-tip density (RTD). Fertilizer treatments are: foliar inorganic (FI), foliar organic (FO), and soil inorganic (SI). A separate one-way ANOVA was used to test sample location effect on EcM colonization as well.

CHAPTER III

RESULTS

Specific Aim 1
Assess ectomycorrhizal (EcM) colonization in two varieties of *Carya illinoinensis* after inoculation with *Tuber lyonii*

Both 'Elliott' and '87Mx5-1.7' rootstocks formed ectomycorrhizae with *T. lyonii*. 'Elliott' rootstock showed 49.3% colonization ten months (August 29, 2013) after inoculation, and seven months later (March 20, 2014) showed a reduction to 21.3%. '87Mx5-1.7' rootstocks first measured 32.9% colonization (August 29, 2013) and at the second measurement showed a reduction to 8.9% colonization (March 20, 2014). There was no significant difference ($\alpha = 0.05$) between rootstocks in the number of EcM root-tips (Figure 3.1). There was a general trend for 'Elliott' rootstocks to have a greater number of EcM roots. Both varieties of rootstock showed a general decrease in colonization over time. Growth data were not collected for these plants.

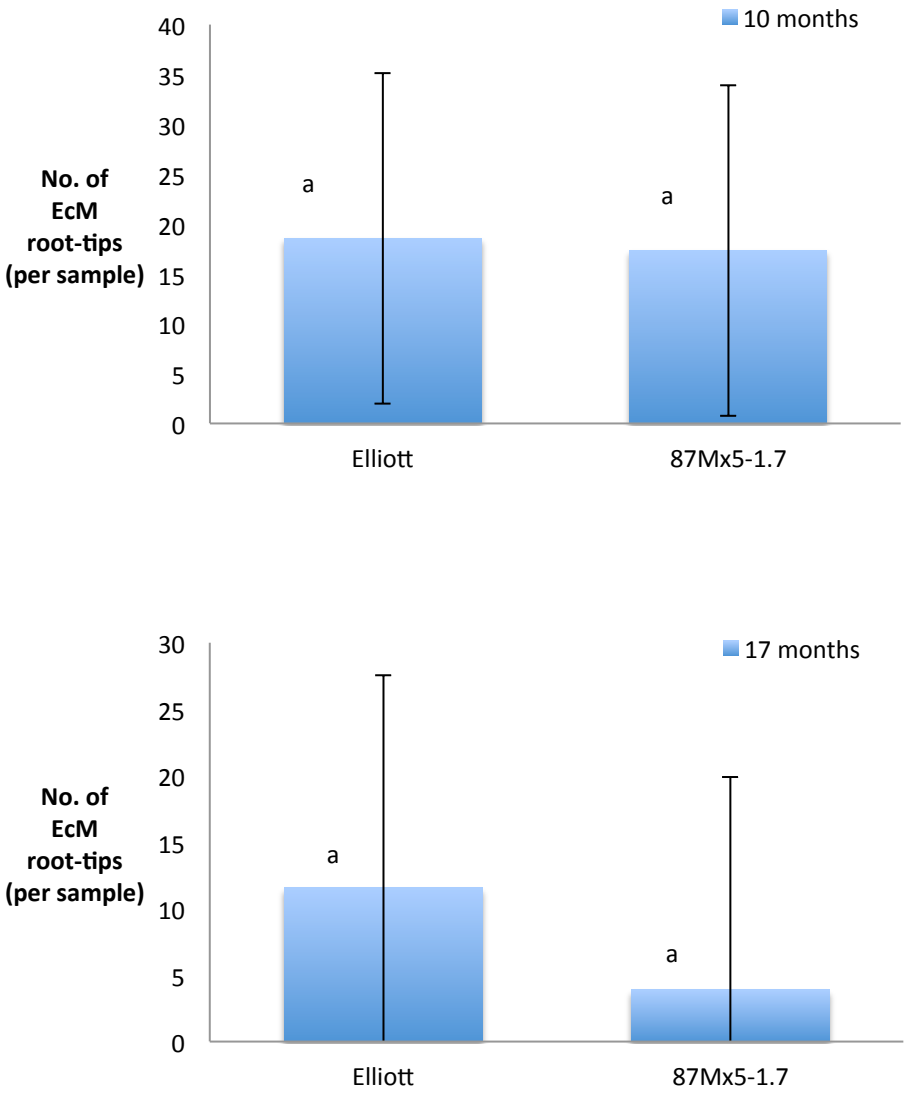

Figure 3.1 Mean number of *Tuber lyonii* colonized root-tips per sample in Experiment 1 shown with LSD bars and groupings. There was no significant difference ($\alpha = 0.05$) between varieties of rootstock at either ten or seventeen months after inoculation.

Specific Aim 2

Determine the effect of vernalization on plans ot Elliott Variety that were either un-inoculated or inoculated with *Tuber lyonii*

Experiment 2 consisted of 24 'Elliott' seedlings that were inoculated on November 12, 2012 and measured for colonization three times. Roots were sampled first at ten months (Sept. 25, 2013), which was before the vernalization treatment. After the vernalization treatment seedlings were sampled at fifteen months (Feb. 23, 2014), and at 21 months (August 17, 2014). Only main effects were found to be significant and listed; interaction effects were not found to be significant. This experiment had low levels of *Tuber lyonii* colonization throughout the study. The plants showed a general decline to almost no colonization at all. The colonization levels were 17.0%, 6.6%, and 0.4% (10, 15, 21 months respectively). The analysis showed no statistical difference ($\alpha = 0.05$) in the amount of EcM root-tips between the vernalized seedlings and the non-vernalized seedlings. The level of EcM colonization from the final analysis at 21 months was sufficiently low that it did not show a statistical difference ($\alpha = 0.05$) between inoculated seedlings and un-inoculated seedlings in regard to the amount of EcM root-tips (Figure 3.2). Vernalized seedlings showed no differences in colonization compared to the non-vernalized seedlings.

Sample stratification was analyzed separately to test if sample locations along the root system showed any consistent colonization patterns. This experiment showed a significant reduction in colonization as the experiment progressed, but at ten months when the experiment had its highest levels of colonization, there was a significant difference between sample stratification Level 1 compared to Level 2 and 3 ($p= 0.0036$) (Figure 3.3). This trend did not continue as the experiment progressed, but that could correlate with the loss of EcM colonization.

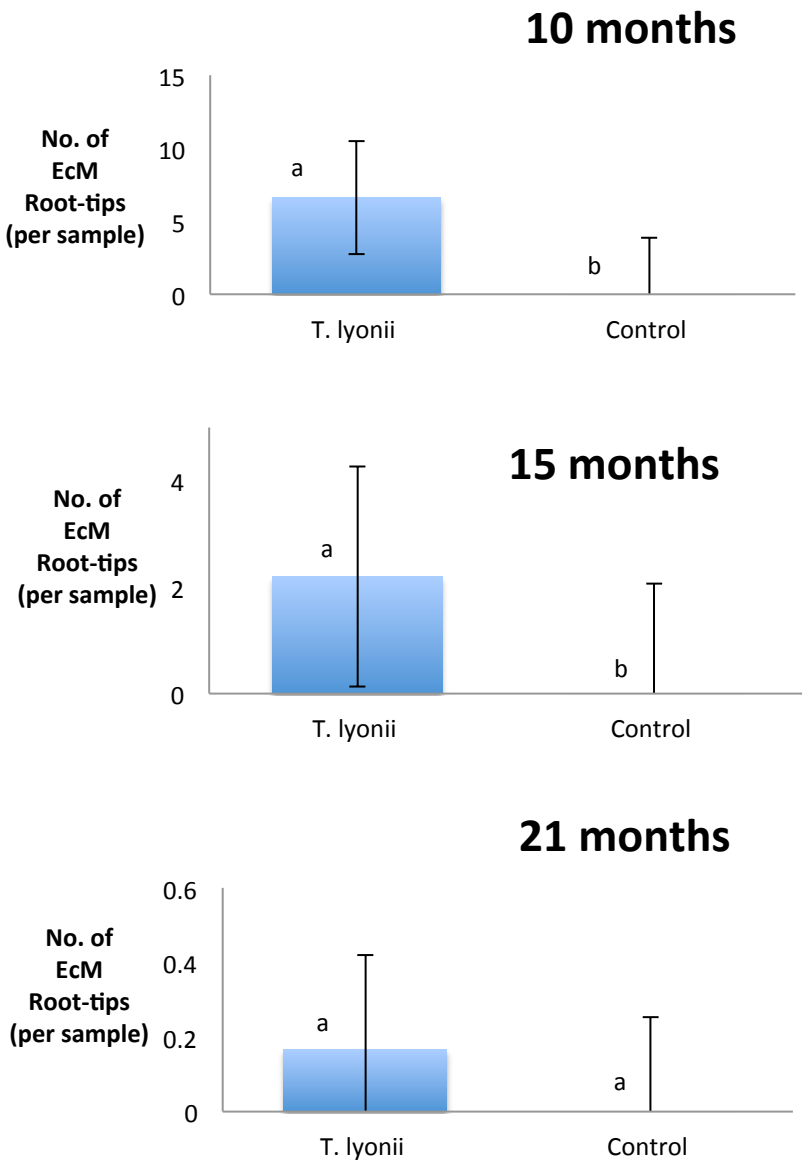

Figure 3.2 Mean numbers of EcM root-tips were recorded at three intervals after inoculation with *Tuber lyonii* in Experiment 2, which was carried out to test the effect of vernalization. A decline in EcM colonization can be seen in the above three charts. Seedling colonization was first analyzed with mean of 6.6 EcM root-tips per sample and at the final analysis had a mean of 0.17 EcM root-tips per sample. The final analysis at 21 months showed no statistical difference in colonization between inoculated and un-inoculated seedlings (p= 0.1892).

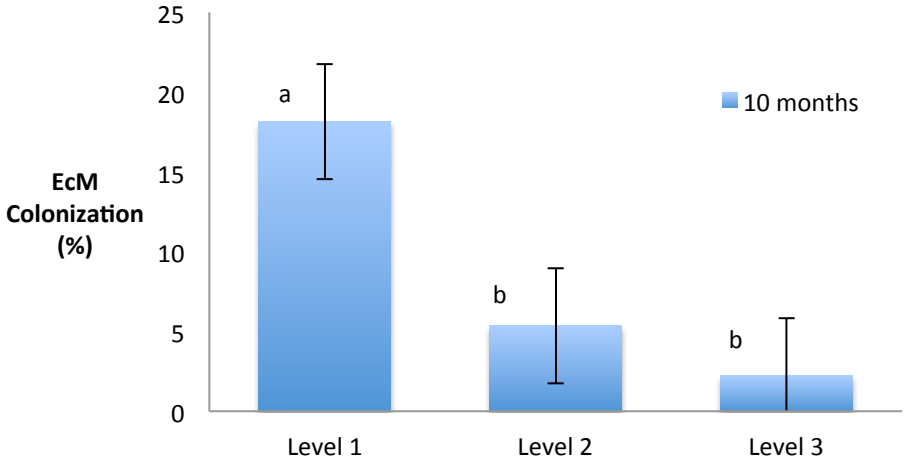

Figure 3.3 Mean colonization percentages to compare three levels of sample stratification in Experiment 2 shown with LSD bars and groupings. At ten months after inoculation, there was a significant difference in the mean colonization of Level when compared to Levels 2 and 3 (p= 0.0036).

