Influence of *Leptographium terebrantis* S.J. Barras and T.J. Perry on *Pinus taeda* L. physiology, growth and productivity

by

John Kobina Mensah

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Approved by

Dr. Lori Eckhardt, Chair, Professor of Forestry and Wildlife Sciences
Dr. Mary A. Sword Sayer, Research Plant Physiologist
Dr. Emily Carter, Research Soil Scientist
Dr. Zhaofei Fan, Associate Professor of Forestry
Dr. Ryan Nadel, Assistant Research Professor

Abstract

Loblolly pine (*Pinus taeda* L.) is the predominant tree species in forest plantations across the southeastern U.S., but over the past several decades, cases of declining loblolly pine have been reported in localized areas in central parts of Alabama and Georgia. Bark beetle vectored fungus *Leptographium terebrantis* is frequently isolated from roots of the declining loblolly pine trees and has been implicated as one of the agents contributing to the decline and or mortality. Although the fungus has been shown to be pathogenic to seedlings, saplings and roots of mature loblolly pine trees, its ability to contribute to growth decline is unknown. This study examined the effect of *L. terebrantis* infection on loblolly pine growth and hypothesized that *L. terebrantis* infestation will induce sapwood occlusions, affect physiological functions, reduce leaf area and cause growth decline in loblolly pine trees. Using *L. terebrantis* colonized sterile toothpicks at differential inoculum densities; the sterilized toothpicks served as useful substrates for the production and transfer of *L. terebrantis* propagules into living tissues of loblolly pine. The pathogen compromised xylem function by causing phloem lesions, sapwood occlusions and significantly reduced specific hydraulic conductivity. These modifications, however, did not impose moisture stress on the foliage of young loblolly pine trees and the trees survived by producing new growth devoid of the pathogen infestation. In mature loblolly pine trees, the pathogen infestation affected moisture content and foliar mineral nutrition. Nitrogen concentration decreased below the sufficiency level and was most severe at high inoculum density relative to the control trees. In contrast, manganese concentration increased and was most elevated in the high inoculum treatment trees coupled with elevated calcium. *L. terebrantis*

infestation limited carbon fixation caused a reduction in leaf area and the ratio of leaf area to sapwood area. Collectively, the physiological and morphological changes coupled with moderate drought caused growth decline which was more pronounced at high inoculum density resulting in loblolly pine mortality. At lower inoculum densities the loblolly pine trees completely tolerated the pathogen by producing new sapwood to sustain the trees physiological processes. The results of the study demonstrate that *L. terebrantis* is not passively associated with declining loblolly pine trees but can negatively influence loblolly pine growth.

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Table of Contents

List of Tables

List of Figures

CHAPTER I

Introduction and Literature Review

1.1 Forest Estate of Southern United States

Forests and woodlands account for 36% (822.5 million acres) of the land area of the U.S. (Oswalt et al., 2019) distributed across 9 forest regions. Among the forest regions, the southern forests occupy the largest forest land area and constitute approximately 32% of the total forested area of the U.S. Moreover, the region has the largest planted timberland (71%) with Alabama possessing the highest timberland (33%) within the region and country (Oswalt et al., 2019). The region contains endemic pine species such as loblolly (*Pinus taeda* L.), longleaf (*Pinus palustris* Mill.), shortleaf (*Pinus echinata* Mill.), and slash (*Pinus elliottii* Engelm.). Hardwood species within the region include *Quercus* spp (oaks), *Carya* spp (hickory), *Fraxinus* spp (ash), *Acer* spp (maple), *Magnolia* spp (magnolia), *Populus* spp (poplar), *Juglans* spp (walnut) etc. Most of the forested areas are owned privately (58%), and 42% in public ownership. The federal, state and local governments control 31%, 9% and 2% respectively of the public forested land areas (Butler et al., 2016; Oswalt et al., 2019). As a result of the enormous forest resources, the region is commonly referred to as the "wood basket" of the country. The large forested area coupled with high productivity make southern forests a significant portion of the total U.S. carbon budget, accounting for 36% of the sequestered forest carbon (Turner et al., 1995).

Although the region constitutes about 2% of total global forest cover, it contributes significantly to wood supply on country and on global level. The region produces approximately

60% and 16% of the U.S. and world's wood resources, respectively (Prestemon and Abt 2002; Smith et al., 2009; Wear and Gries 2012). The region's role in the provision of timber resources is projected to increase for decades as a result of improved tree genetics, silvicultural interventions and competitive returns on investment in forest plantations (Fox et al., 2007; Jokela et al., 2010). In 2010, planted pine was estimated at 19%, or approximately 39.5 million acres, of southern forests, and is projected to increase to 24-36% by 2060 (Huggett et al., 2013). The forest estate also contributes to the socio-economic development of the region with over one million jobs and over $50 billion of labor income generated in 2011 (Brandeis and Hodges, 2015). Therefore, any factor that affects the growth and sustainability of the forest estate could affect the country's wood resources.

1.2 Loblolly pine (*Pinus taeda* L.)

Within the southeastern forest estate, loblolly pine is the most productive and extensively planted tree species (Schultz, 1997). Loblolly pine belongs to the family *Pinaceae*, with worldwide distribution, but native to northern temperate regions. The tree species grows on the Coastal Plain and Piedmont regions of the U.S, with its native range spanning 15 states from southern New Jersey south to central Florida and west to Texas. Within its native range, it constitutes more than half of the standing pine volume in the region (Schultz, 1997; Neale and Wheeler, 2004). It grows on a wide variety of soils but performs better on soils with poor to moderate surface drainage. Loblolly pine grows in pure stands and in mixtures with shortleaf, longleaf and slash pines, and hardwoods (Baker and Langdon, 1990; Schultz, 1997; Neale and Wheeler, 2004).

Historically, loblolly pine occupied approximately 2.2 million hectares prior to the arrival of Europeans (Schultz, 1997). However, after settlement most of the hardwood dominated forests

were converted to agricultural lands, particularly cotton. Cotton farms were negatively affected by the boll weevil (*Anthonomus grandis* Boheman), and farms abandoned which facilitated the spread of loblolly pine. Subsequently, loblolly pine became dominant and occupied about 13.4 million hectares of forest land in less than a century in the southern U.S. (1800-1900) (Schultz, 1997).

Loblolly pine constitutes about 80% of the commercial forest areas in the south (Smith et al., 2004). The dominance of loblolly pine in forest plantation is largely attributed to its fast growth and ability to thrive on different sites (Baker and Langdon, 1990). Each year, about one billion loblolly pine seedlings are planted (McNabb and Enebak, 2008) to sustain the economic and ecological contribution of the tree species. It serves as the cornerstone of the pulp and paper industry, and provides cost-effective feedstock for lignocellulosic ethanol production (Frederick et al., 2008). Ecologically, the tree species provides food and habitat for game and nongame wildlife species with immeasurable aesthetic and recreational values (Schultz, 1997). Loblolly pine production is considered as a promising tool to curb greenhouse gas levels through carbon sequestration (Gough and Seiler, 2004).

1.2.1 Biology and growth of loblolly pine

Loblolly pine is monoecious with both male and female flowers occurring on the same plant; they mature reproductively in about 12 years old. Flowering is initiated in July and August but the reproductive structures are not differentiated into recognizable structures until late September to October. Fertilization takes place in spring of the following year (Baker and Langdon, 1990) and by October of the 2nd year cones mature and seeds ripen, about 26 months after strobili are initiated (Baker and Langdon, 1990). Although loblolly pine is a prolific seed

producer, seed production is affected by tree genetic make-up, stand conditions, climatic factors or physiographic region.

Seeds produced go through a dormant period prior to germination, which lasts longer than any other southern pines. Once the dormant period has elapsed and germination is initiated, it grows rapidly and consistently throughout a stand (Baker and Langdon, 1990; Schultz, 1997). Growth is initiated in spring after bud break and continues to late October. By mid-August, leaf area peaks, terminal and lateral branch growth are complete, and the majority of new roots are established (Baker and Langdon 1990, Dougherty et al., 1994, Sword et al., 1996, Emhart et al. 2006). Natural senescence of foliage produced in the previous year occurs between September and November (Dougherty et al., 1995, Sampson et al., 2003).

In natural stands, growth rates differ and individual trees express early dominance when growing under the best microsite conditions (Baker and Langdon, 1990). Growth differentiation occurs at early stages on a good site but delayed on poor sites, separating trees into different crown classes. The most vigorous individuals become dominant as the stand ages, whilst the least vigorous form the intermediate crown class (Baker and Langdon, 1990). Loblolly pine forms a relatively short taproot and extensive shallow lateral roots which spread farther than crowns and have grafting capabilities in closely spaced plantations (Baker and Langdon, 1990). It is moderately tolerant to shade at early stages of growth but becomes intolerant with age and classified as shade intolerant (Schultz, 1997).

1.2.2 Factors affecting loblolly pine growth

Growth and productivity of loblolly pine are affected by several factors such as competition, genetics, biotic and environmental stressors e.g CO_2 concentration, air pollution,

temperature, moisture stress, and soil nutrients (Kramer, 1986; Gower et al., 1994). Competing vegetation adversely affects growth, particularly in natural forests (Baker and Langdon, 1990; Schultz, 1997). It has been estimated that competing vegetation accounts for an average loss of 25% and 14% of volume production in natural and plantation forests, respectively, across the southern region (Baker and Langdon, 1990). Borders and Bailey (2001) also found that controlling vegetation improved yield compared to annual fertilization.

Temperature and moisture stress are among crucial factors that affect loblolly pine growth (Teskey et al., 1987; Seiler and Johnson, 1988; Wertin et al., 2010; Maggard et al., 2016). Moisture stress affects trees physiology by limiting stomatal opening (Brodribb and Holbrook, 2003), CO_2 uptake, photosynthesis and carbon allocation (Dewar et al., 1994; Gower et al., 1994). Reduction in soil moisture affects net photosynthesis (Wertin et al., 2010), and among the dominant southern pines, loblolly pine has been shown to demand more water and nutrient (Baker & Langdon, 1990). Teskey and Will (1999) found a similar amount of biomass accumulation in loblolly pine seedlings grown at 25 and 30 ^{o}C, but a reduction in biomass at 35 ^{o}C. These factors affect vigor and predispose trees to attack by biotic agents.

Biotic agents such as insects and fungal pathogens affect loblolly pine growth (Connor and Wilkinson, 1983; Baker, 1972). The southern pine beetle (*Dendroctonus frontalis* Zimmermann) is the most destructive among the bark beetles and attacks stems of both stressed and healthy trees within its native range and other ornamental settings (Thatcher and Barry, 1982). Regeneration weevils such as *Hylobius pales* (Herbst) and *Pachylobius picivorus* (Germar) (Eckhardt et al., 2007) and beetle species such as *Hylastes salebrosus* Eichoff and *H. tenuis* Eichoff also feed on roots of stressed loblolly pine (Eckhardt, 2004, Matusick, et al., 2013). Loblolly pine suffers from attacks by pine tip moths (*Rhyacionia* spp.) during the initial

stages of growth (< 5 years) (Fettig et al., 2000). Fungal diseases including fusiform rust (*Cronartium quercuum* f. sp. *fusiforme),* root rot (*Heterobasidion irregulare* Fr.,) Armillaria root-rot (*Armillaria* spp.), heart rot (*Phellinus pini* Tho. Ex. Fr.) (Baker and Balmer, 1983b) and pitch canker (*Fusarium circinatum*) (Nirenberg and O'Donnell 1998) also affect loblolly pine growth.

1.3 Forest health and underlying processes

Forest health, as defined by Teale and Castello (2011), is the ability of the forest to meet a landowner's objectives and capable of sustaining itself with respect to its size and structure. Within a forest stand, tree growth and development is determined by its physiological and biochemical processes (Sharkey and Bernacchi, 2012; Smith and Dukes, 2013). These processes are influenced by resource availability and stand conditions (Kozlowski et al., 1991).

Photosynthesis, the major physiological and biochemical process takes place in the crown, the source of primary productivity and an indicator of tree health. Zhao et al. (2012) indicated that within the southern U.S., healthy loblolly pine trees have a crown ratio of ≥ 40%. Hence, trees with large dense crowns are considered as healthy and vigorous, whereas those with sparsely foliated crown have little or no growth (Zarnoch et al., 2004).

The size of the crown is often measured as leaf area index (LAI), the ratio of projected foliage area (m^2) to ground area (m^2) (Weiss et al., 2004). LAI regulates photosynthetically active radiation interception, transpiration, carbon uptake and growth efficiency (Landsberg and Gower, 1997). As a function of tree productivity, LAI is strongly associated with basal area growth (Jacobi et al., 1988; Gower et al., 1992; Vertessy et al., 1995) and volume increment (Hamilton, 1969). Albaugh et al. (1998) reported peak LAI of 0.63 and 1.2 for 8 and 11 yr old

loblolly pine plantation, respectively, growing on site with 30 yr average annual precipitation of 1210 mm. Samuelson et al. (2014) also reported peak LAI of 2.5 m^2 m^{-2} in September for loblolly pine plantation at age 8 on site with annual precipitation of 1109 mm.

The ability of the crown to perform its functions, and sustain tree health depends on resources such as CO_2, water, mineral nutrients and light (Kozlowski et al., 1991; Vose et al., 1988, Albaugh et al., 1998, Samuelson et al., 2014). For instance, fertilization has been shown to increase LAI from 2.5 to 3.4 m^2 m^{-2} (Samuelson et al., 2014), and trees with a large LAI demand more resources for sustainance. However, under scarce resources, growth supporting processes may be impaired and carbon sequestration and allocation patterns altered (Gower et al., 1992). This may compromise the tree health and predispose trees to insect and pathogen attack.

1.4 Factors affecting forest health

Forest health is affected by several factors including forest insects and pathogens, fire, air pollutants, water and mineral nutrients (Landsberg et al., 2017; Millar and Stephenson, 2015; Tkacz et al. 2008; Percy and Ferretti, 2004). Water and dissolved mineral nutrients are essential resources for tree growth, survival and productivity. Water absorption, transport, and transpiration are important physiological processes for tree function (Landsberg et al., 2017). Water absorbed by feeder roots is essential for crown carbon fixation, and any limitations may reduce root growth (Sword et al., 1998; 1996). Differences in soil water potential and tree tissues create a potential gradient which enables water movement (Whitehead and Jarvis, 1981; Landsberg et al., 2017). The flow of water through the stem is driven by rates of transpiration, stomatal conductance of the foliage and ratio of leaf area per stem area (Landsberg et al., 2017).

Water transport occurs largely in the outer sapwood and with time the inner sapwood loses part of its hydraulic function (Domec et al., 2002; Ford et al., 2004). For instance, in mature loblolly pine trees, Ford et al., (2004) noted that 50 to 60% of the total stem sapflow occurs in the outer 12% of the sapwood area. Philips et al., (1996) also noted a 59% reduction in sap flux in loblolly pine from outer to inner sapwood. Loss of hydraulic function is affected by several abiotic and biotic factors. Abiotic factors such as drought, freezing and high temperatures induce the formation of air bubbles in the xylem conduit, causing cavitation and loss of conductivity (Cochard et al., 1996). Biotic agents including vascular pathogens growing in the xylem interferes with hydraulic functions (Olivia et al., 2014). Fungal mycelia, oleoresin and other secondary metabolites clog xylem conduit causing tissue occlusions (Paine et al., 1997; Franceschi et al., 2005). Metabolites such as oxalic acid in occluded sapwood also contribute to embolization and desiccation of the sapwood (Coutts, 1977; DeAngelis et al., 1986).

Hydraulic dysfunction and water deficits cause stomatal closure, and affect the amount of water transpired (Flexas et al., 2006; Chaves et al., 2009). Moreover, stomatal closure affects carbon intake, allocation for growth and synthesis of secondary metabolites (Linder, 1987; Gower et al., 1992; Ericsson et al., 1996). Gholz et al. (1990) indicated that reduction in leaf area development is a major response of trees to water stress. Under water stress, the carbon allocation patterns shift in favor of below ground biomass and cause an increase in root to shoot ratio (Ericsson et al., 1996). Thus moisture stress imposed by vascular wilt pathogens may contribute to reduction in leaf area and primary productivity of infected trees.

Nutrient availability strongly influences biomass production and allocation in pine forests (Gower et al., 1994, Albaugh et al., 1998). Gower et al. (1994) noted that foliage production is greater in fertile soils than nutrient-poor soils, and biomass allocation to belowground is greater

in nutrient poor soil. Nonetheless, the direction of C shift is influenced by the specific mineral nutrients with N, P or S deficits related to an increase in root fraction while a shortage of K, Mg and Mn reduces root biomass (Ericsson and Kahr, 1995).

1.5 Forest Decline Concept

Forest decline is an episodic event characterized by premature, progressive loss of tree and stand vigor over a period of time with no obvious evidence of a single identifiable causal factor. (Manion and Lachance, 1992). It is a global issue that has eliminated millions of trees and continues to threaten the existence of forests worldwide. Decline causes economic and ecosystem losses and occurs at local, landscape, regional, national and continental levels across the globe (Woo, 2009; Adams, 2012). Several abiotic and biotic factors have contributed to forest stands experiencing decline (Contreras-Hermosilla, 2000). Among these factors are acid rain, air pollution, climatic factors, competition, nutrient deficiency, insects and pathogens (Houston, 1981; Smith, 1992). According to Manion (1991), declines are characterized by symptoms that include root necrosis, shortened internodes, yellowing and loss of foliage, dieback of twigs and branches, reduced growth, and increased prevalence of root decay.

In Africa, declines have been reported in atlas cedar (*Cedrus atlantica*) in Algeria (Bentouati, 2008); in quiver tree (*Aloidendron dichotomum)* in Namibia (Foden et al., 2007) and mountain acacia (*Brachystegia glaucescens*) in Zimbabwe (Tafangenyasha, 2001). Unlike other African countries where declines have been reported, forest decline is not prevalent in Ghana. However, in the mid-seventies, widespread die-back of Ofram (*Terminalia ivorensis*) occurred in the natural forest of Ghana and Ivory Coast (Ofosu-Asiedu and Cannon, 1976).

In North America, forest decline has eradicated thousands of hectares of forested lands including aspen (*Populus tremuloides*) decline in western U.S. and Canada (Worrall et al., 2013), oaks (*Quercus* spp.) in eastern North America (Bendixsen et al., 2015), whitebark pine (*Pinus albicaulis*) in western North America (Wong and Daniels, 2017), pinon pine (*Pinus edulis*) in southern U.S. (Gaylord et al., 2015) and loblolly pine (Brown and McDowell, 1968; Eckhardt et al., 2007). Arguably, the continent has suffered unprecedented damage of forest declines. Over 300000 ac of oaks forest land was negatively affected by decline in the Ozark Mountains of northern Arkansas and southern Missouri in 1999 and 2000 (Starkey et al., 2000; Heitzman, 2003). These declines were attributed to a number of interacting factors such as drought and outbreak of red oak borer (*Enaphalodes rufulus* Haldeman) which resulted in stand mortality and modification of the landscape.

1.5.1 Theories of Forest Decline

Several theories have been proposed to explain the forest decline phenomenon. These include the following: 1) the environmental stress and secondary organism theory (Houston, 1981; 1992), 2) chain reaction theory (CRT) (Sinclair and Hudler, 1988; Manion, 1991), 3) the climate change or climate perturbation theory (Auclair et al., 1992), 4) air pollution theory (Schutt and Cowling, 1985) and 5) ecological theory (Mueller-Dombois et al., 1983; Mueller-Dombois, 1992). These theories emphasize the fact that no single factor is responsible for a decline but several factors which differ among the theories.

