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PHYSIOLOGICAL RESPONSE OF *PINUS TAEDA* TREES TO STEM INOCULATION WITH *LEPTOGRAPHIUM TEREBRANTIS*

by

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3.1. ABSTRACT

Leptographium terebrantis S.J. Barras and T.J. Perry is an opportunistic root pathogen that compromises the xylem function of infected trees and is commonly associated with *Pinus taeda* L stands that experience an unexplained loss of vigor in the southeastern U.S. To understand the relationship between *L. terebrantis* inoculation density, sapwood occlusion, and sapwood function characterized by hydraulic conductivity and moisture content, an artificial inoculation study was conducted in young *P. taeda* trees in a naturally regenerated stand over a 24-week period in south central Alabama. Four levels of increasing stem inoculation were used as a surrogate for comparable levels of woody root inoculation followed by an evaluation of pathogen-induced occlusion, sapwood function, and fascicle physiology. Occlusion of old sapwood intensified as *L. terebrantis* inoculum density increased, but occlusion was absent in current-year sapwood. Occlusion reduced sapwood hydraulic conductivity and moisture content but did not interfere with stomatal conductance. The vertical spread of *L. terebrantis* was correlated with losses of sapwood hydraulic conductivity and moisture content due to occlusion. Results demonstrate that the sapwood function of *P. taeda* is tolerant of the pathogen vascular occlusion when stand conditions sustain adequate carbon fixation for occlusion-free stemwood growth.

3.2. INTRODUCTION

Insect pests promote forest decline by their attraction to physiologically compromised trees and heightened population growth driven by inciting climatic factors (McDowell et al., 2008; Marchetti et al., 2011; Haavik et al., 2015). They, in turn, may vector pathogens that further compromise tree vigor and timber value (Lowell et al. 2010). Such is the case for southern pine

decline (SPD) when *Leptographium terebrantis* S.J. Barras and T.J. Perry is vectored by root-feeding bark beetles (*Hylastes* spp.) (Hess et al., 2005; Eckhardt et al., 2007). Once the root pathogen is introduced, fungal advancement induces secondary metabolite production in the outer xylem, and reduces woody root hydraulic function (Franceschi et al., 2005; Oliva et al., 2014). Similar hydraulic dysfunction of *Pinus* spp. in response to insect-vectored fungal pathogens has been documented (Butnor et al., 1999; Lee et al., 2006; Sallé et al., 2008). The resulting loss of hydraulic function contributes to water limitations that may reduce carbon assimilation and allocation to both growth and carbohydrate reserves in the whole tree (Joseph et al., 1998; Sallé et al., 2008; Aguadé et al., 2015).

In evaluating climate-linked tree mortality, Allen et al., (2010) suggest drought-related tree mortality parallels Manion's chain of events in forest decline in tree species that primarily rely on stomatal control for drought avoidance (Manion, 1991). The large-scale mortality of *Populus tremuloides* Michx. (quaking aspen) across western North America during the past decade is an example of a landscape-scale forest decline incited by acute drought and exacerbated by site and stand conditions, insect pests, and diseases (Worrall et al., 2013; Dudley et al., 2015). The loss of *Quercus rubra* L. (northern red oak) in the Ozark and Ouachita Mountain forests of Arkansas also resembles a forest decline triggered by drought and worsened by *Enaphalodes rufulus* (Haldeman) (red oak borer) infestation and *Armillaria* root rot (Haavik et al., 2015). Alternatively, tree mortality events during drought may occur exclusively by hydraulic failure, or by inadequate gas exchange for the sustained supply of secondary metabolites to defend against insect pests and pathogens favored by a causal change in climate (McDowell et al., 2008).

Recent assessments of tree mortality have not detected spatial patterns indicative of large insect or disease hot-spots in *Pinus* forests of the southeastern United States (Potter and Paschke, 2016). Nevertheless, sparse and chlorotic tree crowns, reduced radial growth, and deterioration of fine roots culminating in tree mortality have been observed among isolated groups of mature *Pinus taeda* L. (loblolly pine) trees since the mid-1950s (Otrosina et al., 1999; Hess et al., 2005; Eckhardt et al., 2007). The first cases of this problem were reported by Brown and McDowell (1968) on the Oakmulgee Ranger District of the Talladega National Forest in Alabama. Subsequent reports confirmed the decline in growth of localized *P. taeda* trees in plantations across the central portion of the southeastern U.S. (Eckhardt et al., 2007; Eckhardt et al., 2010). Several root pathogens were implicated as contributing to these losses of *P. taeda* vigor (Roth and Peacher, 1971; Miller, 1979; Mistretta and Starkey, 1982). However, the root pathogen most commonly isolated from declining *P. taeda* trees was *Leptographium* spp. either alone or in association with *Phytophthora cinnamomi* Rands, *Pythium* spp., or *Heterobasidion annosum* (Fr.) Bref. (Otrosina et al., 1999; Hess et al., 2005). Presently, unexplained poor *P. taeda* vigor in conjunction with isolation of *Leptographium* spp. from woody root tissue indicates the likelihood of SPD. To adjust timber harvest schedules where *L. terebrantis* root disease is found, commercial landowners need to equate the incidence of *L. terebrantis* with the likelihood of stemwood growth loss and tree mortality.

This problem is most widespread in the west-central portion of Georgia south of Columbus, and in the upper coastal plain and lower Piedmont regions of Georgia and Alabama (Eckhardt et al., 2007; Eckhardt et al., 2010). Eckhardt and Menard (2008) reported a positive correlation between SPD severity in Georgia and Alabama and slope and aspect features indicative of water

limitations to tree growth (Fekedulegn et al., 2003; Tromp-van Meerveld and McDonnell, 2006; Dyer, 2009). Edaphic factors that worsen water deficit have also been observed as underlying components of other tree mortality events (Klos et al., 2009; Dudley et al., 2015). Current understanding indicates that SPD syndrome emanates from predisposing, inciting, and contributing factors which act in a concerted manner to reduce tree vigor and defense capacity and cause tree mortality (Manion, 1991; Eckhardt et al., 2010). The predisposing factors of SPD are long-term, underlying circumstances and may include climatic and edaphic conditions or genetic traits that exert physiological stress on individual trees, but alone do not cause tree mortality. Inciting factors are short-term conditions that decrease tree growth when combined with predisposing factors. Examples of inciting factors include short-term severe drought and defoliation by insects or fire. Contributing factors are opportunistic biotic agents such as insect pests and plant pathogens that accelerate the decline of trees already weakened by predisposing and inciting conditions (Manion, 1991; Jurskis, 2005).

