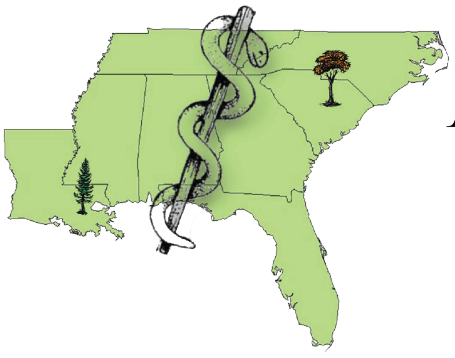

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NEW ROOT GROWTH AND ECTOMYCORRHIZAL COLONIZATION OF FINE ROOTS IN LOBLOLLY PINE AS AFFECTED BY THE INOCULATION OF *LEPTOGRAPHIUM TEREBRANTIS*

by
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4.1. ABSTRACT

Leptographium terebrantis is reported to affect the roots of loblolly pine trees throughout the southeastern United States. However, it is unknown if this fungus can affect fine roots that are ectomycorrhizal. From 2017 to 2019, a field study in a mature loblolly pine stand in Eufaula, AL was conducted to investigate root growth and ectomycorrhizal colonization responses to an on-site stem inoculation of loblolly pine trees with *L. terebrantis*. The post-inoculation analyses accounted for variations due to the inoculation treatment, measurement dates, and depths of observation. Root growth was studied for a period of 23 months using minirhizotrons and a root periscope and expressed as root length density (RLD). Fine roots were collected in September 2017 to determine percentage ectomycorrhizal colonization by the gridline intercept method. Though the treatment effect on analyzed parameters was insignificant till December 2018, seasonal and depth variation in cumulative RLD was evident. Significant differences in cumulative root length density among treatments was found in February 2019. In 2017 and 2018, root growth was rapid from April to October and February to October respectively. Over the entire study period, minimum amount of roots were observed near the soil surface and a maximum amount was observed at the 28.3 cm depth. Difference in cumulative RLD between the treatment and control pairs of trees within treatment plots were mostly insignificant. Ectomycorrhizal colonization varied by depth and was highest at the 20-30 cm depth. Our study showed that loblolly pine ectomycorrhizae thrive in acidic soil.

4.2. INTRODUCTION

In the 1950s, Brown & McDowell (1968) observed premature decline and death of loblolly pine (*Pinus taeda* L.) trees on the Oakmulgee District of the Talladega National Forest, AL. The infected trees exhibited fine root mortality and presence of stress cones for one year before they died. The results were consistent with the observations of Hess et al. (2002) who found that loblolly pine experienced a decrease in fine root biomass that was highly correlated with the occurrence of tree decline in Central Alabama, suggesting that fine root mortality is a sign of this problem. Yet it was reported that a reduction in the number of fine roots of declining loblolly and longleaf pine infected with *Leptographium terebtantis* Barras & Perry was followed by tree deterioration and death (Eckhardt &

Menard, 2009). Eckhardt et al. (2007) observed that root mortality was higher on sites with declining loblolly pine trees than on sites with healthy loblolly pine trees.

Throughout the world, ophiostomatoid fungi have affected the root system and overall tree health of pine species resulting in tree death (Otrosina et al., 2002). These fungi with *Leptographium* anamorphs have been isolated from the woody roots of declining pines throughout the southeastern United States (Otrosina et al., 2002; Menard et al., 2006; Eckhardt et al., 2007). The ophiostomatoid fungal root pathogen, *L. terebrantis*, is one of the causal agents associated with declining loblolly pine trees (Eckhardt, 2003). Eckhardt et al. (2007) reported the isolation of abundant *L. terebrantis* from the woody roots of declining loblolly pine which indicates that in the future, this fungus may compromise tree health and productivity. This pathogen causes xylem occlusions and phloem lesions in woody roots of loblolly pine trees (Matusick & Eckhardt, 2010). Pathogenic infection in the xylem may subsequently decrease the basipetal supply of carbon (C) in the phloem (Oliva et al., 2014). Without the regular supply of C to the root system and symbiotic fungi, mycorrhizal function is likely to be hindered (Högberg et al., 2002).

Correlation between forest decline and root system deterioration among coniferous and hardwood species has been reported around the world. Together, moisture stress and *Leptographium* and *Armillaria* root disease were determined as major factors contributing to the death of fine roots and subsequent decline of western white pine (*Pinus monticola* Douglas ex D. Don) in the Inland Empire (Auclair et al., 1990; Hennon, 1990; Manion, 1991). Poorly drained soils at low elevations, together with more than 50 species of fungi and a bark beetle species (Coleoptera: Scolytidae) were associated with fine root mortality in declining Alaskan yellow cedar (*Chamaecyparis nootkatensis* D.Don) trees (Hennon et al., 1990a,b). Mosca et al. (2017) reported that pedunculate oak (*Quercus robur* L.) trees experiencing severe decline had decreased ectomycorrhizal diversity and abundance. These results are consistent with those of Corcobado et al. (2014) who found that declining holm oak (*Quercus ilex* L.) trees affected by *Phytophthora cinnamomi* Rands were colonized by a less diverse ectomycorrhizal population and had fewer ectomycorrhizal root tips compared to non-declining trees.

Roots can be affected directly by various root diseases and indirectly by adverse environmental and anthropogenic factors (Atkinson, 1991; Smucker, 1993; Eissenstat et al., 2000). Root disease pathogens such as *L. terebrantis* inhibit the vascular transport of water, mineral nutrients, and carbohydrates which leads to root deterioration (Oliva et al., 2014). Also, because maintenance of a forest tree root system requires approximately 20-47% of photosynthetically fixed C (Smucker, 1993), factors that reduce C fixation in the tree crown have the potential to restrict C allocation to the tree root system. The ectomycorrhizal network that includes colonized fine roots, extramatrical hyphae, and rhizomorphs contributes to water and nutrients uptake, protection of the root system from unfavorable environmental conditions and biotic stress, and maintenance of soil physical structure (Amaranthus, 1998). Ectomycorrhizae in return receive nearly 20-25% of the C fixed in tree crowns (Söderström & Read, 1987; Högberg & Högberg, 2002; Hobbie, 2006). Due to the mutualistic association of forest trees with ectomycorrhizal fungi, the impact of tree decline doubtlessly influences the fungal partner (Sapsford et al., 2017). Past studies have focused on either tree decline or mycorrhizal processes but not on the mycorrhizal processes of declining trees (Sapsford et al., 2017). Though there is substantial review on the potential effects of climate change on mycorrhizal fungal diversity (Bellgard & Williams, 2011), we have limited information to assess how forest health affects mycorrhizal communities (Egli, 2011).

A field study was installed in 2016 to investigate how the crown and root system of plantation loblolly pines respond to on-site stem inoculation with *L. terebrantis*. Over the course of five years, it is anticipated that pathogen spread in the stem xylem will adversely affect the translocation of soil

resources to the crown. Subsequent reductions in leaf area and whole-crown C fixation will result followed by a decrease in carbohydrate supply to the root system and ectomycorrhizal network. We hypothesize that after the inoculation treatments and the appearance of significant inoculation treatment effects on tree leaf area or stemwood growth, new root growth expressed as root length density as well as the ectomycorrhizal colonization of fine roots will decrease in response to *L. terebranitis* infection. The present objectives are to (i) describe the installation of minirhizotron root tubes, (ii) document the efficacy of each tube for continued study, (iii) summarize root length density observations that were made twelve times at a 2 month interval after the inoculation treatment, and (iv) assess if ectomycorrhizal colonization differed among the treated trees 6 months after the inoculation treatment.

4.3. MATERIALS AND METHODS

4.3.1. Site description

The research site was located near Eufaula, Alabama, in Barbour County in a loblolly pine plantation managed by Rayonier Inc. (32°1'13.10"N, 85°12'31.76"W). The plantation is located within the east Gulf coastal plain physiographic region of Alabama. The study site was dominated by fine sandy loam soil. Loblolly pine seedlings were planted in January 2003 to establish the plantation which was intensively managed until the time of study establishment (Alan Wilson, personal communication).

Fifteen treatment plots were established in 2015 (Figure 4.1). The average area of each plot was 76.38 m2. At the time of study establishment, the trees were 14 years old and when the inoculation treatments were established, the trees were 16 years old. In 2014, prior to the study establishment, thinning was done so each plot retained one pair of trees in parallel rows, approximately 1 m apart within the planting row and 3.048 m apart between planting rows (Figure 4.2). A metal tag was attached to each tree denoting the tree number and a diameter band was attached to the tree at breast height. A weather station (WatchDog 2000, Spectrum Technologies Inc.) was installed to record air temperature, solar radiation, relative humidity, precipitation, and wind speed of the study area.

The experimental design consisted of 15 plots arranged in a completely randomized design (CRD) with 5 treatments and 3 replicates (Figure 4.1). The treatments for the study were: control (no inoculum or wound), wound (no inoculum), low inoculation, medium inoculation, and high inoculation which were randomly assigned to five trees in one plantation row of each 15 pair-lined plots (Figure 4.1, Figure 4.2).

4.3.2 Experiment design and inoculation treatment

Two preliminary small-scale experiments were conducted before inoculating the trees near Eufaula, Alabama primarily, to (i) select an appropriate virulent fungal isolate of *L. terebrantis* and (ii) to determine the levels of low, medium, and high inoculation treatment. To select the most virulent *L. terebrantis* isolate from 42 isolates, an extensive seedling inoculation experiment was done according to Devkota & Eckhardt (2018). From the study, ATCC accession no. MYA-3316 was found as the most virulent *L. terebrantis* isolate to loblolly pine in comparison to 41 other isolates.

To identify the levels of inoculation treatment, a preliminary field inoculation experiment was carried out at the Solon Dixon Forestry Education Center, Andalusia, Alabama. Following the sterilization of toothpicks at 121°C and 0.103 MPa for 60 minutes, *L. terebrantis* isolate (ATCC

accession no. MYA-3316) was grown in toothpicks imbibed with malt extract agar for approximately 24 days at 23°C in the dark (Devkota et al., 2018).

The inoculation treatment of trees near Eufaula, Alabama was carried out on March 13 and 14, 2017. The treatments were applied in accordance with Devkota et al. (2018), and based on the response of loblolly pine to different densities of virulent *L. terebrantis* from the Andalusia, Alabama study (Devkota et al., 2018).

The treatments for the study were: control (no inoculum or wound), wound (no inoculum), low inoculation, medium inoculation, and high inoculation. Five randomly chosen trees in one plantation row of each pair-lined plot received one of the five inoculation treatments. Control trees were left untouched. To apply rest of the treatments, selected trees were wrapped in plastic transparencies before drilling the holes. Plastic transparencies were marked with pre-determined inoculation points for different inoculation treatments with a permanent marker. A 5 mm deep hole was drilled at each predetermined inoculation point. The holes were drilled perpendicular to the surface of the stem using a sterilized 1.5 mm drill bit. One toothpick was inserted per hole. The toothpick insertion method simulated fungal transfer from maturation feeding activities of root-feeding bark beetles. Toothpicks were left inserted, clipped down to the bark, and covered with duct tape. Wounded trees had one fungus-free toothpick inserted per 1.2 cm ground-line diameter. Trees that received low, medium, or high inoculation treatments had one toothpick infected with *L. terebrantis* inserted per 10.0 cm, 2.4 cm, and 1.2 cm of ground- line diameter, respectively. The inoculation points were radially equidistant from each other. Each inoculation point was replicated 4 times vertically and equally spaced.

4.3.3. Root growth study

4.3.3.1 Tube installation

Understory vegetation was removed manually to facilitate easier root tube installation and to reduce the interference of roots of competing vegetation with pine root growth. Clear acrylic tubes were chosen for installation as they have higher transparency and durability, and less effect on pigmentation and survival rates of white roots compared to other plastics, e.g. butyrate (Withington et al., 2003). For the minirhizotron tube placement, four trees (2 inoculated trees and 2 control trees) with a comparable diameter at breast height (dbh) were chosen in each plot (Figure 4.5) to minimize the influence of tree size on root growth. The tubes were installed on plots 1 and 2 on October 25, 2016, plots 3 and 8 on October 26, 2018 and on plots 7, 5 and 8 on October 27, 2016. Tubes were installed on the remaining eight plots on November 17 and 22, 2016.

Two pairs of holes were drilled on either side of each tree, outside the planting row, and 152.4 cm away from the base of the bole. Augured hole were drilled toward the tree at a 45° angle relative to the soil surface with a drill bit, extension bar, and gas powered auger. Prepared tubes were inserted into the augured holes (Figure 4.5, Figure 4.6). Each tube was 82 cm in length and had a 3.81 cm inner diameter. Their circumference was scored at eight 10 cm increments with 10 cm of tube length beyond the first scored line on the upper end of the tubes, and 2 cm of the tube length beyond the scored line on the bottom end of the tubes. Before installation, the end of the tube that went into the ground was sealed by a rubber plug to prevent water from seeping into the tube. Scored lines corresponded to 0, 7.1, 14.1, 21.2, 28.3, 35.4, 42.4 and 49.5 cm below the ground level after tube installation. To prevent light from entering the tube, the 10 cm length at the upper end of the tube was covered in black tape (Figure 4.7).