Growth data were collected at three-week intervals on all plants throughout the experiment. Two dates were chosen for analysis, one before vernalization (Nov. 2, 2013) and the second after vernalization (July 29, 2014). Seedling height and seedling stem diameter were used to measure growth. The two vernalization treatments did not show any significant differences ($\alpha = 0.05$) in growth. When inoculation treatments were analyzed, seedling height and stem diameter only showed a statistical difference between treatments at nineteen months. *Tuber lyonii* inoculated seedlings had significantly larger ($p= 0.0106$) stem diameters (Figure 3.4).

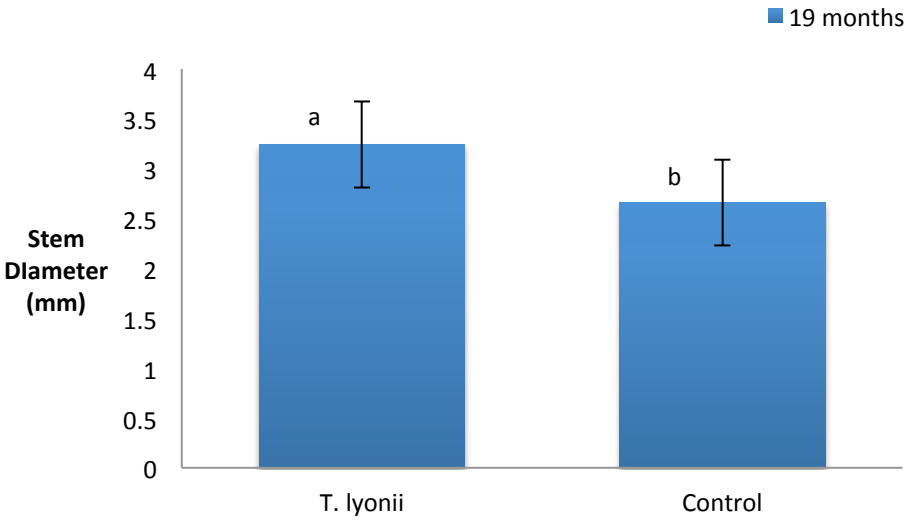

Figure 3.4 Mean stem diameter of *Tuber lyonii* inoculated and un-inoculated control seedlings from Experiment 2, shown with LSD bars and groupings. *T. lyonii* inoculated seedlings were found to have a statistically greater stem diameter compared to control seedlings at 19 months after inoculation (p= 0.0106).

Specific Aim 3
Evaluate plant growth and EcM colonization in response to interactions of plant variety and fungicide treatments

Experiment 3 consisted of 40 seedlings of both 'Elliott' and '87Mx5-1.7' varieties, half of which were inoculated with *T. lyoniii.* Half of these plants were also treated with Stratego 250 EC at six months (Oct. 12, 2013).

Colonization data were collected twice after the fungicide treatment: first, when the seedlings were eleven months (March 28, 2014) and second at eighteen months (Sept., 14, 2014). Main effects as well as interaction effects were analyzed, but only main effects were found to be significant. 'Elliott' seedlings were 11.6% colonized at eleven months and 27.1% colonized at eighteen months. '87Mx5-1.7' colonization was first measured at 27.4% and later at 31.8%. There was no significant difference ($\alpha = 0.05$) in colonization between the two rootstocks at either sampling period. Fungicide treatment showed no statistically relevant effect ($\alpha = 0.05$) on colonization (Figure 3.5). Despite there being no statistical significance between the two treatments, the fungicide treatments showed an interesting trend with colonization plants that received fungicide only showing a slight increase in colonization (11.7%, 12.2%). These results can be compared with the non-fungicide treatment, which originally showed 7.8% colonization at the first analysis and concluded with 17.3% colonization at the second analysis.

Sample stratification levels were also analyzed for differences in colonization in Experiment 3, LSD groupings were used to show significant groupings at $\alpha = 0.05$. A significant pattern was apparent at all three dates of analysis: five months, eleven months, and eighteen months. Level 1 samples were shown to be significantly different from Levels 2 and 3 throughout the experiment ($p= 0.0176$, $p= 0.0001$, $p= 0.0014$). Levels 2 and 3 were grouped together at five months and eighteen months, but all three levels were significantly different from one another at eleven months (Figure 3.6).

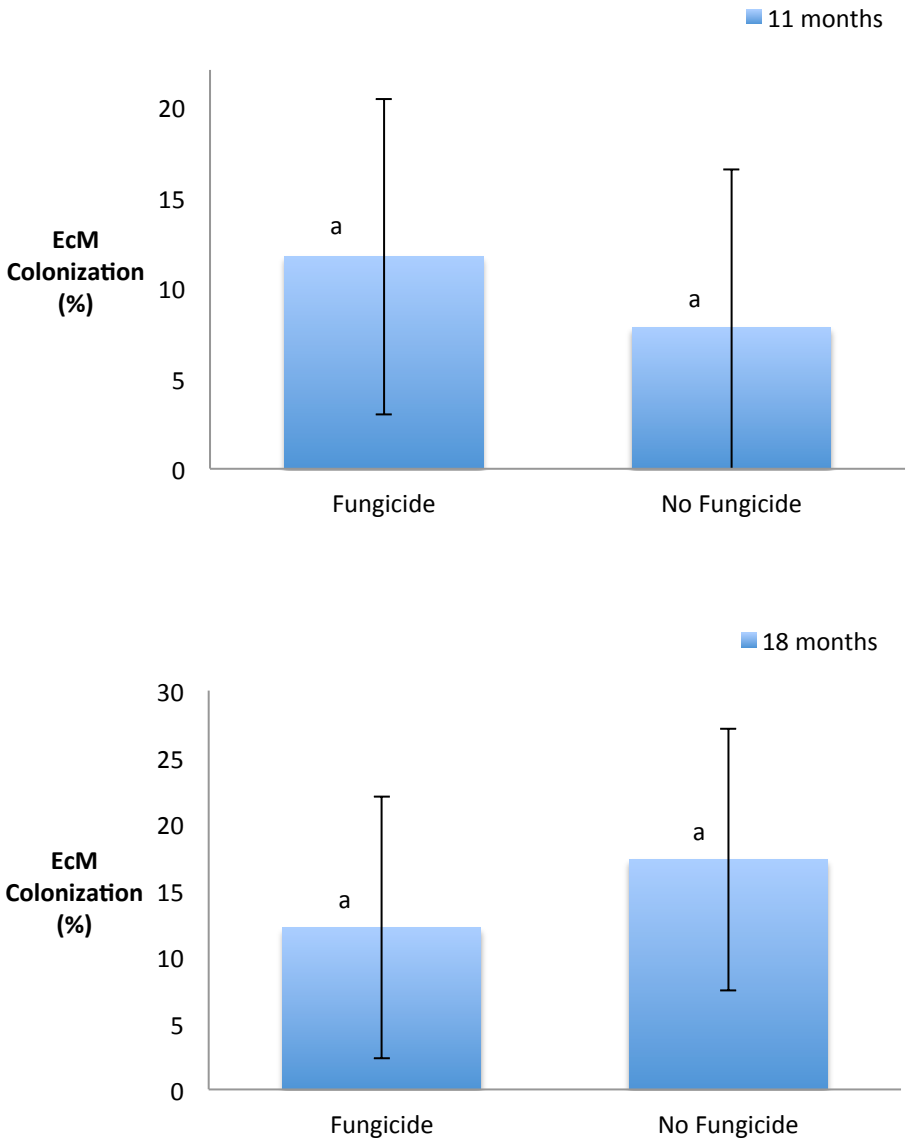

Figure 3.5 Mean EcM colonization percentages of seedlings treated with Stratego 250 EC compared to seedlings not treated with any fungicide in Experiment 3. LSD bars and groupings are shown. There was no statistical difference in either treatment at either eleven or eighteen months after inoculation (p= 0.3757, p=0.3039), but a slight trend could show a minor effect of fungicide on colonization.

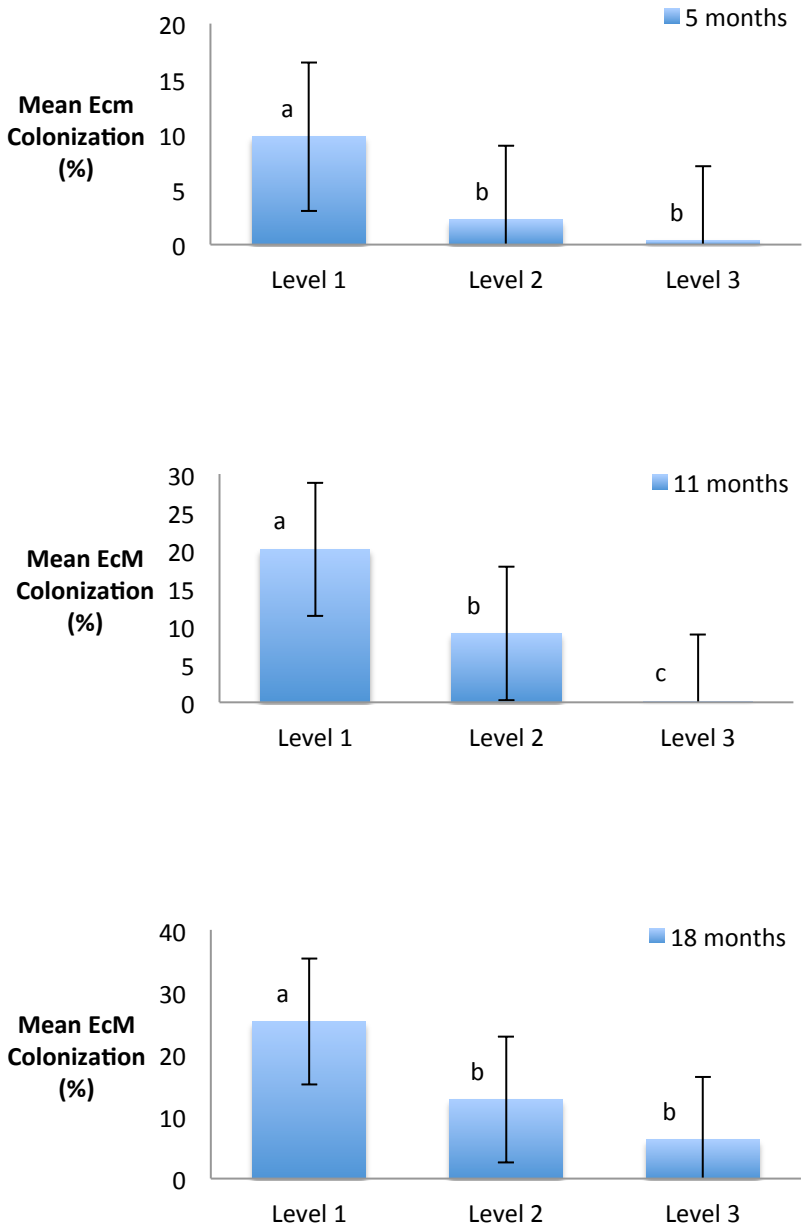

Figure 3.6 The mean colonization percentage of three sample stratification levels in Experiment 3, which was designed to test the effect of a fungicide application. The graphs are shown with LSD bars and groupings. A significant pattern is apparent in each of the three analyses. The results indicate Level 1 samples to have significantly greater EcM colonization compared to Levels 2 and 3 ($p= 0.0176$, $p= 0.0001$, $p= 0.0014$). Levels 2 and 3 are grouped together throughout the experiment, except at eleven months, when all three levels are significantly different from one another.