Leptographium terebrantis has been shown to be pathogenic to artificially inoculated *P. taeda* seedlings (Singh et al., 2014; Devkota and Eckhardt, 2018; Devkota et al., 2018b), and it is commonly isolated *in situ* from the woody roots of mature *P. taeda* that exhibit SPD symptoms (Eckhardt et al., 2007; Matusick et al., 2013). In a study of root pathogen behavior in *Pinus palustris* Mill. and *P. taeda*, Matusick et al., (2016) noted that *L. terebrantis* infested both woody roots and stems. In *Pinus*, a primary purpose of xylem tissue is water transport regardless of its stem or woody root origin (Eissenstat and Van Rees, 1994; Hacke and Sperry, 2001). Thus, stem infection with *L. terebrantis* may be used as a surrogate for woody root infection with *L. terebrantis* to study how this pathogen affects tree physiological processes.

The present study was conducted to provide information about how *P. taeda* sapwood xylem function is affected by four increasing levels of artificial inoculation with *L. terebrantis*. Our first objective was to determine the relationships between pathogen inoculation density, sapwood occlusion, and sapwood function characterized by hydraulic conductivity and moisture content. Second, with stemwood growth and stomatal conductance as indicators of tree vigor, the impact of pathogen-compromised sapwood function on tree vigor was evaluated as *L. terebrantis* infestation advanced over 24 weeks. We hypothesized that positive relationships would be found between *L. terebrantis* inoculation density, sapwood occlusion and the loss of sapwood function. We further hypothesized that pathogen-induced loss of sapwood hydraulic conductivity would cause more negative fascicle predawn water potentials and a decrease in stomatal conductance.

3.3. METHODS

3.3.1. Study Site and experimental design
The study was conducted in a naturally regenerated *P. taeda* stand near Andalusia, AL, USA at the Solon Dixon Educational Center (31.1427ºN, 86.6963ºW) on a complex of two soil series, Dothan sandy loam and Malbis fine sandy loam. These sandy loam soils contain an argillic horizon and are characterized by moderate to moderately low saturated hydraulic conductivities (Clapp and Hornburger, 1978; Watts et al., 1982; Rawls et al., 1998). The stand was approximately 5- to 7-yr-old and contained young trees of *Pinus echinata* Mill. (shortleaf pine), *Pinus elliottii* Engelm. (slash pine), and *Pinus palustris* Mill. (longleaf pine) as a minor component. In May to October 2016 when this study was conducted, mean daily air temperature

was 25.0 °C which was similar to the 30-year average between 1986 and 2016 of 24.5 °C (NOAA, 2019) (Figure 1). The location received 1197 mm of annual precipitation in 2016 with 41% during the 5-mo period of this study between May and October (NOAA, 2019). Compared to the 30-yr average annual precipitation between 1986 and 2016, precipitation in 2016 and during the 5-mo period of this study were 15% and 30% less than normal, respectively.

One hundred young *P. taeda* trees free of competition for sunlight on at least three sides of the crown and with a ground-line diameter (GLD) of 6.3 cm ($\pm$1.3 cm) and total height of 4.5 m ($\pm$0.4 m) were identified and selected for the study. Each tree was randomly assigned to one of five inoculation treatments which were no inoculation (Control), and four levels of increasing inoculation density (IP): 2IP, 4IP, 8IP, and 16IP. The 2IP, 4IP, 8IP, and 16IP inoculation densities represented one *L. terebrantis* colonized toothpick per 10.0, 5.0, 2.5, and 1.3 cm around the circumference of the stem bark, respectively (Devkota et al., 2019). Inoculation points were equidistant from each other and repeated three times at a 1.3 cm interval below the initial inoculation point. Thus, the 2IP, 4IP, 8IP, and 16IP inoculation densities corresponded to 8, 16, 32, and 64 total inoculation points, respectively. From the 20 trees per inoculation treatment, subsets of five trees were randomly assigned to four treatment periods (8, 16, 20, and 24-wk post-inoculation) when fascicle physiology measurements were conducted. Following fascicle physiology measurements at 16, 20, and 24-wk post-inoculation, trees were destructively harvested to assess sapwood occlusion, stem hydraulic conductivity, and stem moisture content.

3.3.2. Inoculation method
Wooden toothpicks were sterilized at 121 °C for 30 min and soaked overnight in malt extract broth (MEB) (BD Bacto ™ Malt Extract, BD Biosciences, San Jose, CA). Sterile Petri plates, 9 cm in diameter, were prepared with approximately 20 ml of sterile malt extract agar (MEA). The agar was split into two equal halves and fifteen sterilized toothpicks imbibed with MEB were arranged on the half agar medium (Devkota et al., 2019). This enabled growth of, and entire colonization of the toothpicks by the fungus. A 5 mm diameter agar disc of actively growing, 2-wk-old *L. terebrantis* was placed centrally on the agar in the Petri plates. Inoculated Petri plates were incubated at 25°C in darkness for 21 days. The isolate of *L. terebrantis* (LOB-R-00-805/ MYA-3316) used in this study was obtained from roots of declining *P. taeda* trees at Talladega National Forest, Oakmulgee Ranger District, AL (Eckhardt et al., 2007; Devkota and Eckhardt, 2018) and maintained on MEA slant cultures at 4°C at the Forest Health Dynamics Laboratory at Auburn University. Devkota and Eckhardt (2018) identified this isolate as one of the most virulent to *P. taeda* seedlings among 42 isolates of *L. terebrantis*. Moreover, this isolate was used in a study to confirm sterilized toothpicks as a suitable substrate for fungal growth and sporulation (Devkota et al., 2019).

For trees that received the 2IP, 4IP, 8IP, or 16IP inoculation densities, the dead cork of the bark was scraped around the circumference of the stem between 10 cm and 15 cm above the ground line with a 20.3 cm long ironton straight draw shave (Northern Tool + Equipment, Burnsville, MN, USA). To ensure proper inoculum placement, a stencil sheet identifying inoculation points was wrapped around the stem. Inoculation points, approximately 1.2 mm in diameter and 5 mm deep, were drilled into the stem through the stencil sheet. Trees were inoculated by inserting toothpicks containing inoculum into the holes within 5 min of drilling. After inoculation, the protruding ends of the toothpicks were cut, and the inoculation zone of the stem was sealed with duct tape. Between five and nine trees were inoculated within an hour and inoculations were

completed within one day (May 5, 2016). The trees were monitored at a 2-wk interval in the field for symptoms of disease such as foliage discoloration and oleoresin exudation from the edge of the taped inoculation zone.