Once installed, the taped end protruding from the soil surface was plugged with a black, size 7 rubber stopper, which was 25 mm in length and had a 37 mm top diameter and a 30 mm bottom diameter (VWR Catalog no. 59580-262). The stopper was used to prevent the entry of dust, water, and insects inside the tube. The tubes were then covered with a green plastic pot of 17.8 cm bottom diameter (Kord Plastics, Toronto, Canada) (Figure 4.7). The tube insertion areas were redressed with pine straw to avoid interference with insect activity and prevent erosion.

4.3.3.2 Root growth measurements

New root (<2mm diameter) measurements started approximately six months after minirhizotron root tube installation and one month post-inoculation. Root growth measurements started on April 13, 2017, and were repeated approximately at 2-month intervals until February 10, 2019. Root initiation was monitored using an optical root periscope (JRD Merrill Speciality Equipment, Logan, UT) that had a fiber optic light powered by a battery. Specifically, for each of the eight scored lines around the circumference of root tubes, numbers of pine root intersections were counted. Loblolly pine roots were visually identified and differentiated from the roots of other plants on the basis of color (white when first visible which changed to reddish-brown and then to brown as the root aged) (Sword et al., 2000), diameter, branching pattern, lifespan, and presence of ectomycorrhizal infection on distal root tips (Pregitzer et al., 2002, Sword & Haywood, 2006).

Root data were expressed as root length density (RLD) according to the method described by Newman (1996). The Newman equation, $R= (\pi \times N \times A)/ (2 \times H)$ was applied to the new root data, where R is the total root length (cm), N is the number of root intersections with scored lines, A is the area of tube being assessed, and H is the length of the scored line which is the tube circumference. RLD was calculated as: RLD=R/A, where RLD is root length density (cm/cm2), R is root length (cm), and A is the area of tube being assessed.

4.3.4. Ectomycorrhizae study

4.3.4.1 Ectomycorrhizae sampling

Soil samples for assessing ectomycorrhizal colonization were collected on September 21 and 27, 2017. Ten soil cores, 6 cm × 50 cm, were removed from each plot with a pneumatic soil core sampler (Figure 4.9). The cores were taken from the planting row approximately 76 cm from either side of the stem of the five intensively measured treated trees in each treatment plot (Figure 4.10). The samples were sealed, placed in a cooler, and transported to the USDA Forest Service, Auburn, Alabama where they were stored at 4°C until being processed.

4.3.4.2 Ectomycorrhizae Processing

Each soil core was cut into 10 cm increments. From our previous minirhizotron observations on April-August, 2017, we found that an insignificant amount of new roots were present in the top 10 cm of the soil. In most plant species, the presence of mycorrhizae is related to the abundance of roots and this suggests that few ectomycorrhizae were present at the 0-10 cm depth (Jentschke & Godbold, 2001). Additionally, there was a lot of debris that interfered with the wet sieving of surface soil. Hence, the top 10 cm of each soil sample was discarded from the analysis. Using a soil sieve with 0.5 mm openings (No. 35), roots were washed free of soil by running tap water (Horton & Bruns, 1998; Roberts & Anderson, 2001) in the Forest Health Dynamics Laboratory (FHDL), Auburn University, Auburn, Alabama. The washed roots were compostited by depth (10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm) in each treatment plot for a total of 60 samples. Composited samples were stored in Ziploc bags at 4oC until being processed.

To assess the percentage of fine root colonization by ectomycorrhizal fungi, one hundred 1 cm root segments were randomly selected from each of the 60 composited root samples (4 depth intervals × 15 plots). When 100 1-cm root segments were not available in a sample, all the roots were analyzed. Roots were cleared with 10% KOH, rinsed and stored in 50% glycerol until being analyzed (Brundrett et al., 1996). Ectomycorrhizal and non-ectomycorrhizal root tips were visually identified and quantified with a dissecting microscope at a magnification of 10-40X, and using the gridline intercept method (Brundrett et al., 1996). The percentage ectomycorrhizal colonization was calculated according to Danielsen et al. (2013) as:

EC (%) = (ET / ENT) × 100, where, EC is percentage ectomycorrhizal colonization, ET is number of ectomycorrhizal root tips, and ENT is the number of ectomycorrhizal plus non-ectomycorrhizal root tips.

4.3.5. Statistical analysis

4.3.5.1 New root growth

Initially, per-tube data were analyzed to check if the minirhizotron tubes were behaving normally. The tubes that had no root counts at any depth could not be used and therefore were deleted during the analysis. From a total of 240 tubes, 120 and 115 tubes were deleted during the month of February 2018 and month of February 2019 analyses, respectively. The tubes were deleted either due to death of the tree, an absence of fine roots at any depths, or due to the presence of water inside the tube. The tubes that were tight or had a tight rubber stopper which obstructed periscope insertion were also deleted (Table 4.1). From the remaining tubes, the legitimate outliers were deleted on a tube-by-tube basis during each measurement date. The scatterplot function of MS Excel was used to plot the data of each tube by depth. Observation of an abnormally high number of root counts at a certain depth was determined as an outlier and removed before data analysis.

The statistical analyses were completed using SAS version 9.4 (SAS Institute Inc. 2010, Cary, NC). For each measurement date, a two-way ANOVA (PROC GLM) was used to test if the cumulative RLD among trees that received different inoculation treatments varied significantly at different depths (Table 4.2). Cumulative RLD was averaged by inoculation treatment and measurement interval and also by inoculation treatment and depths to test the variation in cumulative RLD among depths and measurement dates, respectively by one-way ANOVA (PROC GLM). The differences among treatments and depths were considered significant at $P \leq 0.05$ unless otherwise noted. Tukey's Multiple Range test (PROC GLM) was used to compare the means of inoculation treatments, across depths, and measurement dates.

Net RLD among measurement dates were calculated by the difference between pairs of data points in two consecutive measurement intervals. Since, we took measurements bimonthly, RLD differences were divided by 2 and reported as cm cm-2 month-1. Negative net RLD values were reported as 0.

The cumulative RLD of untreated trees (Figure 4.5, unfilled blue symbols) was compared with that of treated trees (Figure 4.5, unfilled purple symbols) within the treatment plots by a paired *t*-test (PROC TTEST). The trees that received low inoculation treatment in plot 4 were dead due to *Ips* sp. infestation. So, all plot 4 data was excluded while performing the paired *t*- test. The cumulative RLD of treated trees and control trees were averaged by depth within each treatment plots prior to the analysis.

4.3.5.2 Ectomycorrhizal colonization

Initially, a two-way ANOVA (PROC GLM) was used to test if ectomycorrhizal colonization among trees varied significantly by inoculation treatment, soil depth, or interaction between inoculation treatment and soil depth. These results and soil chemical properties results in Chapter 2 indicated that soil chemical properties may have impacted the response variable. As a result, soil pH (pH_{salt} from Chapter 2) was used in correlation (PROC CORR) analyses to determine the relationship between ectomycorrhizal colonization and soil pH at different soil depths. Correlation analyses were conducted among average ectomycorrhizal colonization and soil pH at 10-50 cm (4 depth intervals), and then by depth interval (10-20 cm, 20-30 cm, 30-40 cm, and 40-50 cm).

Because ectomycorrhizal colonization was significantly correlated with soil pH, soil pH was used as a covariate during data analysis to increase the precision in determining the effect of inoculation treatment on ectomycorrhizal colonization. A two-way ANCOVA (PROC GLM) with soil pH as the covariate was used to test the main effects of inoculation treatment and soil depth, and the interaction effect of treatment × depth on ectomycorrhizal colonization. The correlations, differences among treatments, and across depths were considered significant at $P \leq 0.05$ unless otherwise noted. Tukey's Multiple Range test (PROC GLM) was used to compare the means of treatments.

4.4 RESULTS

4.4.1. Stand environment and new root growth

Stand environment data from our on-site weather station for July 2018, August 2018, December 2018, January 2019, and February 2019 were not available and therefore are not included in comparisons between the climate during this study and average climatic conditions over the past 25 years. Compared to the 25-year average between 1994 and 2018 (NOAA, 2019) of Barbour County, Alabama, precipitation and air temperature from March 2017 through November 2018 were reduced by 19.8% and increased by 0.7%, respectively. The majority of this rainfall deficit occurred during the period of less root growth between October 2017 and February 2018 when precipitation was 50.6% less than normal (Figure 4.11).

From a total of 240 minirhizotron tubes, 120 and 125 tubes were used during the month of February 2018 and the month of February 2019 analyses, respectively (Table 4.1). During each measurement date between April 2017 and February 2019, cumulative RLD was significantly affected by depth (Table 4.2). Cumulative RLD in June 2017 was significantly different among treatments with a significantly higher value associated with trees that received the medium inoculation treatment compared to the low (P=0.0165) or high inoculation treatments (P=0.0278) (Table 4.2, Figure 4.12i). Cumulative RLD in February 2019 was significantly different among treatments with a significantly higher value associated with control trees compared to the wound (P=0.0222) or high inoculation treatments (P=0.0379) (Table 4.2, Figure 4.12ii). A significant treatment × depth interaction was observed during June 2018 ($P \leq 0.05$) and December 2018 ($P \leq 0.1$) measurements (Table 4.2, Figure 4.13).

New roots were not observed at the soil surface during April 2017, June 2017, August 2017, and February 2019. During April 2017, June 2017, and October 2017, maximum cumulative RLD was observed at the 14.1 cm depth. During August 2017, December 2017, February 2018, April 2018, October 2018, and December 2018, maximum cumulative RLD was observed at the 28.3 cm depth. During December 2017, June 2018, August 2018, and February 2019, maximum cumulative RLD was observed at the 35.4 cm depth (Figure 4.14, Figure 4.15). Statistically significant differences

in cumulative RLD among depths during each measurement date from April 2017 to February 2019 are shown in figures 4.14 and 4.15 and presented in tables from 4.3 to 4.6.

The sum of RLD among depths, averaged by inoculation treatment, varied significantly across measurement dates (F=10.84, P<0.0001), with peaks in October 2017 and 2018. These RLD values increased from April to October 2017 and again from February to October 2018 respectively. A plateau in cumulative RLD took place from June 2018 to February 2019 representing the return of root system to an equilibrium condition (Figure 4.16i). During 2017 and 2018, net RLD decreased from June to August in addition to October 2017 to February 2018 and October 2018 to February 2019 (Figure 4.16ii).

When averaged by inoculation treatment and measurement interval, cumulative RLD varied significantly across depths (F=66.32, P<0.0001). Differences across depths are shown in Figure 4.17, and statistically significant differences between depth pairs are presented in Table 4.7. Minimum cumulative RLD was observed at the soil surface and maximum cumulative RLDwas observed at the 28.3 and 35.4 cm depths with a gradual increase in cumulative RLD from 0 cm to the 28.3 cm depth and then a gradual decrease in cumulative RLD from 35.4 cm to the 49.5 cm depth (Figure 4.17).

Except for the within-plot comparison of wound and control trees in February 2019, the difference in cumulative RLD was not significant for within-plot comparisons of treated and control trees (Table 4.8). In February 2019, a significantly lower value of cumulative RLD was associated with wound trees compared to the control trees (P=0.0045) within the treatment plot (Figure 4.18).

4.4.2. Ectomycorrhizal colonization of loblolly pine fine roots

Ectomycorrhizal colonization was significantly different among treatments (Table 4.9). Ectomycorrhizal colonization was significantly lower in the plots with trees inoculated with the medium inoculation treatment compared to plots with wounded trees (P=0.0397) and plots with trees inoculated with the low inoculation treatment (P=0.0033) (Figure 4.19). However, ANCOVA results indicated that ectomycorrhizal colonization was significantly affected by soil pH as a covariate, as well as depth, but not inoculation treatment or its interaction with depth (Table 4.11). Ectomycorrhizal colonization responses to depth resulted in significantly greater values at the 20-30 cm depth compared to the 40-50 cm depth (P=0.0109) (Figure 4.21). Ectomycorrhizal colonization across treatments and soil depths was 34.6%.

The ANCOVA results indicate that the ectomycorrhizal colonization response to inoculation treatment was not due to inoculation treatment but was due to covariance between ectomycorrhizal colonization and soil pH. These results are supported by a negative correlation between ectomycorrhizal colonization and soil pH (Table 4.10). When analyzed by depth, significant negative correlations between ectomycorrhizal colonization and soil pH were found at the 10-20 cm and 30-40 cm depths at a 0.05 significance level, and at the 20-30 cm depth at a 0.1 significance level (Table 4.10, Figure 4.20). Significant negative correlations between ectomycorrhizal colonization and soil pH and ANCOVA results with a significant effect of soil pH but not inoculation treatment on ectomycorrhizal colonization indicate that ANOVA results of ectomycorrhizal colonization risked misinterpretation of the treatment effect.