According to the environmental stress and secondary organism theory, environmental stress such as drought adversely affects healthy trees and secondary organisms attack the weakened trees. Houston (1981) emphasized that if the stress factor is removed at the early stages of decline and the secondary organism is absent, the host recovers from the decline.

The CRT is a three-step factor theory composed of predisposing, inciting and contributing factors which act together to cause decline (Sinclair & Hudler, 1988). Manion (1991) modified the CRT into the decline spiral model (DSM). Within the DSM, the three factors identified by Sinclair and Hudler (1988) act in a specific sequential order to produce a gradual deterioration of stand health. According to DSM, the predisposing factors are long-term underlying factors that put permanent stress on trees such as genetic, climate, soil type or site conditions. The inciting factors are relatively short-term conditions which act by aggravating the stress imposed by the predisposing factors. They may be both physical and biological in nature such as drought and insect defoliation. The contributing factors are biological agents that further aggravate the deterioration of a predisposed tree and cause growth reduction (Jurskis, 2005; Manion, 1991).

The climate perturbation theory suggests that forest decline mechanisms are underpinned by climate change. Auclair et al., (1992) indicated that extreme freezing and or moisture fluctuations due to climate change can cause xylem cavitation injuries. The water stress associated with cavitation injuries cause diebacks and mortalities in affected forest stands. Schutt & Cowling (1985) noted that cumulative stress from air pollutants such as sulphur dioxide, nitrogen oxide, and ozone, and acid deposition account for the air pollution theory. Acid deposition often increases soil acidity and aluminum levels that are toxic to fine roots and mycorrhizae. The deposited acid also depletes soil of mineral nutrients such as nitrogen, sulphur, phosphorus, potassium, calcium, and magnesium which cause chlorotic foliage and affect forest health.

The ecological theory identified simplified forest structure, edaphically extreme sites, periodic recurring perturbations and biotic agents as the underlying causes of forest decline (Mueller-Dombois et al., 1983; Mueller-Dombois, 1992). According to the theory, simplification

of forest structure by few canopy species can degrade the soil through nutrient extraction and acidification. Moreover, extreme sites, for example, serpentine soils in Oregon may support dieback of susceptible species. Periodic perturbations such as drought also stresses forest stand and predispose them to biotic agents to hasten decline and mortality (Mueller-Dombois, 1992).

1.5.2 Southern Pine Decline

Southern pine decline has been previously referred to as pine decline, loblolly pine die-off, loblolly pine decline (Brown and McDowell, 1968; Eckhardt et al., 2010; Coyle et al., 2015), however, the term pine decline (PD) will be adopted herein after. Historically, the first case of PD was described in 1959 in Oakmulgee and Tuscaloosa Ranger Districts of Talladega National Forest in Alabama (Brown and McDowell, 1968). Subsequently, PD incidence has been reported in localized areas of southeastern U.S. from Alabama to South Carolina in the Atlantic and East Gulf Coastal Plains and Piedmont physiographic regions (Eckhardt et al., 2010).

Currently PD incidence has been reported in over 100 counties across the southeastern U.S. as indicated in Figure 1(FHC, 2017). Risk mapping analysis also indicated widespread occurrence of PD across the pine regions in the southeastern U.S. (Meyerpeter, 2012). It occurs on public lands under multiple management objectives (Hess et al., 2002; Menard, 2007) and nonindustrial private lands and forest industries (Eckhardt et al., 2010) where mature loblolly and shortleaf (*Pinus echinata* Mill) pine mistures are predominant. Pine decline is characterized by the formation of sparse and chlorotic crowns, reduced radial growth, short chlorotic needles, fine root deterioration, and isolation of root fungal pathogens (Eckhardt and Menard, 2008; Eckhardt et al., 2010).

The PD follows the DSM concept described by Manion (1991). Eckhardt et al., (2010) indicated that PD is caused by multiple stressors such as anthropogenic disturbances, site quality, climate variation, land use, insect and root pathogens, and mature or old stands. Various pathogens such as *Phytophthora cinnamomi*, *Heterobasidion annosum*, and *Leptographium* spp. have been implicated as contributing factors of PD. Nonetheless, root feeding bark beetles and their associated *Leptographium* spp. are the most dominant contributing agents associated with the declining pines (Brown and McDowell, 1968; Ostrosina et al., 1997; Eckhardt et al., 2007).

Contrary to the potential risk of the decline, other researchers have indicated a lack of discernible patterns of PD in the southeastern region (Coyle et al., 2015). They attributed the cause of PD to natural occurrences within affected forest stands. Again they noted that empirical data that gives quantitative description of the pine decline is nonexistent (Coyle et al., 2015). This suggests that research is needed to unravel the pattern of PD and establish the role of the contributing agents identified by Eckhardt et al., (2007) in the PD phenomenon.

1.6 Bark beetles and their fungal associates

Bark beetles (Curculionidae: Scolytinae) are among the most destructive pests worldwide (Wood, 1982). They attack woody plants, feed and construct galleries in the phloem under the bark and lay their eggs. The most common bark beetles in the southeastern U.S. pine ecosystem are the southern pine beetle (*Dendroctonus frontalis*) (SPB), black turpentine beetle (*D. terebrans*) and the three species of Ips (*Ips avulsus, I. calligraphus,* and *I. grandicollis*). Among the bark beetles, the SPB is the most destructive pest that attacks both healthy and weakened pine trees. It has been estimated that SPB has killed trees valued at $1.5 billion (Price et al., 1998) but neither SPB nor Ips species have been implicated in pine decline. Other bark beetles

Hylastes tenuis, *H. salebrosus* and weevil species (*Hylobius pales*, *Pachylobius picivorus*) have been isolated from declining loblolly pines (Eckhardt, 2004; Matusick, et al., 2013).

Characteristically, the bark beetles serve as vectors of fungi (Beaver et al., 1989; Klepzig and Six, 2004; Harrington, 2005). The beetles vector fungi either in mycangia or on the exoskeleton (Six, 2003) and facilitate host infection through wounding. The fungal associates of the beetles serve as food for larvae and adult beetles, and modify the substrate for their development (Six, 2003; Harrington, 2005). The bark beetles and their fungal associates form a complex system that contributes to pine disorders (Jacobs and Wingfield, 2001) and pine decline (Eckhardt, 2004; Matusick, et al., 2013).

1.6.1 Ophiostomatoid fungi

The ophiostomatoid fungi are members of the Ascomycota in diverse ecological niches and are commonly associated with bark beetles (Seifert et al., 2013). These fungi are mostly pathogens of conifers (Repe and Jurc, 2010). They are noted for blue or bluish-gray stains in the sapwood of conifer trees and logs (Yamaoka, 2017) and black-stain root disease of conifers (Witcosky et al., 1986). The fungi produce slimy masses of spores at the top of long conidiophores (Wingfield et al., 1993) which aid their dispersal by attaching to the surface of the bark beetles. Ophiostomatoid fungi are characterized by dark pigmented hyphae which cause blue stains of plant tissue (Jacobs and Wingfield, 2001). Although the blue stains do not affect the structural integrity of the wood, it degrades the quality and economic value of wood (Uzunovic et al., 1999).

Taxonomic classification of the fungi has gone through several changes (Yamaoka, 2017). Based on molecular phylogenetic analysis of the sequences of the nuclear large subunit

(LSU) and internal transcribed spacer (ITS) region of rDNA, De Beer and Wingfield (2013) reclassified ophiostomatoid fungi into two orders: Ophiostomatales and Microascaleles. The Ophiostomatales contain six genera: *Ophiostoma* s.l., *Ceratocystiopsis, Fragosphaeria, Graphilbum*, *Raffaelea* s.str., and *Leptographium* s.l. (including *Grosmannia*). The genera *Ophiostoma* and *Leptographium* are predominant with 134 and 94 identified and accepted species respectively (De Beer, et al., 2013b). The Microascales consists of six genera: *Ceratocystis, Graphium, Knoxdaviesia, Sphaeronaemella, Cornuvesica,* and *Custingophorah* monotypic.

Members of the genus *Leptographium* have been identified as economically important in several pine ecosystems (Harrington and Cobb, 1983; 1988; Hansen et al., 1997; Hess et al., 1999; Zhou et al., 2002). Some *Leptographium* species are saprophytes found in the soil or on decaying plant material, and some are tree pathogens (Harrington and Cobb 1988). In the southeastern U.S., species such as *L. terebrantis*, *L. procerum, L. serpens*, and *L. lundbergi* have been commonly associated with loblolly pine undergoing growth decline (Ostrosina et al., 1997; Hess et al., 1999; Eckhardt et al., 2004; Matusick et al., 2013). Among the several *Leptographium* spp, *L. terebrantis* has been shown to be pathogenic to loblolly pine (Devkota andEckhardt, 2018).

1.6.2 *Leptographium terebrantis* S.J. Barras and T.J. Perry

Leptographium terebrantis is an ophiostomatiod fungus which was first isolated from the black turpentine beetle, *Dendroctonus terebrans* (Olivier), in infected loblolly pine stands (Barras and Perry, 1971). It has also been isolated from weevil species such as *Hylobius pales* (Herbst), *Pachylobius picivorus* (Germar), root bark beetles: *Hylastes tenuis* (Eichoff), *H. salebrosus* (Eichoff) (Eckhardt, 2004; Matusick, et al., 2013). Within the southeast, the foremost

pine species that are infected are loblolly, longleaf, and slash (Otrosina et al., 2002, Eckhardt et al., 2007). The fungus is also associated with other pine ecosystems such as Scots pine (*Pinus. sylvestris* L.) (Highley andTattar, 1985), lodgepole pine (*P. contorta* Dougl) (Morrison & Hunt, 1988), red pine (*P. resinosa* Ait.) (Klepzig et al., 1991), and Douglas-fir (*Pseudotsuga menziesii*) (Harrington, 1988).

Leptographium terebrantis, as described by Jacobs and Wingfield (2001), has single or groups of up to four conidiophores with primary, secondary, tertiary and quaternary cylindrical branches. The stipe on which the branches are borne have cross-walls but the branches are aseptate. Additionally, no rhizoid-like structures connect the conidiophores to the substrate. The fungus produces conidia basipetally with a truncated base in a gelatinous sticky mass. The hyphae are hyaline with dark green color, aerial hyphae are seen occasionally, and the numerous conidiospores are formed on the entire colony (Barras and Perry, 1971). The cell wall is made up of chitin, glucan, and rhamnose and is tolerant of high concentrations of cycloheximide (Jacobs and Wingfield, 2001).

Other *Leptographium* species such as *Leptographium truncatum* and *L. procerum*, and *Grosmannia* species such as *G. alacris,* and *G. huntii* are associated with southern pine decline. Among the isolated species, *L. terebrantis* has been shown to be one of the most pathogenic fungi to loblolly pine seedlings and saplings (Matusick & Eckhardt, 2010; Singh et al., 2014, Matusick et al., 2016). , *L. terebrantis* has exhibited varying degree of pathogenicity depending on the host and the prevailing environmental conditions (Morrison and Hunt 1988). The fungus causes lesions in the phloem and resin-soaking of the xylem in seedlings and mature trees of several conifers (Harrington and Cobb, 1983; Wingfield, 1983; Rane and Tattar, 1987).

1.7 Fungal infection and colonization of host plant tissues

Pine trees under stress emit volatile organic compounds (monoterpenes) which attract bark beetles (Kelsey et al., 2014). Additionally, warm temperatures associated with drought cause a rise in beetle population (Bentz et al., 2010; Creeden et al., 2014). Bark beetles feed on phloem tissues of large roots and the lower portion of the stem and create wounds that cause phloem girdling. The wounds serve as the inoculation points for fungal propagules vectored by the beetles, and under favorable conditions, the spores germinate and initiate infection. The emerging hyphae penetrate and proliferate in the xylem tissues, induce secondary metabolites production and cause tissue occlusion.

Vascular occlusions have been shown to be common structural modifications made by many plant species in response to pathogen infection. The occlusions affect water storage and conductivity of the affected tissues (Butnor et al., 1999; Guérard et al., 2000; Sallé et al., 2008; Mensah et al., 2020*).* Butnor et al., (1999) showed that diseased trees of eastern white pine (*Pinus strobus* L) exhibited vascular occlusions, had lower sapwood moisture contents, and reduced hydraulic conductivity. Additionally, production of toxins into the xylem stream reduced moisture content (Coutts, 1977; DeAngelis et al., 1986), which negatively affects physiological functions for growth.

Following infection and colonization by ophiostomatoid fungi, cellular functions are altered as the fungi feed on the content of xylem sap and sugars from the cell-wall (Yadeta and Thomma, 2013). This has immediate effects on trees physiological processes such as foliage wilting (Hubbard et al., 2013; Olivia et al., 2014) and sudden mortality (Yamaoka et al., 1995) of the corresponding infected parts or the entire tree. Studies conducted by Hubbard et al., (2013) on *Pinus contorta* found a decline in transpiration within a few days after beetle infestation. The

pre-dawn water potential dropped significantly as water transport to the canopy declined by 60% relative to healthy trees; this was attributed to the fungus associated with the beetle. The disruption of tree water balance through vascular plugging with resin has been hypothesized to cause tree death (Reed et al., 2014).

1.8 Defensive barriers to fungal infection

Loblolly pine has well developed physical and chemical defenses against invasion by insect pests and pathogens (Franceschi et al., 2005). Physical barriers imposed by the bark act in concert with chemical compounds to prevent attack by pathogens as most pathogens require wounds to invade their host. The physical barriers in pines consist of thick outer bark composed of dead cells, waxes and thick leaf epidermal cell walls, as well as lignified and suberized cells throughout the tree (Franceschi et al., 2005). Beneath the bark are schlerenchyma layers which are composed of massive, irregularly-shaped stone cells (Wainhouse et al., 1997; Franceschi et al., 2005). These barriers limit pathogen penetration by physical repulsion of the invaders.

The chemical compounds are secondary metabolites which are made up of anti-herbivory and toxic compounds, such as calcium oxalate crystals (Franceschi, 2001; Franceschi and Nakata, 2005), tannins (Kraus et al., 2003), phenolics (Krekling et al., 2004), and terpenes (Franceschi et al., 2005). These compounds are produced by specialized cells to inhibit or kill invaders that overcome the physical barriers. Chemical compounds are activated upon disruption of the physical barriers by beetles and other pests and which cause secretory cells to produce high volumes of terpenoid compounds to accumulate and plug the wound (Franceschi et al., 2005). Subsequently, the volatile components of the exudates evaporate, causing the resin diterpenes to crystallize and completely seal the wound (Langenheim, 1994; Keeling and Bohlmann, 2006). Examples of induced defenses include oleoresin flow in a loblolly pine wound

(Ruel et al., 1998), a hypersensitive reaction which causes the death of affected tissues and delayed resistance involving long-term complex changes due to cell division and differentiation (Cook and Hain, 1985).

1.9 Mechanisms of fungi induced mortality

The function of pathogenic fungi in tree mortality can be summed up based on two hypotheses collectively referred to as the classical paradigm (Six and Wingfield, 2011). The first hypothesis states that the virulent fungi colonize xylem tissues and block water conduction (Paine et al., 1997) with mycelia and secondary metabolites. Secondly, fungi play a critical role in overcoming tree defenses that cause mortality by stimulating induced chemical production in the phloem and the chemical become depleted (Lieutier et al., 2009).

Underneath these hypotheses are the underlying mechanisms of tree mortality: hydraulic dysfunction and carbon starvation as a result of drought (Sala et al., 2010; McDowell, 2011; Reed et al., 2014; Oliva et al., 2014; Sevanto et al., 2014). Olivia et al., (2014) noted that mortality in trees infected by vascular wilt pathogens may be triggered by hydraulic failure. Carbon starvation triggered by water deficit or hydraulic dysfunction can cause tree mortality (Oliva et al., 2014; Sevanto et al., 2014).

Studies involving artificial inoculations with blue-stain fungi or that measured transpiration response following beetle infestation suggest that the fungal infection kills trees relatively quickly (Yamaoka et al., 1995; Hubbard et al., 2013). Hubbard et al., (2013) noted that blue stain fungi rather than phloem-feeding bark beetle appear to be the primary cause of mortality in lodgepole pine (*Pinus contorta* Dougl). Wingfield (1986) reported mortality in *Pinus strobus* seedlings following artificial inoculations with *L. terebrantis*. The fungus ability to cause

mortality in mature trees has not been established while others have attributed mortalities in pine stands to bark beetles (Six, 2003; Six and Wingfield, 2011).

1.10 Problem statement

Pests and diseases pose a threat to forest growth and productivity worldwide (Wingfield et al., 2015; Freer-Smith et al., 2017) with significant economic consequences. In the U.S., approximately $7 billion worth of forest products are lost due to plant pathogens each year (Hall and Moody, 1994; USBC, 2001). According to Pimentel et al., (2005), alien-invasive plant pathogens cause about $2.1 billion loss in forest products annually.

Within the southeastern U.S., PD has been identified as a problem of southern pines such as loblolly, shortleaf and longleaf. Pine decline has been reported in localized areas in several counties in Alabama and it is commonly associated with mature pines especially loblolly pine trees (Brown and McDowell, 1968; Eckhardt, 2004). *L. terebrantis* is frequently isolated from roots of declining loblolly pine trees and studies have established that it is pathogenic to loblolly pine seedlings and saplings (Nevill et al., 1995; Matusick and Eckhardt, 2010; Singh et al., 2014; Matusick et al., 2016). However, its impact on mature tree growth is not fully established whether following infection the pathogen will continue to invade the host or the host tolerates the pathogen. Again the potential of *L. terebrantis* to initiate and cause growth decline and/or mortality in mature loblolly pines trees is unknown. Therefore, it is important to understand the loblolly pine and *L. terebrantis* pathosystem, and the effect of the interactions on growth and productivity of loblolly pine.

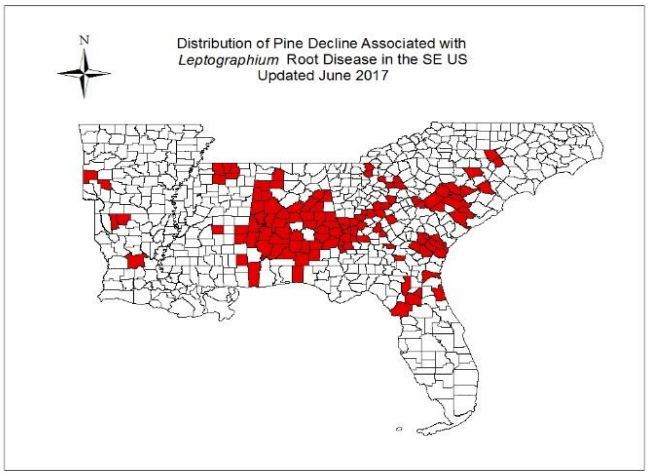


Figure 1. 1: Distribution of pine decline associated with *Leptographium* root disease in the southeastern U.S.

1.11 Inoculation Approach

The relationship between insect-vectored fungi and their host remains poorly understood (Six and Wingfield, 2011) but artificial inoculations are commonly used to elicit host response to pathogen invasion. To simulate natural inoculation of ophiostomatoid fungi by bark beetles, different inoculation approaches have been adopted (Harrington and Cobb, 1983; Wingfield, 1986). Common inoculation methods have involved cork-borer techniques to remove a circular disc of bark tissue in single or multiple points inoculations (Klepzig et al., 2005; Lee et al., 2006) and removal of bark flap (Strobel and Sugawara, 1986) to insert agar plugs with active fungal mycelia. Substrates such as wooden blocks and toothpicks (Wingfield, 1986; Nevill et al., 1995) are used to culture fungus in inoculation studies. However, other inoculation methods such as spore suspensions in wound inoculations (Otrosina et al., 2005), root dips in fungal mycelium (Hessburg and Hansen, 2000) are not used frequently.