3.3.3. Predawn water potential and stomatal conductance

Predawn fascicle water potential and stomatal conductance at 8, 16, 20, and 24 wk after inoculation on May 5, 2016 were measured on the five trees per treatment that were randomly assigned to be destructively harvested at 24-wk post-inoculation. Predawn fascicle water potential (PWP) was measured by a pressure chamber (PMS Instrument Corp., Corvallis, OR, USA) as described by Tyree and Hammel (1972). At least two mature fascicles were collected from the upper one-half of the crown of each measurement tree before sunrise between 01:00 and 05:00. Fascicles were detached, placed in plastic zip-lock bags containing a moist paper towel, and stored in an ice chest containing ice. All PWP (MPa) measurements were completed by 30 min after the last sample was collected. Fascicles used for PWP measurements were weighed to the nearest 0.01g (W_W), soaked in distilled water overnight, reweighed in the morning to obtain turgid weights (W_T), dried at 70 ^oC to equilibrium, and reweighed (W_D).

Fascicle relative water content (RWC_F, %) was determined by the equation:

$$RWC_F = [(W_W - W_D) / (W_T - W_D)] * 100. \hspace{2cm} (1)$$

Stomatal conductance to water vapor (g_w) was measured using a leaf porometer (model SC-1, Decagon Devices, Inc., Pullman, WA, USA) with an accuracy of $\pm 10\%$. Prior to each g_w (mmol m^{-2} s^{-1}) measurement, the porometer was calibrated using the manufacturer's guidelines to ensure that the sensor head and the prevailing environmental conditions were in thermal equilibrium. The g_w measurements were taken between 11:00 and 14:00 on a single sunny day without cloud cover. During each measurement, fascicles (4 to 6) of the first flush produced in 2015 attached to a shoot in the upper one-half of the crown were arranged in a horizontal plane to completely cover the sensor screen. Then the sensor block was gently clamped, and measurements were completed within 30 sec. An effort was made to minimize fascicle overlap in the clamped sensor block. Data were the mean of two or three measurements at different fully sunlit locations in the crown.

3.3.4. Sapwood occlusion and specific hydraulic conductivity

For trees used in sapwood occlusion and hydraulic conductivity measurements at 16, 20, and 24-wk post-inoculation, stems were cut at ground level and again at 50.0 cm above ground level by a chainsaw, and stem segment ends were painted with rubber sealant. Sealed stem segments were wrapped with plastic sheeting, stored on a bed of ice and transported to the laboratory. To assess stem occlusion, duct tape was removed from the inoculation zone and the 10 cm section of each stem segment with the inoculation zone centrally located was permanently marked. The rubber sealant on either end of the stem segment was removed and a 5.0 cm length was cut from terminal and basal ends. Remaining stem segments were cut at 1.0 cm intervals until occluded sapwood was observed. When sapwood occlusion was not observed before the 10 cm stem section containing the inoculation zone was reached, stem occlusion assessment by 1.0 cm stem intervals was resumed after hydraulic conductivity determinations were completed. Occlusion was identified by a darkened sapwood appearance (Solheim and Krokene, 1998; Lee et al., 2006). Occluded stem lengths were determined to the nearest mm by an average of observations on opposite sides of the stem segment. After occluded stem lengths were determined, a 10.0 cm

section of each stem segment with the inoculation zone centrally located was identified. Following the protocol of Butnor et al., (1999), this 10.0 cm section was excised, debarked, weighed (W_1) to the nearest 0.1 g, wrapped in plastic, and refrigerated until hydraulic conductivity measurements.

Prior to each series of hydraulic conductivity measurements, deionized water was degassed, acidified with 0.1 M hydrochloric acid, and stored in a 15 l reservoir elevated 1.0 m above the laboratory floor. On the day of measurements, the basal end of each 10.0 cm stem section was affixed to a rubber tube (5.0 cm in diameter) by a pipe clamp. The rubber tube was also fitted to the valve of the reservoir containing degassed and acidified deionized water. Pressure (10.0 KPa) from the raised reservoir was allowed to force water through the stem section (Butnor et al., 1999; Melcher et al., 2012) for 5-10 min until a constant flow rate was achieved. After this equilibration period, the volume of water that flowed through stem sections was collected at three 5 min intervals into a pre-weighed beaker and the average flow rate was calculated. Each set of three intervals was completed within a 20 min period after a constant flow rate was attained. After detaching the stem sections from the rubber tube, the cross-sectional area of the sapwood (A_s) on the basal end of stem sections was traced onto a transparent plastic sheet and the traced area was quantified by a planimeter (Lasico®, Los Angeles, CA, USA).

Native, stem specific hydraulic conductivity (K_s, Kg m^{-1} MPa^{-1} s^{-1}), was calculated as:

$$K_s \, (\text{Kg m}^{-1} \text{MPa}^{-1} \text{s}^{-1}) = (QL) / (PA_s) \tag{2}$$

where Q is the average flow rate (Kg s^{-1}), L is the length of the stem section (m), P is the pressure applied to the stem section (MPa), and A_s is the cross-sectional area of the sapwood (m^2) (Butnor et al., 1999; Melcher et al., 2012). After K_s determinations, stem sections were oven-dried at 70 ^{o}C to equilibrium and weighed (W_2). The moisture content (MC_s) of stem sections was expressed as a percentage of oven-dried weight. The 10 cm long stem sections were cut into seven 1.3 cm discs by a band saw. Total and occluded sapwood areas of the basal side of each disc were traced onto a transparent plastic sheet and total and occluded sapwood areas of the seven discs per stem section were determined by a planimeter. Similarly, after the 24-wk post-inoculation K_s and sapwood area determinations, the basal side of each disc was assessed for the area of new sapwood that grew after artificial inoculation. Occluded and new sapwood areas were expressed as a percentage of total sapwood areas of the seven discs per stem section.

3.3.5. Data analysis

Values of PWP, RWC_F, g_w, K_s, MC_s, stem occluded length, and percentages of stem segment sapwood area that were occluded and new were assessed for normality and equal variance assumptions. Natural logarithm and logit transformations were applied to occluded stem length and percentage occluded sapwood area, respectively, to satisfy the assumptions of normality and homogeneity of variance. All variables except new sapwood area were evaluated by a completely randomized split plot in time experimental design with five replications using analyses of variance (ANOVA) and the Mixed and GLM procedures of SAS statistical software (SAS Institute, Version 9.4, Cary, NC, USA). The whole plot effect was inoculation density (Control, 2IP, 4IP, 8IP, 16IP or 2IP, 4IP, 8IP, 16IP), and the subplot effect was treatment period since inoculation (8, 16, 20, and 24 wk or 16, 20, and 24 wk). Similarly, the percentage of new sapwood area at 24-wk post-inoculation was evaluated by a completely randomized experimental

design with five replications. Treatments were the 2IP, 4IP, 8IP, and 16IP inoculation densities. Significant main and interaction effects were further evaluated by a pair-wise comparison among means using the post-hoc Tukey's Honest Significance Difference Test (HSD) for multiple comparisons. The linear relationship between occluded stem length and stem moisture content (MC_s) at 24-wk post-inoculation and those between MC_s and occluded sapwood area at 16-, 20-, and 24-wk post-inoculation were assessed by regression. Regression parameters of pairs of significant MC_s-occluded sapwood area lines were compared by the general linear test using the REG procedure of SAS statistical software (Neter and Wasserman, 1974). Probabilities of a greater F value and mean comparisons were considered significant at an α-level of 0.05.