Growth data were collected at three-week intervals on all plants throughout this experiment. Two dates were chosen for analysis. Once, before vernalization, six months from inoculation (Oct. 23, 2013) and second, post vernalization, fifteen months from inoculation (July 30, 2014). 'Elliott' seedlings showed higher growth rates when compared to '87Mx5-1.7'. 'Elliott' seedlings were significantly taller ($p = 0.0001$) in the first analysis at six months, but not at the second analysis at fifteen months (Figure 3.7). 'Elliott' seedlings had significantly larger ($p= 0.0008$, $p= 0.0330$) stem diameters at both dates of analysis when compared to '87Mx5-1.7' (Figure 3.8).

When analyzing the effects of inoculation, inoculated plants of both rootstock varieties showed statistically significant difference ($\alpha = 0.05$) in both seedling height and stem diameter. Seedling height was only significant at fifteen months ($p= 0.0009$), with *Tuber lyonii* inoculated seedlings having a higher mean seedling height and a separate LSD grouping (Figure 3.9). Stem diameter was significantly different between inoculation treatments at both analysis dates, *T. lyonii* inoculated seedlings having higher mean stem diameter values ($p= 0.0043$, $p= 0.0330$) (Figure 3.10).

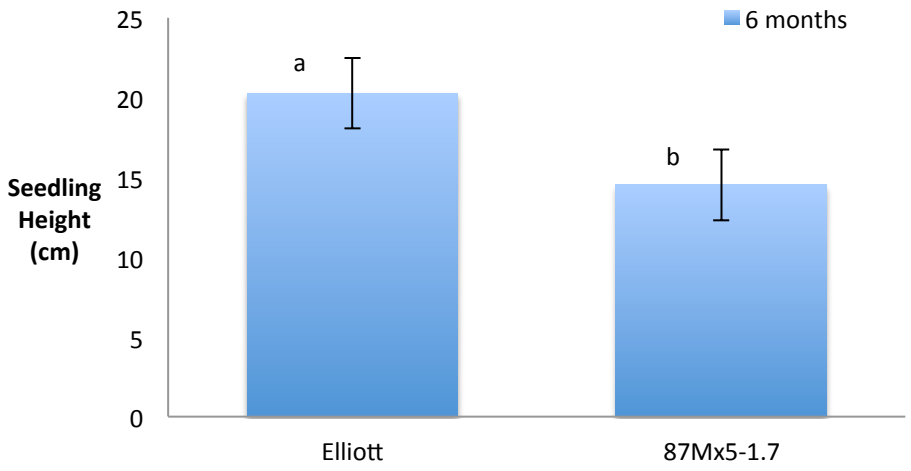

Figure 3.7 Mean seedling heights of two varieties of rootstock from Experiment 3, designed to test a fungicide application. The graph is shown with LSD bars and groupings. At six months from inoculation there is a statistical difference in height (p= <0.0001), indicating that 'Elliott' seedlings are taller than '87Mx5-1.7'.

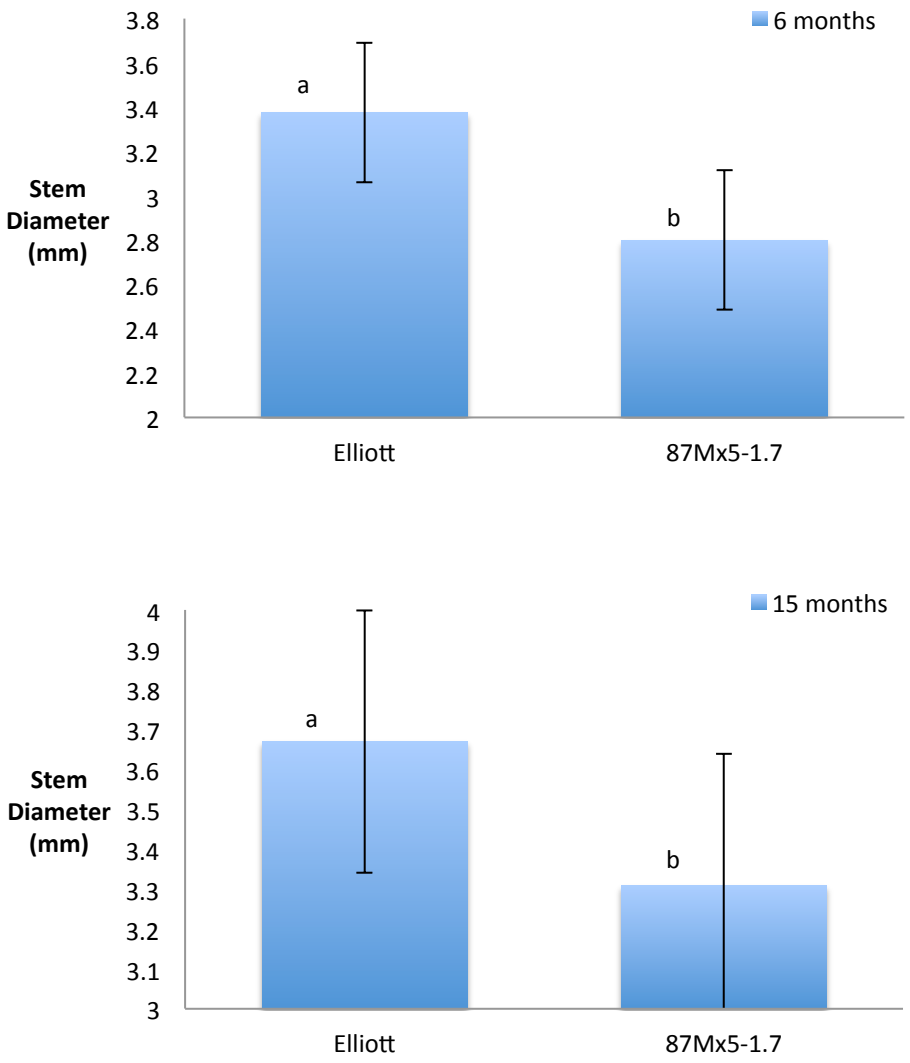

Figure 3.8 The mean stem diameter of two rootstock varieties in Experiment 3, shown with LSD bars and groupings. 'Elliott' seedlings have a higher seedling stem diameter at both six and fifteen months after inoculation when compared to '87Mx5-1.7' (p= 0.0008, p= 0.033).

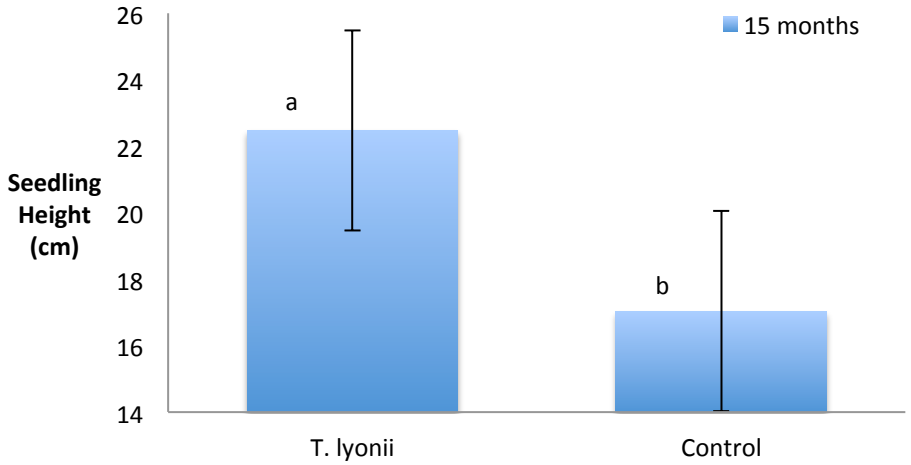

Figure 3.9 Mean height of *Tuber lyonii* inoculated seedlings and un-inoculated seedlings from Experiment 3, shown with LSD bars and groupings. At 15 months a statistical difference (p= 0.0009) is shown between the mean seedling heights of the two treatments.

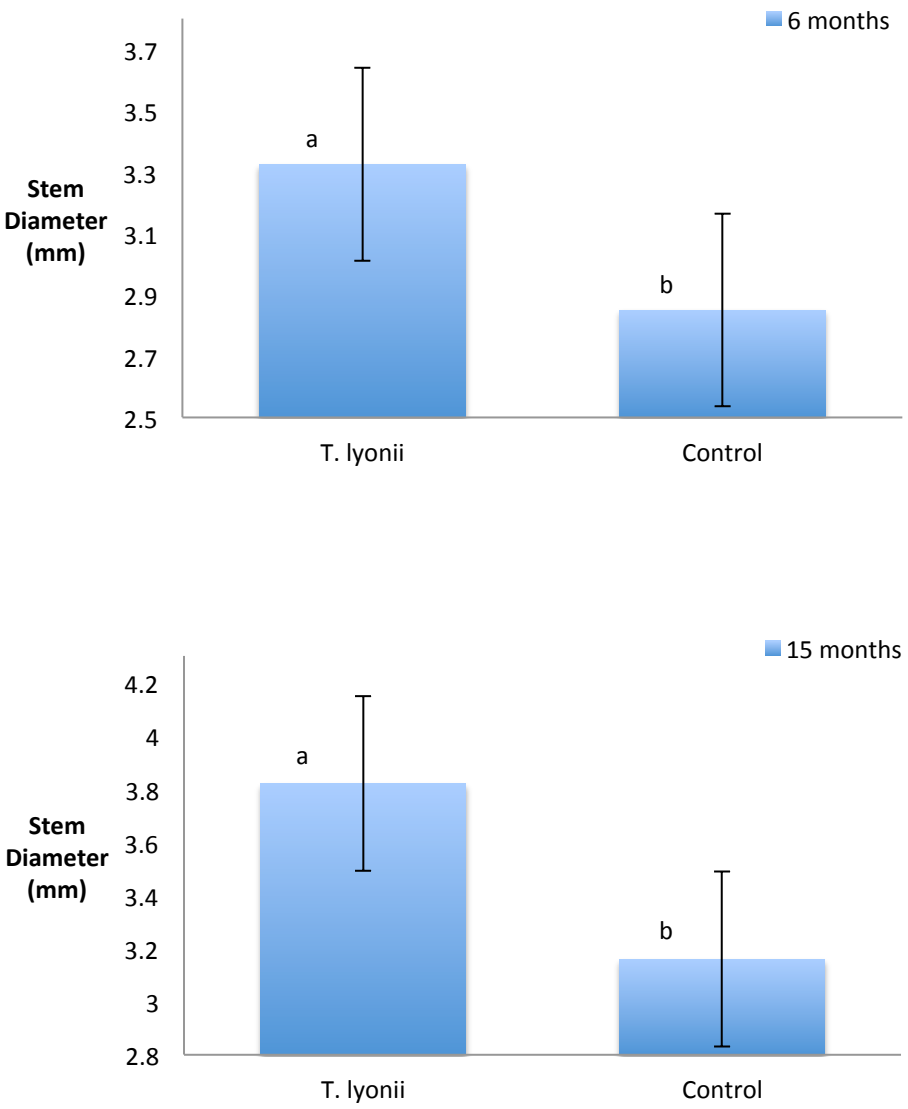

Figure 3.10 Mean stem diameters of *Tuber lyonii* inoculated and un-inoculated seedlings from Experiment 3, shown with LSD bars and groupings. *T. lyonii* inoculated seedlings were found to have significantly greater stem diameters (p= 0.0043, p= 0.0330) at both six and fifteen months after inoculation.

Specific Aim 4

Evaluate plant growth and EcM colonization in response to interactions of plant variety and fertilizer treatments

Experiment 4 consists of 60 seedlings, half 'Elliott' and half '87Mx5-1.7'. One half of both rootstocks were inoculated with *T. lyonii*. The seedlings were then split evenly among three fertilizer treatments: Foliar Inorganic (FI), Foliar Organic (FO), and Soil Inorganic (SI). Both main effects and interaction effects were analyzed, but only main effects were found to be significant.