4.5 DISCUSSION

This research was designed to study if artificial stem inoculation with *L. terebrantis* affects the root growth or ectomycorrhizal colonization of mature loblolly pine trees. However, it appeared that stem

inoculation with *L. terebrantis* did not affect loblolly pine new root growth or ectomycorrhizal colonization within 2 years of inoculation. Cumulative RLD in June 2017 was significantly higher among trees inoculated with the medium inoculation treatment compared to the low or high inoculation treatments. Nevertheless, this observation is probably not because of an inoculation treatment effect as the difference among treatment levels was insignificant among other measurement dates until February 2019. Specifically, significantly lower values of cumulative RLD in wounded trees and trees inoculated with high inoculation treatment were found compared to the control trees in February 2019. Also in February 2019, a within-plot comparison of cumulative RLD between control and either wounded or inoculated trees revealed significantly lower cumulative RLD associated with wounded trees compared to control trees. Based on ectomycorrhizal colonization responses to soil pH in this study, perhaps these RLD responses are also attributed to a microsite variable. Significant treatment × depth interaction was found during June and December 2018 representing a trend towards a decrease in cumulative RLD with an increase in inoculum load at the 42.4 cm depth.

New roots were not observed at the soil surface during the first three measurement dates or in February 2019. Interestingly, maximum numbers of new roots were observed at either the 14.1, 28.3, or 35.4 cm depths during different measurement dates. It is, however, uncertain if the diverse pattern of root growth among depths during different measurement dates is typical for our site. Regardless, a large proportion of new roots were observed at the 0-28.3 cm depth (approximately 72%, 70%, 71%, 65%, 57%, 59%, 59%, 57%, 76%, 60% 72%, and 56% in April2017, June 2017, August 2017, October 2017, December 2017, February 2018, April 2018, June

2018, August 2018, October 2018, December 2018, and February 2019, respectively). Averaged over the entire study period, maximum cumulative RLD values were observed at the 28.3 and 35.4 cm depths, with 60% and 78% of cumulative RLD occurring at or above the 28.3 and 35.4 cm depths, respectively. Our results are consistent with those of Hendricks et al. (2006) who found that a higher percentage of fine roots in a longleaf pine (*Pinus palustris* Mill.) forest was present in the upper 30 cm of the soil profile.

New pine root growth in the southeastern Unites States is characterized by accelerated root elongation and persistence in spring through early fall followed by a period of low root elongation in fall through early spring (Sword et al., 1998; King et al., 2002; Coleman & Aubrey, 2018). Simultaneously, the timing of fine root mortality is variable and may be correlated to water and mineral nutrient availabilities (King et al., 2002). In the present study, there were four periods of interest with regard to the relationship between RLD and climate. These were the periods of high new root growth and maintenance between March and October 2017 and 2018 and the periods of less new root growth between October 2017 and February 2018 and 2019.

New root production was rapid from April 2017 to October 2017, decreased from October 2017 to February 2018, rose again from February 2018 to October 2018, and decreased afterward suggesting that new root growth occurred in two distinct phases. The first phase was when new root growth was rapid from April 2017 to October 2017 and February 2018 to October 2018, with a peak in October. This observation is supported by King et al. (2002) who reported maximum root growth in a loblolly pine plantation in spring through fall. Sword & Haywood (2006) reported similar results in a longleaf pine stand where maximum root elongation took place from May through October.

The second phase corresponds to a period of reduced root growth that occurs naturally during late fall through early spring (Sword et al., 1998; King et al., 2002; Coleman & Aubrey, 2018). In addition to the natural occurrence of this period of low root growth, three conditions may have contributed to reduced new root growth. First, measurements between April 2017 and October 2018 were during the

equilibration period when the root system and soil were recovering after destructive installation of minirhizotron tubes. Pritchard et al. (2008) suggested that this pulse of root growth might be due to the production of a large number of new roots to compensate for the death of roots during destructive minirhizotron tube installation. Philips et al. (2006) also reported that root production was rapid for the first six months after minirhizotron tube installation and then decreased afterward in a 2-year minirhizotron study.

Second, since moisture stress affects root growth (Kaufmann, 1968; Torreano & Morris, 1998), a rainfall deficit from October 2017 through February 2018 resulted in 50.6% less rainfall than normal which may have decreased soil moisture and subsequently, limited new root growth (Sword et al., 1996, Torreano & Morris, 1998). A third possible factor contributing to slow root growth after October 2017 is the stand temperature which was 6.6% higher than normal from October 2017 through February 2018. This condition may have contributed to an increase in soil evaporation (Pritchett, 1979; Wells et al., 1979; Neary et al., 1999), thus aggravating low soil moisture in the rooting zone.

Nineteen months after minirhizotron tubes installation, new root growth was steady by June 2018, thus representing the return of tree root systems to an equilibrium condition (Rewald & Ephrath, 2013). Following the minirhizotron tube installation, the tree root system might take anywhere between 6 months to 3 years to return to an equilibrium condition (Johnson et al.,2001; Strand et al., 2008). A study by Ruess et al. (2003) in a black spruce (*Picea mariana* L.) forest reported that the root distribution along minirhizotron tubes took two years or more to equilibrate. As equilibration is achieved, the magnitude of difference between RLD over time during phases one and two of the new root growth cycle will likely be less.

A significant difference in ectomycorrhizal colonization was observed between the 20-30 cm and 40-50 cm depths representing a trend towards a decrease in ectomycorrhizal colonization as soil depth increased. This observation is supported by Trautwig et al. (2017) who suggested that fine root colonization with ectomycorrhizal fungi in loblolly pine stands decreases with soil depth. Maximum ectomycorrhizal colonization at the 20-30 cm depth may be due to a higher prevalence of new root surface area for ectomycorrhizal colonization at the 21.2 - 35.4 cm depth. The present results are supported by Jentschke & Godbold (2001) who suggested that the presence of mycorrhizae is related to the abundance of roots.

More acidic soil favored ectomycorrhizal colonization in our commercial loblolly pine stand. Soil pH in plots that received the wound treatment or the low inoculation treatment averaged 4.2, and the soil pH in plots that received the medium inoculation treatment averaged 4.5. Ectomycorrhizal colonization was greater among plots receiving the wound or low inoculation treatment compared to those receiving the medium inoculation treatment. The present results are supported by those of Mikola (1973) who proposed that most ectomycorrhizal fungi thrive in acidic soil. Furthermore, Marx (1990) reported that ectomycorrhizal development at pH 5.8 was more than four times higher than that at pH 6.7.

In conclusion, since significant differences among inoculation treatments were not observed until February 2019, a longer observation period may have been required before detection of root responses to artificial *L. terebrantis* inoculation. Also, ectomycorrhizal colonization may not be affected until root system damage partially manifested as a decrease in new root growth has occurred due to the inoculation treatment. The 42.4 cm depth may be the most reliable depth to observe an inoculation treatment effect on new root growth in the future since the trend of decrease in cumulative RLD with an increase in inoculum load was observed at this depth during June and December 2018. Regardless of whether the effect of a root pathogen on new root growth and ectomycorrhizal colonization is direct or indirect, knowledge of the interaction between root pathogens and the growth and maintenance of

the root system and mycorrhizal network is critical to understanding how root pathogens affect tree productivity.

Table 4.1. Number of minirhizotron tubes used and deleted during analyses at the end of the first and second year of the new root growth study in a stand of mature loblolly pine.

Time	Minirhizotron tubes			
	Total	Used for analysis	Deleted	Cause for deletion
February 2018	240	120	120	No roots at any depth (109 tubes) Two dead trees (8 tubes) Stuck rubber stopper (3 tubes)
February 2019	240	125	115	No roots at any depth (98 tubes) Two dead trees + one tree harvested (12 tubes) Stuck rubber stopper (4 tubes) Water in the tube (1 tube)

Table 4.2. Probabilities of a greater F-value from a two-way ANOVA for the cumulative root length density of a mature loblolly pine near Eufaula, Alabama after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.

Time	Effect	df	*F*-value	*P>F*
April 2017	Treatment (T)	4	0.66	0.6173
	Depth (D)	7	5.02	<0.0001
	T×D	28	0.57	0.9630
June 2017	Treatment (T)	4	3.40	0.0091
	Depth (D)	7	6.35	<0.0001
	T×D	28	0.82	0.7328
August 2017	Treatment (T)	4	1.77	0.1329
	Depth (D)	7	10.36	<0.0001
	T×D	28	1.00	0.4715
October 2017	Treatment (T)	4	1.92	0.1052
	Depth (D)	7	2.73	0.0084
	T×D	28	1.13	0.2947
December 2017	Treatment (T)	4	0.96	0.4307
	Depth (D)	7	3.29	0.0019
	T×D	28	1.14	0.2846
February 2018	Treatment (T)	4	0.82	0.5147
	Depth (D)	7	3.27	0.0021
	T×D	28	0.99	0.4765
April 2018	Treatment (T)	4	0.32	0.8616
	Depth (D)	7	4.55	<0.0001
	T×D	28	0.84	0.7040
June 2018	Treatment (T)	4	1.15	0.2864
	Depth (D)	7	5.57	<0.0001
	T×D	28	1.50	0.0396
August 2018	Treatment (T)	4	1.19	0.3140
	Depth (D)	7	5.16	<0.0001
	T×D	28	1.01	0.4524
October 2018	Treatment (T)	4	1.73	0.1406
	Depth (D)	7	5.19	<0.0001
	T×D	28	1.35	0.1106
December 2018	Treatment (T)	4	1.21	0.3055
	Depth (D)	7	6.53	<0.0001
	T×D	28	1.38	0.0954
February 2019	Treatment (T)	4	4.01	0.0032
	Depth (D)	7	4.53	<0.0001
	T×D	28	0.79	0.7781

Table 4.3. *P*-value for significantly different cumulative root length densities among depths of a mature loblolly pine stand near Eufaula, Alabama during April 2017, June 2017, and August 2017 after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.

Depth pairing	April 2017	June 2017	August 2017
7.1 cm vs. 49.5 cm	0.0424	0.0506	0.0194
14.1 cm vs. 21.2 cm	0.0424		
14.1 cm vs. 42.4 cm	0.0160	0.0141	
14.1 cm vs. 49.5 cm	0.0009	0.0007	0.0564
21.2 cm vs. 49.5 cm			0.0004
28.3 cm vs. 7.1 cm			0.0123
28.3 cm vs. 14.1 cm			0.0478
28.3 cm vs. 42.4 cm			0.0001
28.3 cm vs. 49.5 cm			<0.0001
35.4 cm vs. 49.5 cm			0.0067

Table 4.4. *P*-value for significantly different cumulative root length densities among depths of a mature loblolly pine stand near Eufaula, Alabama during October 2017, December 2017, and February 2018 after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.

Depth pairing	October 2017	December 2017	February 2018
7.1 cm vs. 0 cm	0.0167		
14.1 cm vs. 0 cm	0.0006		0.0423
14.1 cm vs. 49.5 cm	0.0824		
21.2 cm vs. 0 cm	0.0228		0.0106
28.3 cm vs. 0 cm	0.0025	<0.0001	<0.0001
28.3 cm vs. 7.1 cm			0.0653
28.3 cm vs. 49.5 cm		0.0651	0.0125
35.4 cm vs. 0 cm	0.0044	<0.0001	<0.0001
35.4 cm vs. 14.1 cm		0.0430	
35.4 cm vs. 49.5 cm		0.0228	0.0306
42.2 cm vs. 0 cm			0.0117

Significance tested at $P< 0.05$ and $P<0.1$ by Tukey's Multiple Range test

Table 4.5. *P*-value for significantly different cumulative root length densities among depths of a mature loblolly pine stand near Eufaula, Alabama during April 2018, June 2018, and August 2018 after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.

Depth pairing	April 2018	June 2018	August 2018
14.1 cm vs. 0 cm	0.0047	0.0006	0.0653
21.2 cm vs. 0 cm	0.0180	0.0263	0.0060
28.3 cm vs. 0 cm	0.0005	<0.0001	0.0008
35.4 cm vs. 0 cm	0.0010	<0.0001	<0.0001
35.4 cm vs. 49.5 cm			0.0163
42.2 cm vs. 0 cm		0.0135	0.0027

Table 4.6. *P*-value for significantly different cumulative root length densities among depths of a mature loblolly pine stand near Eufaula, Alabama during October 2018 and December 2018 after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.

Depth pairing	October 2018	December 2018
14.1 cm vs. 0 cm	0.0002	0.0071
21.2 cm vs. 0 cm	0.0039	0.0088
28.3 cm vs. 0 cm	<0.0001	<0.0001
28.3 cm vs. 7.1 cm		0.0129
35.4 cm vs. 0 cm	0.0005	<0.0001
35.4 cm vs. 7.1 cm		0.0488
42.2 cm vs. 0 cm	0.0043	0.0006

Table 4.7. *P*-value for significantly different cumulative root length densities among depths of a mature loblolly pine stand near Eufaula, Alabama during the study period from April 2017 through February 2019 after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.