The choice of an inoculation method depends on the developmental stage of the host: seedling, sapling or mature trees, and the organs involved: root, stem, and foliage. Although ophiostomatoid fungi are associated with mature trees, seedlings and saplings are commonly used in pathogenicity studies to partly exert control over environmental conditions. Conifers are known to have well-developed resin duct systems through which oleoresins are mobilized to sites attacked by beetles and fungi. The development of the duct system depends on age and growth rate and is less developed in young trees or saplings (Nebeker et al., 1993).

Bark beetles attack roots and lower stem segments of stressed trees. In mimicking attacks by beetles, inoculations are conducted on roots and stems. Stem inoculations are often used as a surrogate because of difficulties associated with mature tree root inoculations (Nagy et al., 2006; Matusick et al., 2016). Inoculation of mature trees, under natural conditions, has been predicted to be more useful for investigating disease development and progression since mature trees can react differently than seedlings to infection (Johansson et al., 2004). In this dissertation, toothpicks colonized with *L. terebrantis* mycelia and spores were used as an inoculant to elicit response in mature loblolly pine trees.

1.12 Lesion and occlusion measurement

Fungal infection, colonization, and growth following inoculation can be assessed by observing symptoms and signs expressed by the host. The host response to the invading pathogen includes the formation of localized dark resinous lesions (Matusick et al., 2016); necrosis of inner bark, chlorotic needles, reduced water potential (Rane and Tatter, 1987); sapwood occlusion (Butnor et al., 1999; Matusick et al., 2016); changes in monoterpene and carbohydrates composition (Cook and Hain, 1985) and mortality (Harrington and Cobb, 1983; Wingfield, 1983).

The fungi cause necrotic lesions which extend radially and vertically from the point of inoculation. Post inoculation assessment has emphasized vertical lesion length (Cook and Hain 1985; Paine et al., 1988) with little emphasis on the relationship between vertical lesion length and depth (Parmeter et al., 1992). The choice of the vertical lesion as a means of assessing pathogenicity has been attributed to limited sapwood depth particularly in seedlings. The sapwood occlusion is commonly assessed by dipping the infected tissue in a dye solution and measuring the area which the dye could not permeate (Parmeter et al., 1992).

1.13 Summary

The root pathogen *Leptographium terebrantis* isolated from roots of loblolly pines exhibiting symptoms of decline in localized areas of southeastern U.S. has been shown to be pathogenic to loblolly pine seedlings, saplings and mature roots. Infections cause lesions and occlusions, and interfere with host hydraulic functions. However, the potential of the pathogen to cause pine decline symptoms still remains uncertain, regardless of its frequent association with declining loblolly pine. Considering the economic significance of loblolly pine to the southeastern states economies, it is important to understand how the root pathogen interacts with the host to influence its growth and productivity. This would enable improved management practices in affected forest stands to minimize economic losses associated with loblolly pine decline.

CHAPTER II

Pinus taeda saplings response to *Leptographium terebrantis* differential inoculum density

2.1 Abstract

Bark beetle-vectored ophiostomatoid fungi, *Leptographium terebrantis*, is inoculated on the roots and lower stems of stressed *Pinus* species during the feeding activity of bark beetle. To determine the exact host response following inoculation, it is critical to challenge the host with a realistic amount of fungal inoculum. We examined loblolly pine saplings response to *L.terebrantis* colonized sterile toothpicks and identify potential fungal inoculum densities for further studies in mature trees. The toothpicks served as a substrate for fungal growth and sporulation and the inoculation showed their utility in eliciting host's response to the pathogen. The inoculated fungus caused sapwood occlusions in *P. taeda* saplings. The volume of occluded, visually damaged sapwood increased by 1.96 cm^3 per radial inoculation point. This indicates that *L. terebrantis* colonized sterilized toothpicks can be used to elicit the host response in simulating inoculation of beetle vectored fungi.

2.2 Introduction

Ophiostomatoid fungi are an ecologically and economically important group of fungi worldwide (Seifert et al., 2013). These fungi are associated with bark beetles that carry their spores on an outer cuticular surface or in specialized structures called mycangia (Harrington, 2005; Six, 2003). Bark beetles are required for the dissemination of fungal spores from tree to tree. Host wounding, caused by the bark beetles during host feeding and boring, creates the necessary gateway for fungal entry into the host vascular tissue. Introduced fungi can grow rapidly in the xylem tissue, disrupting water transport, exploiting host resources and weakening host defenses, which can lead to host mortality under certain circumstances (Horntvedt et al., 1983; Jacobs and Wingfield, 2001).

Host–pathogen interactions are commonly explored by introducing fungal mycelia or spores in the healthy host tissue under controlled (laboratory) or semi-controlled (glasshouse or field-grown trees) conditions. A variety of methods and protocols have been used. The most commonly used techniques to explore ophiostomatoid fungi and their host interaction include creating a wound using a cork borer in field-grown trees (Solheim et al., 1993; Yamaoka et al., 2000; Lee et al., 2006) or with a sterile razor blade to create a bark flap in seedlings to insert a plug of agar containing mature fungal mycelia (Devkota et al., 2018b).

These artificial inoculation techniques have consistently been used to successfully infect host tissue and to determine the resulting tissue necrosis and occlusion in the host (Parmeter et al., 1989; Kuroda, 2005; Devkota et al., 2018a; Devkota et al., 2018b). Wounds caused by both the cork borer and bark flap methods may, however, contribute to local tissue necrosis and occlusion of vascular tissue (Yamaoka et al., 2000; Matusick and Eckhardt, 2010). Additionally, the common wounding methods are inadequate for examining the effects of mass inoculation of

fungi, which occurs when several beetles attack and inoculate the host with the associated ophiostomatoid fungi.

In addition to causing localized wounds that are more extensive than what occurs naturally, the commonly used inoculation methods introduce unrealistically large quantities of actively growing fungal mycelium into the host tissue. For example, in artificial inoculation experiments involving *Leptographium* spp., mycelial plugs of 5–12 mm diameter are commonly used to inoculate the host tree (Yamaoka et al., 2000; Fäldt et al., 2006; Matusick et al., 2016). The beetle vector, *Dendroctonus frontalis* Zimmermann (southern pine beetle), of *L. terebrantis* is, however, about 2–4 mm long and 1–1.5 mm wide (Thatcher, 1981). Due to practical concerns, including the experimental length (commonly 8 weeks), these inoculation methods have been developed to ensure timely results. Inoculation methods may, however, greatly contrast with the natural inoculation process. Hence, there is a need for a alternative inoculation approach that can more closely mimic pathogen introduction in the host during bark beetle attack.

Host damage resulting from the beetle–fungi complex is a function of fungal virulence, inoculation density (the number of the fungal inoculation points per unit of bark) and inoculum load (Horntvedt et al., 1983; Solheim, 1992), among other factors. The attack density of the bark beetles above which tree mortality may occur is expressed as a critical attack threshold (number of attacks per m^2 of bark) (Lieutier et al., 2009). For example, the threshold is 50–120 for *Dendroctonus ponderosae* Hopkins on *P. contorta* Dougl, (Raffa and Berryman, 1983; Raffa, 2001) and 850 for *Ips acuminatus* Gyllenhal on *P. sylvestris L.* (Guérard et al., 2000). Critical thresholds of inoculation density have been determined as 300–400 for *L. wingfieldii*, and 400–

800 for *Ophiostoma minus* (Hedgcock) H. & P. Sydow on *P. sylvestris* L. (Långström et al., 1993; Solheim et al., 1993).

In these experiments, however, the size of the inoculation wounds was many times larger than the width of the beetle vector, likely causing unnaturally high levels of primary tissue damage as a result of wounding. Moreover, these experiments are often limited to inoculation per surface area of host stem and generally do not consider the frequency of inoculation points at the transverse cross-section, which is important since the ophiostomatoid fungi spread radially in ray parenchyma tissues (Ballard et al., 1982; 1984) and cause vascular tissue occlusion (Oliva et al., 2014).

Thus, consideration of inoculation at different radial densities may be important for examining the response of conifers to bark beetle-vectored ophiostomatoid fungal inoculation. Inoculations using fungal cultured toothpicks have been used in the past, including pathogenicity test of *L. terebrantis* in *P. strobus* L. seedlings (Wingfield, 1986). The toothpicks may serve as a reliable means for inoculum transfer as the width of the beetle (1–1.5 mm) is comparable to that of the toothpick (1–2 mm). Toothpick or point inoculations may be a useful method for understanding the response of *Pinus* species to multiple or mass fungal inoculations. Despite numerous studies that have been conducted involving the inoculation of ophiostomatoid fungi in conifers (Yamaoka et al., 2000; Fäldt et al., 2006; Davydenko et al., 2017), the intricate relationship between these fungi and their host may not have been adequately understood due to the lack of an efficient inoculation technique which can simulate the natural fungal inoculation by bark beetles (Guérard et al., 2000).

To advance the adoption of artificial inoculation method that closely mimics natural inoculation by bark beetles, we utilized *L. terebrantis* colonized toothpicks to determine the

pathogenicity of the pathogen on *P. taeda* saplings and identify potential inoculum densities for further studies in mature trees.

2.3 Materials and methods

2.3.1 Fungal isolate culturing

The *L. terebrantis* isolate (ATCC accession no. MYA-3316) (Figure 1) used for the study was isolated from the roots of *P. taeda* undergoing growth decline in Talladega National Forest, Oakmulgee Ranger District, AL, USA (Eckhardt et al., 2007). The fungal isolate utilized in the study was highly pathogenic to *P. taeda* host as compared to 41 other *L. terebrantis* isolates (Devkota and Eckhardt, 2018) isolated by Eckhardt et al., (2007).

L. terebrantis isolate cultured on presoaked toothpicks in Malt extract broth (MEB) were used for inoculation. Malt extract agar (MEA) plates (100 × 15 mm) were prepared, and the half segment of the media was removed from the plate to allow one end of the toothpick free from agar contact. Fifteen sterilized toothpicks were horizontally arranged equidistant in the MEA plates. Four mycelia agar plugs of 5-mm-diameter discs of actively growing *L. terebrantis* isolate were placed adjacent to the end of the toothpicks in each MEA plate. These petri plates were incubated at 23°C for 24 days in the dark so as to allow complete sporulation of the fungi onto the toothpicks.

2.3.2 *Pinus taeda* sapling stem inoculation

To determine whether the toothpick point inoculations can effectively be transferred to living tissue, an experimental plot was established in naturally regenerating *P. taeda* stand (approximately 7 years old) with mean annual precipitation of 1523.74 mm near

Andalusia, Alabama (31.1427° N, 86.6963° W). In May 2016, a total of 108 healthy *P. taeda* saplings were selected and then distributed across three replicate groups. The average groundline diameter of *P. taeda* saplings was 6.4 (±1.3) cm.

To quantify the damage caused by varying densities of inoculations, four inoculum densities were determined, including two, four, eight and sixteen inoculation points (IP) in the cross-section referred to as inoculum density (Figures 2 and 3).The points were radially equidistant from each other. Each inoculation point was repeated four times vertically and evenly spaced at 1.2 cm apart, as it was the best distance between inoculation points determined from an initial stem segment inoculation experiment. Each inoculum load was treated to stems of six randomly chosen trees within each replicate at the height of 15 cm above the ground level.

To determine the tissue damage caused by the inoculation method and toothpick alone, three trees per treatment within each replicate were inoculated with sterile toothpicks to serve as controls. In order to apply the treatments consistently, clear transparent sheets with an overlaid grid were designed and used for each inoculation treatment. Each template was wrapped around and fixed to the stem of sample trees. A drill bit of 1.5-mm-diameter was used to drill an approximately 5-mm-deep hole to reach to the phloem of the stem segments. The free end of a toothpick with sporulating fungus was inoculated in each drilled hole and the protruding ends of the toothpicks were clipped. The inoculation zone was sealed with duct tape to prevent contamination due to external contaminants.

2.3.3 Post Inoculation assessment

Eight weeks after inoculation, the trees were cut at ground level and 15 cm above the inoculation zone and transported to the laboratory on ice. To determine the presence of infection, the percentage of the radial area of tissue occlusion, the length and volume of occluded sapwood

tissue, and the degree of the infection across various levels of inoculation density were assessed. The occlusion length was determined by scraping the bark of the stem segment and cutting both ends of the stem until occluded tissue was identified.

Each treated stem segment was sectioned into four small segments using a band saw. The area of the fungal occlusion of each cross-section was traced onto a transparent sheet and the area was measured using a Lasico® Planimeter (Lasico®, Los Angeles, CA, USA). The total volume of the occluded tissue per sapling segment was determined from the occluded area and length.

2.3.4 Re-isolation

From each tree a 5 mm section of the stem tissue around the inoculation point was plated on selective media (MEA containing 800 mg L^{-1} of cycloheximide and 200 mg L^{-1} of streptomycin sulphate) to confirm the re-isolation of the inoculated fungus from the host tissue and then incubated at 23°C. After 14 days, fungal cultures resulting from plating were morphologically identified and re-isolation of *L. terebrantis* was recorded.

2.3.5 Data analysis

Data were analyzed using a general linear model (GLM) in SAS statistical software (SAS Institute, 9.4 versions, Cary, NC). The control treatment was excluded from the analysis due to absence or negligible occlusions in the host. Data were first checked for normality and equal variance using Shapiro-Wilk and Levene test respectively. Data for occlusion length and volume from the saplings were log transformed prior to the analysis. Pair-wise comparisons were undertaken using the Post Hoc Tukey's test on the four fungal treatments at $\alpha = 0.05$.

2.4 Results

The inoculation with fungal cultured toothpicks yielded significant infection in the living saplings. The sterilized toothpicks (control) failed to cause infection, and the amount of the tissue damaged from the control treatment was negligible. Externally, the fungal inoculated points were coated with resins, suggesting an active host response. Upon transverse sectioning of the inoculated tree segments into circular discs, dark brown occluded tissues were observed around inoculation points (Figures 2.2 and 2.4). Notwithstanding the infection of *P. taeda* saplings by *L. terebrantis* and subsequent production of occluded tissues, no symptoms of dieback or mortality were observed in the sapling crowns during the study period.

Pathogenicity was established by detecting both the presence of occluded tissues and successful re-isolation from inoculated stems. Percentage of tissue occlusion area, occlusion length and volume increased with increasing radial inoculum density (Table 2.1 and Figure 2.2). Occluded area was significantly different ($F_{(3, 68)} = 22.84$, $p = <0.0001$) among treatments (Table 2.1). The highest and lowest inoculum densities caused occlusions of 45.6% and 9.0%, respectively. Comparatively, the treatment with 16 inoculation points (IP) caused 14.1% more tissue occlusion than treatment 8IP (t-value= 2.99, $p = 0.0039$), whereas treatment 4IP caused 9.7% more tissue occlusion than treatment 2IP (t-value= 2.06, $p = 0.0431$) (Figure 2.4). Differences in occlusion length were significant ($F_{(3, 68)} = 11.27$, $p = <0.0001$), and the trend observed was similar to that of percentage tissue occlusion among the treatments. The 16IP treatment recorded 27.92 mm more occlusion length than treatment 8IP (t-value= 5.80, $p = 0.0060$), whereas treatment 4IP recorded 25.33 mm higher than treatment 2IP (t-value = 2.57, $p = 0.0122$). The maximum mean occlusion length observed is 144.4 mm (Table 2.1) relative to average sapling height of 5 m.

The volume of occluded tissue was significantly different between inoculation treatments ($F_{(3, 68)}$ = 36.43, p = <0.0001). Treatment 16IP recorded 53.9 cm^3 more volume occlusion than treatment 8IP (t-value= 6.28, p = <0.0001), whereas treatment 4IP recorded 17.4 cm^3 higher occlusion volume than treatment 2IP (t-value= 2.01, p = 0.0463). Length, area and volume of occluded tissue positively correlated with inoculum density (ID) (Table 2.2). The inoculum density correlated best with the volume of the occluded tissue, but accounted for 61% of the variation observed, followed by percentage tissue occlusion area as indicated in table 2.2. The occlusion length showed a weak association with the inoculum density and accounted only for 32% of the variation (Table 2.2).

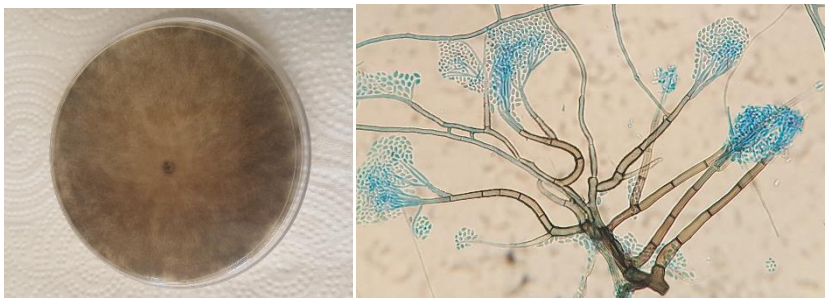


Figure 2. 1: *L. terebrantis* (a) Pure culture on MEA and
(b) Conidiophore bearing conidia cultured on MEA

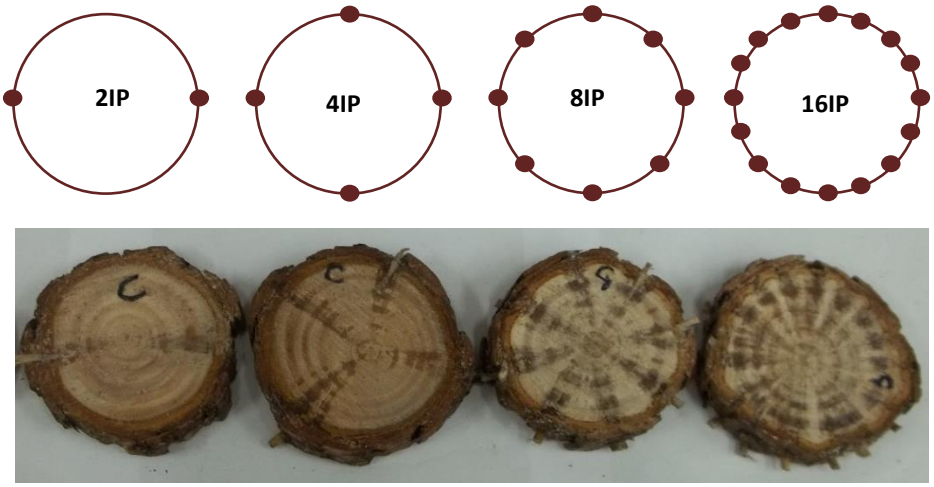


Figure 2. 2: Radial position of inoculation points of four different inoculum densities of *Leptographium terebrantis* in young *Pinus taeda* trees followed by tissue occlusions caused by those inoculum densities (2, 4, 8, and 16 inoculation points from left to right) in the bottom (Note: 2IP: two inoculation points, 4IP: four inoculation points, 8IP: eight inoculation points, and 16IP: sixteen inoculation points).

Figure 2. 3: *Pinus taeda* sapling inoculated at 16 radial points x 4 vertical points with sterilized toothpicks (control).