Colonization data were collected three times throughout the experiment, at four months (Aug. 10, 2013), eleven months (March 20, 2014), and sixteen months (August 28, 2014). Both 'Elliott' and '87Mx5-1.7' rootstocks showed similar levels of colonization, and did not show any significant difference ($\alpha = 0.05$) between varieties. Fertilizer treatment did significantly affect colonization levels. In the first analyses FI had significantly higher ($p= 0.0401$) levels of colonization than both FO and SI. At the second analyses FI was significantly grouped with higher levels of colonization than SI, but FO was similar to both treatments ($p= 0.1054$). The third analyses showed that both FI and SI had significantly higher ($p= 0.0002$) levels of colonization than FO. FI treatment consistently had the highest levels of colonization among the three treatment types throughout the experiment (Figure 3.11).

Root-tip density (RTD, the number of fibrous-roots / total length of sample) was also analyzed to look for differences between inoculated and un-inoculated seedlings as well as between both varieties of rootstock. Between varieties the RTD showed a consistent trend with '87Mx5-1.7' seedlings having a higher level of RTD. This trend was only significant ($p= 0.0017$) at the third analyses (Figure 3.12). When analyzed by inoculation the un-inoculated seedlings showed a significantly higher ($p= 0.0199$, $p= 0.0198$)) level of RTD throughout the experiment, excluding the final analyses where both treatments were not significantly different but still showed the same trend (Figure 3.13). The analyses of RTD in respect to fertilizer treatment showed treatments FI and FO having significantly higher ($p= 0.0006$, $p= 0.0006$, $p= 0.0019$) levels of RTD

compared to SI (Figure 3.14).

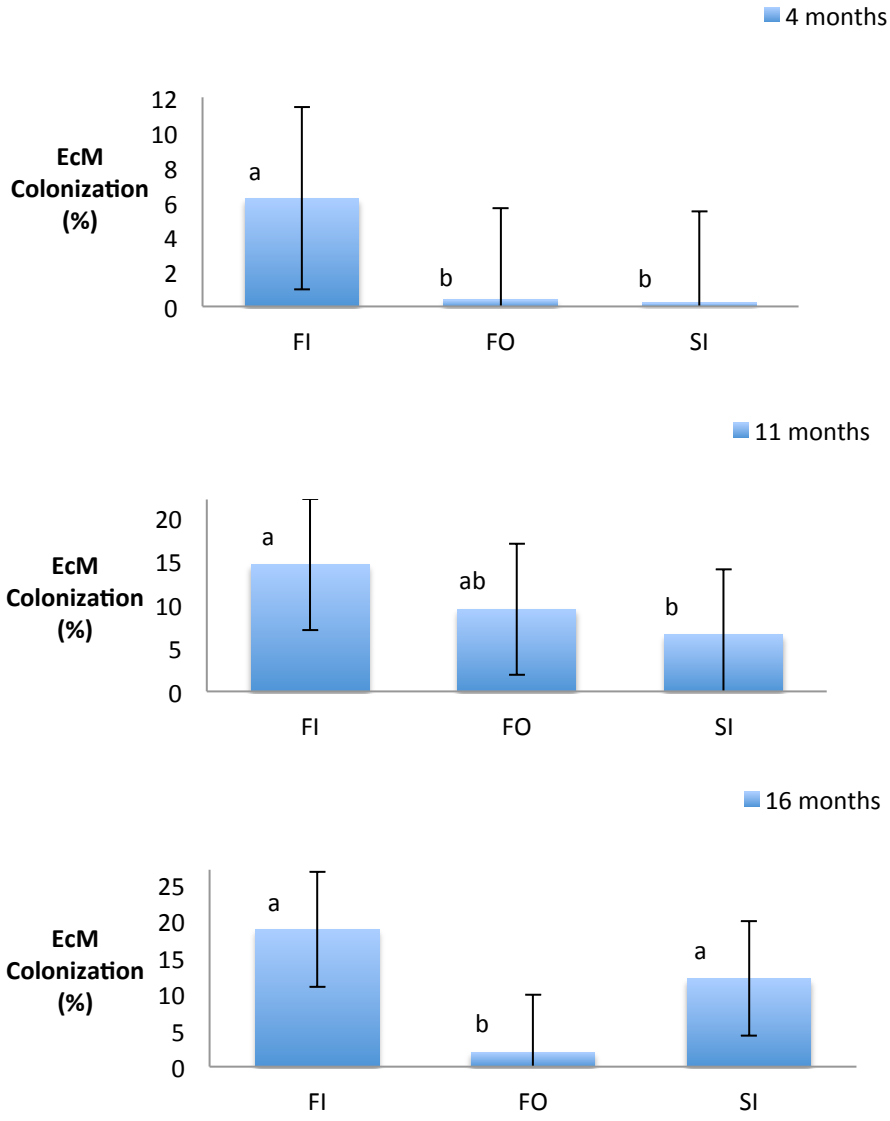

Figure 3.11 Mean colonization percentages for the three fertilizer treatments in Experiment 4, which was designed to test three fertilizer treatments. At four months after inoculation FI showed a statistically significant elevation in EcM colonization (p= 0.0401). At eleven months FI is statistically grouped differently from SI (p= 0.1054), but FO is similar to the other two treatments. At sixteen months FI and SI are shown to be similar to each other, but grouped separately from FO (p= 0.0002). Fertilizer treatments are: foliar inorganic (FI), foliar organic (FO), and soil inorganic (SI).

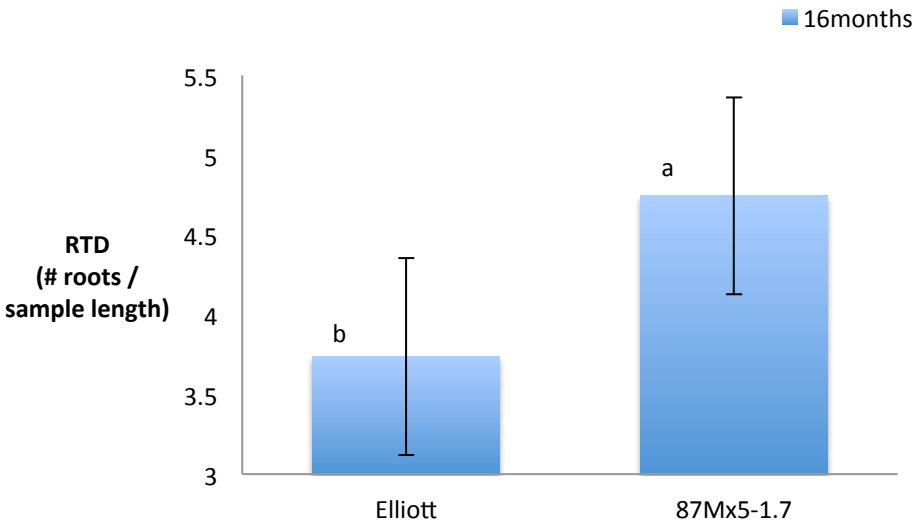

Figure 3.12 Mean root-tip densities (RTD) of two varieties of rootstock from Experiment 4 are shown with LSD bars and groupings. '87Mx5-1.7' had consistently higher mean RTD compared to 'Elliott' at the first two analyses, but the difference was only significant at sixteen months (p= 0.0017).

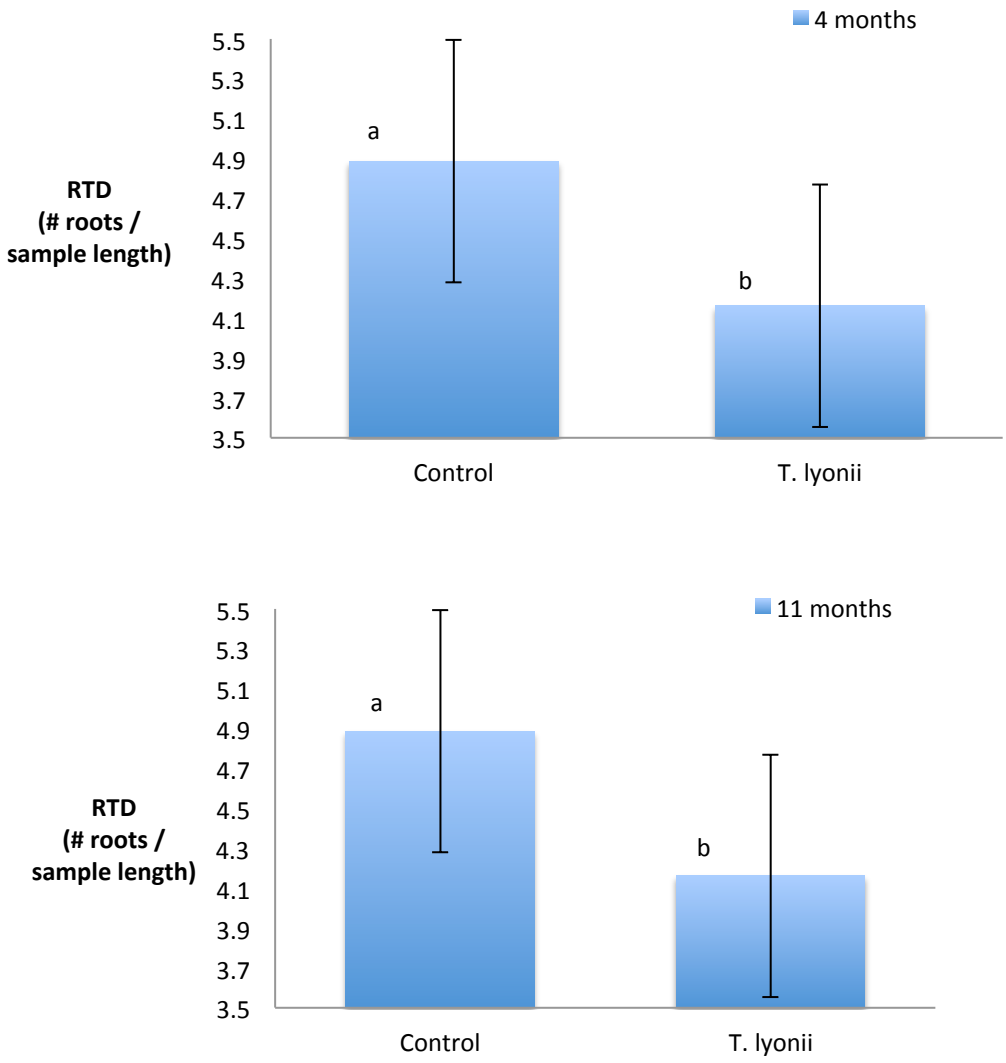

Figure 3.13 Mean root-tip densities (RTD) between *Tuber lyonii* inoculated and un-inoculated control seedlings in Experiment 4, shown with LSD bars and groupings. At four and eleven months control seedlings were found to have significantly greater RTD values when compared to *T. lyonii* inoculated seedlings (p= 0.0199, p= 0.0198).