Depth pairing	P-value
7.1 cm vs. 0 cm	<0.0001
7.1 cm vs. 49.5 cm	0.0560
14.1 cm vs. 0 cm	<0.0001
14.1 cm vs. 7.1 cm	0.0010
14.1 cm vs. 49.5 cm	<0.0001
21.2 cm vs. 0 cm	<0.0001
21.2 cm vs. 49.5 cm	<0.0001
28.3 cm vs. 0 cm	<0.0001
28.3 cm vs. 7.1 cm	<0.0001
28.3 cm vs. 14.1 cm	0.0049
28.3 cm vs. 21.2 cm	<0.0001
28.3 cm vs. 42.4 cm	<0.0001
28.3 cm vs. 49.5 cm	<0.0001
35.4 cm vs. 0 cm	<0.0001
35.4 cm vs. 7.1 cm	<0.0001
35.4 cm vs. 14.1 cm	0.0333
35.4 cm vs. 21.2 cm	0.0003
35.4 cm vs. 42.4 cm	<0.0001
35.4 cm vs. 49.5 cm	<0.0001
42.4 cm vs. 0 cm	<0.0001
42.4 cm vs. 49.5 cm	<0.0001
49.5 cm vs. 0 cm	0.0230

Table 4.8. Within-plot comparison of cumulative root length density by paired *t*-test for mature loblolly pine near Eufaula, Alabama after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.The cumulative root length density of trees that received the control (C) treatment was compared with the cumulative root length density of trees that received either wound (W), low (L), medium (M), or high (H) inoculation treatments.

Date	Treatment-Control pair	df	*t*-value	*P*>|*t*|
April 2017	W-C	23	-0.140	0.1739
	L-C	15	-1.34	0.2004
	M-C	23	0.36	0.7187
	H-C	23	-0.26	0.7944
June 2017	W-C	23	-1.54	0.1379
	L-C	15	-1.37	0.1896
	M-C	23	1.23	0.2327
	H-C	23	-1.28	0.2122
August 2017	W-C	23	-0.71	0.4867
	L-C	15	0.71	0.4860
	M-C	23	1.11	0.2793
	H-C	23	0.71	0.4860
October 2017	W-C	23	-0.149	0.1490
	L-C	15	-0.80	0.4366
	M-C	23	1.79	0.0865
	H-C	23	-1.82	0.0826
December 2017	W-C	23	-1.27	0.2168
	L-C	15	-0.87	0.3965
	M-C	23	0.74	0.4695
	H-C	23	-0.23	0.8188
February 2018	W-C	23	-0.06	0.9543
	L-C	15	-0.00	1.0000
	M-C	23	1.53	0.1384
	H-C	15	0.52	0.6104
April 2018	W-C	23	-0.81	0.4267
	L-C	15	1.47	0.1623
	M-C	23	0.07	0.9418
	H-C	23	1.01	0.3222
June 2018	W-C	23	-1.61	0.1212
	L-C	15	1.47	0.1623
	M-C	23	0.65	0.5199
	H-C	23	0.20	0.8453
August 2018	W-C	23	-1.43	0.1661
	L-C	15	-0.12	0.9030
	M-C	23	1.03	0.3123
	H-C	23	1.60	0.1235
October 2018	W-C	23	-0.22	0.8313
	L-C	15	1.16	0.2648
	M-C	23	0.21	0.8366
	H-C	23	1.24	0.2267
December 2018	W-C	23	-1.03	0.3125
	L-C	15	0.14	0.8915
	M-C	23	1.61	0.1205
	H-C	23	1.48	0.1516
February 2019	W-C	23	-3.15	0.0045
	L-C	15	0.98	0.3422
	M-C	23	0.94	0.3570
	H-C	23	1.35	0.1904

Table 4.9. Probabilities of a greater *F*-value from a two-way ANOVA for ectomycorrhizal colonization of fine roots in a mature loblolly pine stand near Eufaula, Alabama after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017.

Source	df	*F*-value	*P>F*
Treatment (T)	4	4.3156	0.0054
Depth (D)	3	0.9947	0.4051
T × D	12	1.1861	0.3256
Error	40		

Table 4.10. Pearson's coefficient of correlation between ectomycorrhizal colonization and soil pH along with their respective *P*-value at different soil depths.

Soil depth	n	r	*P*-value
10-50 cm	60	-0.3659	0.0040
10-20 cm	15	-0.6539	0.0082
20-30 cm	15	-0.4824	0.0686
30-40 cm	15	-0.6563	0.0079
40-50 cm	15	-0.3239	0.2389

Significance tested at $P< 0.05$ and $P<0.1$

Table 4.11. Probabilities of a greater *F*-value from a two-way ANCOVA for ectomycorrhizal colonization of fine roots in a mature loblolly pine stand near Eufaula, Alabama after an onsite stem inoculation treatment with *Leptographium terebrantis* in March 2017. Soil pH was used as a covariate in the model.

Source	df	*F*-value	*P>F*
Soil pH	1	10.35	0.0026
Treatment (T)	4	1.27	0.2989
Depth (D)	3	3.77	0.0182
T × D	12	1.63	0.1220
Error	39		

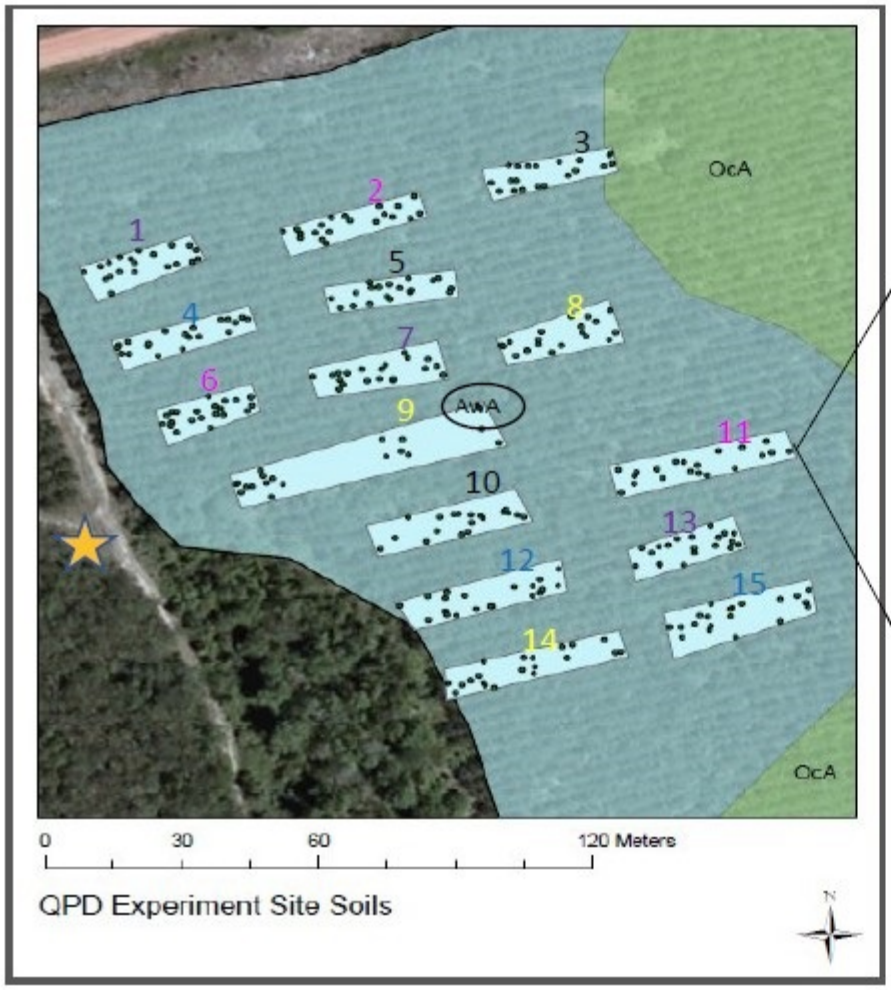

Figure 4.1. Map of the study site near Eufaula, Alabama. Treatment plots are represented by rectangles and soil series are represented by background color (AwA: Annemaine-Wahee complex). Small circles represent pre- thinning tree locations. Filled yellow star indicates the location of weather station.

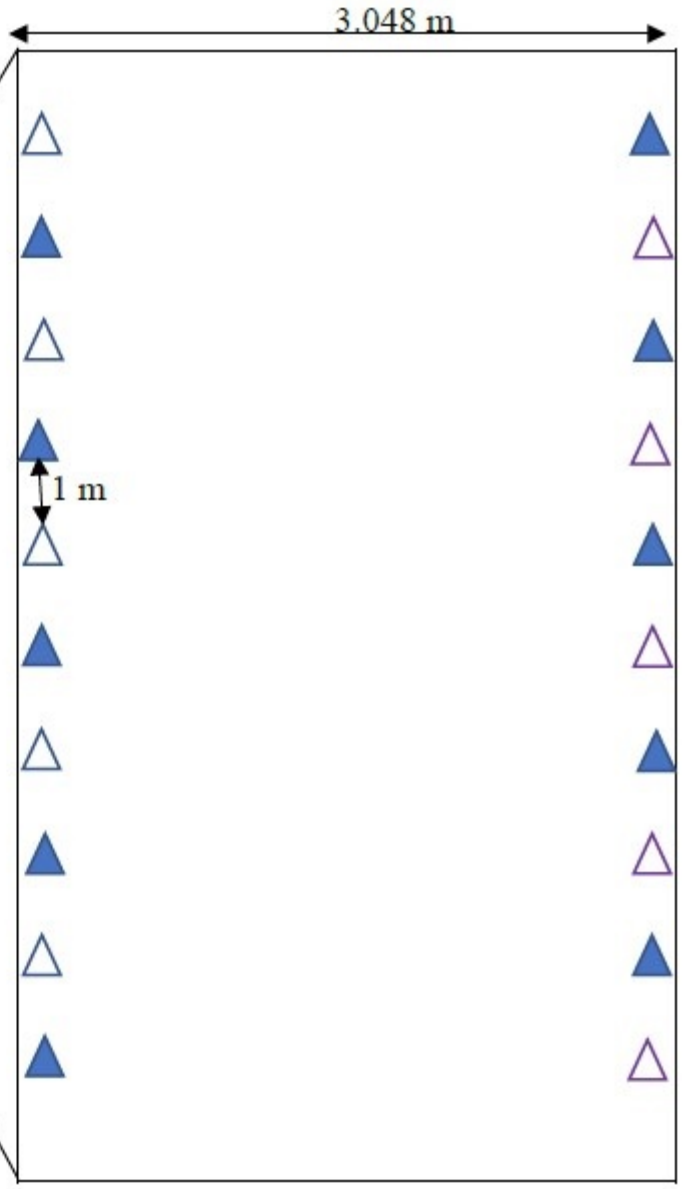

Figure 4.2. Post-thinning field layout of tree arrangement and distance between them in each treatment plot. Unfilled purple triangles indicate inoculated trees, unfilled blue traingles indicate control trees, and solid blue traingles indicate other trees.

Figure 4.3. Toothpicks with *Leptographium terebrantis* inserted at 16 radial points of four rows in loblolly pine saplings at the Solon Dixon Center, Andalusia, Alabama. Toothpicks were clipped to facilitate covering the inoculation zone with a duct tape.

Figure 4.4. Toothpicks with *Leptographium terebrantis* inserted in loblolly pine tree at the study site near Eufaula, Alabama.

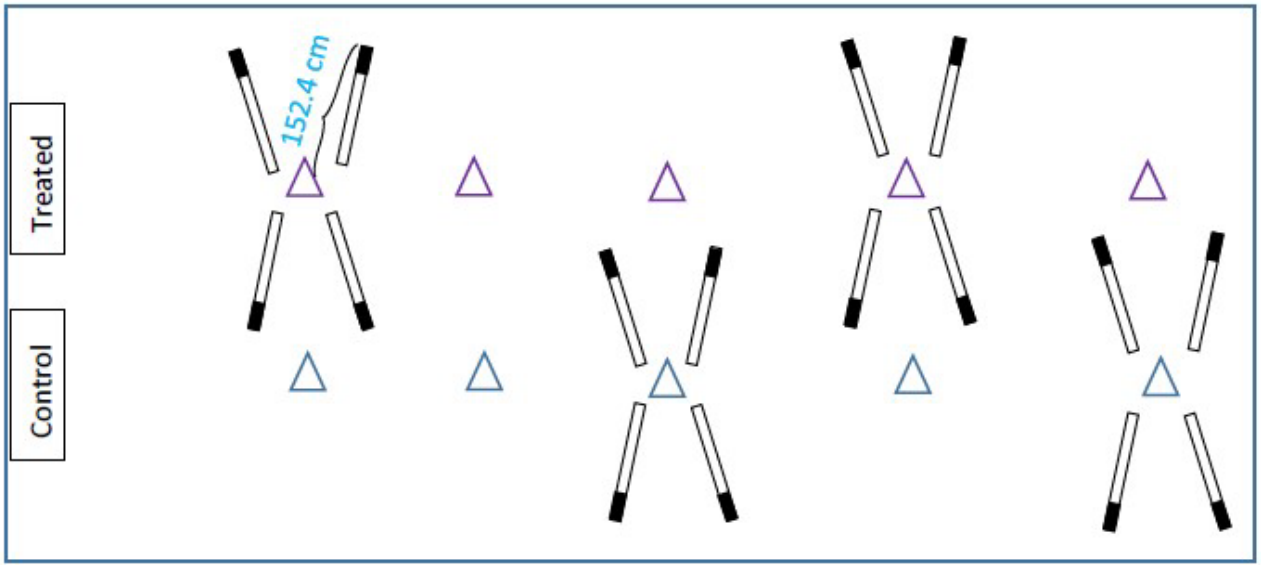

Figure 4.5. Field layout of minirhizotron tubes in each plot. The distance from the upper portion of the tube to the base of the tree is 152.4 cm. Unfilled purple triangles indicate inoculated trees and unfilled blue traingles indicate control trees.