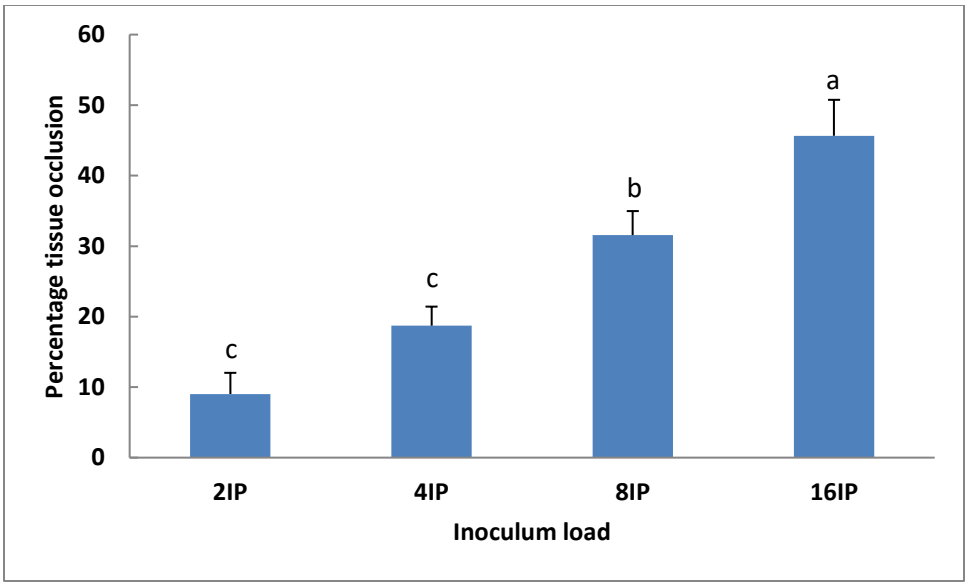


Figure 2. 4: Mean tissue occlusion caused by *Leptographium terebrantis* at different inoculum loads in filed trees. Different letters indicate significant differences in percentage of tissue occlusion caused by different inoculation points at $\alpha = 0.05$. Error bars represent 95 % confidence intervals.
(Note: 2IP: 2 inoculation points, 4IP: four inoculation points, 8IP: eight inoculation points, 16IP: sixteen inoculation points).

Table 2. 1: Mean and standard errors of occlusion length and volume associated with different inoculation points.

Treatment	Sample size	Occlusion length ± SE (mm)	Occlusion volume ± SE (cm^3)
2IP	72	87.30 ± 5.03a	12.47 ± 1.31a
4IP	72	112.63 ± 7.87ab	29.91 ± 3.19b
8IP	72	116.44 ± 6.64b	52.20 ± 5.11c
16IP	72	144.36 ± 7.88c	106.20 ± 10.43d

(Note: 2IP: Two inoculation points, 4IP: Four inoculation points, 8IP: Eight inoculation points, and 16IP: Sixteen inoculation points, SE: Standard error).

Table 2. 2: Associations between tissue occlusions and *Leptographium terebrantis* inoculation points in *Pinus taeda* saplings.

Linear regression equations	Sample size	*P* > *F*	R-square
Occlusion= -4.42 + 12.27(IP)	72	<0.0001	0.4986
Ln(Occlusion length) = 4.31 + 0.156(IP)	72	<0.0001	0.3275
Ln(Occlusion volume) =1.79 + 0.677(IP)	72	<0.0001	0.6134

(Note: IP: Inoculation points).

2.5 Discussion

L. terebrantis colonized toothpicks served as a useful substrate for fungal inoculum transfer unto loblolly pine saplings. Inoculation with the colonized toothpicks resulted in the production of lesions, occlusions and resins in the host. Colonized wooden toothpicks have been successfully used as an artificial vector in insect-vectored ophiostomatoid fungi inoculation studies under laboratory and field conditions as reported in a few other toothpick inoculation studies (Wingfield, 1986; Takahashi et al., 2010). Fungal colonized wooden toothpicks have been used in studying the pathogenicity of fungi such as the novel ophiostomatoid fungi in the branches of *Euphorbia ingens* E.Mey. ex Boiss trees (Van der Linde et al., 2016), saplings of *Quercus crispula* Blume (Kusumoto et al., 2012), and in logs of *Quercus* species (Kusumoto et al., 2015). Toothpick inoculation may provide a more realistic and uniform estimate of host tissue damage following ophiostomatoid fungal inoculation. Future studies, including comparisons of inoculation experiments utilizing both bark beetle vector and toothpick inoculation, should be conducted in parallel to understand the efficacy of utilizing toothpicks for both point and mass inoculations.

The increase in radial inoculation points increased occlusion length, area and volume. This is consistent with the earlier finding of Fernandez et al., (2004) who reported unstained sapwood area of *Pinus sylvestris* to decrease with increasing inoculum density of *Ophiostoma ips* (Rumbold) Nannfeldt. Furthermore, they reported that higher inoculum densities resulted in yellow-green coloration in needles as opposed to no symptoms at lower densities. *Leptographium wingfieldii* Morelet was also found to cause more sapwood occlusion in *P. sylvestris* trees relative to *Ophiostoma canum* (Münch) Syd. & P. Syd. and *Ophiostoma minus* (Hedgcock) H. & P. Sydow (Solheim et al., 2001) but did not cause mortality in the inoculated trees. Nonetheless, Solheim et al., (1993) found that *L. wingfieldii* and *O. minus* were able to kill *P. sylvestris* trees when the inoculation points were 800 per m^2 on whole tree basis. They also found mortality to occur at a lower inoculum density (400 inoculations per m^2) when the trees were subjected to pruning stress. Mass inoculation loads have been found to cause the sudden decline of tree health (Horntvedt et al., 1983; Christiansen, 1985; Solheim and Krokene, 1998).

The saplings survival in our study can be attributed to the fact that the critical inoculum threshold was not attained, and moreover, the study duration may not have been long enough to cause massive sapwood occlusion necessary to cause hydraulic dysfunction in the saplings. Comparatively, the inoculation densities in the current study are lower relative to the critical attack threshold. The inoculation densities utilized in the study were to understand the relationship between inoculation density and associated host tissue occlusion. The fungal inoculation by bark beetles on an ecological scale may be more detrimental as beetles usually attack previously stressed trees (Kelsey et al., 2014). Vascular-inhabiting fungal invasion in an embolized drought-stressed tree might cause complete hydraulic failure and plant mortality

(Oliva et al., 2014). The inoculation threshold beyond which tree cannot regain its health can be precisely determined by increasing the radial density of the fungal inoculation points.

The results from this study will act as a baseline for future inoculation studies investigating the long-term impact of different inoculation densities on mature *P. taeda* tree health. In conclusion, fungal colonized toothpicks can be utilized in artificial inoculation as an efficient and uniform vector for mass and point inoculation studies. To determine the efficacy of this technique in mimicking the natural inoculation by the beetles, experiments including bark beetle vector should be conducted in parallel. Likewise, parallel experiments including the wounding of bark for mass inoculation and toothpick inoculation should be conducted to develop a standard reproducible and uniform technique that can be used for inoculation of beetle-vectored ophiostomatoid fungi. Future inoculation studies, utilizing colonized toothpicks of *L. terebrantis*, should be conducted over a longer period to allow for the development of symptoms and mortality, if they are to occur.

CHAPTER III

Physiological response of *Pinus taeda* L. trees to stem inoculation with *Leptographium terebrantis*

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 (±1.3 cm) and total height of 4.5 m (±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:

$$RWC_F = [(W_W - W_D) / (W_T - W_D)] * 100. \quad (1)$$

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 ±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:

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

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 ^oC 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).

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
Occluded sapwood area[3]	I	3	21.3104	< 0.0001
	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

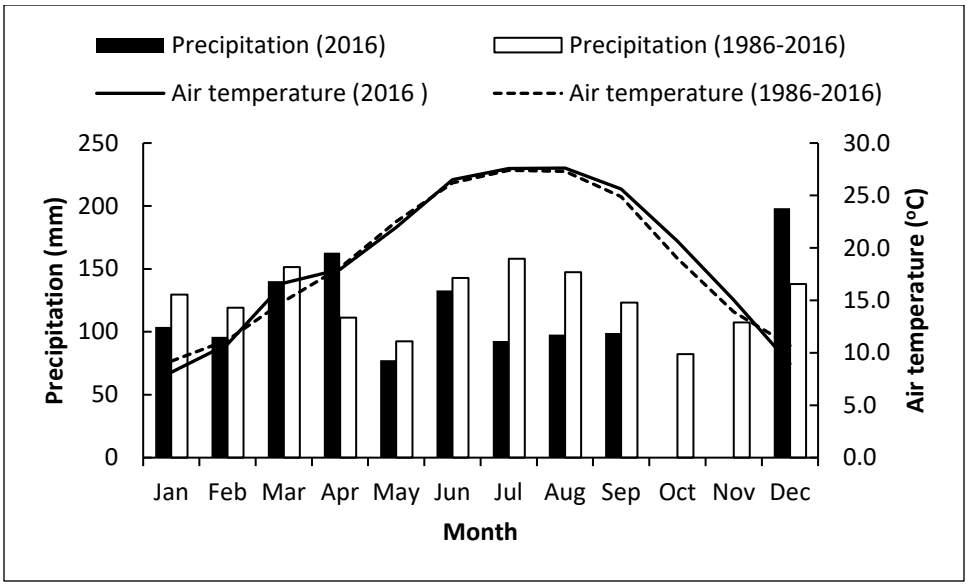


Figure 3. 1: Monthly precipitation and average daily air temperatures in 2016 and during the 30-year period between 1986 and 2016 at the study site near Andalusia, AL (NOAA 2019). Stem inoculation was done on May 5, 2016 followed by post-inoculation assessments 8, 16, 20 and 24 weeks (June-October) afterward.

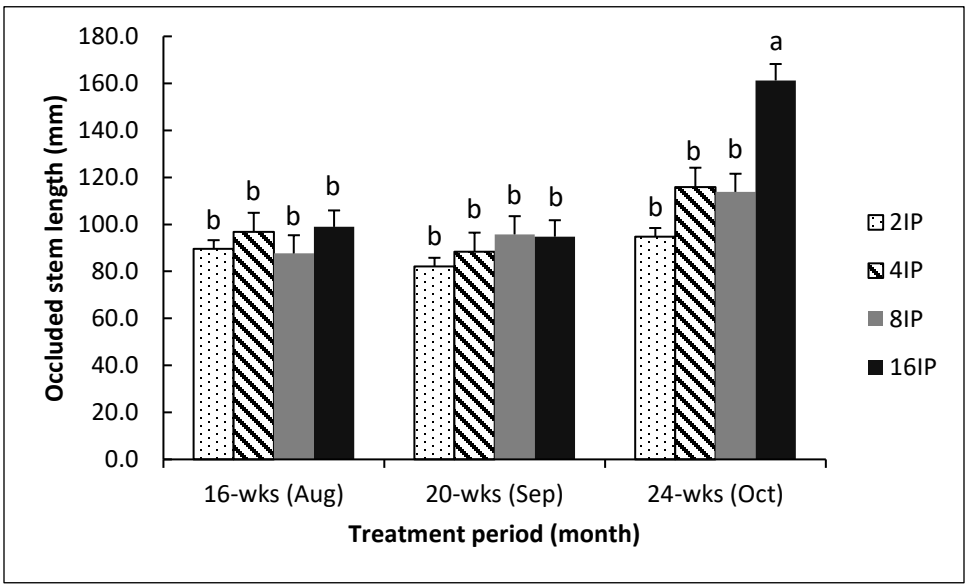


Figure 3. 2: Occluded stem length after three treatment periods, 16, 20, and 24 weeks after inoculation with four *L. terebrantis* inoculation densities (2IP, 4IP, 8IP, 16IP). Means associated with different lower-case letters are significantly different at an α-level of 0.05 by the Tukey HSD test for multiple comparisons. Bars represent one standard error of the mean.

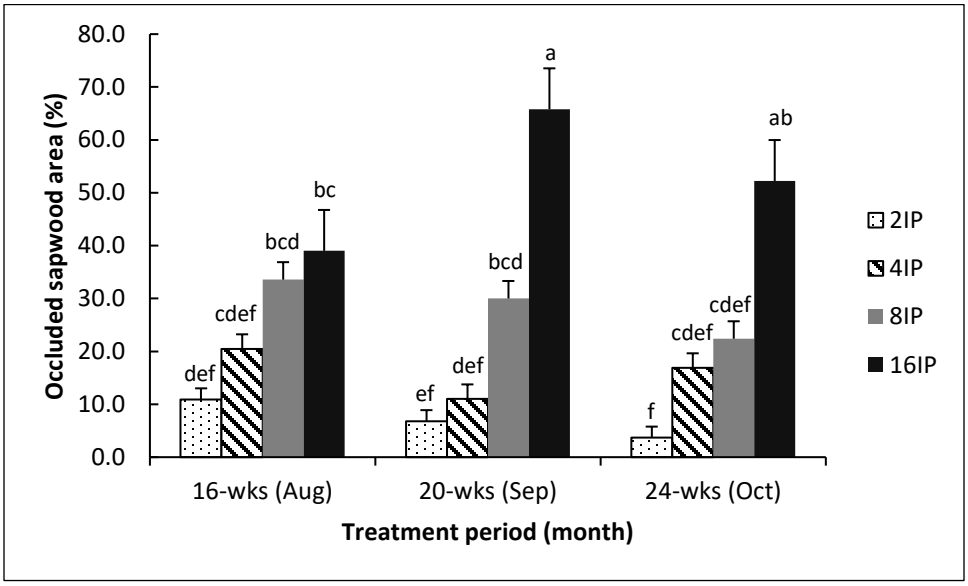


Figure 3. 3: Percentage occluded sapwood area of stem segments after *L. terebrantis* inoculation at four densities (2IP, 4IP, 8IP, 16IP) and three treatment periods, 16, 20 and 24 weeks. Means associated with different lower-case letters are significantly different at an α-level of 0.05 by the Tukey HSD test for multiple comparisons. Bars represent one standard error of the mean.

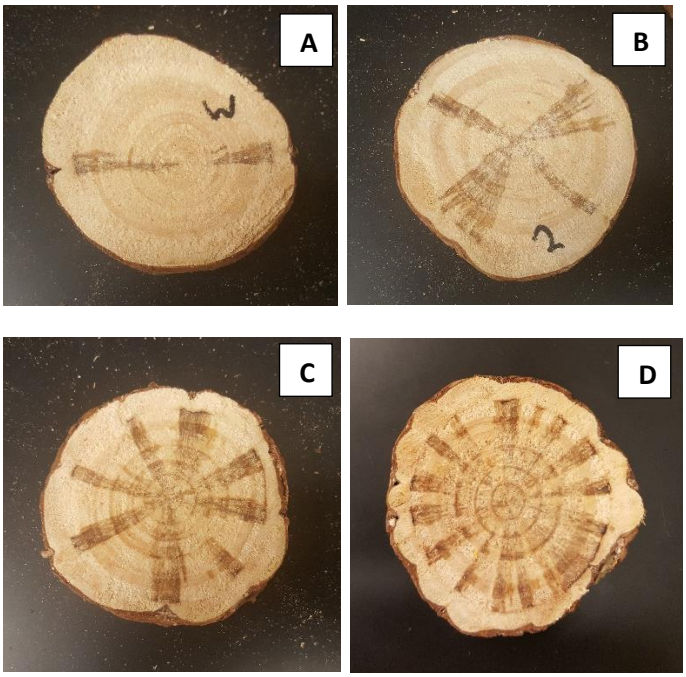


Figure 3. 4: Cross-sections of stem segment discs: A, B, C, and D received the 2IP, 4IP, 8IP, or 16IP inoculation density, respectively. Deep brown color indicates occluded sapwood due to artificial inoculation with *L. terebrantis.* Note the un-occluded sapwood area around the circumference of the discs that grew in the 24-week period.

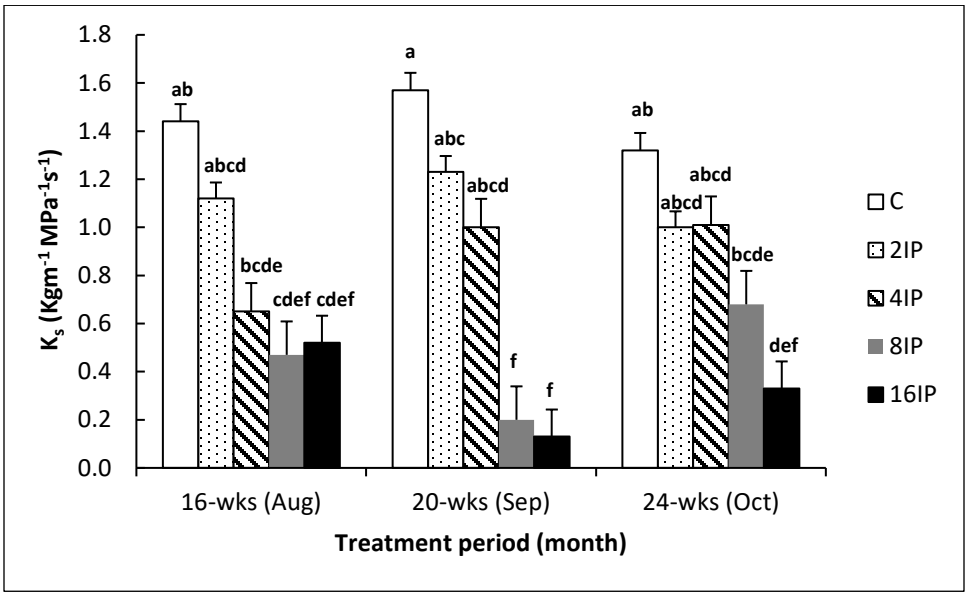


Figure 3. 5: Native, specific hydraulic conductivity (K_s) of stems in response to four *L. terebrantis* inoculation densities (2IP, 4IP, 8IP, 16IP), or no inoculation (C). Data were collected at three treatment periods, 16, 20, and 24 weeks after artificial stem inoculation of *P. taeda* trees. Means 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.

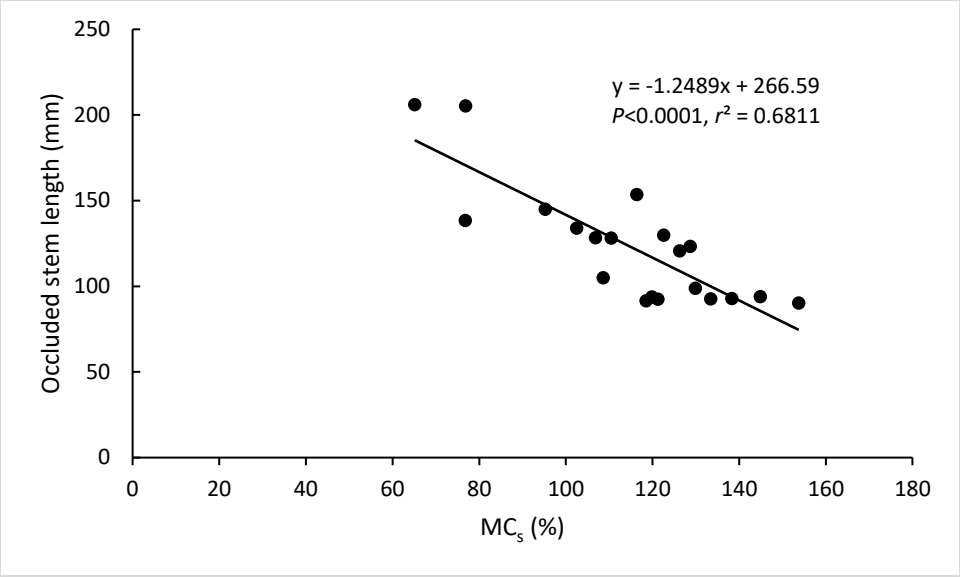


Figure 3. 6: Linear relationship between occluded stem length and stem moisture content (MC_s) among *P. taeda* trees 24 weeks after inoculation with one of four densities of *L. terebrantis* (2IP, 4IP, 8IP, 16IP).