3.4. RESULTS
Throughout the 24 weeks after inoculation, trees were monitored every 2 weeks in the field for symptoms of disease such as foliage discoloration, oleoresin exudation from the edge of the taped inoculation zone, and mortality. No indications of disease were detected in the field during this period. After duct tape was removed at all treatment periods, however, harvested stems showed oleoresin exudation from the majority of inoculation points.

Neither PWP nor RWC_F were significantly affected by the main effects of inoculation density or treatment period but their interaction was significant (PWP: $p = 0.0013$; RWC_F: $p = 0.0142$). Mean comparison tests did not indicate significant differences among PWP by inoculation densities and treatment periods with values that ranged between -0.31 to -0.51 MPa during the study. Mean comparison tests indicated four significant differences in RWC_F that occurred by inoculation densities and treatment periods. Values of RWC_F were significantly lower at the 20-week treatment period compared to the 8-week treatment period for the 2IP (18%) and 8IP (13%) trees. A similar significant decrease in RWC_F was observed for the 8IP (15%) and 16IP (12%) trees between the 8- and 24-week treatment periods.

Fascicle g_w was significantly affected by treatment period ($p < 0.0001$) and inoculation density ($p < 0.0001$) but not their interaction. Values of g_w were significantly different among the four treatment periods with maximum g_w (190.5 mmol m^{-2} s^{-1}) at 16-weeks post-inoculation in August 2016, and minimum g_w (56.9 mmol m^{-2} s^{-1}) at 24-weeks post-inoculation in October 2016. Across the four treatment periods, Control tree g_w averaged 99.1 mmol m^{-2} s^{-1} and was significantly less (21%) than average g_w (126.1 mmol m^{-2} s^{-1}) among trees receiving the 2IP, 4IP, 8IP, and 16IP inoculation densities.

Occluded stem length was significantly affected by interaction between inoculation density and treatment period (Table 1). Occluded stem length did not differ among inoculation densities at the end of the 16- and 20-week treatment periods (Figure 2). At the end of the 24-week treatment period, a significantly higher occluded stem length was observed with the 16IP inoculation density compared to 2IP, 4IP, and 8IP inoculation densities. The stem occluded length of the 16IP trees at 24-week was also significantly greater than those of all four inoculation densities at the 16- and 20-week treatment periods.

Occluded sapwood areas of stem segments used for K_s determinations were significantly affected by interaction between inoculation density and treatment period (Table 1). At the end of the 16-week treatment period, 16IP trees had significantly more occluded sapwood area compared to

2IP trees (Figure 3). Four weeks later at the 20-week treatment period, the 16IP trees had significantly more occluded sapwood area than the 2IP, 4IP, or 8IP trees, and the 8IP trees had significantly more occluded sapwood area than the 2IP trees. This trend in sapwood area occlusion was also observed at the 24-week treatment period with significantly more occluded sapwood area among the 16IP trees compared to the 2IP, 4IP, and 8IP trees. The occluded sapwood area of the 16IP trees increased significantly between 16 and 20 weeks post-inoculation but did not increase between 20 and 24 weeks post-inoculation.

By the end of the 24-week treatment period, a distinct zone of new sapwood area that was not occluded was apparent in all stem segment discs regardless of inoculation density (Figure 4). The percentage of new sapwood area of stem segments at the 24-week treatment period was not significantly different among the inoculation densities (2IP: 6.2 (mean) ± 0.3 (standard error) %, 4IP: 6.7 ± 0.6%, 8IP: 6.8 ± 0.3%, 16IP: 7.3 ± 0.5% of total sapwood area).

Values of K_s were significantly affected by interaction between inoculation density and treatment period (Table 1). By the end of the 16-week treatment period, the K_s of 8IP and 16IP trees was less than that of Control trees. Also at this time, K_s was not significantly different among the 2IP, 4IP, 8IP, and 16IP trees but its trend at subsequent treatment periods was established (Figure 5). Four weeks later at the end of the 20-week treatment period, K_s was significantly reduced among the 8IP and 16IP trees compared to the Control trees and those receiving the 2IP and 4IP inoculation densities. By the end of the 24-week treatment period, a significant decrease in K_s was only observed in trees receiving the 16IP inoculation density compared to Control trees.

Values of MC_s were significantly affected by inoculation density and treatment period but not their interaction (Table 1). Across the three treatment periods, the MC_s of the 16IP trees was significantly lower by 20% compared to average MC_s among the Control, 2IP, 4IP, and 8IP trees. Averaged across inoculation densities, MC_s at the end of the 24-week treatment period was significantly greater (30%) than that at the end of the 20-week treatment period. At 24 weeks, a significant linear relationship was found between occluded stem length and stem MC_s ($r^2 = 0.6811$, $p < 0.0001$) (Figure 6). Linear relationships between the natural logarithm of MC_s and percentage of occluded sapwood area were significant at 20 ($p < 0.0001$, $r^2 = 0.5734$) and 24 ($p < 0.0001$, $r^2 = 0.6967$) weeks. The y-intercepts but not the slopes of these 20 and 24 week lines were significantly different (y-intercepts: $p < 0.0001$) (Figure 7).

3.5. DISCUSSION

Leptographium terebtantis is most commonly vectored by root feeding bark beetles (*Hylastes tenuis* (Eichoff), *H. salebrosus* (Eichoff)) rather than stem feeding bark beetles (*Ips* and *Dendroctonus* spp.) (Eckhardt et al., 2007; Matusick et al., 2013). We utilized artificial stem inoculation as a surrogate for *Leptographium* root disease and as such, our results cannot be directly applied to predict loss of tree vigor after *L. terrebrantis* is vectored by root-feeding bark beetles. However, our observations provide insight about the physiological mechanisms and conditions that hinder or augment the consequences of wilt pathogen activity in forest stands. Specifically, we emphasize the impact of wilt pathogens on stem hydraulic conductivity and moisture content and the role of available water and current-year sapwood in sustained stomatal conductance and sapwood growth.