Figure 4.6. (i) Drilling holes using an extension bar and drill bit, **(ii)** Numbers (1-4) indicate the point where tubes were installed around a single tree.

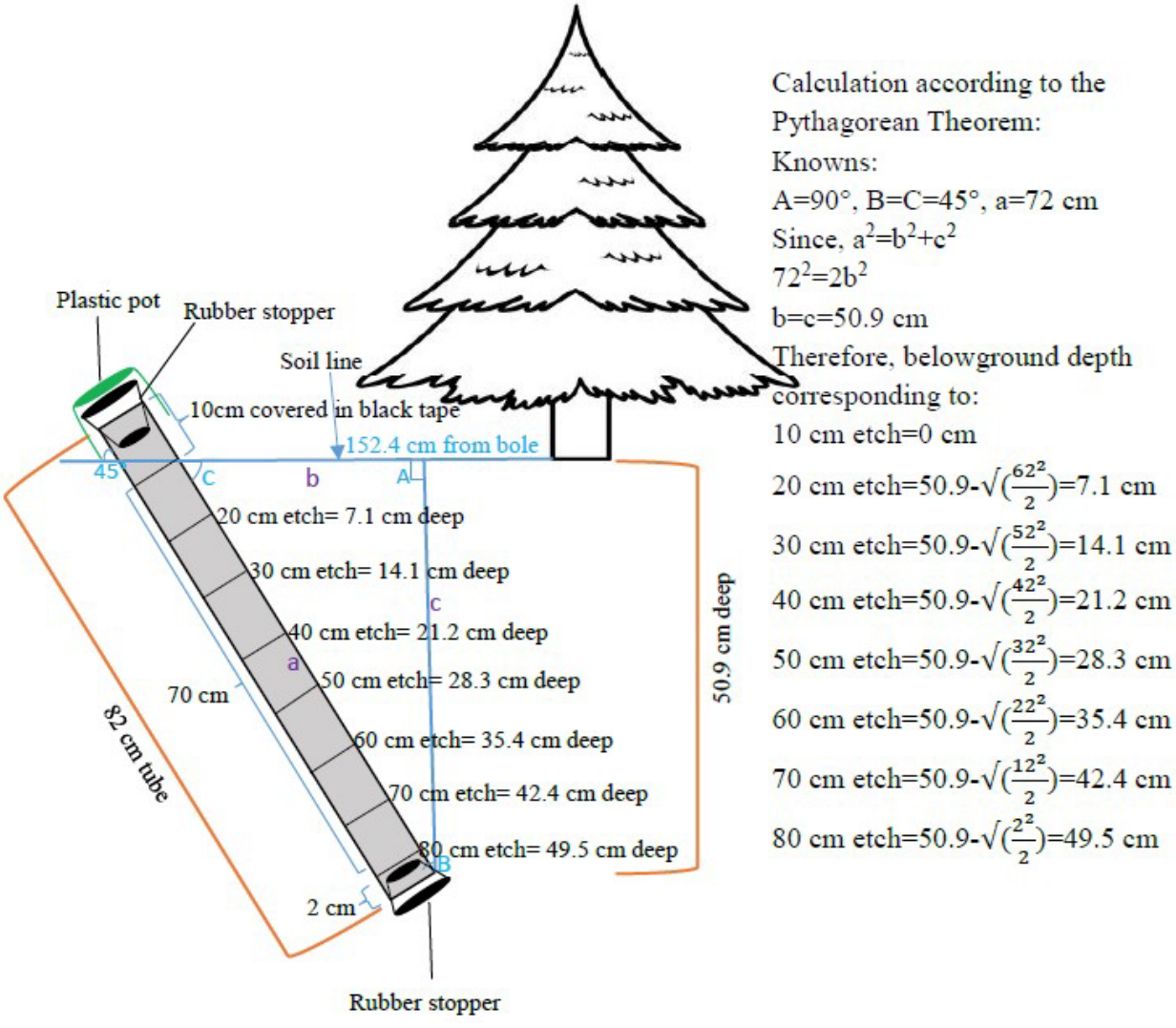

Figure 4.7. Field layout of minirhizotron tubes showing the belowground depth corresponding to each scored line on the tubes.

Figure 3.8. (**i)** Observing root intersections with an optical periscop e. The black rectangular object on the ground is a battery which is connected to the periscope to power the light, **(ii)** the faint line noted by the red arrow is a new root.

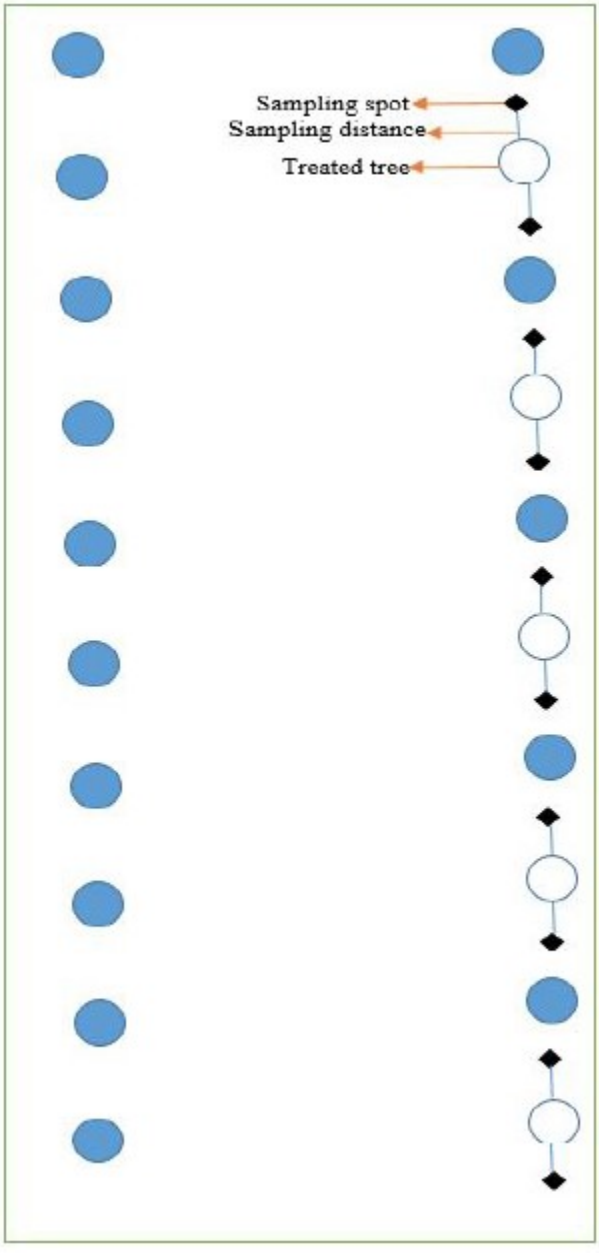

Figure 4.9. Field protocol for ectomycorrhizae sampling. Diamond shapes indicate points of soil core collection from either side of treatment trees in each plot.

Figure 4.10. Soil core with approximately 50 cm deep soil sample.

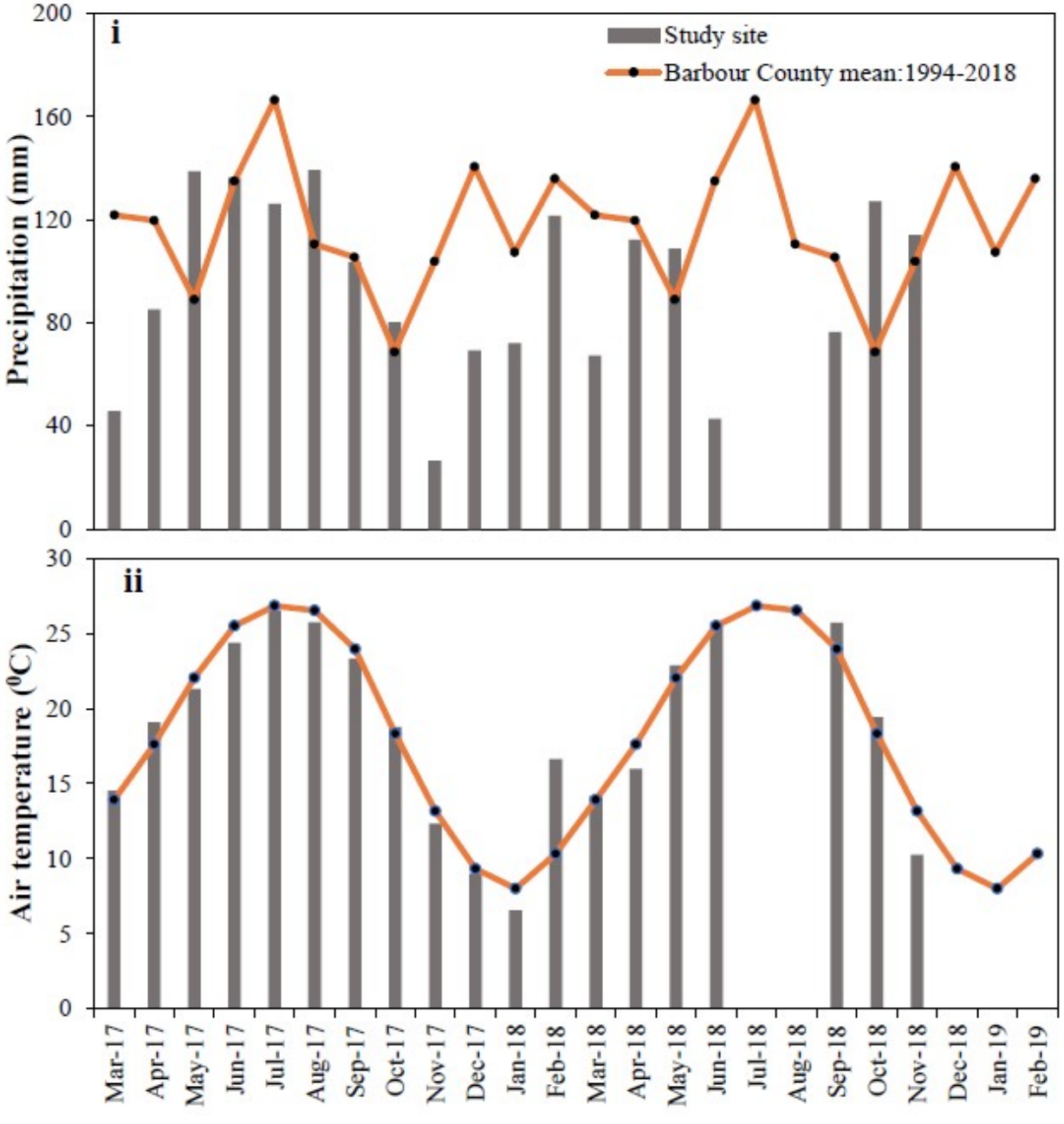

Figure 4.11. (i) Mean monthly precipitation (mm) between 1994 and 2018 in Barbour County, Alabama and total monthly precipitation between March 2017 and November 2018 in a mature loblolly pine stand near Eufaula, Barbour County, Alabama, and **(ii)** Mean monthly air temperature (0C) between 1994 and 2018 in Barbour County, Alabama and monthly temperature (0C) between March 2017 and November 2018 in a mature loblolly pine stand near Eufaula, Barbour County, Alabama. Data were not collected in July 2018, August 2018, December 2018, January 2019 and February 2019.