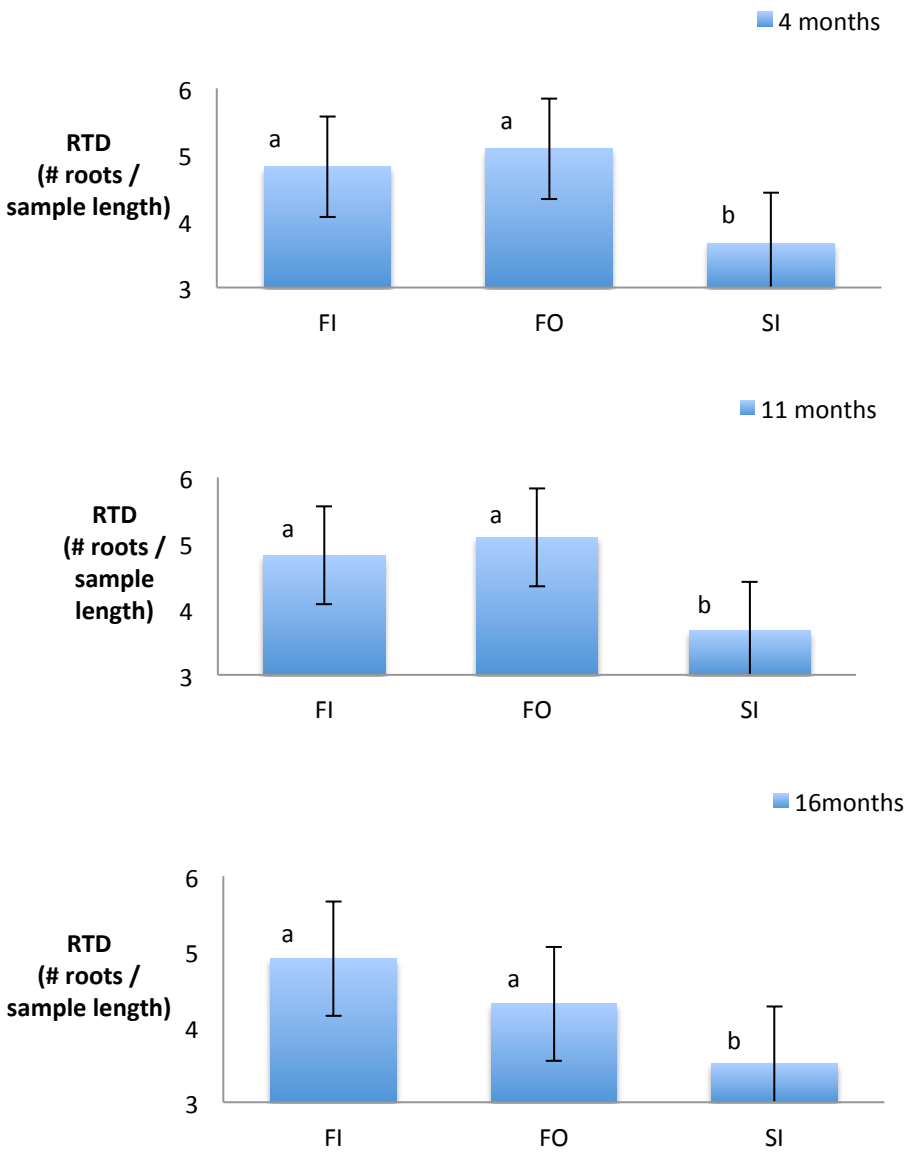

Figure 3.14 Mean root-tip densities (RTD) of three fertilizer treatments from Experiment 4, shown with LSD bars and groupings. A trend is apparent with two significantly different groupings throughout the experiment (p= 0.0006, p= 0.0006, p= 0.0019). FI and FO treatments had significantly higher RTD values compared to SI treatments.

Sample stratification levels were also analyzed for differences in colonization in Experiment 3, LSD groupings were used to show significant groupings at $\alpha = 0.05$. A trend was shown with Level 1 samples, those closest to the inoculation site, having higher mean colonization levels. At eleven months Level 1 samples were statistically different from Level 3 samples ($p= 0.0574$). Level 2 samples were similar to the other two levels (Figure 3.15).

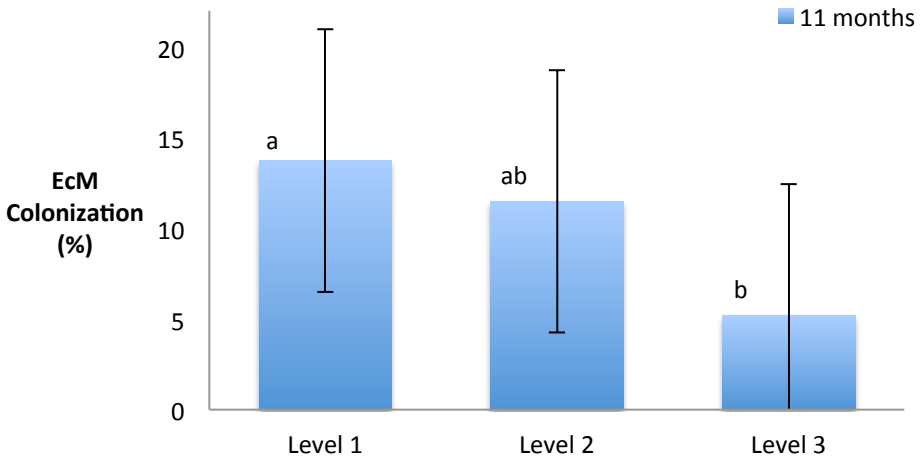

Figure 3.15 Mean colonization percentages of three sampling stratification levels from Experiment 4 with LSD bars and groupings. At eleven and sixteen months there is a consistent trend showing Level 1 samples to have higher means of colonization. This trend is only significant at eleven months where Level 1 is shown to be grouped differently by LSD from Level 3, Level 2 being similar to the other two levels (p= 0.0574).

Growth data were collected at three-week intervals on all plants throughout this experiment. Two dates were chosen for analysis, once before vernalization at seven months after inoculation (November 4, 2013) and the second after vernalization at fifteen months after inoculation (July 31, 2014). Seedling height (cm) and stem diameter (mm) were used to measure seedling growth. Amongst the two varieties, 'Elliott' showed statistically higher ($p = <0.0001$) levels of growth at seven months (Figure 3.16). A statistical difference ($p = 0.0017$) in stem diameter between the two rootstocks was observed at the first sampling date. The second date showed no statistical difference in stem diameter between the two varieties of rootstock, but the trend continued with 'Elliott' rootstocks having a larger mean diameter ($p = 0.1337$) (Figure 3.17). When analyzed by inoculation treatment there was no statistical difference ($\alpha = 0.05$) between the two treatments in regard to height or stem diameter at the first analyses. The second analyses showed a significant difference ($\alpha = 0.05$) in height between the two inoculation treatments with inoculated seedlings having a higher mean height (Figure 3.18). Stem diameter was also found to be statistically different at seventeen months when comparing the two inoculation treatments with inoculated seedlings, having a higher mean stem diameter (Figure 3.19). When analyzed by fertilizer treatment there was no difference in height at the first sampling period. The second analyses at fifteen months showed that the SI treatment was significantly taller ($p = <0.0001$) than both FO and FI treatments (Figure 3.20). The first analyses of stem diameter showed SI was significantly larger than FO, with FI being similar to the other two treatments? ($p = 0.1015$). The second analyses showed that SI was significantly larger ($p = <0.0001$) than both FI and FO (Figure 3.21).

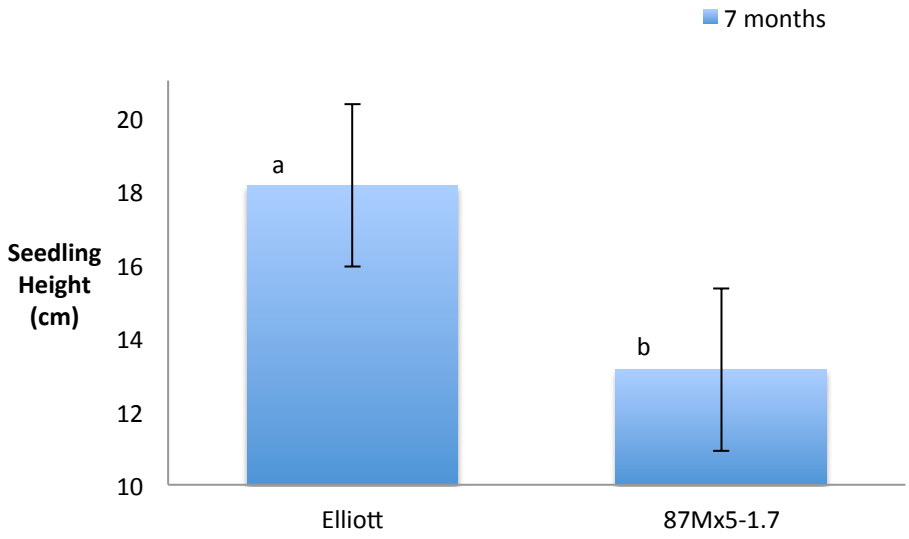

Figure 3.16 Mean seedling height of two rootstocks in Experiment 4 shown with LSD bars and groupings. 'Elliott' was found to have a significantly higher mean seedling height when compared to '87Mx5-1.7' at seven months (p= <0.0001), the trend continued but was not significant at fifteen months.

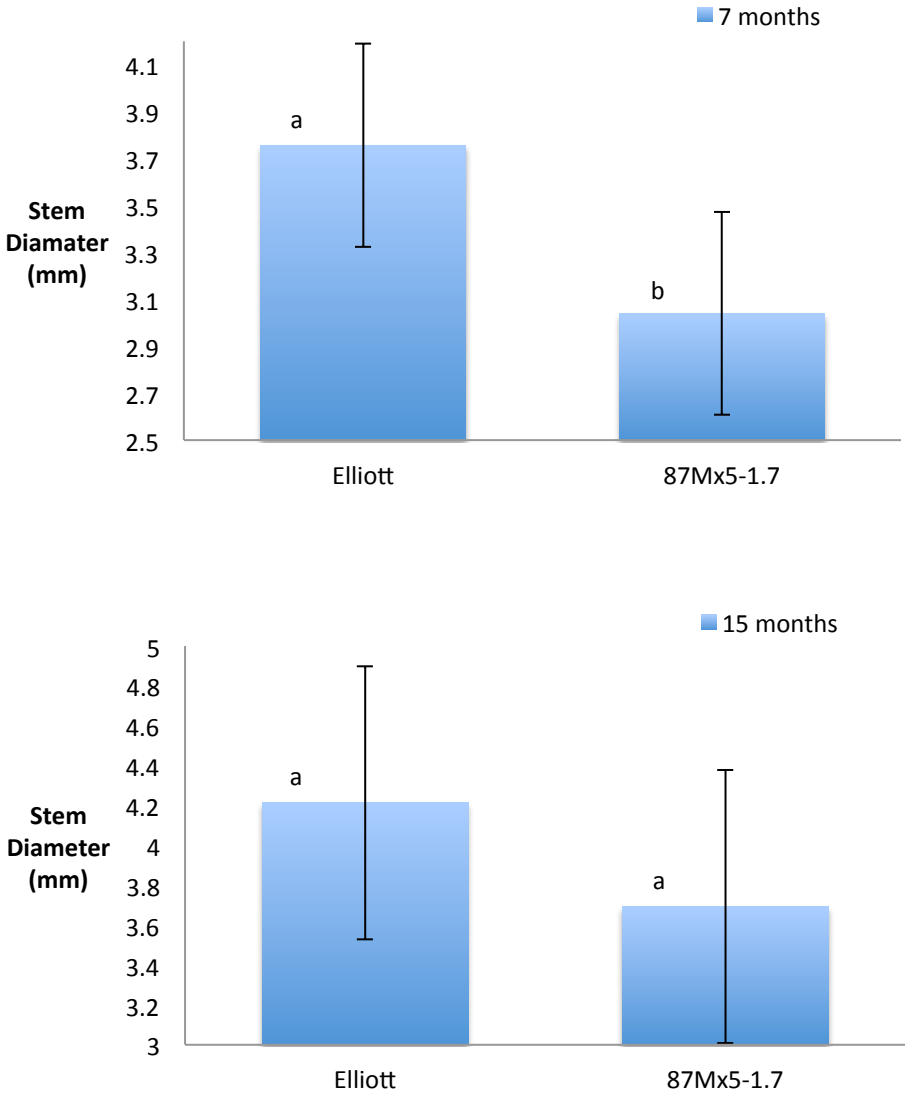

Figure 3.17 Mean seedling diameter of two rootstocks in Experiment 4, shown with LSD bars and groupings. At seven months 'Elliott' showed significantly higher mean stem diameter ($p= 0.0017$) compared to '87Mx5-1.7'. The trend continued on to fifteen months, but there was not a difference at the $\alpha = 0.05$ level ($p= 0.1336$).