The effect of *L. terebrantis* on *P. taeda* stems as inoculation density increased was evident in occluded sapwood area among the 2IP, 4IP, 8IP, and 16IP trees. This response was apparent by 16 weeks post-inoculation, and four weeks later at 20 weeks post-inoculation, peak occluded sapwood area was evident in the 16IP trees. Although a similar trend in occluded sapwood area was observed at 24 weeks post-inoculation, the robust nature of this response was absent. A similar pattern of sapwood occlusion in response to fungal inoculation density was reported in *Pinus contorta* Dougl. ex Loud. var *latifolia* Engelm. ex S. Wats (lodgepole pine) and *Pinus sylvestris* L. (Scots pine) (Croisé et al., 1998; Solheim et al., 2001; Lee et al., 2006). Croisé et al., (1998) noted that at a high inoculum density, *Leptographium wingfieldii* Morelet caused significant sapwood damage by occlusion in Scots pine but at a low inoculum density, only a small fraction of sapwood occlusion occurred.

Comparable symptoms of sapwood occlusion and lesions occurred when the stem and mature, woody roots of *P. taeda* were inoculated with different ophiostoimatoid fungal species (Matusick et al., 2016; Devkota et al., 2018a). However, others have found that plant organ responses to fungal pathogens differ. For instance, in the southeastern United States, Matusick et al., (2016) found that fungal species such as *Grosmannia huntii* (R.C. Rob. Jeffr.) Zipfel, Z.W. de Beer and M.J. Wingf., *G. alacris* (T. A. Duong, Z. W. de Beer and M. J. Wingf.), *Heterobasidion irregulare* (Fr.) Bref. and *L. procerum* (W.B. Kendr.) M.J. Wingf. caused more damage in mature, woody roots compared to stems of *P. taeda.* On the contrary, *L. terebrantis* caused more stem damage by occlusion compared to mature woody roots in *P. taeda* (Matusick et al., 2016).

Activation of inducible defenses and deposition of oleoresin in the vicinity of the invaded sapwood to isolate the pathogen from further advancement into host tissues is a carbon-demanding process (Paine et al., 1997; Franceschi et al., 2005). One factor causing occluded sapwood area to intensify by September (week 20) may have been the seasonal carbon dynamics of *P. taeda*. Visual assessment of stem discs used for occluded area measurements indicated that vigorous stem growth took place across all inoculation densities. Since *P. taeda* sapwood growth is initiated in early spring and continues to sequester carbon through late October (Lorio, 1986; Baker and Langdon, 1990; Blanche et al., 1992; Emhart et al., 2006), competition for carbon between sapwood growth and occlusion was likely throughout the duration of this study. By mid-August to September, however, terminal and lateral branch growth of *P. taeda* are completed and peak leaf area is achieved (Baker and Langdon, 1990; Dougherty et al., 1994; Emhart et al., 2006). Furthermore, the majority of *P. taeda* new roots are established between April and October (Sword-Sayer and Tang, 2004; Coleman and Aubrey, 2018). Therefore, the amount of carbon supplied to defense between August and September may have increased because of diminished carbon demands in the crown and root system.

Infection by *L. terebrantis* reduced stem hydraulic conductivity and increased occluded sapwood area. This inverse relationship between K_s and sapwood occlusion caused by pathogen invasion is well established (Butnor et al., 1999; Guérard et al., 2000; Sallé et al., 2008). For example, artificial inoculations of 7- to 8-year-old naturally regenerated *Pinus sylvestris* L. (Scots pine) with *Ophiostoma brunneo-ciliatum* Math caused a 55% loss of K_s at an inoculation point density of 1000 m^{-2} around the stem and caused about 60% sapwood occlusion (Guérard et al., 2000). Similar to occluded sapwood area, we observed that K_s exhibited greater differences among inoculum densities at 20 rather than 24 weeks post-inoculation. We attribute the observed pattern of diminished K_s response to sapwood occlusion over time to several factors that include

available water across the duration of the study, seasonal gas exchange and leaf area dynamics, and the influence of un-occluded, current-year sapwood on calculation of occluded sapwood area.

The duration of time between inoculation and the appearance of inoculation density effects differed between occluded sapwood area and occluded stem length. The inoculation zone was a section of stem circumference approximately 5 cm wide. Regardless of inoculation density, occluded stem length extended from this area by an average of 4.0 cm at 20-weeks post-inoculation. Among all but the highest inoculation density, the extent of occluded stem length was maintained through 24-weeks post-inoculation. However, between 20- and 24- weeks post-inoculation, the 16IP trees experienced a 60% increase in occluded stem length. In contrast to occluded stem length, occluded sapwood area and K_s were most severely affected by inoculation density 20 weeks after inoculation with a sustained but less robust response by 24-weeks post-inoculation.

Vertical spread of the pathogen may be explained by internal conditions of the xylem between the 20- and 24-week treatment periods. Vertical spread of the pathogen between 20- and 24-weeks in the 16IP trees coincided with an increase in occluded sapwood area particularly at 20 weeks post-inoculation. Oleoresin deposition displaces moisture in sapwood leading to drier sapwood conditions as well as a loss of stem moisture content. Butnor et al., (1999) reported a significant drop in sapwood moisture content in mildly symptomatic and diseased *Pinus strobus* L. infected with *Leptographium procerum* (Kendrick) Wingfield. We observed a similar response among the inoculated trees at the 24-week treatment period with a significant inverse relationship between occluded stem length and MC_s as indicated in Figure 6.

As a vascular wilt pathogen, *L. terebrantis* prevents normal sapwood function by colonizing xylem conduits and preventing water transport (Oliva et al., 2014). Additionally, deposition of an inducible chemical defense compounds such as oleoresin (Franceschi et al. 2005) into the xylem has the potential to isolate the pathogen from further advancement into host tissues (Paine et al., 1997; Franceschi et al., 2005). Occluded sapwood may also contain oxalic acid and other secondary metabolites that contribute to embolization and desiccation of the sapwood (Coutts, 1977; DeAngelis et al., 1986).

In general wilt pathogens thrive *in vivo* where they and their toxic metabolites reduce xylem moisture content and facilitate further pathogen spread if not isolated by occlusion (DeAngelis et al., 1986; Croisé et al., 1998; Oliva et al., 2014). Greater damage to trees by vascular wilt pathogens commonly occurs when plant tissues are dry due to disruption of water transport and storage (Croisé et al., 1998; Oliva et al., 2014). In our study, *L. terebrantis* was not sequestered by oleoresin deposition in the 16IP trees, and it spread into the relatively dry sapwood adjacent to the inoculation zone. We propose that favorable conditions for the vertical spread of *L. terebrtantis* were due to the effect of occlusion on MC_s and low precipitation between July and October.