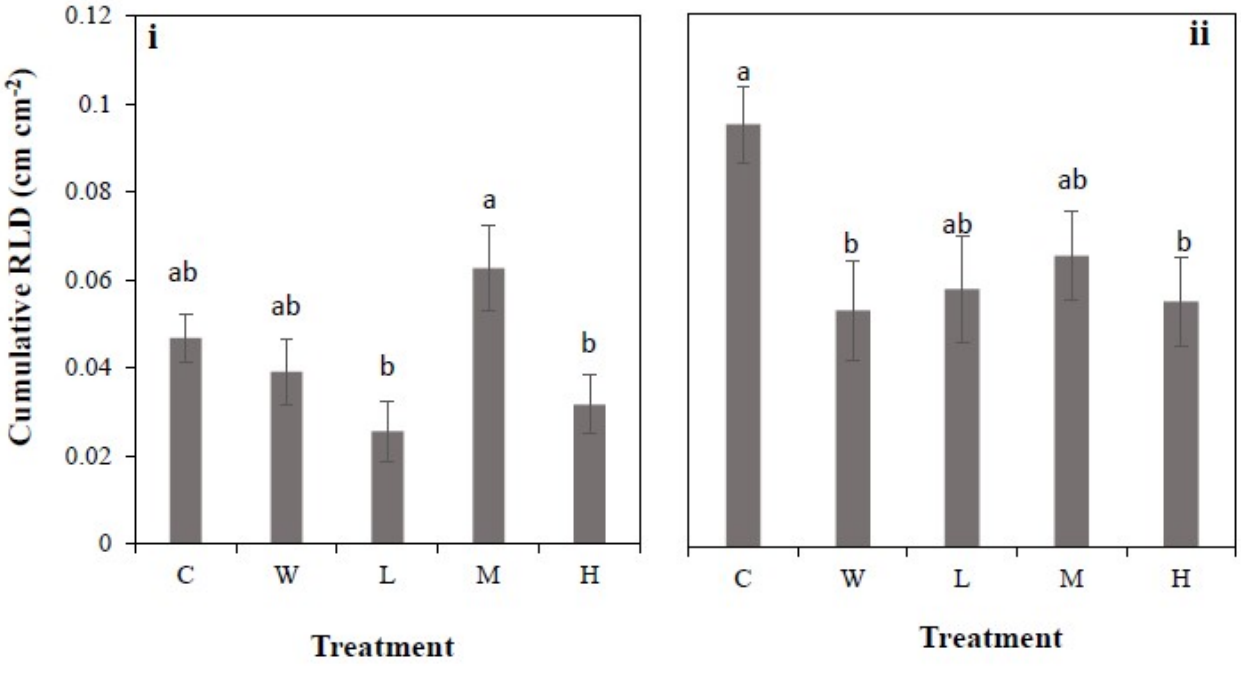

Figure 4.12. Average cumulative root length density (RLD) among treatments during the **(i)** June 2017 and **(ii)** February 2019 measurement intervals. Treatm nts were control (C), wound (L), low (L), medium (M), and high (H) inoculation. Error bars represent the standard error of the mean. In each figure, means associated with a different lower case letter are significantly different by Tukey's Multiple Range test.

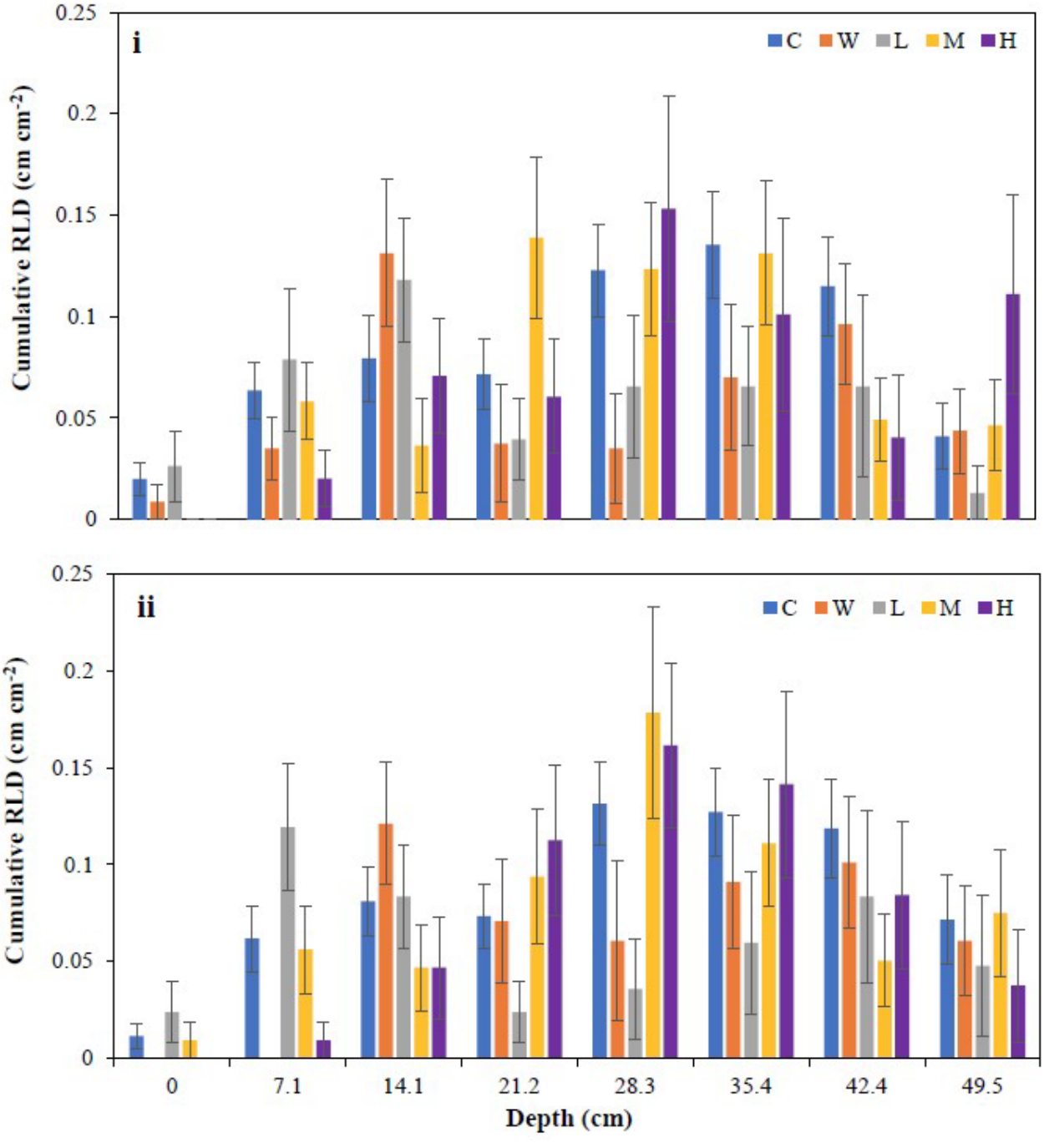

Figure 4.13. Average cumulative root length density (RLD) among treatments at different depths during the **(i)** June 2018 and **(ii)** December 2018 measurement intervals. Treatments were control (C), wound (L), low (L), medium (M), and high (H) inoculation. Error bars represent the standard error of the mean. In each figure, means associated with a different l wer case letter are significantly different by Tukey's Multiple Range test.

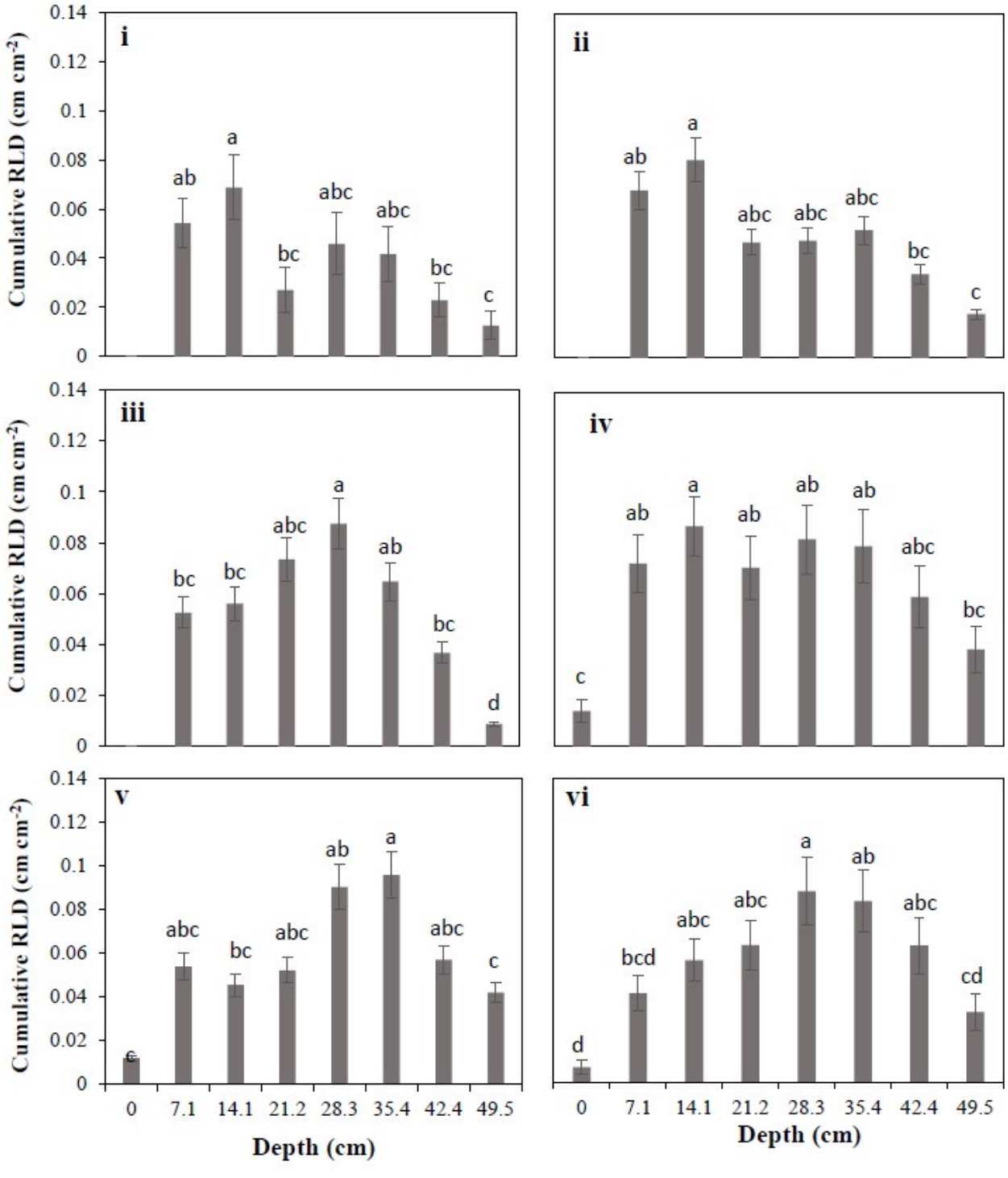

Figure 4.14. Average cumulative root length density (RLD) by depths during the **(i)** April 2017, **(ii)** June 2017, **(iii)** August 2017, **(iv)** October 2017, **(v)** December 2017, and **(vi)** February 2018 measurement intervals. Error bars represent the standard error of the mean. In each figure, means associated with a different lower case letter are significantly different by Tukey's Multiple Range test.