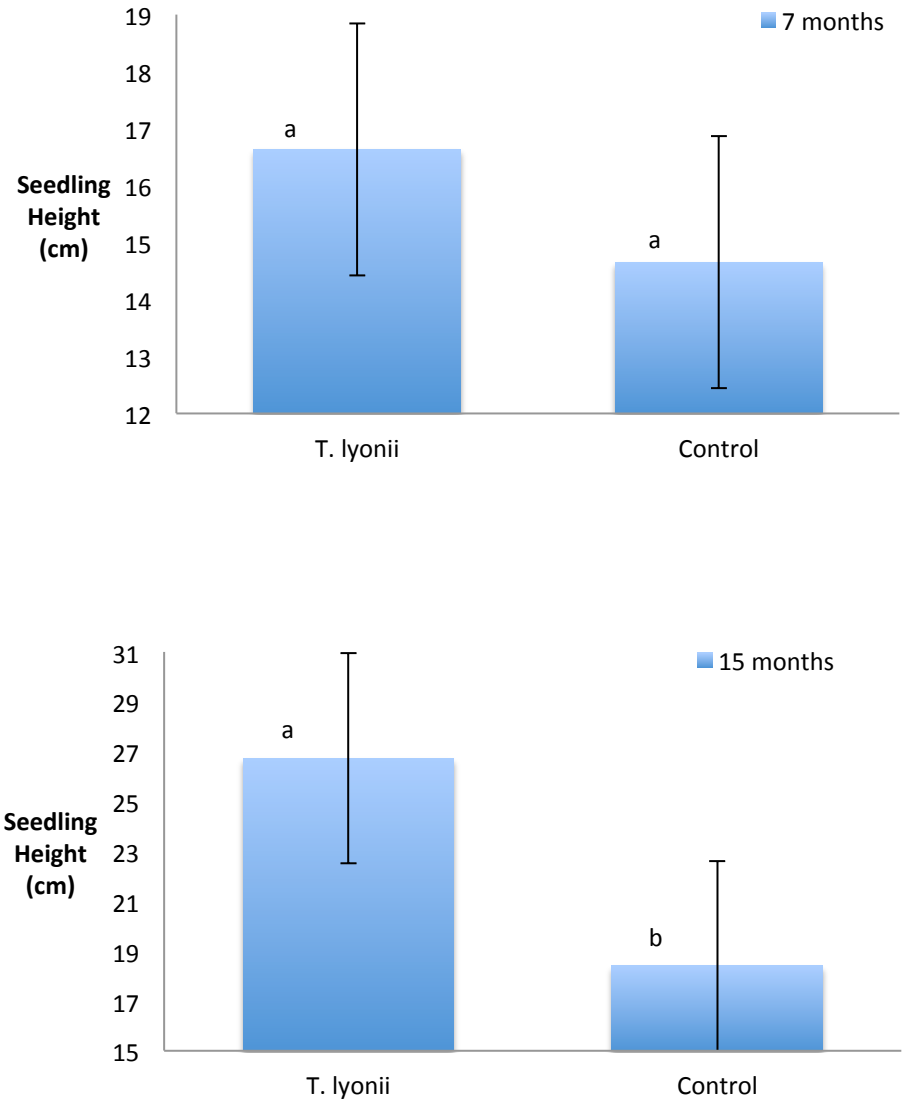

Figure 3.18 Mean seedling height of two inoculation treatments in Experiment 4, shown with LSD bars and groupings. There was a strong trend throughout the experiment showing that *T. lyonii* inoculated plants had greater mean seedling height when compared to control plants. At seven months the trend was not significant at the $\alpha = 0.05$ level, but had a $p = 0.0778$ which is significant at the $\alpha = 0.10$ level. At fifteen months the two inoculation treatments were significantly different ($p = 0.0002$), indicating that inoculated plants have larger mean seedling height.

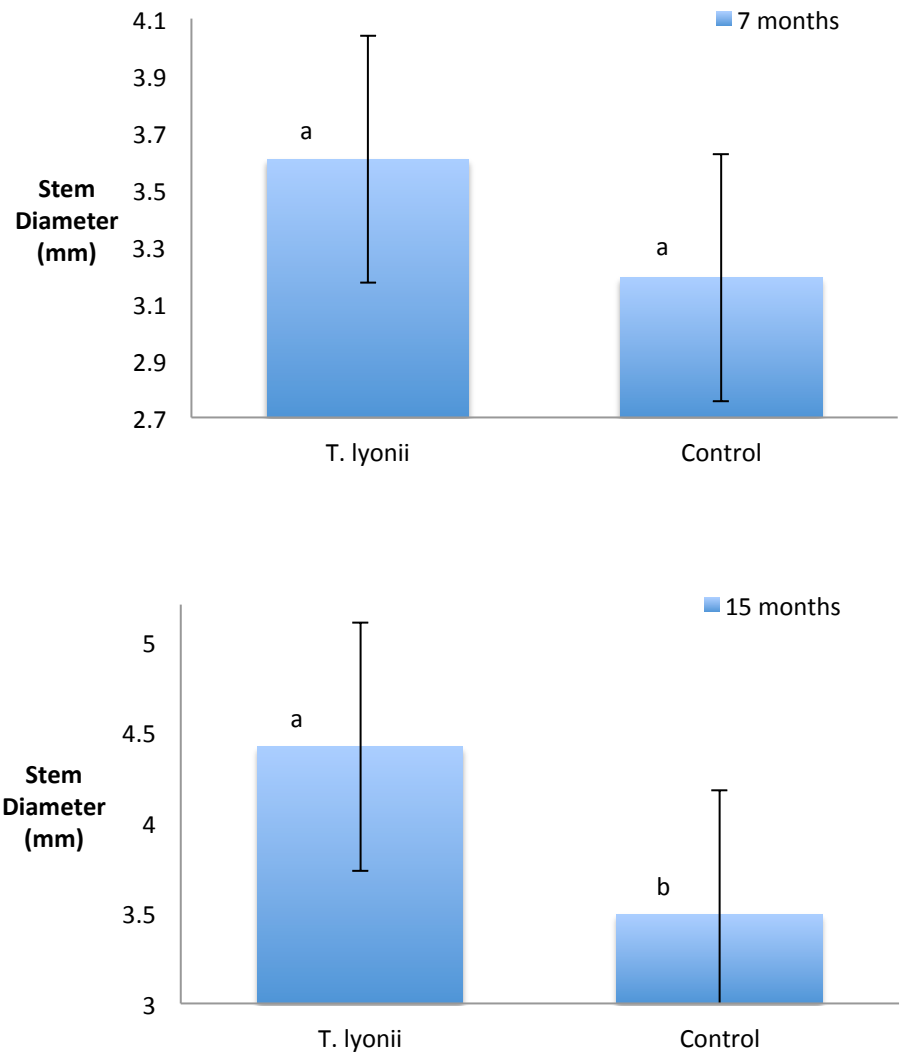

Figure 3.19 Mean seedling stem diameter of two inoculation treatments in Experiment 4 with LSD bars and groupings. There was a strong trend throughout the experiment indicating that *T. lyonii* inoculated plants had larger mean stem diameters compared to control plants. At seven months the trend was not significant at the $\alpha = 0.05$ level, but had a $p = 0.0592$. At fifteen months the two inoculation treatments were significantly different ($p = 0.0091$), indicating that inoculated plants have larger mean stem diameter.

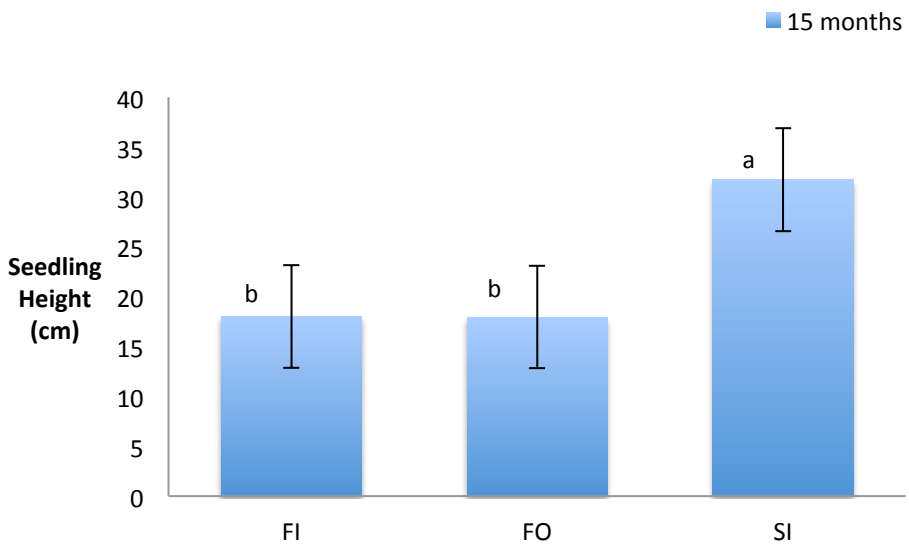

Figure 3.20 Mean seedling height among three fertilizer treatments from Experiment 4, shown with LSD bars and groupings. Seedling height was only significant at fifteen months (p= <0.0001), indicating significantly greater height in the SI treatment. Fertilizer treatments are: foliar inorganic (FI), foliar organic (FO), and soil inorganic (SI).