By its contribution to sustained K_s and stem moisture content, current-year, un-occluded sapwood has the potential to counteract the negative effect of older sapwood occlusion on stem hydraulic function. The role of new sapwood as a buffer against the poor function of older, occluded sapwood depends, however, on tree water demand and amount of current-year sapwood growth. As occluded sapwood area increases, the role of current-year sapwood in maintaining

adequate K_s and stem moisture content increases. Because water deficit may reduce annual stemwood growth in plantation *P. taeda* (Klos et al., 2009; Maggard et al., 2016; Ingwers et al., 2018), un-occluded, current-year sapwood is not a reliable buffer against the pathogen-induced loss of stem hydraulic function during drought. Furthermore, if similar patterns of occluded and un-occluded sapwood occur after *Leptographium* infection of the stem and woody roots, water deficits that limit radial root growth risk a deterioration of root system hydraulic function.

The inner sapwood of *P. taeda* loses a fraction of its hydraulic function over time (Phillips et al., 1996; Domec and Gartner, 2002; Ford et al., 2004). This natural decrease in sap velocity by sapwood depth in conifers is attributed, in part, to a corresponding increase in xylem resistance to hydraulic transport with sapwood depth (Spicer and Gartner, 2001; Ford et al., 2004). Phillips et al., (1996) evaluated the radial profile of sap velocity in 12-year-old *P. taeda* that were similar in stature to those in our study. They found at sapwood depths greater than 2 cm, sap flux density averaged 41% less than in the outer 2 cm of sapwood and attributed this difference to the presence of juvenile xylem at radial depths greater than 2 cm. In their survey of tree age during transition from juvenile to mature xylem formation, Clark et al., (2006) reported that the age of mature xylem formation in *P. taeda* is variable and ranges from age 6 years to over 20 years. It is clear that the majority of sapwood in our study was composed of juvenile xylem. However, it is also possible that transition from juvenile to mature xylem formation was underway during the year of our study.

Mature xylem produced in the year of this study and a subsequent difference in the ratio of mature and juvenile xylem within the functional sapwood of inoculated and Control trees may explain why greater g_w was observed in inoculated trees compared to Control trees. Because occlusion excluded some juvenile xylem from the functional sapwood, inoculated trees may have been characterized by a higher ratio of mature to juvenile xylem in the functional sapwood compared to Control trees. During our study, PWP ranged between -0.31 to -0.51 MPa which is indicative of sufficient water for normal gas exchange in *P. taeda* (Samuelson et al., 2008, Tang et al., 2003). We hypothesize that higher g_w among inoculated trees compared to Control trees was expedited by the combined effect of lower xylem resistance to hydraulic transport in functional sapwood and ample available water, indicated by relatively high PWP throughout the study.

3.6. CONCLUSIONS
We used artificial stem inoculation with *L. terebrantis* to assess hydraulic function and stemwood growth responses of young *P. taeda* trees to infestation by a wilt pathogen. The young *P. taeda* trees tolerated *L. terebrantis* infection when stand conditions provided adequate carbon for the production of new sapwood and defense chemicals that occluded infected xylem. At the same time, relationships between occluded sapwood area and both stem hydraulic conductivity and moisture content suggested that there were underlying risks to *L. terebrantis* infection and subsequent sapwood occlusion. In support of our first hypothesis, we observed decreases in stem hydraulic conductivity and moisture content as occluded sapwood area increased, and a positive correlation between the vertical spread of the pathogen and loss of stem moisture content.

Elevated stomatal conductance during most of the study which is indicative of high rates of carbon fixation, and current-year sapwood that was devoid of the pathogen provided a means of sustained vigor despite infection of older sapwood with *L. terebrantis*. These observations indicate that not only is carbon fixation and allocation to sapwood important to the production of merchantable stemwood, but it may also be vital to maintenance of crown physiology when the hydraulic function of older sapwood is compromised by a wilt pathogen. Therefore, under the favorable growing conditions of this study we reject our second hypothesis that pathogen induced loss of hydraulic conductivity will decrease stomatal conductance. Further research is needed to determine thresholds of *L. terebrantis* infection in woody roots that together with stand conditions, have the potential to either sustain or compromise woody root sapwood function and, in turn, affect tree vigor and stemwood production. Our observations indicate that this effort will benefit by attention to root xylem formed both before and after pathogen infestation.

Table 3.1. Mean squares and probabilities of a greater *F*-value ($P > F$) for occluded stem length, occluded sapwood area, specific hydraulic conductivity (K_s), and stem moisture content (MC_s). Measurements were conducted at three treatment periods that were 16, 20, or 24 weeks after stem inoculation of *P. taeda* trees with four *L. terebrantis* inoculation densities.

Variable	Source of variation	df[1]	Mean square	*P > F*
Occluded stem length[2]	Inoculation Density (I)	3	0.158	< 0.0001
	Treatment period (T)	2	0.4718	< 0.0001
	I x T	6	0.0593	0.0044
	I	3	21.3104	< 0.0001

|				
Occluded sapwood area[3]	T	2	0.624	0.1942
	I x T	6	1.441	0.0029
K_s	I	4	319.34	< 0.0001
	T	2	1.2	0.8358
	I x T	8	20.43	0.006
MC_s	I	4	2498.75	< 0.0001
	T	2	6791.08	< 0.0001
	I x T	8	381.42	0.1001

[1]df: degrees of freedom.

[2]Occluded stem lengths were transformed to natural logarithm values to ensure the data were normally distributed.