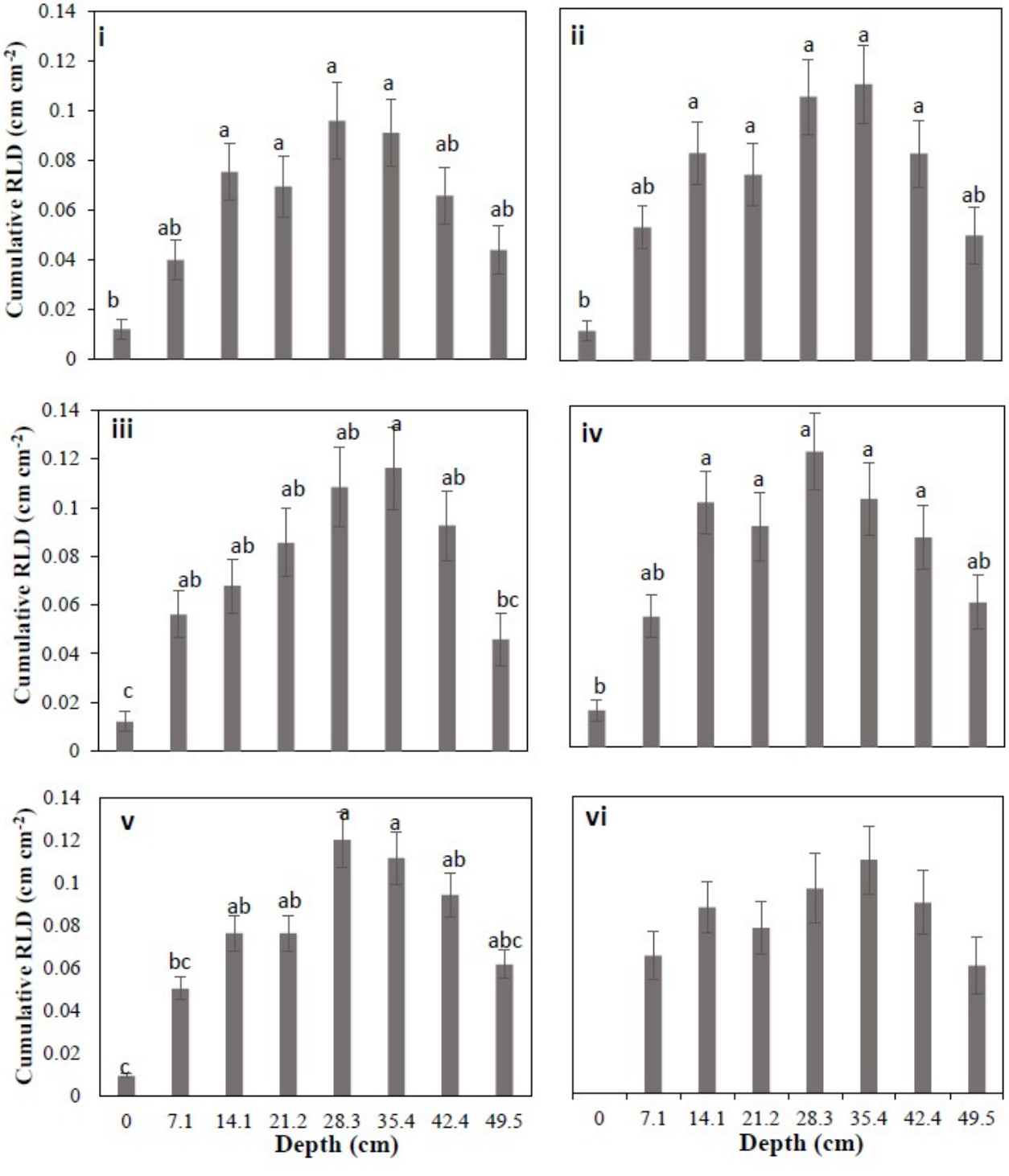

Figure 4.15. Average cumulative root length density (RLD) by depths during the **(i)** April 2018, **(ii)** June 2018, **(iii)** August 2018, **(iv)** October 2018, **(v)** December 2018, and **(vi)** February 2019 measurement intervals. Error bars represent the standard error of the mean. In each figure, means associated with a different lower case letter are significantly different by Tukey's Multiple Range test.

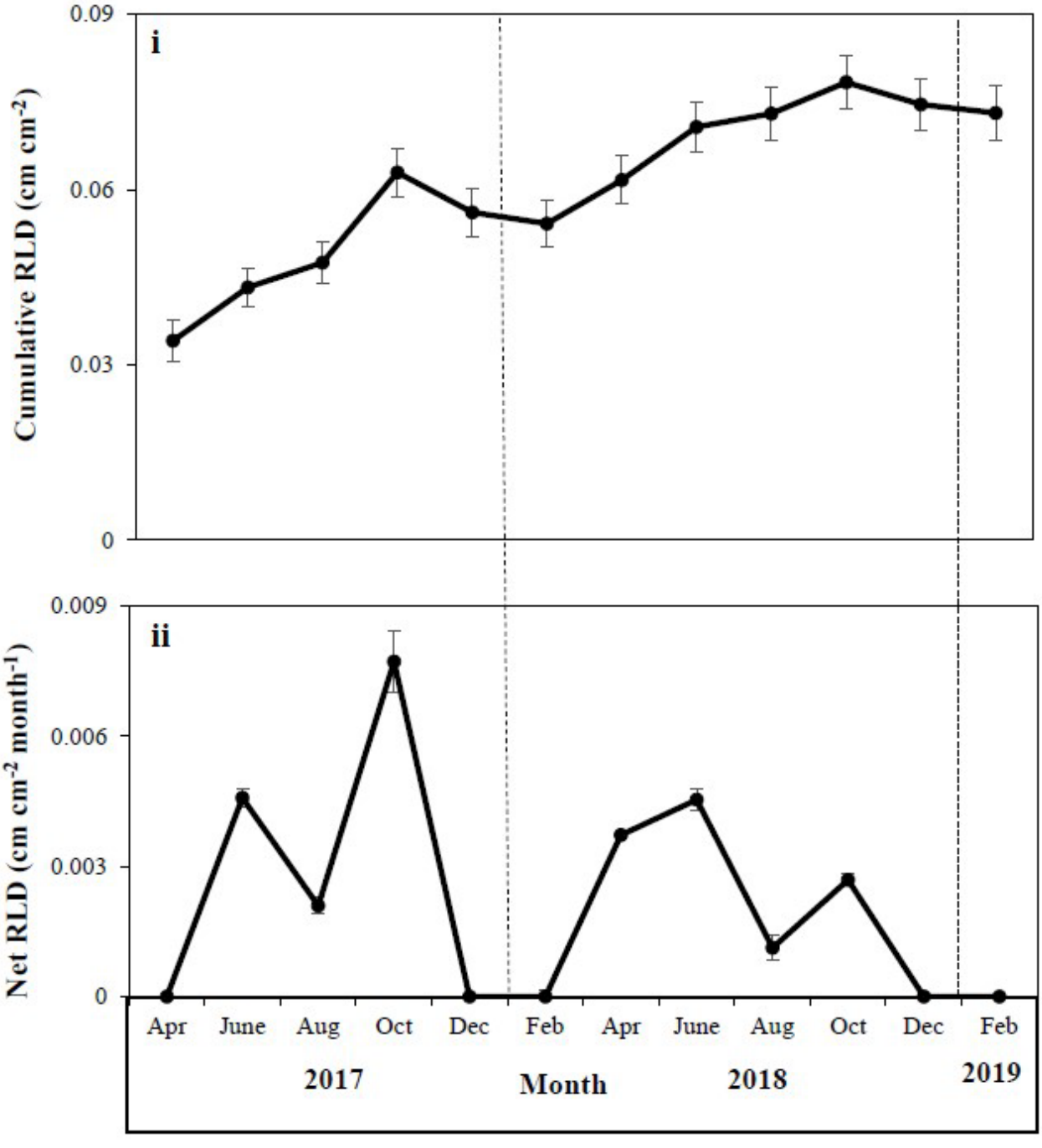

Figure 4.16. Average **(i)** cumulative root length density (RLD) and **(ii)** net root length density (RLD) of mature loblolly pine from April 2017 through February 2019 at the 0 cm to 49.5 cm depth. Error bars represent the standard error of the mean.

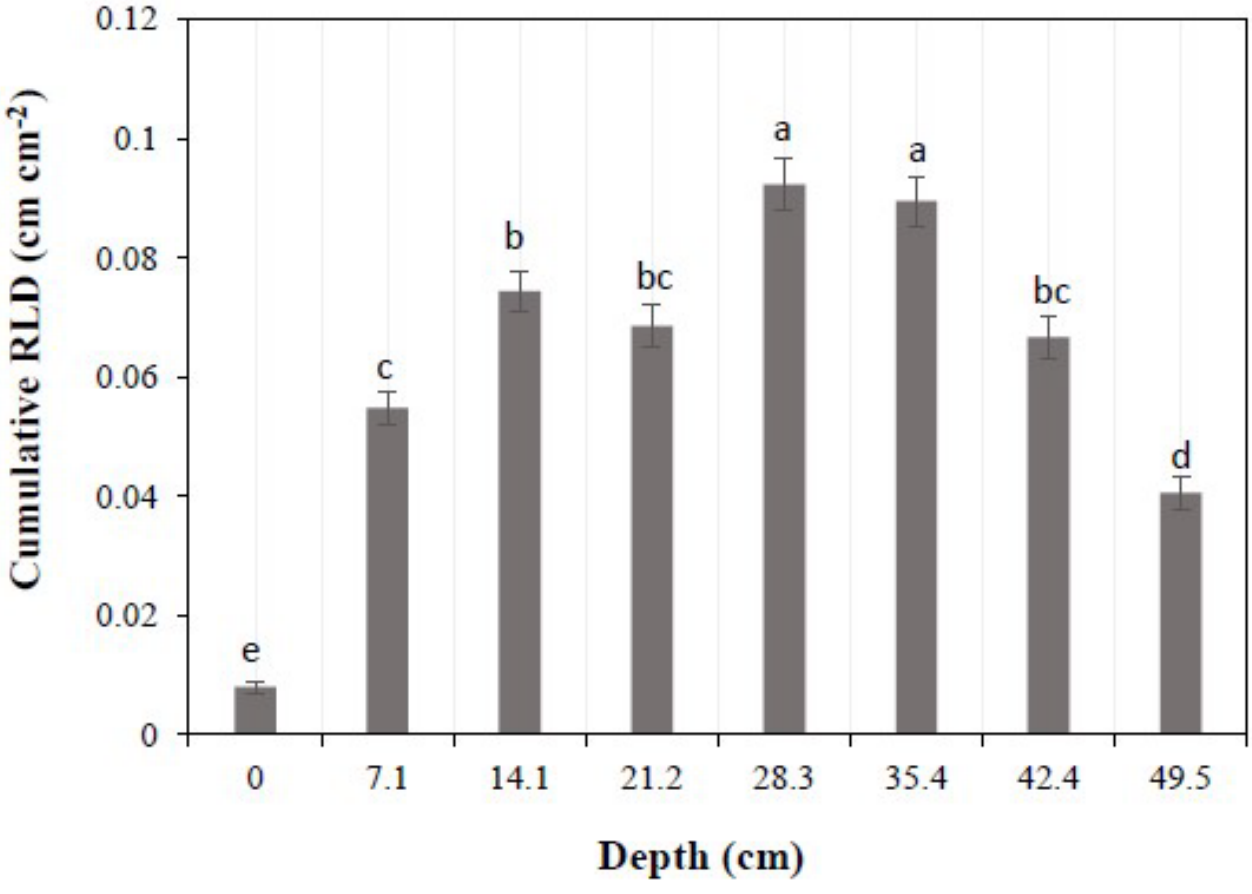

Figure 4.17. Average cumulative root length density (RLD) of a mature loblolly pine at different depths during the study period from April 2017 through February 2019. Error bars represent the standard error of the mean. In each figure, means associated with a different lower case letter are significantly different by Tukey's Multiple Range test.

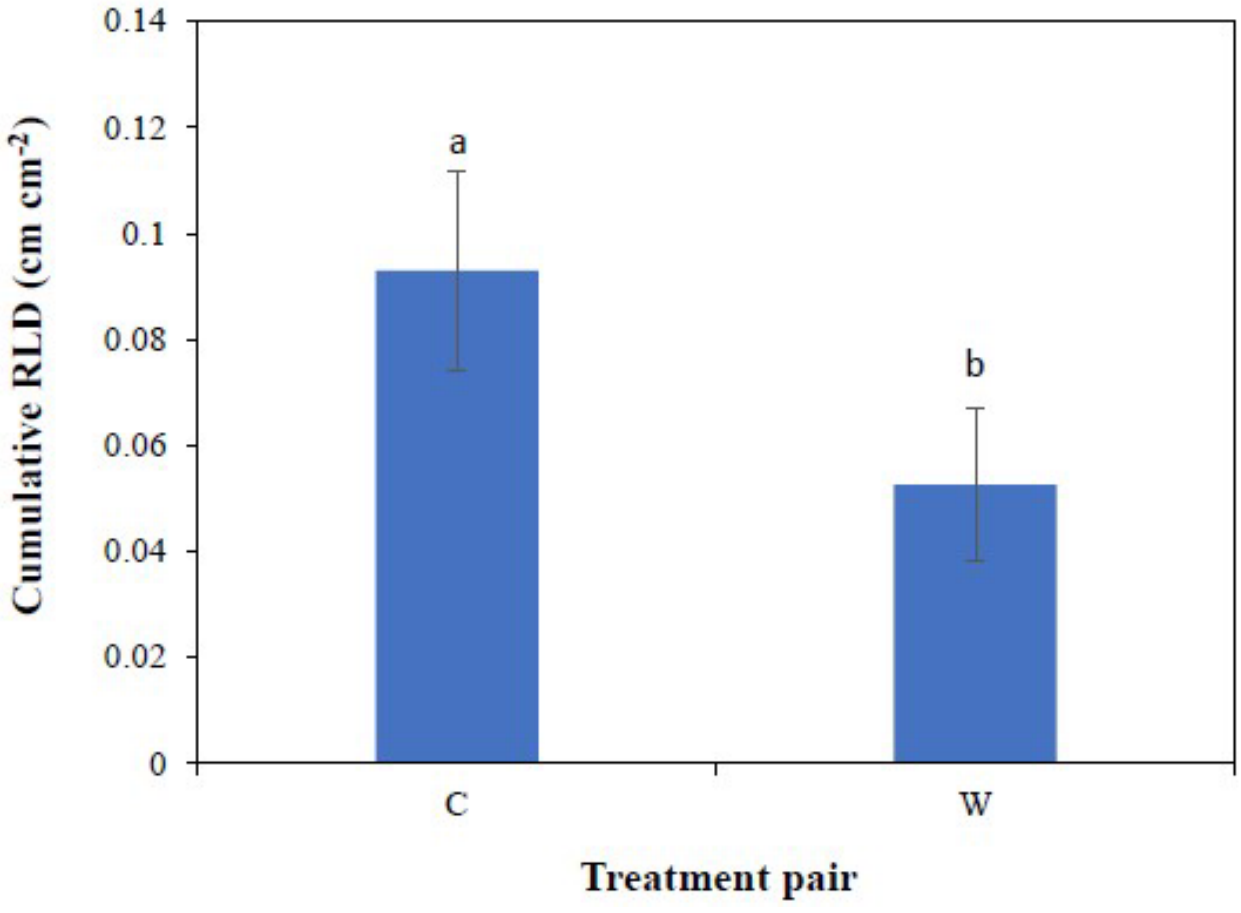

Figure 4.18. Within plot comparison of average cumulative root length density (RLD) of control (C) and wound (W) treatments during the February 2019 measurement. Error bars represent the standard error of the mean. In each figure, means associated with a different lower case letter are significantly different by Tukey's Multiple Range test.