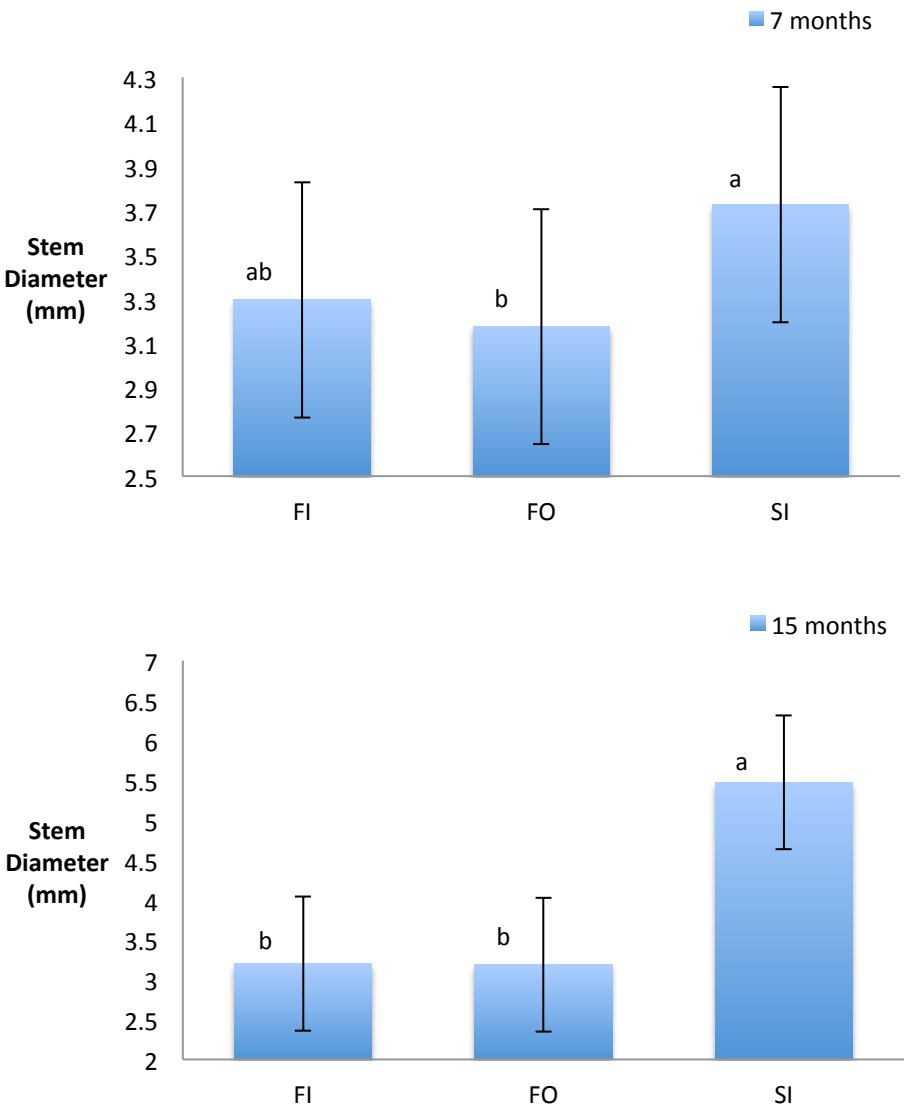

Figure 3.21 Mean seedling stem diameter among three fertilizer treatments from Experiment 4 with LSD bars and groupings. At seven months SI treatment showed significantly greater mean stem diameter compared to FO, but FI was similar to both groupings (p= 0.1015). At fifteen months SI showed significantly greater mean stem diameter than either FO or FI (p= <0.0001). Fertilizer treatments are: foliar inorganic (FI), foliar organic (FO), and soil inorganic (SI).

CHAPTER IV

DISCUSSION

Tuber lyonii Colonization

The mean number of EcM root-tips per sample and the mean percentage of EcM colonization were used to compare the amount of EcM colonization between two *Carya illinoinensis* rootstocks. Three different experiments tested the effects of vernalization, fungicide application, and three different fertilization techniques. These treatments were chosen because of their relevance to woody- perennial nursery propagation and because of the need for more information to educate a developing nursery industry specializing in inoculated rootstocks.

Experiment 1 consisted of two separate experiments testing the inoculation process and viability of two different rootstocks of *Carya illinoinensis*. A different technician had set up this experiment, as well as Experiment 2, and slight differences in technique might have occurred. The seedlings were grown in 11.5 by 15 cm containers, long enough to allow extensive root circling to occur, which may have affected growth and colonization (Figure 4.1). There was a high mortality rate that could have been caused by insufficient fertilization as well as pest pressure in the greenhouse. The remaining 27 plants, 21 ‘Elliott’ and 6 ‘87Mx5-1.7’ seedlings, were transplanted into larger containers and placed under the same fertilization treatments as the other experiments in this experiment in February of 2013. Both rootstocks were colonized by *Tuber lyonii* at the first analysis in August of 2013 and seven months later showed a reduction in mean colonization, particularly in ‘87Mx5-1.7’ rootstocks (Figure 3.1). There was no significant difference ($\alpha = 0.05$) in colonization between the two rootstock varieties.

Rootstock variety selection is an important aspect of pecan nursery production, due to the regional rootstock preferences of pecan growers. Our results indicate that there is not a significant difference between the two rootstock varieties chosen,

'Elliott' and '87Mx5-1.7', in respect to *Tuber lyonii* root colonization. Further studies are currently being carried out at Texas Tech University testing three additional rootstock varieties. Assuming that the trend is repeated by the current studies, this will be advantageous for the specialty inoculated seedling nursery industry giving them freedom to select rootstock varieties for different regions without having to be concerned about variety related colonization reductions.

Experiment 2 had an unexplained decrease in colonization over the period of the experiment, which could have affected the results of the experiment (Figure 3.2). There were no significant differences ($\alpha = 0.05$) in colonization means when comparing the rootstock varieties or the two vernalization treatments. At 21 months there was no significant difference in the mean colonization values when comparing inoculated seedlings to un-inoculated seedlings ($p= 0.1892$) (Figure 3.2). Further studies need to be conducted studying the effect of vernalization on colonization. A potentially important observation is that most *Tuber* spp. produce their fruiting bodies in the winter while the host tree is dormant. A result of this might be the inevitable winter deposition of spores via mycophagy and the subsequent mycorrhization could be affected in some way by plant dormancy and vernalization. Artificial inoculation techniques could potentially be benefitted from inoculating dormant seedlings during the winter.

Figure 4.1 Root circling occurs in pecan seedlings when they are grown in containers. The extent of circling is influenced by the size of the transplanted seedling and the container. The period of time a seedling remains in a container also affects root circling. This observation was particularly obvious in plants grown for Experiment 1 that were grown in 11.5 x 15 cm containers for longer than eight months.

Experiment 3 showed that there were no statistically significant ($\alpha = 0.05$) effects of a systemic fungicide, Stratego 250 EC, on mean colonization values. A slight trend could be observed with fungicide treated seedlings having similar mean EcM colonization percentages at eleven months and eighteen months ($p= 0.3757$, $p=0.3039$). A further study with a higher number of replicates might be able to detect an effect. This is a good result and shows that some systemic fungicides can be used to treat inoculated seedlings without concern that it will effect colonization levels. More experiments will need to be run testing a wide range of fungicides for their effect on *Tuber lyonii* colonization. The experiment also showed that there was no difference in mean colonization values between 'Elliott' and '87Mx5-1.7' rootstocks.

Statistical differences in mean EcM colonization percentages among the three different fertilizer treatments were observed in Experiment 4. At four months after inoculation the FI treatment group showed significantly higher ($p= 0.0401$) mean EcM colonization percentages compared to FO and SI, which were grouped together. At eleven months the FI treatment group was significantly grouped separately from SI treatments, but FO was similar to the other two treatments ($p= 0.1054$). At sixteen months FI and SI were significantly grouped together ($p=0.0002$), but separate from FO treatments (Figure 3.11). The FI fertilization method had the highest mean EcM colonization percentage in all three analyses, indicating that FI treatment is the most desirable fertilization technique for inoculated rootstock production. FO treatments could have been affected by inconsistencies in the selected brand of organic fertilizer. Two different bottles of concentrate were purchased, the later apparently having a higher concentration of stock fertilizer. The change in concentration caused partial and full defoliation among the FO treatment until the concentration of fertilizer was reduced. The defoliation may have caused reductions in overall plant health, resulting in an impact on EcM colonization. The SI treatment was grouped below FI treatment in the first two analyses, but in the last analyses it was in the same statistical grouping as FI treatment. The introduction of fertilizer to the rhizosphere has been shown to impact the EcM community structure (Lilleskov et al. 2001). The application of SI

treatment negatively affected mean colonization levels of *Tuber lyonii* EcM colonization in the first two analyses, and in the last analyses SI still had a lower mean EcM colonization percentage compared to FI treatment.

This experiment testing fertilization techniques was a successful first step in the process of finding an optimal fertilization technique for producing *Tuber lyonii* inoculated pecan seedlings. The FI (foliar inorganic) fertilization technique was preferable to the more traditional SI (soil inorganic) drench technique, in regard to EcM colonization. The FO (foliar organic) technique suffered from slight foliage burn caused by the fertilizer and inconsistencies in the concentration of stock fertilizer received from the manufacturer. Elevated EcM colonization in FI treatments was likely caused by the inefficiency of nutrient absorption via the stomata (foliar absorption) when compared to the nutrient absorbance of the root system. Because of the deficit in nutrients, the FI treated seedlings were more likely to form EcM root-tips with *Tuber lyonii*. Further studies could build on this information to find a more efficient fertilization technique. A relevant future study could test varying fertilizer concentrations of SI drench techniques as well as varying application frequencies. A similar experiment could also be used to test for EcM colonization among various reduced fertilizer concentrations and varying rates of application of foliar fertilizer treatments.

Carya illinoinensis **Seedling Growth**

Mean stem diameter (mm) and seedling height (cm) were analyzed to compare growth between two *Carya illinoinensis* rootstocks, ‘Elliott’ and ‘87Mx5-1.7’, with two different inoculation treatments, *Tuber lyonii* inoculated and un-inoculated. Three different experiments also tested the effects of vernalization, fungicide application, and three fertilizer treatments. These treatments were chosen because of their relevance to woody- perennial nursery propagation and because of the need for more information to educate a developing nursery industry specializing in inoculated rootstocks.

Experiment 2 had an unexplained decrease in colonization over the period of the experiment, which could have affected the results of the experiment (Figure 3.2). The only significant growth difference was at nineteen months from inoculation and showed that *T. lyonii* inoculated seedlings had higher mean stem diameters compared to un-inoculated seedlings. There were no statistical differences in regard to EcM colonization between vernalized and non-vernalized seedlings on the dates analyzed. Further analysis of the complete data set could show some growth differences at other stages of seedling development, particularly as seedlings break dormancy in the spring. Inoculated plants seemed to break dormancy faster and produce new leaves more vigorously (personal observation). Leaf count data were collected but not analyzed due to subsequent partial defoliations caused by black pecan aphids, *Melanocallis caryaefoliae.* Further analysis of the complete data set could identify some growth differences caused by vernalization at dates of analysis directly after the plants broke dormancy.

Experiment 3 showed statistical differences in seedling growth amongst both rootstock and inoculation treatments. ‘Elliott’ seedlings had higher levels of growth in respect to mean stem diameter and mean seedling height. At six months from inoculation (Oct. 23, 2013) ‘Elliott’ seedlings had greater seedling height when compared to ‘87Mx5-1.7’ ($p = <0.0001$) (Figure 3.7). At both six months and fifteen months from inoculation ‘Elliott’ seedlings had greater stem diameters compared to ‘87Mx5-1.7’ ($p = 0.0008$, $p = 0.0330$) (Figure 3.8). ‘Elliott’ consistently showed higher levels of above ground growth compared to ‘87Mx5-1.7’ which indicated that it has higher seedling vigor when compared to ‘87Mx5-1.7’. It was observed that ‘87Mx5-1.7’ seedlings had longer taproots and more extensive fibrous root systems when compared to ‘Elliott’ seedlings (personal observation). This could be interpreted as a survival strategy used by ‘87Mx5-1.7’ seedlings in their arid native habitat of northern Mexico. ‘87Mx5-1.7’ seedlings seem to allocate more energy for seedling root production, which would benefit the plant in its native habitat by increasing its ability to assimilate nutrients in a nutrient poor environment. Reduced seedling size would

also decrease the amount of leaves on each plant and reduce the loss of water through transpiration. As a result of this enhanced root network '87Mx5-1.7' seedlings may experience more vigorous growth later in development when compared to 'Elliott' seedlings.