[3]Occluded sapwood areas were transformed to logit values to ensure the data were normally
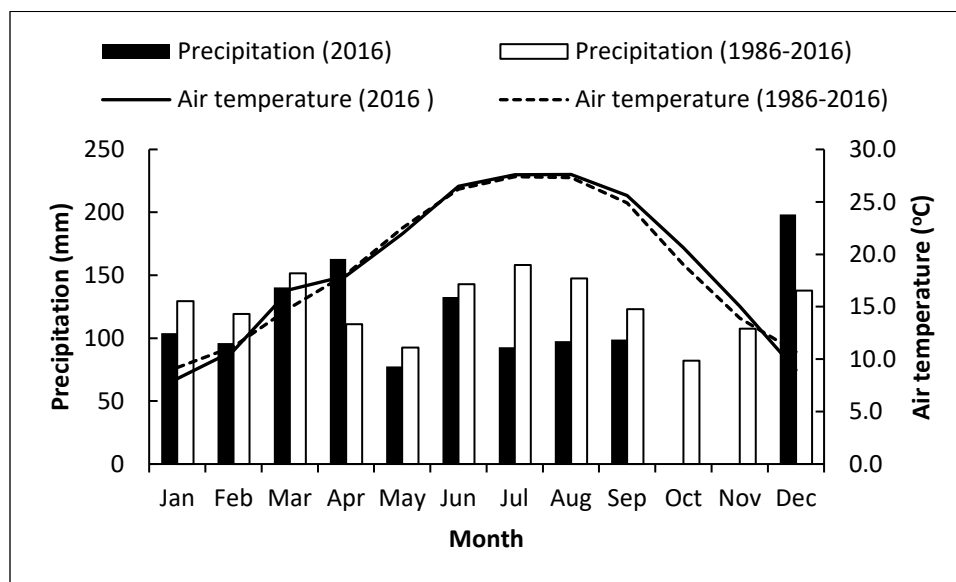
Figure 3.1. Monthly precipitation and average daily air temperatures in 2016 and during the 30-year period between 1986 and 2016 at the study site near Andalusia, AL (NOAA 2019). Stem inoculation was done on May 5, 2016 followed by post-inoculation assessments 8, 16, 20 and 24 weeks (June-October) afterward.
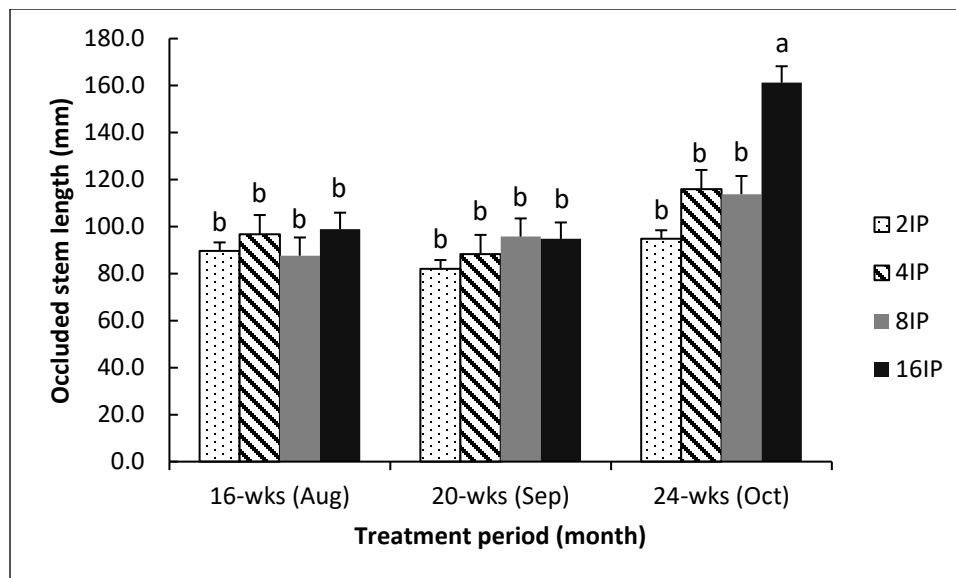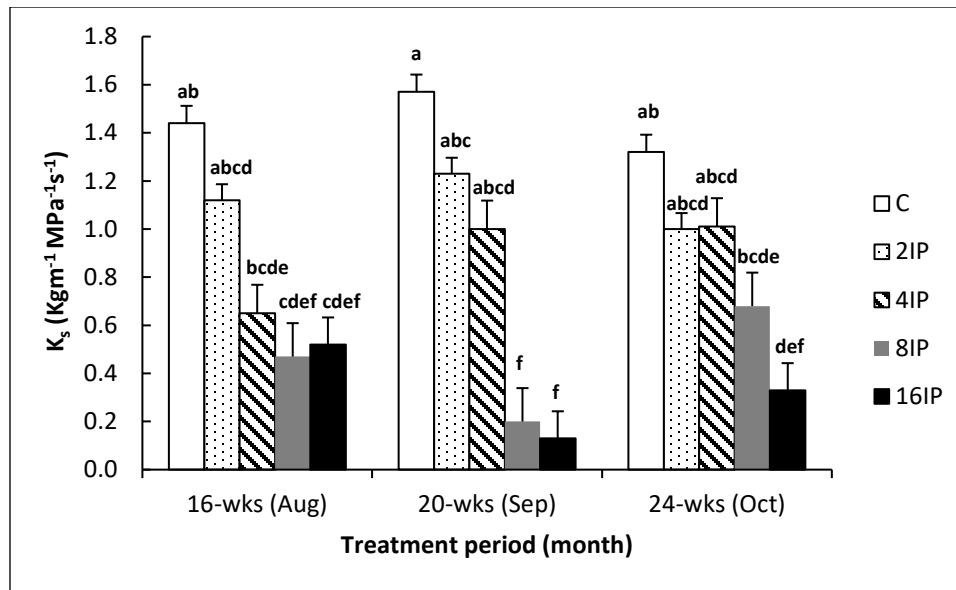
Figure 3.2. Occluded stem length after three treatment periods, 16, 20, and 24 weeks after inoculation with four *L. terebrantis* inoculation densities (2IP, 4IP, 8IP, 16IP). Means associated with different lower-case letters are significantly different at an α-level of 0.05 by the Tukey HSD test for multiple comparisons. Bars represent one standard error of the mean.
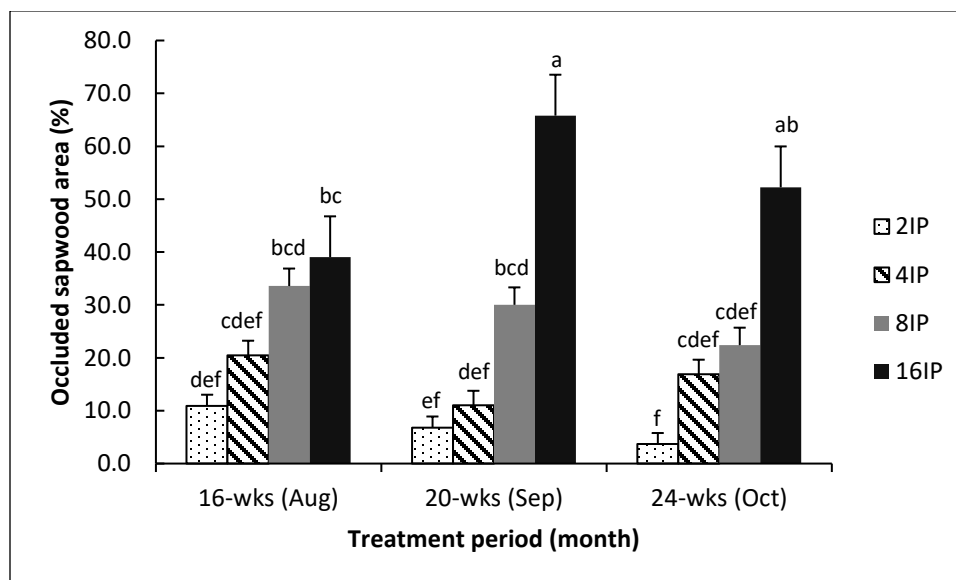