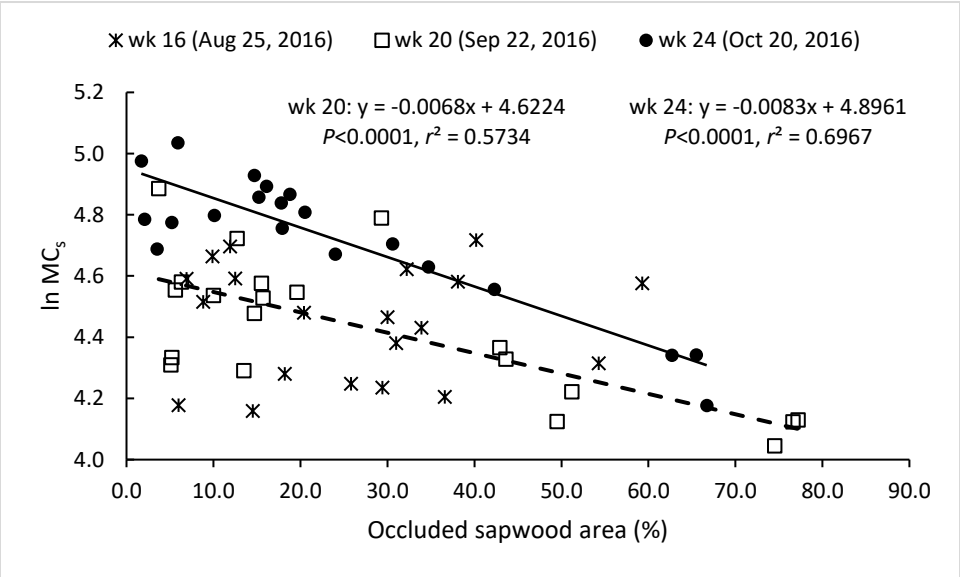


Figure 3. 7: Relationships between the natural logarithm of stem moisture content (ln MC_s) and percentage of occluded sapwood area among *P. taeda* trees at 16, 20, or 24 weeks after artificial stem-inoculation with *L. terebrantis*.

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.

CHAPTER IV

Effect of *Leptographium terebrantis* and drought on foliage, new root dynamics and stemwood growth in plantation *Pinus taeda* L.

4.1 Abstract

The course of the bark beetle-vectored fungus, *Leptographium terebrantis* S. J. Barras and T. J. Perry, in stemwood growth losses of declining pines of the southeastern U.S. was assessed. The study was installed in a 13-year-old loblolly pine (*Pinus taeda* L.) plantation near Eufaula, Alabama, U.S. Artificial inoculation with sterile toothpicks colonized by *L. terebrantis* at varying inoculum densities was used to elicit host growth responses. The root pathogen compromised xylem function and caused a reduction in foliage moisture content, leaf area (A_L) and the ratio of A_L to tree sapwood area (A_S). Decreases in relative stemwood growth were more pronounced in trees receiving the high inoculum treatment relative to those receiving the low, medium, or wound-control treatments. This decline in stemwood growth was associated with 7-months of water deficit suggesting that in the loblolly pine and *L. terebrantis* pathosystem an additional factor of water deficit is required to enable the pathogen's role in stemwood growth loss. Thus, presence of *L. terebrantis* in pine forests of the southeastern U.S. which are vulnerable to water deficit has the potential to widen the gap between predicted and actual stemwood production.

4.2 Introduction

Loblolly pine (*Pinus taeda* L.) is the principal tree species grown in forest plantations across the southeastern U.S. (Schultz, 1997). To sustain economic and ecological contributions of this tree species in the region, about one billion loblolly pine seedlings are planted each year (McNabb and Enebak, 2008). Dominance of this tree species in the region is projected to increase for decades (Huggett et al., 2013) as a result of improved genetics and silvilcultural treatments that enhance plantation productivity. Over the past six decades, loblolly pine growth loss and mortality (declines) have been reported in localized areas of central Alabama and Georgia (Eckhardt et al 2010; Forest Health Cooperative, 2017).

Forest declines are caused by complex interactions between pests and abiotic factors (Manion, 1991; Manion and Lachance, 1992), and several theories have been formulated to explain the forest decline concept (Sinclair and Hudler, 1988; Manion, 1991; Auclair et al., 1992; Houston, 1992; Mueller-Dombois, 1992). Common among these theories is the supposition that no single factor causes forest decline but it is a multiplicity of factors that act in a sequential order for a decline outcome. The decline spiral model (DSM) proposed by Manion (1991) is the most common among the forest decline theories. The DSM reclassified the cause of decline from the chain reaction theory (Sinclair and Hudler, 1988) to the systematic occurrence of predisposing, inciting, and contributing factors.

According to the DSM, predisposing factors are long-term, underlying conditions such as genetic potential, climate, and soil quality that put permanent physiological stress on trees. Inciting factors are relatively short-term conditions such as drought or insect attack including

defoliation that worsen the stress imposed by predisposing factors (Manion, 1991; Bigler et al., 2006; Liu et al., 2013; Williams et al., 2013). For example, Bigler et al., (2006) noted that the high mortality of Scots pine (*Pinus silvestris* L.) in the Rhône Valley and other dry areas of the European Alps during the early 20th century was incited by several years of drought.

Predisposing and inciting factors establish favorable conditions for contributing factors of decline which are biotic in nature such as population increases in stem- and root-feeding bark beetles and the subsequent spread of their associated fungi. These factors worsen physiological stress (Manion, 1991; Jurskis, 2005) and may accelerate growth loss and mortality. For example insects such as lesser pine shoot beetle (*Tomicus piniperda* L., *T. minor* Hart.), the pine processionary moth (*Thaumetopoea pityocampa*, Denis and Schiff.) and the six toothed bark beetle (*Ips sexdentatus,* Boern.) contributed to the Scots pine mortality in the Rhône valley (Rigling and Cherubini, 1999; Bigler et al., 2006)

In North America, forest declines threaten sustainable timber production across thousands of acres. Over 300,000 acres (approximately 121,405 hectares) of *Quercus* spp. (oak) were negatively affected by decline in the Ozark Mountains of northern Arkansas and southern Missouri in 1999 and 2000 (Starkey et al., 2000; Heitzman, 2003). Declines reported in these locations were attributed to interacting factors that included drought and outbreaks of red oak borer (*Enaphalodes rufulus* Haldeman) that led to stand mortality and modification of the landscape. Other forest declines in North America include aspen (*Populus tremuloides* Michx) in the western U.S. and Canada (Worrall et al., 2013), whitebark pine (*Pinus albicaulis* Engelm.) in western North America (Wong and Daniels, 2017), pinyon pine (*Pinus edulis* Engelm.) in the western U.S. (Gaylord et al., 2015), and loblolly pine (Brown and McDowell, 1968; Eckhardt et al., 2007) decline in the southern U.S.

Since the 1950s, loblolly pine decline (LPD) has been reported in several Alabama counties (Brown and McDowell, 1968; Eckhardt et al., 2007; Eckhardt et al., 2010). Stands of loblolly pine exhibiting decline symptoms are characterized by sparse crowns, short and chlorotic needles, reduced radial growth, woody roots with resinous bark wounds and stained sapwood, and tree mortality (Hess et al., 1999; Eckhardt et al., 2007). Root-feeding bark beetles that vector *Leptographium* spp. are commonly found in declining loblolly pines (Eckhardt et al., 2004; Eckhardt et al., 2007).

In accordance with the DSM (Manion, 1991), the bark beetle-fungal complex of LPD acts as a contributing factor to decline after trees have been predisposed. The role of stem-feeding bark beetles in pine mortality is well known and attributed to the tunneling beneath the bark and subsequent larvae production which girdles the tree and disrupts the transport of photosynthates from the foliage to the roots (Millar et al., 2012; Hicke et al., 2016; Berner et al., 2017). Similarly, root-feeding bark beetles damage the vascular cambium and conducting tissues near the point of woody root entry (Paine et al., 1997). Further disruption of xylem and phloem occurs by the spread of vectored fungal associates and the net effect may lead to stemwood growth loss and tree mortality. These fungal associates are not considered to be aggressive pathogens unless they overcome host defenses leading to an advancement of sapwood occlusion (Six, 2003; Six and Wingfield, 2011). *Leptographium terebrantis* is one of several bark beetle-vectored fungi commonly isolated from woody roots of loblolly pine exhibiting symptoms of LPD.

Several studies have shown that *L. terebrantis* may be pathogenic by inducing sapwood occlusion in loblolly pine seedlings, saplings, and mature trees under greenhouse or field conditions (Matusick et al., 2016; Devkota and Eckhardt, 2018; Mensah et al., 2020). At the

same time, tree growth and physiological responses to *L. terrebrantis* are variable despite distinct signs of pathogenesis (Mensah et al., 2020). Thus, the contribution of *L. terebrantis* infection to LPD when vectored by root-feeding bark beetles is unknown. *Leptographium terebrantis* grows into xylem tissues and disrupts water and mineral nutrient transport from the soil to the crown (Oliva et al., 2014; Mensah et al., 2020). This compromises xylem function by limiting water conductance and reduces both stomatal function and carbon fixation. Poor xylem function risks inadequate carbon for normal foliage, stem, and root system growth and constitutive chemical defense production. For example, Viiri et al., (2001) noted the reduction in total soluble carbohydrates near the site of fungal infection when Norway spruce (*Picea albeis* L.) was inoculated with the bark beetle-associated fungus, *Ceratocystis polonica* (Siemaszko) C. Moreau**.** Disruption of xylem can lead to mortality in mature trees (Tyree and Zimmermann, 2002).

In this study, we assessed the annual stemwood growth of loblolly pine trees grown in commercial plantation. These trees were either non-inoculated or inoculated at one of three densities with *L. terebrantis*, to determine the potential for this pathogen to affect stemwood growth. In addition, seasonal assessments of new root growth, and destructive measurements of leaf area and stem sapwood area 34 months after inoculation provide knowledge about the effect of *L. terebrantis* infection during the progression of LPD. We hypothesized that *L. terebrantis* infection has the potential to impair whole-crown carbon fixation such that tree leaf area, new root production, and stemwood growth become carbon-limited. We further hypothesize that this response is not apparent until together, site conditions and a threshold of *L. terrebrantis* infection cause carbon limitations that cannot be tolerated by the tree.

4.3 Methods

4.3.1 Study site and experimental design

The study was located in a loblolly pine plantation near Eufaula, Alabama, U.S. in Barbour County (32°1'13.10"N, 85°12'31.76"W). The plantation was situated on the East Gulf Coastal Plain physiographic region and the humid subtropical climatic zone. Soil series identified within the study area included Annemaine and Wahee. Their taxonomic classification is a fine, mixed, semi-active, thermic Aquic Hapludult and fine, mixed, semi-active, thermic Aeric Endoaquult, repectively. Annemaine is the predominant soil series, consisting of a fine sandy loam surface and clayey subsoil, and moderately well drained. Wahee contains a clay loam subsoil overlain by fine sandy loam surface and poorly drained (Trayvick, 2005; Ditzler et al., 2017). Average annual precipitation and air temperature of the area are 1407 mm and 18.1 ^{o}C, respectively (NOAA, 2020). The plantation was established in 2003 at1.2 m x 3.0 m spacing using open-pollinated seedlings and third-row thin at 12 years age in 2014. The study site received nitrogen and phosphorus fertilization at planting but no herbicide or pesticide control after planting and has a site index of 22 m at 25 years.

Fifteen plots containing two rows, 3.0 m apart, of 10 trees per each row were established in the plantation at age 13 years in December 2015 in a completely random experimental design with three replications and five inoculation treatments. All plot trees were permanently identified by numbered metal tags and outfitted with a manual dendrometer band (D1, UMS GmbH, Munich, Germany) installed at 1.4 m above the ground line (DBH) on five randomly chosen trees in each row per plot. A weather station (WatchDog 2000, Spectrum Technologies Inc., Aurora, IL, U.S.) was installed adjacent to the study site to monitor local precipitation, air temperature, solar radiation, relative humidity, and wind speed.

Inoculation treatments were applied to the five randomly chosen measurement trees that were fitted with dendrometer bands in one of the two rows per plot. Treatments of the study included a no inoculation or wounding (control), no inoculation but sterile toothpick wounding (wound), and three levels of increasing fungal inoculum density (low, medium, high). Inoculum densities were selected based on earlier studies that established the relationship between number of *L. terrebrantis* toothpick inoculum points, occluded radial area of the stem (Devkota et al., 2019), and stem hydraulic conductivity in loblolly pine (Mensah et al., 2020). The treatments were applied by a procedure similar to that described by Devkota et al., (2019) with modification due to differences in tree size. For each tree, the number of inoculation points was marked on a stencil sheet adhered to the inoculation zone to ensure proper inoculum placement around the stem circumference. Three series of inoculation points were identified at 1.2 cm, 2.4 cm and 3.6 cm below the initial inoculum point (Devkota et al., 2019). The low, medium, and high inoculum densities received three series of 5-8, 20-28, or 40-58 *L. terebrantis*-colonized toothpicks, respectively, around the circumference of the lower stem in March 2017. The wound treatment was applied similar to the high inoculum treatment.

4.3.2 Minirhizotron tube installation

Four clear acrylic tubes, 82 cm in length and 3.81 cm inner diameter, were installed around each of two trees of comparable DBH per row and plot (two treated and two untreated trees). Each tube was installed at a 152.4 cm distance from the base of the bole and at a 45^{o} angle toward the tree with two tubes installed on each side of the original planting row. The circumference of the tubes was scored at eight 10 cm increments corresponding to 0, 7.1, 14.1, 21.2, 28.3, 35.4, 42.4 and 49.5 cm below ground level as described by Duwadi (2019). The 10 cm length of the upper end of the tube was covered with a black tape to prevent light from

entering the tube and plugged with a #7 rubber stopper to prevent invasion of the tube by insects or water. The tubes were then covered with small plastic pot as described by Duwadi (2019).

4.3.3 Inoculation method

Prior to treatment application, the dead cork of the bark was scraped around the circumference of the lower stem between 20 cm and 30 cm above the ground line with a 20.3 cm long ironton straight draw shave (Northern Tool + Equipment, Burnsville, MN, USA). The inoculation points, approximately 1.2 mm in diameter and 5 mm deep, were drilled into the trees stems through the identified points on the stencil sheet placed between 23 cm and 27 cm above ground level.

To prepare for treatment application, wooden toothpicks, sterilized at 121 °C for 30 min and soaked overnight in malt extract broth (MEB) (BD Bacto ™ Malt Extract, BD Biosciences, San Jose, CA), were inoculated with *L. terrebranits* or not inoculated and incubated in the dark at 23 ^{o}C for 24 days as described by Devkota et al., (2019). Trees were inoculated in March 2017 by inserting toothpicks containing *L. terebrantis* inoculum (mycelium and spores) 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 to prevent contamination (Devkota et al., 2019, Mensah et al., 2020).

4.4 Measurements

4.4.1 Stem growth

Tree diameter at breast height (DBH) expressed as cm was determined from measurement tree dendrometer bands every month from January 2016 to February 2017 before inoculation treatments were applied. These measurements continued between March 2017 and

December 2019 after inoculation treatments were applied. Total measurement tree height was quantified and expressed as m by a TruPulse 200 Rangefinder-Hypsometer (Laser Technology Inc, Centennial, CO, USA) in January each year between 2016 and 2020. Measurement tree basal area (BA) was determined by equation (1),

$$BA = (\pi D^2)/4 \quad (1)$$

where D is DBH expressed as cm. Annual tree basal area increment (BAI) expressed as cm^2, was calculated in 2016 through 2019 as the difference between current year (BA_2) and previous year (BA_1) tree basal areas in January.

Relative stem radial growth (RG) of the measurement trees was determined as the ratio of BAI and BA_1 from 2016 to 2019 (Johnson and Abrams, 2009). Annual outside-bark stem volume of the measurement trees was estimated as described by Burkhart (1977) for loblolly pine and expressed as m^3.

4.4.2 Root growth

New root growth was assessed and counted with the aid of an optical root periscope (JRD Merrill Speciality Equipment, Logan, UT) that had a fiber optic light powered by a battery. New root (< 2 mm diameter) growth measurements started 6 months after minirhizotron root tube installation and one-month post-inoculation in April 2017 and were repeated at 3-month intervals until trees were harvested in January-February 2020. During new root assessments, the number of pine roots that intersected the seven scored lines below ground level of each tube were visually identified and counted. New roots were identified by color and diameter (initially white/translucent and turning reddish-brown with time). New root growth, calculated as root

length density (RLD) and expressed as cm cm^{-2} according to Newman (1996), was determined by equation (2),

$$RLD = R/A \quad (2)$$

where R is total root length (cm) expressed by the equation, $R = (\pi * N * A)/(2 * H)$, N is the number of roots intersecting scored lines, A is the area of the minirhizotron tube between two scored lines, and H is the length of a scored line. Cumulative RLD (CRLD) by tube was calculated as the sum of seven RLD values between ground level and the 49.5 cm depth and tree CRLD was calculated as the average of four CRLD values by tree.

4.4.3 Leaf area, sapwood area and tissue moisture content

In January and February 2020, the five treated measurement trees in one of two rows per plot were felled at the ground line with a chainsaw. Green foliage was removed from branches and weighed within 2 to 4 h after tree harvest to determine live foliage fresh weight per tree (W_1). Green foliage was transported to the laboratory. Three subsamples, each containing between 15 and 25 three-needle fascicles were randomly sampled by tree to estimate the ratio of total leaf area and foliage dry mass per tree. Subsequently, the remaining fresh foliage was oven-dried at 70 ^{o}C to a constant weight (W_2). The moisture content (MC) of the foliage at the time of tree harvest was expressed as percentage of oven-dried weight by the equation, $MC = [(W_1 - W_2)/W_2] * 100$.

The total leaf area of each subsample of fascicles was determined by volume displacement of bundles of two fascicles as described by Johnson (1984) using equation (3),

$$A = 2L[1 + \frac{\pi}{n}]\sqrt{(Vn/\pi L)} \quad (3)$$

where A is total surface area (cm^2), L is cumulative green needle length (cm), V is volume displaced by the fascicle bundle (cm^3), and n is the number of needles per fascicle bundle. The total leaf area of fascicle subsamples was calculated as the sum of A among fascicle bundles and projected leaf area by fascicle subsample was expressed as A divided by 3.142 (Grace 1987). Fascicle subsamples were oven-dried at 70°C to a constant weight and their specific leaf area (SLA) was calculated as the ratio of projected leaf area and dry weight ($cm^2\ g^{-1}$). Tree SLA was estimated as the mean of three SLA values by tree and projected leaf area by tree (A_L) was calculated as the product of tree SLA and W_2.