Inoculation treatments in Experiment 3 also showed an effect on seedling height and stem diameter. *Tuber lyonii* inoculated seedlings had greater seedling height and stem diameter when compared to un-inoculated seedlings. Mean seedling height was significantly different at fifteen months when inoculated plants had a higher mean value compared to un-inoculated seedlings ($p= 0.0009$) (Figure 3.9). Seedling stem diameter among the two inoculation treatments was analyzed and showed different statistical groupings at both six and fifteen months. *T. lyonii* inoculated seedlings had higher seedling growth vigor at both dates. This result indicates a strong trend in higher growth rates by both 'Elliott' rootstocks and *T. lyonii* inoculated rootstocks. This confirms the initial hypothesis that *T. lyonii* inoculated seedlings would have higher seedling growth and vigor. Increased growth vigor caused by the inoculation with *T. lyonii* is beneficial in two ways. One, inoculation simply makes the plant grow faster while generally benefitting plant health. Two, increased growth vigor can be used to convince pecan growers who otherwise think that truffles are pathogenic fungi that harm their trees and reduce crop yield.

Experiment 4 seedlings showed strong growth patterns that were similar to the growth data collected in Experiment 3. Between the two varieties of rootstock, 'Elliott' had greater seedling height and stem diameter. 'Elliott' seedling height at seven months was larger ($p= <0.0001$) than '87Mx5-1.7' seedlings, a result similar to Experiment 3 (Figure 3.16). Stem diameter was also analyzed for both varieties of rootstock at seven and fifteen months. At seven months from inoculation 'Elliott' seedlings had greater stem diameters when compared to '87Mx5-1.7' seedlings (Figure 3.17). This trend continued at fifteen months, but was not significant at $\alpha = 0.05$ level ($p= 0.1336$). The rootstock results from Experiment 4 showed similarity to the results from the other two experiments indicating that 'Elliott' rootstocks have

more vigorous seedling growth in regard to both seedling height and stem diameter.

The inoculation treatments in Experiment 4 indicated differences between *Tuber lyonii* inoculated seedlings and un-inoculated seedlings when analyzing growth. At fifteen months *T. lyonii* inoculated seedlings showed significantly greater mean height when compared to un-inoculated seedlings ($p= 0.0091$) (Figure 3.18). Stem diameters of inoculated seedlings were also shown to be significantly greater than the un-inoculated seedlings at fifteen months (Figure 3.19). The results of Experiment 3 regarding the effect of *T. lyonii* inoculation conform to the results of the other two tests indicating a significant increase in inoculated seedlings growth.

Three fertilizer treatments were also analyzed for their effect on seedling growth in Experiment 4. As hypothesized, SI fertilizer treatments had greater levels of seedling growth. At fifteen months after inoculation, SI treated seedlings were taller compared to FO and FI treated seedlings (Figure 3.20). SI treatments were also had greater stem diameters at both seven and fifteen months after inoculation compared with FO and FI treatments (Figure 3.21). Importantly, increased seedling growth in the SI treatment came at the cost of *Tuber lyonii* colonization, which is an important consideration for inoculated rootstock producers.

Experiment 4 was the only experiment to show statistical differences when analyzing RTD (root-tip density) among rootstock varieties, inoculation treatments, and fertilizer treatments. ‘87Mx5-1.7’ seedlings had higher RTD values compared to ‘Elliott’ (Figure 3.12). This is an understandable result because ‘87Mx5-1.7’ is a rootstock from northern Mexico that is being studied because of its resistance to drought. It is logical that a drought tolerant variety would have a more extensive, or dense, fibrous root network in order to maximize water and nutrient absorption during infrequent rains. This is an interesting result when comparing the general growth characteristics of the two rootstock varieties in question. Stem diameter and height analyses alone indicate that ‘Elliot’ seedlings have more vigorous aboveground growth compared to ‘87Mx5-1.7’ seedlings. Considering that RTD is higher in ‘87Mx5-1.7’

seedlings, it can be inferred that '87Mx5-1.7' seedlings have more vigorous belowground growth. This conclusion concurs with previous observations made by the author in the above section discussing the growth of seedlings in Experiment 3. '87Mx5-1.7' seedlings seem to employ a survival strategy adapted to harsher climates in which the plant must allocate most of its energy to early root development while neglecting aboveground development.

RTD decreased in response to *Tuber lyonii* inoculation at both four and eleven months after inoculation in Experiment 4 (Figure 3.13). This result is interesting because it shows that root morphology and production may be affected by the presence of an EcM fungus like *T. lyonii*. This result could be an effect of the increased nutrient absorption efficiency of EcM roots compared to un-colonized roots. This increased efficiency might allow the plant to produce fewer roots while still absorbing the same amount of nutrients.

RTD also showed interesting statistical differences among the three fertilizer treatments in Experiment 4. At all three dates FI (foliar inorganic) and FO (foliar organic) treatments had greater RTD values when compared to the SI (soil inorganic) treatment (Figure 3.14). These results show that both of the foliar fertilizer treatments encourage the development of fibrous roots when compared to a soil drench technique. Increased RTD in foliar treatments may be caused by the lack of available nutrients within the plant. This increase in root production could play a role in the increase of colonization percentages observed in FI treated plants. If this hypothesis were true, it would mean that there is an inherent advantage to using foliar fertilizer treatments when producing inoculated seedlings.

Sample Stratification

As the experiment progressed there appeared to be a trend in amount of colonization according to sample stratification level in Experiments 2-4. Level 1 samples in all experiments had greater amounts of *Tuber lyonii* colonization. In Experiment 2, *T. lyonii* colonization declined as time progressed, but there was a significant stratification effect at the first sampling date (10 months). At ten months Level 1 samples had higher mean colonization compared to Levels 2 and 3 (Figure 3.3). Experiment 3 showed the same trend as in Experiment 2, but at all three dates. At five months and eighteen months Level 1 samples were shown to be different from Levels 2 and 3. At eleven months all three stratification levels where shown to be different from one another (Figure 3.6). In Experiment 4 the same significant trend can be observed at eleven months indicating that Level 1 samples have higher levels of *T. lyonii* colonization (Figure 3.16). This trend continued at sixteen months, but is not significant at $\alpha = 0.05$ level.

Sample stratification analyses indicated that Level 1 samples had greater *Tuber lyonii* colonization. These results might have implications for the inoculation technique used in this experiment. An inoculation 'paste' was made from *T. lyonii* ascocarps and applied to the fibrous roots of one-month old seedlings. At this time the fibrous root zone was only five to seven centimeters long. As the seedling grows the fibrous root zone becomes much larger. As the root zone became larger the roots that received the initial spore inoculation end up at Level 1, and the new roots that were produced make up Level 2 and Level 3. Ectomycorrhizae colonize roots in two ways and do so at varying rates depending on the EcM species and genus. One way is via spore contact and subsequent sporulation that results in EcM colonization. The second mode is colonization via mycelia that have been produced by previously colonized root-tips (Fiore-Donna and Martin 2001). The results of the experiment showed that Level 1 samples had higher levels of colonization by *T. lyonii* compared to Levels 2 and 3. Assuming that most of the spores from the initial inoculation had been washed out of the container before the development of Levels 2 and 3, it can be assumed that the resulting colonization in Levels 2 and 3 was from secondary mycelial colonization. Level 2 had slightly higher mean colonization values than Level 3, which may indicate some residual spores at the

time Level 2 roots were developing. This scenario would indicate that *T. lyonii* is a poor mycelial colonizer of Levels 2 and 3 when compared to the effects of the spore colonization on Level 1. To test this, a current experiment at Texas Tech University is comparing the spore 'paste' inoculation technique to a novel three-part application of an aqueous spore solution. The same amount of inoculum is being used in both techniques. This could be important information for inoculated seedling producers who wish to use *T. lyonii* for inoculations. These results justify the use of spore-based inoculum compared to mycelial culturing methods. Due to the secretive nature of the truffle industry there is a need for research and information on commercial inoculation techniques. There are many potential treatments such temperate spore stratification, or chemical degradation by acetate. There are many different inoculum application methods to be tested as well. For example, spore-solution viscosity, multiple inoculation events, and mid-dormancy applications are all viable treatments that may be able to increase colonization percentages.

Conclusion

The experiments described above were designed to assess the effect of some common nursery production practices and orchard management techniques that seemed most relevant to the production of *Tuber lyonii* inoculated *Carya illinoinensis* seedlings. The independent variables for these experiments were rootstock variety, *T. lyonii* inoculation, vernalization, fungicide application, fertilizer treatment and sample stratification. Two types of data were collected to analyze the effect of these variables: growth data and colonization data.

Two *Carya illinoinensis* rootstock varieties were chosen for these experiments, 'Elliott' and '87Mx5-1.7'. There was no difference between the two varieties in regard to *Tuber lyonii* colonization. The two rootstock varieties did reveal growth differences. "Elliott' seedlings had greater above ground growth when measuring stem diameter and height compared to '87Mx5-1.7'. In Experiment 4 '87Mx5-1.7' were found to have higher RTD (root-tip density) values, indicating that '87Mx5-1.7' has a more substantial belowground growth compared to 'Elliott'. The results show two different growth strategies utilized by two rootstock varieties from two very different climates. '87Mx5-1.7' invested energy in early root production, developing a more substantial root-system compare to that of 'Elliott'. Considering that '87Mx5-1.7' is from northern Mexico, it is logical to assume that nutrient limitations like water (as well as mineral nutrients) are an important factor for seed germination and seedling development in arid climates. A strategy to deal with this is to put more energy into developing a substantial root system that will allow the plant to absorb as much water and other nutrients as possible during infrequent rain events. 'Elliott' seedlings had greater aboveground growth compared to '87Mx5-1.7'. 'Elliott' is a variety of pecan from Florida, well adapted to a moist and humid climate. Competition from other plants is more likely to negatively affect 'Elliott' seedlings compared to nutrient limitations like drought.

Tuber lyonii inoculation was tested in all experiments in this study, Experiments 2-4 also had corresponding un-inoculated control plants for comparison. Inoculation

treatment had a significant impact on colonization, with all inoculated plants being colonized successfully. Control plants showed no sign of *T. lyonii* colonization. Inoculation had a consistent impact on seedling growth throughout the all of the experiments. *T. lyonii* inoculated seedlings had greater stem diameter and height throughout the experiment compared to un-inoculated seedlings. RTD was also affected by inoculation treatments in Experiment 4. Inoculated seedlings had lower RTD compared to their un-inoculated counterparts.

Experiment 2 was designed to test the effect of vernalization on *Tuber lyonii* colonization and growth of *Carya illionoinensis* seedlings. This experiment underwent an unexplained atypical decline in *T. lyonii* colonization. By the end of the experiment there was almost no colonization difference between inoculated and un-inoculated seedlings. The experiment should be repeated to test if the results are consistent. Seedling growth was also found to be unaffected by vernalization.

Experiment 3 tested the effect of a systemic fungicide, Stratego 250 EC, on *Tuber lyonii* colonization and seedling growth in *Carya illinoinensis* seedlings. The fungicide was found to have no impact on *T. lyonii* colonization. Seedling growth was unaffected by the application of Stratego 250 EC.

Experiment 4 compared the effect of three fertilizer applications (foliar inorganic [FI], foliar organic [FO], and soil inorganic [SI]) on *Tuber lyonii* colonization and seedling growth. FI treated seedlings had the highest colonization levels throughout the experiment. FO and SI treatments had similar levels of EcM colonization at all dates, excluding the third date where SI treated seedlings had an increase in colonization. As hypothesized, seedling growth was affected by fertilizer treatment. SI treated seedlings were had greater stem diameters as well as heights. Additionally, RTD was found to affect the belowground development of the root system. SI treated seedlings were found to have lower RTD values compared to FI and FO treatments.

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