Figure 3.3. Percentage occluded sapwood area of stem segments after *L. terebrantis* inoculation at four densities (2IP, 4IP, 8IP, 16IP) and three treatment periods, 16, 20 and 24 weeks. Means associated with different lower-case letters are significantly different at an α-level of 0.05 by the Tukey HSD test for multiple comparisons. Bars represent one standard error of the mean.
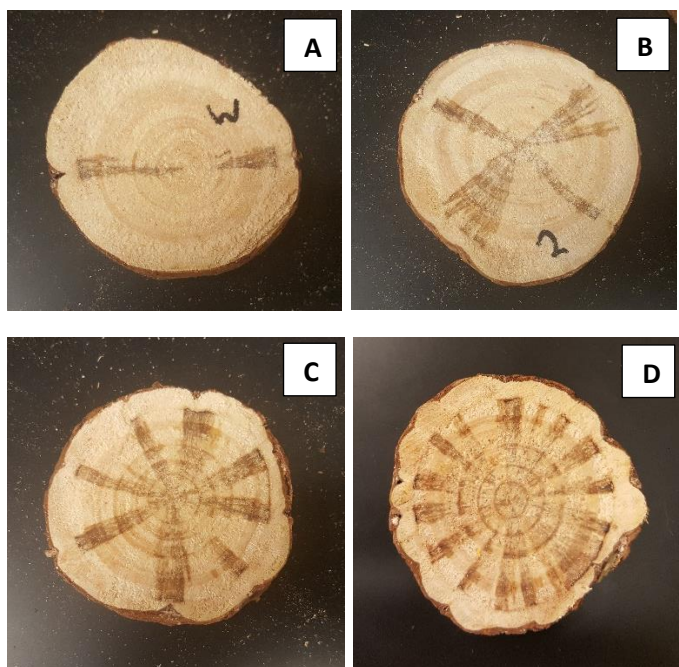
Figure 3.4. Cross-sections of stem segment discs:
A, B, C, and D received the 2IP, 4IP, 8IP, or 16IP
inoculation density, respectively. Deep brown color
indicates occluded sapwood due to artificial
inoculation with *L. terebrantis.* Note the
un-occluded sapwood area around the circumference
of the discs that grew in the 24-week period.

Figure 3.5. Native, specific hydraulic conductivity (K_s) of stems in response to four *L. terebrantis* inoculation densities (2IP, 4IP, 8IP, 16IP), or no inoculation (C). Data were collected at three treatment periods, 16, 20, and 24 weeks after artificial stem inoculation of *P. taeda* trees. Means associated with different lower-case letters are significantly different at an α-level of 0.05 by the Tukey HSD test for multiple comparisons. Bars represent one standard error of the mean.
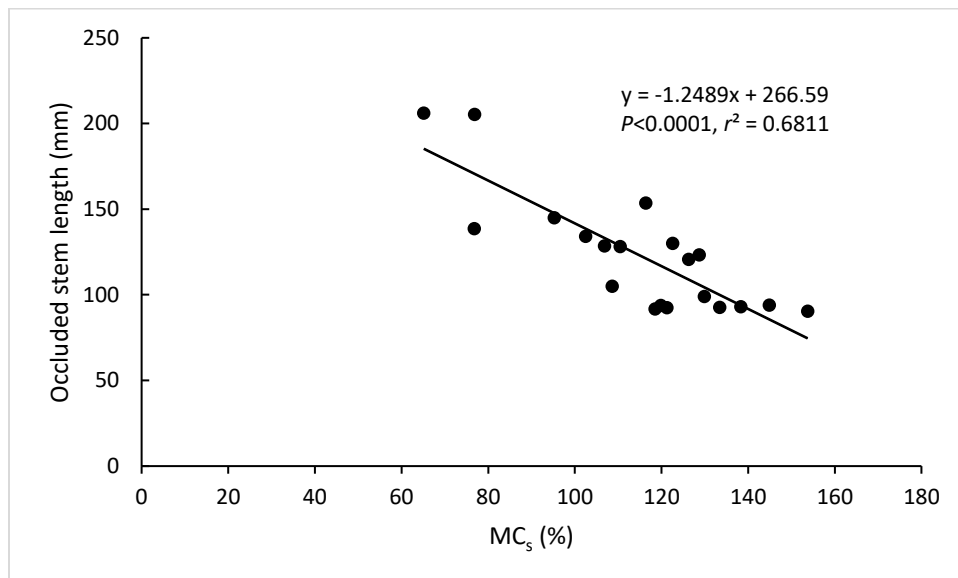
Figure 3.6. Linear relationship between occluded stem length and stem moisture content (MC_s) among *P. taeda* trees 24 weeks after inoculation with one of four densities of *L. terebrantis* (2IP, 4IP, 8IP, 16IP).

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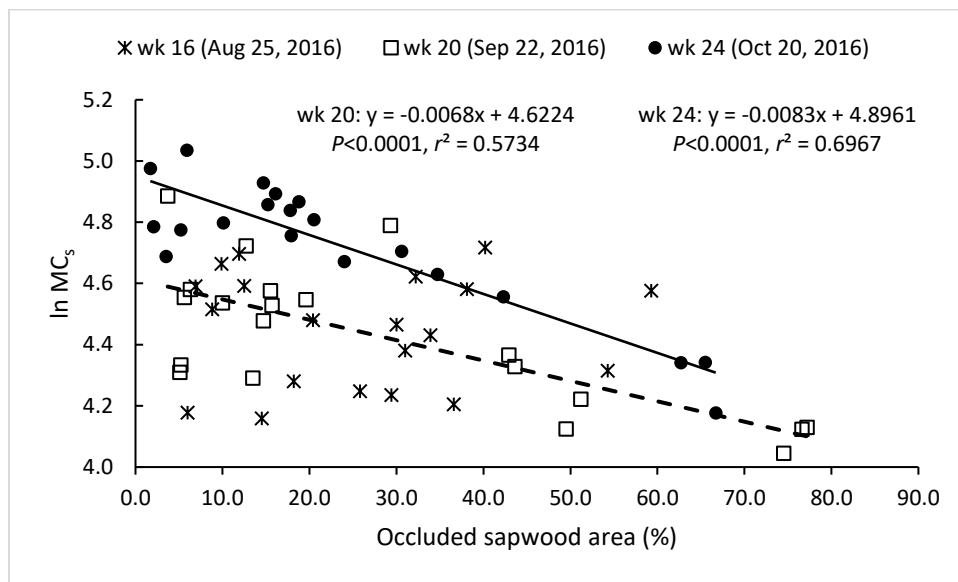
Figure 3.7. Relationships between the natural logarithm of stem moisture content (ln MC$_s$) and percentage of occluded sapwood area among *P. taeda* trees at 16, 20, or 24 weeks after artificial stem-inoculation with *L. terebrantis*.