A wood disc, approximately 5 cm in thickness, was cut with a chainsaw at DBH. The circumference of sapwood area was traced on a transparent sheet and sapwood area (A_S) was determined with a planimeter (Lasico®, Los Angeles, CA, USA). The ratio of A_L and A_S was calculated by tree. Tree growth efficiency (GE) at the time of tree harvest was determined as the BAI in 2019 divided by leaf area (A_L) and expressed as $cm^2\ m^{-2}$

4.5 Data analysis

The main effect of *L. terebrantis* inoculum density, treatment duration and their interaction on growth parameters was analyzed. Values of DBH, total tree height, stemwood volume, relative radial stem growth (RG), tree leaf area (A_L), foliage moisture content (MC), tree growth efficiency (GE), A_L-to- sapwood area (A_S) ratio, and cumulative root length density (CRLD) were assessed for normality and equal variance assumptions. Values of DBH, total tree height, stemwood volume, and RG were evaluated by a completely randomized experimental

design using two-way repeated measures analyses of variance and the Mixed procedures of SAS statistical software (SAS Institute, Version 9.4, Cary, NC, USA) with compound symmetry as the covariance structure.

Similarly, tree A_L, MC, GE, A_L:A_S, and CRLD were analyzed by one-way analyses of variance and the GLM procedures of SAS statistical software (SAS Institute, Version 9.4, Cary, NC, USA). Treatments were control, wound, and low, medium, and high inoculum 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. Linear relationships between A_L, and DBH were assessed by regression. Regression parameters of pairs of significant A_L and DBH 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 marginally significant at an α-level of 0.10 when a biologically related difference was significant elsewhere in the data at an α-level of 0.05.

4.6 Results

4.6.1 Temperature and precipitation at the study area

Annual precipitation during the four-year period was 992.1, 1311.4, 1259.8 and 954.5 mm in 2016, 2017, 2018 and 2019 respectively (Figure 4.1). Prior to tree inoculation in 2017, severe drought occurred in the dormant season of 2016 and in October there was no precipitation. Moderate drought also occurred in 2019 after inoculation and the site received 7 months of less precipitation from March to September with 45% lower than the 30-year average for the area. Overall average monthly air temperature for the measurement period was 18.2, 18.0,

18.1 and 18.4^{o}C in 2016, 2017, 2018 and 2019, respectively. The average monthly air temperature during the 7- month drought period is 22.8 ^{o}C relative to the 30-year average of 22.4 ^{o}C within the same period (Figure 4.1).

4.6.2 DBH, tree height and stemwood volume

At the time of plot establishment in December 2015, mean values of DBH, total height, and stemwood volume by measurement tree were 16.88 ± 0.50 cm, 14.02 ± 0.20 m, and 0.3209 ± 0.023 m^3 respectively. Tree growth was monitored for 14 months prior to treatment application and during this period, no symptoms or signs of disease were observed among the study trees. At the time of treatment application in March 2017, mean plot values of DBH, total height, and stemwood volume by measurement tree were not significantly ($P>0.05$) different among the treatments. Mean values of DBH, total height, and stermwood volume for the treated plot trees are shown in table 4.1. Stem inoculation of loblolly pine trees with *L. terebrantis* did not significantly ($P>0.05$) affect DBH, total height or stemwood volume between treatment application in March 2017 and tree harvest in December 2019. Over the 3-year period since treatment application, DBH, total height, and stemwood volume were significantly affected by year, but not treatment as a main effect. Interaction between treatment and year did not significantly affect DBH but significantly affected total height and had a marginally significant effect on stemwood volume (Table 4.2). Mean total height was 14.8 ± 0.2 m (Figure 4.2a, b and c).

Relative growth (RG) was significantly affected by the main effects of inoculation treatment and year as well as their interaction (Table 4.2). The low and high treatments had the highest (18.5 m) and lowest (16.7 m) height growth, respectively, at the end of 2019. Relative growth peaked in 2017 and declined in the subsequent years and attained minimum value in

2019. The control and medium treatments had highest and lowest RG growth respectively in 2017. Although trend of RG growth declined among the treatments in 2018 and 2019, the high treatment trees had the highest rate of decline (47%) (Figure 4.2d).

At the end of 2017, decline symptoms such as chlorotic and thin crown were not observed among the treatments. In 2018, several trees treated with the medium and high inoculum densities exhibited resinosis above and below the inoculation zone which was sealed with duct tape. In late 2018 and throughout 2019, decline symptoms including chlorotic and thin crowns were manifested in trees treated with the high inoculum density.

4.6.3 Leaf and sapwood areas, foliage moisture content, and tree growth efficiency

Estimates of whole crown projected leaf area (A_L) were significantly affected by inoculation treatment at the end of the study (Table 4.3). Values of A_L were significantly lower among trees receiving the high inoculum density treatment compared to those receiving the wound or control treatments (Figure 4.3). Variables derived from A_L were also significantly affected by inoculation treatment at the end of the study. Specifically, A_L:A_S was significantly greater for the control and wound treatments compared to the high inoculum density treatment and GE was significantly greater for the control treatment compared to the low and high inoculum density treatments (Table 4.4). A marginally significant effect of inoculation treatment on MC was observed at the end of the study ($P = 0.0904$) with 13.7% higher MC among control trees compared to those treated with the high inoculum densities.

A significant ($P < 0.0001$) positive linear relationship was found between A_L and DBH for all inoculation treatments except the high inoculum treatment (control: $P = 0.0005$, $r^2 = 0.84$; wound: $P = 0.0013$, $r^2 = 0.67$; low: $P = 0.0001$, $r^2 = 0.87$; medium: $P = 0.0011$, $r^2 = 0.57$; high: P

=0.5125, r^2 = 0.04). Slope and y-intercepts associated with the control, wound, and low and medium inoculum density treatments were not significantly different from each other. However, the slopes and y-intercepts of the control, wound, and low and medium inoculum density treatments were significantly different from those of the high inoculum density treatment. The slope of this relationship for trees receiving the high inoculum density treatment was significantly lower than those of trees receiving the control (P = 0.0029), wound (P = 0.0030), or low (P = 0.0155), or medium (P = 0.0414) inoculum density treatments. An inverse but similar treatment response was observed for y-intercept with significantly lower values among the control (P =0.0119), wound (P =0.0131), and low (P = 0.0004), and medium (P = 0.0162) inoculum density treatments compared to the high inoculum density treatment.

4.6.4 Root growth

Cumulative root length density (CRLD) was not significantly affected by inoculation treatment in 2017, 2018, or February 2019 (Duwadi 2019). A trend of reduced CRLD was seen with increasing inoculum density in July 2019, but a significant treatment effect was not observed. Three months later in October 2019, CRLD was significantly affected by inoculation treatment (P = 0.0361). Mean CRLD for trees treated with the high inoculum density was 60% lower than that among trees treated with the control, wound, or low or medium inoculum density treatments (Figure 4.5).

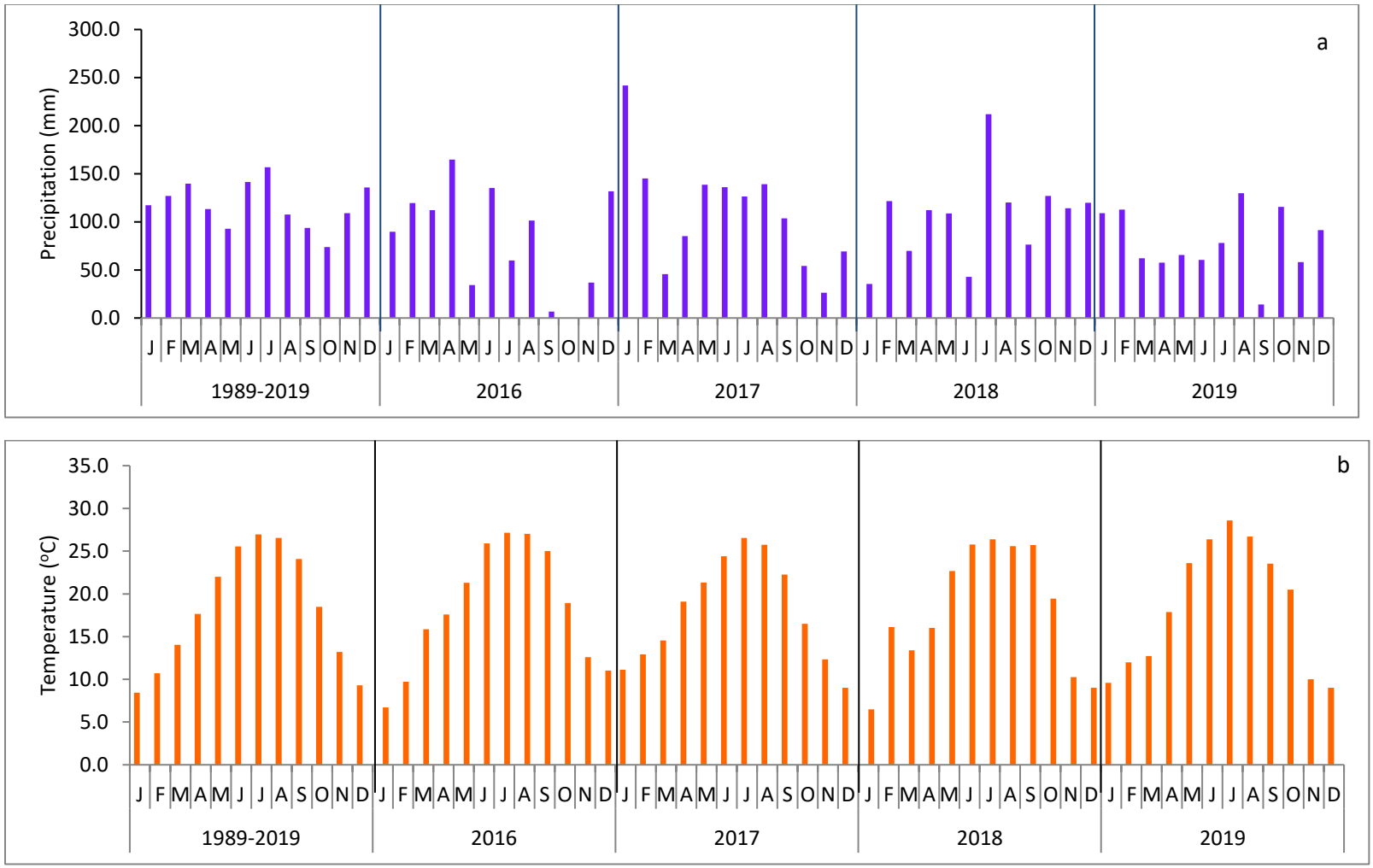


Figure 4. 1: (a) Precipitation at the study site from 2016 to 2019 and 30 year average between 1989 and 2019 (b) daily air temperatures at the study site from 2016 to 2019 and the 30 year average.

Table 4. 1: Mean DBH, total height, and stemwood volume of *P. taeda* measurement trees after plot establishment and 14 months before inoculation treatments were applied.

Treatment	DBH (cm)	Height (m)	Volume (m^3)
Control	17.38±0.34	14.68±0.18	0.3696±0.02
Wound	17.49±0.51	14.58±0.19	0.3564±0.02
Low	17.62±0.85	15.20±0.25	0.3874±0.04
Medium	17.67±0.46	15.10±0.22	0.3755±0.02
High	16.83±0.43	14.53±0.22	0.3300±0.02

Table 4. 2: Probabilities of a greater *F*-value (*P* > *F*) from two-way analyses of variance of DBH, total height, stemwood volume and stem relative growth (RG) thirty-four months (3 years) following stem inoculation of *P. taeda* trees with *L. terebrantis* near Eufaula, AL.

Variable	Source of variation	Df[1]	Mean square	*P* > *F*
DBH	Treatment (T)	4	0.10	0.9826
	Year (Y)	2	283.07	**<0.0001**
	T x Y	8	1.12	0.3515
Tree height	T	4	2.22	0.0697
	Y	2	443.26	**<0.0001**
	T x Y	8	2.75	**0.0062**
Stemwood volume	T	4	0.99	0.4150
	Y	2	227.63	**<0.0001**
	T x Y	8	1.73	0.0915
RG	T	4	2.82	**0.0277**
	Y	2	84.02	**<0.0001**
	T x Y	8	4.94	**<0.0001**

[1] Df, degrees of freedom; *P* > *F*, probability of a greater *F*-value.

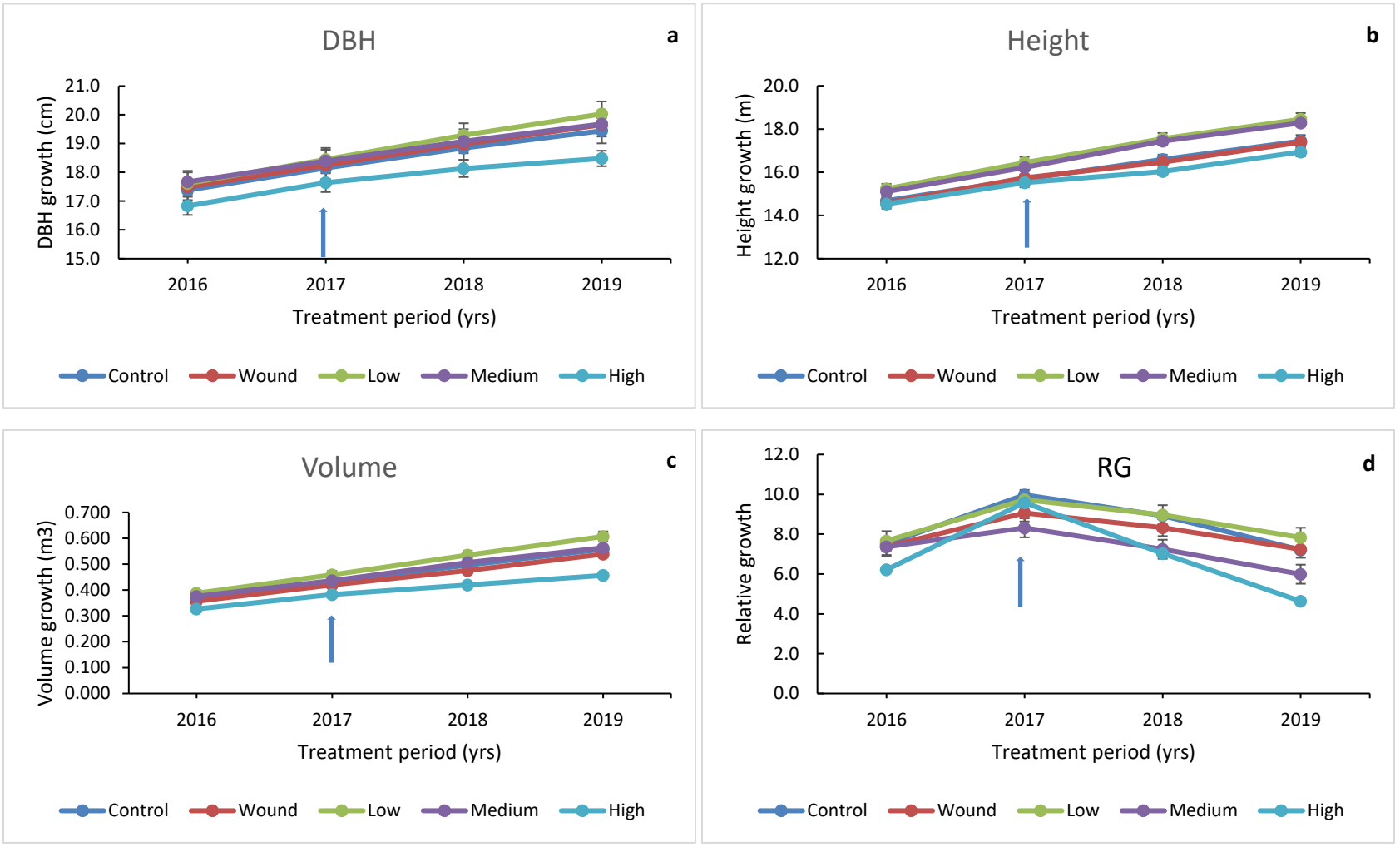


Figure 4. 2: Infuence of *L. terebrantis* on *P. taeda* growth (a) DBH (B) Height (C) Volume and (D) Relative growth. The arrow indicates the period at which pathogen inoculation occurred in March 2017.

Table 4. 3: Probabilities of a greater *F*-value from one-way analyses of variance for tree projected leaf area (A_L), the ratio of A_L to sapwood area (A_L:A_S), tree growth efficiency (GE), and foliage moisture content thirty-four months following stem inoculation of *P. taeda* trees with *L. terebrantis* near Eufaula, AL.

Variable	Df[1]	*F*-value	*P* > *F*
A_L	4	3.07	<0.0001
A_L:A_s	4	2.81	<0.0001
GE[2]	4	4.27	0.0328
MC	4	2.11	0.0904

[1] Df: degrees of freedom; *P* > *F*, probability of a greater *F*-value.
[2] GE is calculated as tree annual increment of basal area divided by tree A_L.

Table 4. 4: Mean ± standard errors of the ratio of tree leaf area to sapwood area (A_L:A_S), tree growth efficiency (GE), and foliage moisture content (MC) thirty-four months following stem inoculation of *P. taeda* trees with *L. terebrantis* near Eufaula, AL.

Treatment	A_L:A_S	GE[1]	MC (%)
Control	0.72±0.06**a**	0.12±0.01**a**	116.3±3.4**ab**
Wound	0.68±0.06**a**	0.09±0.02**ab**	117.8±5.8**a**
Low	0.64±0.05**ab**	0.08±0.01**b**	114.0±3.6**ab**
Medium	0.63±0.04**ab**	0.09±0.02**ab**	110.3±3.0**ab**
High	0.39±0.04**b**	0.09±0.01**b**	100.5±3.5**b**

[1] GE is calculated as tree annual increment of basal area divided by tree A_L.

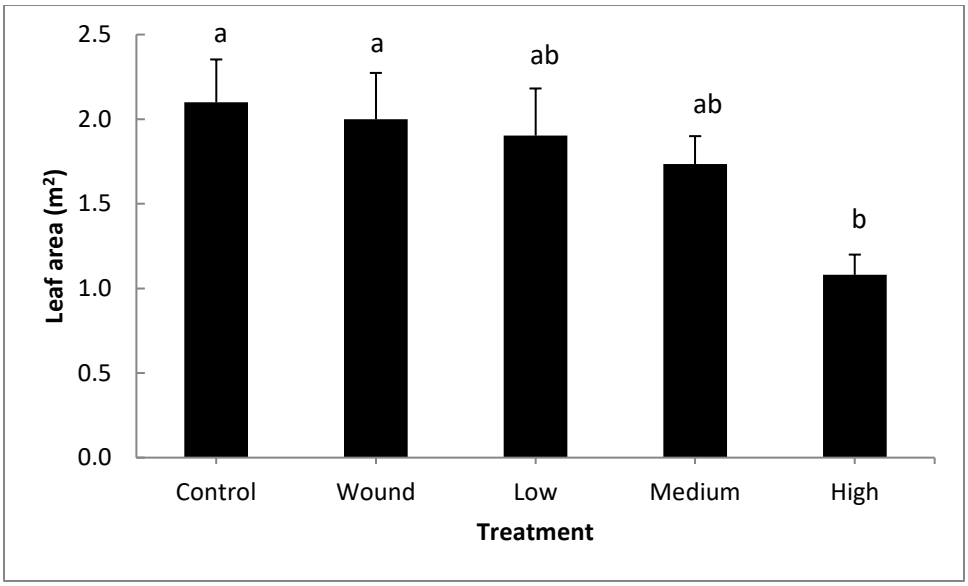


Figure 4. 3: Mean leaf area of *P. taeda* trees inoculated with *L. terebrantis* after destructive sampling in January to February 2020.