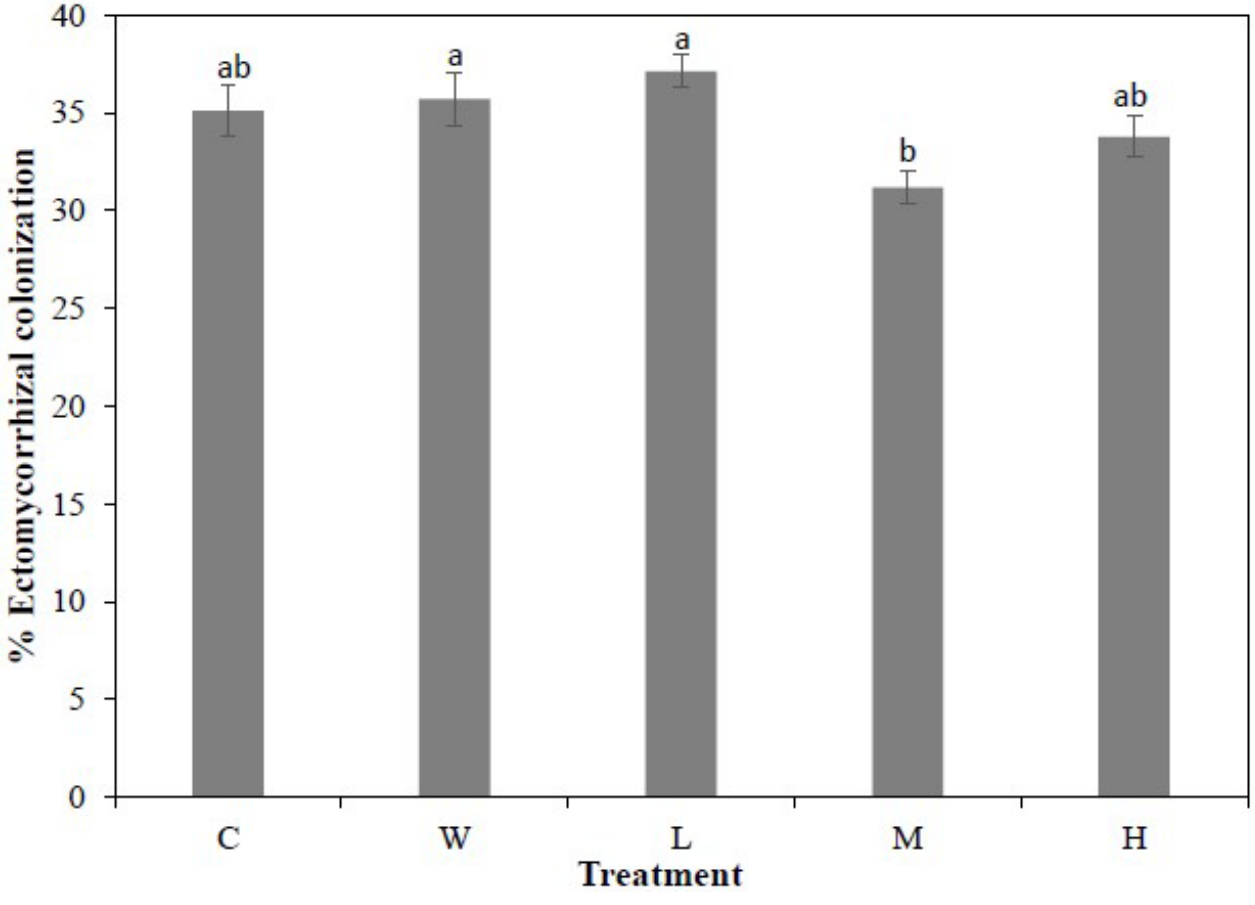

Figure 4.19. Average percentage (%) ectomycorrhizal colonization among treatments during September 2017. Treatments were control (C), wound (L), low (L), medium (M), and high (H) inoculation. Error bars represent the standard error of the mean. In each figure, means associated with a different lo er case letter are significantly different by Tukey's Multiple Range test.

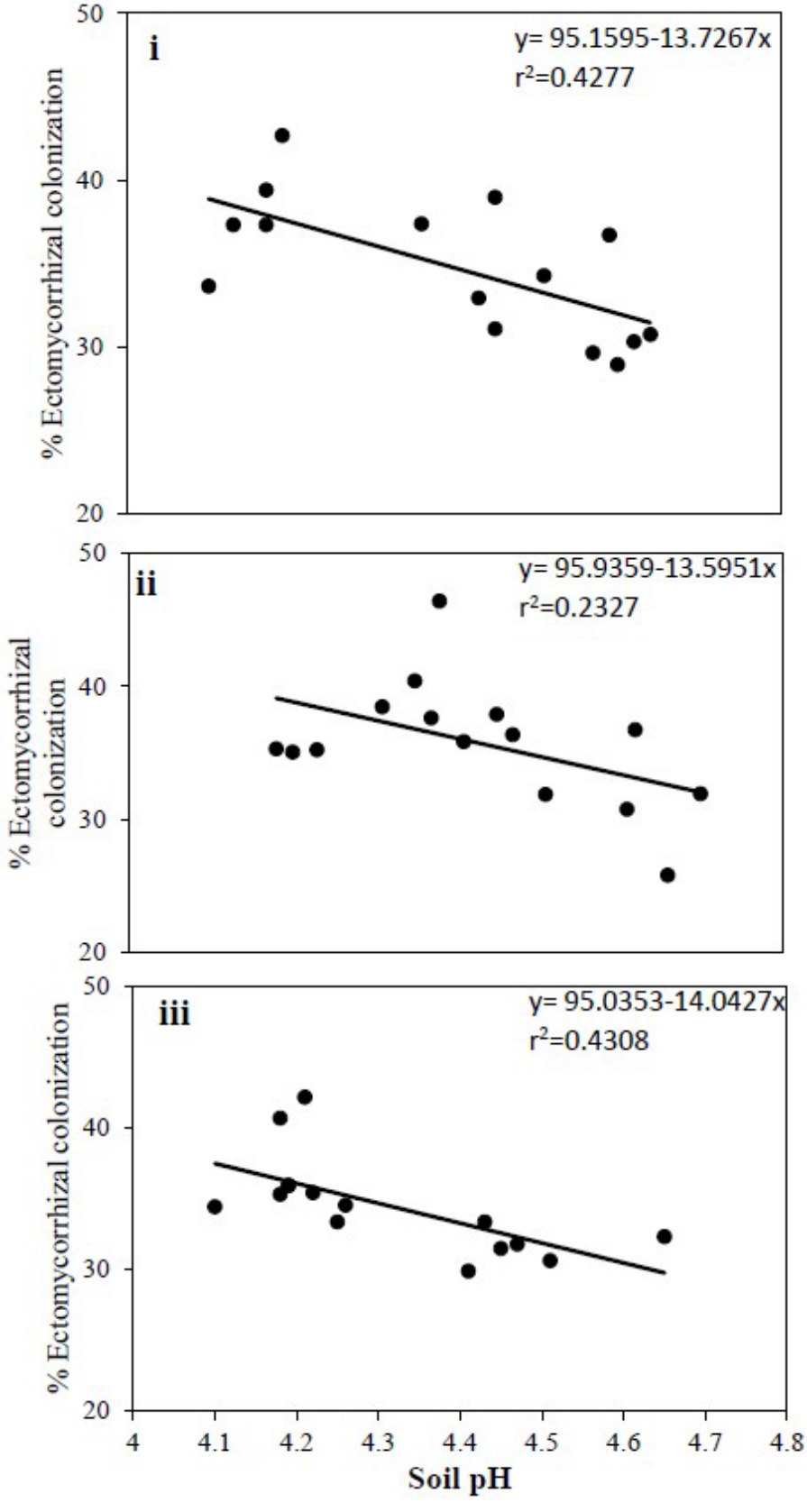

Figure 4.20. Relationship between ectomycorrhizal colonization of loblolly pine fine roots and soil pH at the **(i)** 10-20 cm, **(ii)** 20-30 cm, and **(iii)** 30-40 cm depths near Eufaula, Alabama (n=15, i.e. five treatments× three replicates) during September 2017.

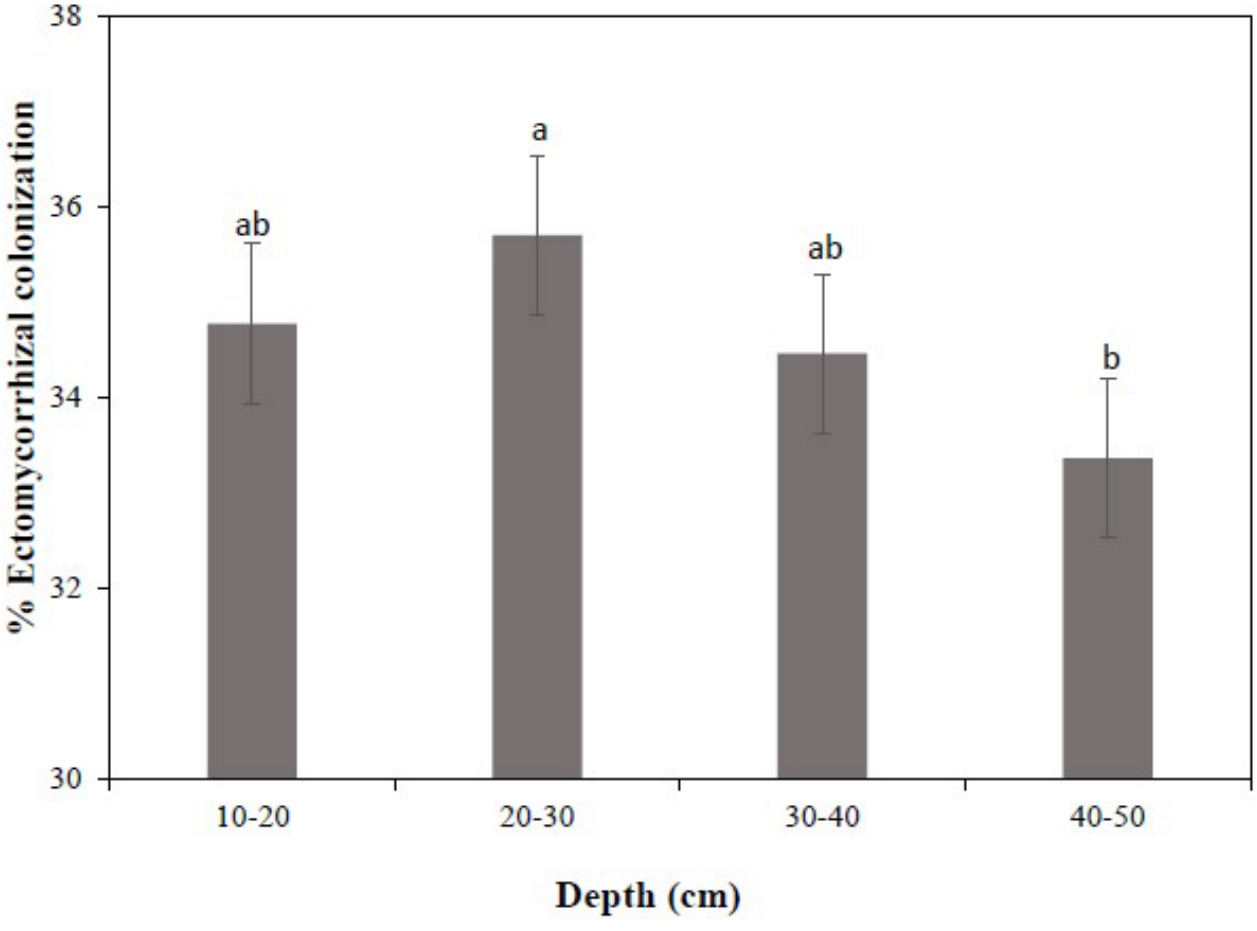

Figure 4.21. Average percentage (%) ectomycorrhizal colonization among depths during September 2017. Error bars represent the standard error of the mean. In each figure, means associated with a different lower case letter are significantly different by Tukey's Multiple Range test.