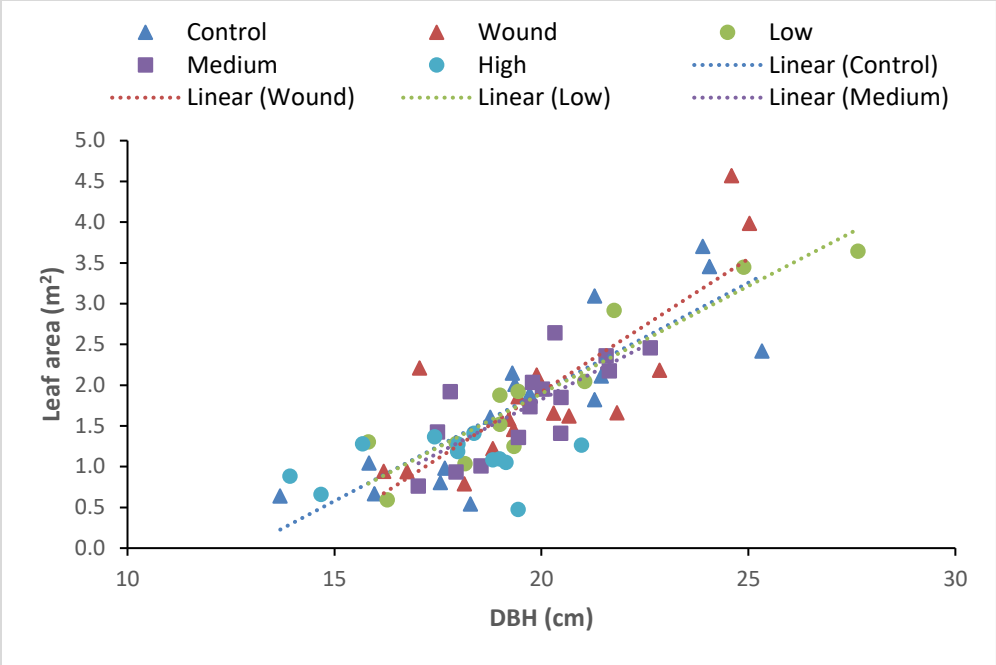


Figure 4. 4: Relationship between leaf area and DBH growth of *P. taeda* following lower stem inoculation with toothpick colonized *L. terebrantis*. Note the non-significant relationship for the high inoculum treatment but the slope of the control, wound, low and medium were significantly higher than the high inoculum treatment.

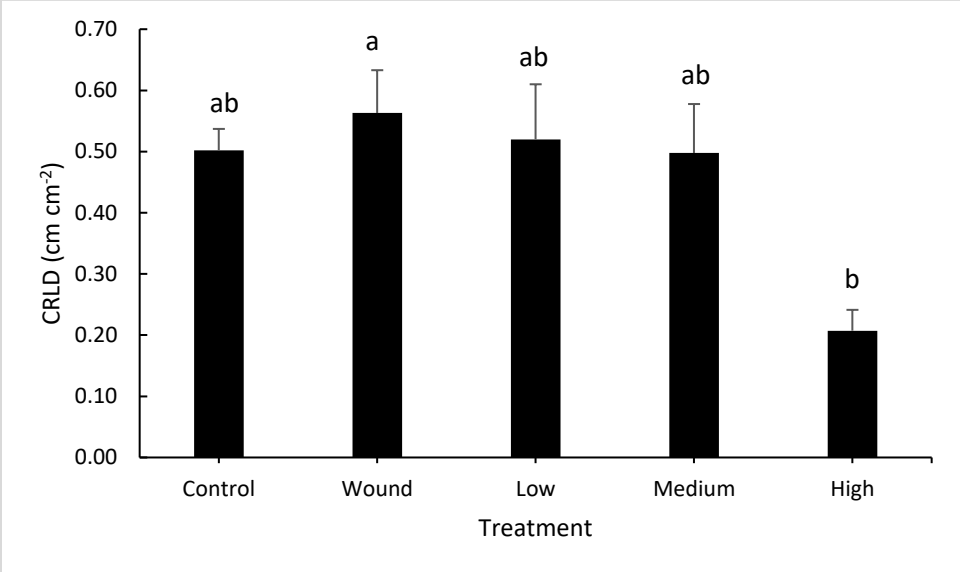


Figure 4. 5: Cumulative root length density (CRLD) in October 2019 from 0 to 50 cm depth following stem inoculation of *P. taeda* with *L. terebrantis.*

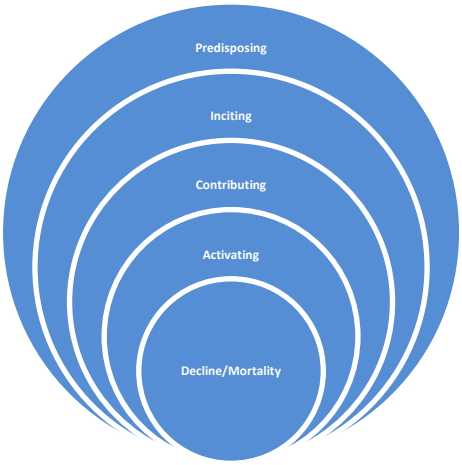


Figure 4. 6: Proposed modified Manion's (1991) model of factors for growth decline in *P. taeda* and *L. terebrantis* pathosystem.

4.7 Discussion

We investigated the potential of *L. terebrantis* to affect *P. taeda* growth and hypothesized that the pathogen infection will impair whole-crown carbon fixation such that tree leaf area, new root production, and stemwood growth become carbon-limited. We further hypothesize that this response is not apparent until together, site conditions and a threshold of *L. terrebrantis* infection can cause carbon limitations that cannot be tolerated by the tree. *Leptographium terebrantis* adversely affected *P. taeda* growth and caused growth decline by compromising xylem function. Decline symptoms however, occur at a critical threshold of pathogen spread and coincided with a seven-months growing season moderate drought. Hydraulic malfunction due to sapwood occlusion caused by the pathogen induced premature foliage senescence and decline in stem growth of *P. taeda* trees.

Leptographium terebrantis infestation caused a reduction in A_L and was most severe at high inoculum density relative to the low, medium, wound and control treatments. Loss of A_L

reduces tree water use, light interception and carbon fixation. Tree growth is a function of A_L and factors that limit A_L reduces tree productivity (Vose and Allen, 1988; Albaugh et al., 1998; Jokela and Martin, 2000). Albaugh et al., (1998) found that in 8-yr-old loblolly pine stand in Scotland County, North Carolina; stem volume increased by 152% with an increase in peak leaf area index (LAI) of 101% after four years of fertilization. Additionally, they established that, irrigation treatment also increased stem volume by 25% with increase in LAI by 16%. The loss of A_L may be attributed to two conditions, drought and poor hydraulic function. First, prolonged drought between March and September of 2019 may have caused premature senescence of older foliage that would normally have senesced only in fall 2019. Premature foliage senescence would have been driven by several factors including drought (Hennessey et al. 1992; Naidu et al., 1993; Warren et al., 2011). Hennessey et al., (1992) noted that the variation in needle-fall patterns in loblolly pine correlates with the droughtiness of the growing season. They found that maximum monthly needle fall occurred earlier in dry years than in wet years when 10-year-old loblolly pine stand was monitored for 5 years after thinning. Second, poor hydraulic function of the stem due to the spread of *L. terebrantis* in the xylem tissues perhaps created water and mineral nutrient limitations to foliage growth which were exacerbated by growing season drought. The pathogen spread must, however, extend over a large cross-sectional area of the conducting sapwood before significant disruption in water transport occurs (Joseph et al., 1998; Liu et al., 2018; Mensah et al., 2020,).

The leaf area of a stem or branch is proportional to cross-sectional sapwood area (A_S) that sustains it (Tyree and Ewers, 1991) and the ratio of A_L:A_S is a key parameter for understanding tree water relations (Whitehead et al.,1984). In this study, the ratio of A_L:A_S was inversely

related to *L. terebrantis* inoculum density, thus, trees inoculated at high inoculum density had the lowest A_L:A_S ratio compared to the low, medium, wound and control trees. As observed with A_L, reduced water availability that was simultaneously due to *L. terebrantis* spread in the sapwood and prolonged growing season drought may explain the lower A_L:A_S ratio in the high inoculum treatment trees. Togashi et al., (2015) noted that in several evergreen species, a positive correlation exist between moisture and A_L:A_S. In a Scots pine stand (*Pinus sylvestris* L), trees growing under moisture stress produce less leaf area per unit conducting sapwood area relative to tree growth in wetter areas (Mencuccini and Grace, 1995). The ratio of A_L to A_S is proportional to hydraulic conductivity (Whitehead et al., 1984), thus it can be inferred that the high inoculum treatment had a lower hydraulic conductivity (HC) relative to the low, medium, wound and control treatments.

A reduction in water transport was manifested in the trend of foliage MC, as it was lowest at high inoculum density. A loss in MC reduces tree vigor as stomatal conductance and photosynthesis are reduced (Drake et al., 2010; Wertin et al., 2010). Mensah et al., (2020) showed that *L. terebrantis* occlusions in young *P. taeda* trees significantly reduced sapwood moisture content in the inoculation zone. Reduction in water transport could adversely affect tree growth and cause branch or crown death during periods of water deficit (Klos et al., 2009; Ganey and Vojta, 2011; Anderegg et al., 2013). Naturally, reduction in resource availability such as moisture stress or drought reduces tree vigor and predisposes tree to attack by bark beetles and their associated fungal pathogens (Lorio Jr et al., 1995; Negron, 2009; Ganey and Vojta, 2011; Hart et al., 2014). It is therefore not surprising that Mensah et al. (Unpublished), found chlorotic crown symptoms and mortality when *P. taeda* trees were inoculated at high *L. terebrantis* inoculum density.

Stem relative growth (RG) correlates with precipitation and in 2017, RG peaked and declined in 2018 and 2019. Among the treated trees, RG was lowest in the high treatment trees prior to pathogen inoculation. But 9 months after inoculation, the high treatment trees had the highest increase (59%) in RG growth compared to less than 30% of the control, wound, low and medium at the end of 2017 (Figure 4.2d). The higher RG growth in 2017 also coincided with maximum precipitation (1311.4 mm) within the study period. This suggests that the contributory role of *L. terebrantis* to *P. taeda* growth decline may not be manifested when precipitation at the study site is similar to the mean annual precipitation of the area.

On the contrary, RG declined in 2018 and 2019 among the treated trees irrespective of the inoculum density or control treatment. This period of growth decline also corresponds with lower precipitation levels/drought in 2018 and 2019. For instance, in 2019, the localized precipitation was approximately 27.2% lower than 2017 and about 30% less the 30 year mean annual precipitation of the area (Figure 4.1). Within this period of drought, growth decline was most severe, 47.5% among the high treatment trees. Furthermore, comparable RG decline was observed for control, wound, and low treatment trees in 2018 and 2019. This suggests that the drought of 2019 was not bad enough to affect RG under minimal (low) *L. terebrantis* infection. This indicates that RG decline will only occur when drought interacts/combined with a critical threshold of *L. terebrantis* inoculum density.

The decline in 2019 RG among trees treated with the high inoculum density coincided with a gradual reduction in cumulative root length density at the 0 to 50 cm soil depth that became significant by October 2019. This loss of new root growth among trees treated with the high inoculum density may have contributed to the loss of RG as a result of compromised function of the tree's absorbing root network. The amount of fine root and ectomycorrhizal

growth is influenced by resource availability (King et al., 2002; Jones et al., 2003; Sayer and Haywood, 2006; Coleman and Aubrey, 2018). King et al., (2002) found that fertilization increased net production of fine and ectomycorrhizal roots in 8-yr-old loblolly pine plantation after three years. In longleaf pine (*Pinus palustris* P. Mill), Sayer and Haywood (2006) observed that severe water limitation during growing season was associated with a delay in peak root growth, and prolonged drought also coincided with a reduction in root starch storage. Similarly, insufficient carbon allocation to the root system in the present study may have reduced new root growth and accelerated fine root and ectomycorrhizae mortality.

In addition to the simultaneous occurrence of poor hydraulic function caused by *L. terebrantis* and growing season drought, reallocation of carbon from stemwood growth to the synthesis of defense chemical compounds may have contributed to RG loss (Klepzig et al., 1995; Schultz et al., 2013; Sampedro, 2014; Villari et al., 2014). Production of defensive compounds increases in response to attack resulting in an increase demand for carbon (Schultz et al., 2013). Carbohydrates support the biosynthesis of plant phenolics and terpenes which are essential in defense against invading pest. Klepzig et al., (1995) found that both phenolics and monoterpenes production in the phloem increased following inoculation of 25-year-old red pine (*Pinus resinosa* Aiton) with *L. terebrantis* relative to non-inoculated trees. In the present study, copious oleoresins were exuded from the inoculation zone of trees treated with the high inoculum density about 5 months after inoculation and this continued till the end of 2019 when a reduction in RG was apparent.

As with tree declines in general, no single factor is known to cause growth loss and according to Manions's model (1991), growth decline occurs after predisposition, inciting, and contributing factors have acted upon the tree sequentially. However, from this study, it is

obvious that notwithstanding the predisposition/inciting of the trees by the 2016 drought prior to inoculation with *L. terebrantis* as a contributing agent, infection require an additional factor of drought before the pathogen can significantly contribute to growth decline. This additional factor for successful interaction between *P. taeda* and *L. terebrantis* pathosystem is suggested as an activating agent (Figure 4.6) to enable the occurrence of tree decline.

4.8 Conclusions

The potential of *L. terebrantis,* a weak wilt pathogen commonly associated with declining pines in the southeastern U.S., to cause growth loss and tree decline was assessed using artificial stem inoculation of plantation *P. taeda* with toothpicks colonized by *L. terebrantis*. The study showed that together with the occurrence of rainfall deficit over a 7-month period during the growing season, this pathogen caused a reduction in foliage moisture content, tree leaf area, and the ratio of tree leaf area and sapwood area. These losses contributed either directly or indirectly to the stemwood growth loss and decline of *P. taeda* trees. This decline was more pronounced in trees treated with the high inoculum density relative to trees treated at lower inoculum densities. A prolonged period of reduced precipitation/drought appeared to act as an activating agent. Thus, in the presence of *L. terebrantis* as a contributing factor for LPD, an additional factor of drought stress (activating agent) is necessary for successful host pathogen interaction. Additionally, *L. terebrantis* must reach critical inoculum threshold before growth decline symptoms can be manifested in loblolly pine trees.

CHAPTER V

Foliar nutrients response of *Pinus taeda* L. to *Leptographium terebrantis* infection

5.1 Abstract

Leptographium terebrantis is a root pathogen commonly associated with declining *Pinus taeda* L. (loblolly pine) in the southeastern U.S. and has been hypothesized to alter *Pinus* foliar nutrition. The influence of *L. terebrantis* inoculation treatments on loblolly pine foliar nutrition and shoot morphology were examined. We found that *L. terebrantis* inoculation significantly affected foliar concentrations of N, Mn, and Fe. Nitrogen concentrations decreased below an adequate level and this reduction was most severe in the high inoculation treatment compared to the control treatment. In contrast, foliar Mn concentration increased within the treatment period and was most elevated in the high inoculation treatment. Shoot length, fascicle number and fascicle density were not affected by inoculation treatment, but mean fascicle length was significantly reduced by the high inoculation treatment.

5.2 Introduction

Mineral nutrient availability is essential for tree growth and productivity. Low soil fertility has been identified as a limiting factor in *Pinus* wood production (Fox et al., 2007a; Chapin et al., 1986). Among the southern pines, insufficient mineral nutrition causes reduced whole-crown leaf area development and hence, low stem growth (Fox et al., 2007). Several studies have shown increases in the radial growth of loblolly pine grown on nutrient-poor sites following fertilization (Albaugh et al., 2004; Jokela et al., 2004; Sayer et al., 2004). Growth losses are attributed to nutrition levels below or above optimum levels that create insufficiencies or imbalances of essential mineral nutrients. In turn, abnormal nutrition may interfere with physiological processes leading to growth loss.

Nutrient-deficient soil is one of several factors that may contribute to growth decline and mortality. Allen et al., (1990) indicated that nutritional limitations occur when nutrients demanded by plants cannot be supplied by the soil or remobilized internally. At the initial stages of tree growth, nutrient demand is low; however, as growth accelerates and tree size increases, the demand for nutrients increases (Miller, 1981). The high requirement and use of nutrients continue until canopy closure or when environmental conditions cannot sustain a high supply of nutrients (Miller, 1981; Piatek and Allen, 1999). Irrespective of the role of nutrients in pine growth and productivity, some available nutrients have greater impacts whilst others have less or variable impacts. For instance, among the macronutrients, nitrogen (N) and phosphorus (P) have been shown to have the greatest influence on loblolly pine growth (Jokela and Martin, 2000; Albaugh et al., 2004). Other macronutrients such as potassium (K) may also improve growth in combination with N and P depending on the site or location (Carlson et al., 2013). In the

southern U.S. macronutrients such as N and P may become deficient at crown closure (Fox et al., 2007).

In order to meet nutrient requirements for growth, some mobile nutrients such as N and P are retranslocated from senescing foliage to active photosynthetic foliage to partially sustain tree growth (Wells and Jorgensen, 1975). But this reallocation may be inadequate to meet the demands of photosynthetically active foliage. Other silvicultural interventions such as multiple fertilizer applications are used by silviculturists for sustainable tree growth (Allen, 2001). Studies have shown that over the years, the acreage of pine forests under fertilization continue to increase. For instance, in 1990 about 200,000 acres of pine plantations were fertilized, and nearly a decade later, the area under fertilization increased 7-fold to about 1.6 million acres (Forest Nutrition Cooperative, 2005).

Regardless of efforts to maximize pine productivity, the presence of root pathogens hinders the ability of pine roots to assimilate and translocate nutrients. The activity of the pathogen within the root sapwood interferes with tree physiology by reducing water and nutrient translocation to foliage. This causes a reduction in the amount of carbon fixed by the needles and allocated to growing shoots leading to a reduction in leaf area (Jokela and Martin, 2000; Albaugh et al., 2004). Ultimately, poor leaf area development risks inadequate carbon allocation to fine root and mycorrhizae maintenance and growth. As a result, root system acquisition of soil resources becomes compromised by both root pathogens and carbon limitations.

The nutritient content of foliage is usually used in assessing the nutritional status of forest stands (Adams and Allen, 1985). It provides an index of the soil supply of, and stand demand for nutrients (Jokela et al., 1991), and may serve as an indicator of nutrient availability in the soil

(Brockley, 2001). Additionally, foliar nutrient ratios have shown promise in identifying nutrient deficient stands (Hockman and Allen, 1990). These ratios are particularly important when either one or both chemical elements are at a deficient level (Marschner, 2011).

Pathogen colonization of the host plant may lead to changes host nutrient status. Sayer et al., (2009) assessed foliar nutrients concentrations in *Pinus palustris* Mill. (longleaf pine) stands that were normal in appearance or exhibited symptoms of low vigor and decline at two military locations, Fort Benning, Georgia and Eglin Air Force Base, Florida. They reported that under both conditions, most macro- and micronutrients were at sufficiency levels. However, foliar Mn concentration was elevated at both sites. Elevated foliar Mn concentration has the potential to disrupt physiological processes tied to carbon fixation and allocation (Mehne-Jakobs, 985; Cakmak and Kirby 2008). Furthermore, Mn has been shown to be toxic to *Pinus elliottii* Engelm. (slash pine) seedlings at 300 mg/kg (Van Lear and Smith, 1990).

Other studies have found changes in nutrient metabolism following pathogen infestation. For instance, host plant N metabolism and tissue concentration may be altered following pathogen infestation (Singh and Bhure, 1974; Walters and Bingham, 2007). Singh and Bhure (1974) found that *Armillaria mellea* (Vahl ex Fr.) Kummer infestation induced reduction in foliar nutrient concentrations of N, P, K, Mg, and Na in coniferous species but caused an increase in foliar Mn, Ca, Fe, and Zn concentrations. In addition, Singh and Bhure (1974) also noted that changes in foliar nutrient levels caused a reduction in the height growth of infected trees. In this study, we assessed the influence of stem inoculation with *L. terebrantis* on *P. taeda* foliar macro- and micro-nutrient concentrations over a 2-year period and subsequent responses of shoot morphology. We hypothesize that stem infestation with *L. terebrantis* will indirectly change

foliar nutrient concentrations and shoot morphology as a result of compromised sapwood function.

5.3 Methods

5.3.1 Study site and experimental design

The study was located in a loblolly pine plantation near Eufaula, Alabama, U.S. in Barbour County (32°1'13.10"N, 85°12'31.76"W). The plantation was situated on the East Gulf Coastal Plain physiographic region and the humid subtropical climatic zone. Soil series identified within the study area included Annemaine and Wahee. Their taxonomic classification is a fine, mixed, semi-active, thermic Aquic Hapludult and fine, mixed, semi-active, thermic Aeric Endoaquult, repectively. Annemaine is the predominant soil series, consisting of a fine sandy loam surface and clayey subsoil, and moderately well drained. Wahee contains a clay loam subsoil overlain by fine sandy loam surface and poorly drained (Trayvick, 2005; Ditzler et al., 2017). Average annual precipitation and air temperature of the area are 1407 mm and 18.1 ^{o}C, respectively (NOAA 2020). The plantation was established in 2003 at1.2 m x 3.0 m spacing using open-pollinated seedlings and third-row thin at 12 years age in 2014. The study site received nitrogen and phosphorus fertilization at planting but no herbicide or pesticide control after planting and has a site index of 22 m at 25 years.

Fifteen plots containing two rows, 3.0 m apart, of 10 trees per each row were established in the plantation at age 13 years in December 2015 in a completely random experimental design with three replications and five inoculation treatments. All plot trees were permanently identified by numbered metal tags and outfitted with a manual dendrometer band (D1, UMS GmbH, Munich, Germany) installed at 1.4 m above the ground line (DBH) on five randomly chosen

trees in each row per plot. A weather station (WatchDog 2000, Spectrum Technologies Inc., Aurora, IL, U.S.) was installed adjacent to the study site to monitor local precipitation, air temperature, solar radiation, relative humidity, and wind speed.

Inoculation treatments were applied to the five randomly chosen measurement trees that were fitted with dendrometer bands in one of the two rows per plot. Treatments of the study included a no inoculation or wounding (control), no inoculation but sterile toothpick wounding (wound), and three levels of increasing fungal inoculum density (low, medium, high). Inoculum densities were selected based on earlier studies that established the relationship between number of *L. terrebrantis* toothpick inoculum points, occluded radial area of the stem (Devkota et al., 2019), and stem hydraulic conductivity in loblolly pine (Mensah et al., 2020). The treatments were applied by a procedure similar to that described by Devkota et al., (2019) with modification due to differences in tree size. For each tree, the number of inoculation points was marked on a stencil sheet adhered to the inoculation zone to ensure proper inoculum placement around the stem circumference. Three series of inoculation points were identified at 1.2 cm, 2.4 cm and 3.6 cm below the initial inoculum point (Devkota et al., 2019). The low, medium, and high inoculum densities received three series of 5-8, 20-28, or 40-58 *L. terebrantis*-colonized toothpicks, respectively, around the circumference of the lower stem in March 2017. The wound treatment was applied similar to the high inoculum treatment.

5.3.2 Soil sampling and chemical analysis

Soil samples for chemical analysis were collected on March 2, 8 and 9, 2017 prior to treatment application. Four soil cores of approximately 6 × 50.8 cm were removed from each plot, capped and transported in a cooler to the USDA Forest Service lab in Auburn, Alabama. Soil samples

were returned within 3 hours of collection and kept at 4°C until processed. Processing consisted of sectioned cores into 10 cm increments and weighed, then sectioned longitudinally and one half dried at 105ºC for 72 hours and the remainder air dried until no change in weight. Coarse materials including stones, root pieces, pine needles and other plant parts were manually removed during processing.

The air-dried soil samples were composited by plot and depth then sieved through a 2 mm opening sieve (No.10). Soil samples were then sent to the Soil Health Assessment Center, University of Missouri, Columbia, Missouri where pH and nutrients were analyzed. Soil pH was determined by water (pH_{water}) (Kalra and Maynard, 1991). Percentage (%) total nitrogen (N_T), and % total sulfur (S_T) were analyzed via combustion analyzer (Kowalenko, 2006). Soil aluminum (Al) and manganese (Mn) (Kachurina et. al, 2008) were extracted with 1M KCl. The quantity of available phosphorus (P), calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), zinc (Zn), copper (Cu), and iron (Fe) were determined according to the Mehlich-3 procedure (Mehlich, 1984) and expressed in mg kg^{-1}.

5.3.3 Inoculation method

Wooden toothpicks sterilized at 121 ℃ for 30 min and soaked overnight in malt extract broth (MEB) (BD Bacto ™ Malt Extract, BD Biosciences, San Jose, CA) were used to culture the fungus in the dark at 23 ºC for 24 days as described by Devkota et al., (2019). Prior to treatment application, the dead outer bark was removed between 20 cm and 30 cm above the ground line with ironton straight draw shave (Northern Tool + Equipment, Burnsville, MN, USA). The inoculation points, approximately 1.2 mm in diameter and 5 mm deep, were drilled into the trees stems through the identified points on the stencil sheet at 27 cm from the ground level. Trees were inoculated by inserting toothpicks containing *L. terebrantis* inoculum

(mycelium and spores) 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 to prevent contamination (Devkota et al., 2019; Mensah et al., 2020).

5.3.4 Needle Sampling, processing and Nutritional Analysis

Pre-treatment foliage was collected in July 2016 from four randomly chosen measurement trees per plot (60). An upper-crown shoot was shot from each chosen tree with a 0.22 caliber rifle and the first flush foliage of 2016 was removed from the internodes. From each foliage sample, approximately 25 3-needle fascicles of the first flush were placed in a paper bag and oven-dried at 70^{o}C to equilibrium (i.e., 72 h). The samples were ground in a Wiley mill to pass through a 0.5 mm mesh screen before being sent to Waypoint Analytical Laboratory (Memphis, TN, U.S.A.) for chemical analysis. Phosphorus (P) concentration was analyzed by combustion (Bryson et al., 2014) whilst nitrogen (N), potassium (K), magnesium (Mg), calcium (Ca), sulphur (S), sodium (Na), boron (B), zinc (Zn), manganese (Mn), iron (Fe), copper (Cu), and aluminium (Al) were determined by a wet digestion standard procedure (Bryson et al., 2014).

Post-treatment sampling and mineral nutrient assessments were done in July 2018 and 2019 as described above with foliage sampled from the first flush of 2018 and 2019, in July 2018 2019, respectively. However, following destructive sampling of the five measurement trees per plot in January and February 2020, the most recently matured flush of 2019 (second flush produced in 2019) was assessed for internode length, number of fascicles, mean fascicle length, and fascicle density (number of fascicles per shoot length).

5.3.5 Analysis

Pre-treatment soil chemistry, and foliar nutrient concentrations, and post-treatment second flush internode length, fascicle number, mean fascicle length, and fascicle density at the time of tree harvest were analyzed by one-way ANOVA with inoculation treatment as the main effect (PROC GLM, SAS Inc., Cary, NC, USA). The main effects of inoculation treatment and treatment duration on post-treatment foliar nutrient concentrations were analyzed by repeated measures ANOVA (Proc Mixed, SAS Inc., Cary, NC, USA) with compound symmetry as the covariance structure. Prior to analysis, each dependent variable was checked for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests respectively. Main and interaction effects were considered significant at $P > F$ values less than 0.05 and significant effects were further evaluated by a pair-wise comparison among means using the post-hoc Tukey honest significant difference (HSD) test for multiple comparisons at $\alpha = 0.05$.

5.4 Results

Pre-treatment soil pH_{water} differed significantly ($P<0.0001$) among the treatment plots (Table 5.1) and was significantly higher (5.4) on the medium inoculum density plot compared to low (5.0) and wound (5.1) and control (5.2) treatment plots. Pre-treatment soil macro- and micro-nutrient levels did not differ significantly by treatment (Table 5.2).

Among the mineral nutrients assessed, pre-treatment foliar concentrations of N, P, and K exhibited the smallest coefficients of variation (CV) which were less than 12% compared to CV values for foliar concentrations of Ca and Na which were greater than 20% (Table 5.3). Prior to treatment, CV values associated with foliar micronutrient concentrations were greater than 20% with those for Mn and B exhibiting the highest CV values of more than 30%.

In response to inoculation treatment and treatment duration, a repeated-measure of analysis variance showed that treatment and its duration but not their interaction significantly affected foliar N concentration (Table 5.4). The foliar concentration of N was highest (1.17%) and lowest (1.04%) for wound and high treatment respectively a year after treatment application (2018). During the second year, the concentration of N reduced to 1.04%, 1.02% and 0.92% and among the wound, control and high, treatments respectively (Table 5.5).

Foliar Mn and Fe concentrations were also significantly affected by inoculation treatment and treatment duration but not their interaction (Table 5.4). Foliar B, Cu, and Al concentrations were significantly affected by treatment duration but not inoculation treatment. Foliar Mn concentration was significantly higher, 417.8 mg kg^{-1} in the high inoculum treatment than the low inoculum treatment, 203 mg kg^{-1} in 2018. The mean Mn concentration in 2019, 360.2 mg kg^{-1} was significantly higher in Mn concentration, 276.7 mg kg^{-1} in 2018. The medium inoculum treatment had a significantly higher Fe concentration, 30.8 mg kg^{-1} in 2019 than the control, 16.4 mg kg^{-1} and wound, 14.3 mg kg^{-1} treatments in 2018. Iron concentration was significantly lower 17.7 mg kg^{-1} in 2018 than 25.6 mg kg^{-1} in 2019. Similar to significant treatment duration effects on foliar Mn concentrations, treatment duration had a marginally significant effect on foliar Ca concentration ($P = 0.0688$) with a 5.3% higher foliar Ca concentration in 2019 compared to 2018.

Inoculation treatment significantly affected mean fascicle length but did not significantly affect internode length, fascicle number, or fascicle density of the second flush of 2019 (Table 5.6). Mean fascicle length of the wound inoculum density treatment was significantly greater than that of the high inoculum density treatment (Figure 5.1). Mean fascicle lengths of the control, wound, low, and medium inoculum density treatments were statistically similar. Mean

internode length, fascicle number, and fascicle density across treatments at the time of tree harvest were 9.8 ± 0.4 cm, 68 ± 3, and 6.9 ± 0.3 fascicles cm^{-1}, respectively.

Table 5. 1: Probabilities of a greater *F*-value **(***P* > *F***)** of pre-treatment soil chemistry variables in March 2017.

Variable[1]	Df[2]	F value	*P* > *F*
pH_{water}	4	9.30	<.0001
N_T	4	0.39	0.8159
S_T	4	1.22	0.3078
P	4	1.18	0.3275
K	4	0.87	0.4845
Mg	4	0.54	0.7099
Ca	4	0.76	0.5567
Na	4	1.25	0.2974
Zn	4	0.93	0.4530
Mn	4	0.56	0.6912
Fe	4	0.27	0.8966
Cu	4	1.24	0.2996
Al	4	1.92	0.1151

[1] pH_{water}, soil pH in water solution; N_T, total combustible nitrogen; total combustible sulphur; P, exchangeable P; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; Zn, zinc ; Mn, manganese; Fe, iron; Cu, copper; and Al, aluminum.
[2] Df, degrees of freedom; *P* > *F*, probability of a greater *F*-value.

Table 5. 2: Mean values of soil nutrients concentration in *P. taeda* tree stand prior to treatment application.

	Inoculation treatment[1]				
Variable	Control	Wound	Low	Medium	High
pH_{water}[2]	5.2 ± 0.1bc[3]	5.1 ± 0.1bc	5.0 ± 0.1c	5.4 ± 0.1a	5.3 ± 0.1ab
N_T (%)	0.04±0.01	0.04±0.01	0.05±0.01	0.03±0.01	0.04±0.01
S_T (%)	0.005±0.001	0.038±0.006	0.005±0.001	0.004±0.001	0.006±0.001
Nutrient (mg kg^{-1})					
P	3.3±0.8	3.3±0.7	5.6±1.2	4.1±0.8	3.5±1.0
K	10.6±2.3	12.5±1.5	10.5±2.4	11.1±2.4	15.5±2.5
Mg	12.6±2.6	12.0±1.6	10.0±2.6	11.3±1.6	14.5±1.6
Ca	36.5±7.7	43.1±8.2	46.9±13.0	40.4±7.2	64.1±9.8
Na	11.1±1.1	9.4±0.5	11.0±0.8	9.4±0.6	11.6±1.3
Zn	0.72±0.06	0.58±0.06	0.63±0.08	0.76±0.08	0.74±0.11
Mn	34.2±6.7	35.6±6.6	24.8±6.4	35.4±6.3	29.0±5.5
Fe	88.5±9.6	85.7±8.5	98.1±11.6	86.5±8.7	88.5±9.1
Cu	0.41±0.08	0.35±0.03	0.47±0.06	0.35±0.03	0.53±0.12
Al	561.7±41.6	560.6±26.3	610.3±42.5	485.8±15.5	605.8±45.5

[1] Control, not wounded; Wound, wounded control; Low, Medium and High are increasing levels of *L. terebrantis* inoculum density assigned for treatment.

[2] pH_{water}, soil pH in water solution; N_T, percentage of dry weight; S_T, percentage of dry weight; P, exchangeable P; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; Zn, zinc ; Mn, manganese; Fe, iron; Cu, copper; and Al, aluminum in mg kg^{-1}.

[3] Means followed by the same lower-case letter are not significantly different by Tukey's Honest Significant Difference test at $\alpha = 0.05$.

Table 5. 3: Descriptive statistics of the foliar mineral nutrition of *P. taeda* in July 2016 prior to stem inoculation with *L. terebrantis*. Foliage was from the first flush produced in 2016.

Nutrient element	N[1]	Mean	SD	CV (%)	Minimum	Maximum
Macronutrients (%)						
N[2]	60	1.09	0.12	11.28	0.86	1.39
S	60	0.10	0.02	17.26	0.05	0.14
P	60	0.10	0.01	8.64	0.09	0.13
K	60	0.42	0.04	9.07	0.31	0.53
Mg	60	0.12	0.02	14.93	0.08	0.16
Ca	60	0.26	0.05	20.52	0.16	0.41
Na	60	0.03	0.01	21.36	0.02	0.04
Micronutrients (mg/Kg)						
B	60	16.98	7.15	42.12	8.00	44.00
Zn	60	27.16	6.05	22.30	15.00	43.00
Mn	60	392.93	127.00	32.32	220.00	766.00
Fe	60	56.93	16.90	29.68	31.00	125.00
Cu	60	4.36	1.04	23.79	3.00	8.00
Al	60	531.79	104.34	19.62	329.00	789.00

[1] N, number of samples; Mean, mean among samples; SD, standard deviation; CV, coefficient of variation; Minimum, minimum value among samples; Maximum, maximum value among samples.

[2]N, nitrogen; S, sulphur; P, phosphorus; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; B, boron; Zn, zinc; Mn, manganese; Fe, iron; Cu, copper; Al, aluminum.

Table 5. 4: Probabilities of a greater *F*-value (*P* > *F*) of foliar nutrient concentrations after stem inoculation of *P. taeda* trees with *L. terebrantis* in March 2017**.** Foliage was sampled in July from the first flush produced in 2018 or 2019.

Effect	Num DF[1]	Den DF[2]	Treatment (T)		Time (Y)		T X Y	
			F Value	***P* > F**	**F value**	***P* > F**	**F Value**	***P* > F**
Macronutrients								
N[3]	4	55	3.18	0.0199	12.21	0.0009	0.67	0.6145
S	4	55	1.61	0.1843	7.49	0.0083	1.32	0.2746
P	4	55	1.26	0.2948	17.3	0.0001	1.37	0.2546
K	4	55	1.16	0.3396	0.24	0.6272	0.8	0.5274
Mg	4	55	1.18	0.3276	0.08	0.7773	0.62	0.6517
Ca	4	55	0.45	0.7715	3.45	0.0688	0.22	0.9274
Na	4	55	1.23	0.3082	0.66	0.4198	1.71	0.16
Micronutrients								
B	4	55	0.79	0.5384	12.45	0.0009	0.17	0.9513
Zn	4	55	0.59	0.6711	0.1	0.7515	0.09	0.9851
Mn	4	55	4.09	0.0055	5.63	0.0212	0.25	0.9073
Fe	4	55	3.64	0.0104	25.14	<.0001	0.48	0.7477
Cu	4	55	1.04	0.3965	400.33	<.0001	0.48	0.7468
Al	4	55	1.28	0.2894	15.44	0.0002	0.74	0.5697

[1]Num DF, degrees of freedom of the numerator; [2]Den DF, degrees of freedom of denominator
[3]N, nitrogen; S, sulphur; P, phosphorus; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; B, boron; Zn, zinc; Mn, manganese; Fe, iron; Cu, copper; Al, aluminum. *P* > *F*, probability of a greater *F*-value

Table 5. 5: Mean foliar nutrient concentrations in July, approximately one (2018) and two (2019) years after stem inoculation of *P. taeda* trees with *L. terebrantis* in March 2017. Foliage was sampled in July from the first flush produced in 2018 or 2019.

Year	Macronutrient (%)	Inoculation treatment[1]					Mean
		Control	Wound	Low	Medium	High	
2018	N[2]	1.07±0.02	1.17±0.05	1.14±0.06	1.12±0.04	1.04±0.03	1.09±0.02
	S	0.06±0.001	0.06±0.004	0.06±0.005	0.07±0.003	0.07±0.006	0.06±0.002
	P	0.09±0.002	0.09±0.004	0.09±0.003	0.08±0.005	0.09±0.004	0.09±0.001
	K	0.41±0.015	0.40±0.017	0.37±0.041	0.37±0.042	0.39±0.017	0.40±0.01
	Mg	0.09±0.002	0.09±0.003	0.10±0.002	0.09±0.008	0.09±0.006	0.09±0.002
	Ca	0.20±0.006	0.19±0.015	0.20±0.021	0.18±0.025	0.18±0.012	0.19±0.005
	Na	0.01±0.001	0.01±0.002	0.01±0.004	0.01±0.003	0.01±0.001	0.01±0.001
	Micronutrient (mg/Kg)						
	B	17.1±0.95	18.2±3.28	16.7±1.45	18.5±1.64	17.2±1.77	17.3±0.70
	Zn	16.2±0.962	16.7±2.512	16.0±3.474	16.7±2.348	17.5±1.190	16.3±0.76
	Mn	252.8±17.1	249.3±43.5	203.0±27.2	380.0±87.3	417.8±63.2	276.7±17.1
	Fe	16.4±0.72	14.3±1.60	22.5±3.79	22.7±2.59	20.2±2.88	17.7±0.76
	Cu	1.1±0.073	1.5±0.224	1.2±0.167	1.2±0.200	1.5±0.224	1.2±0.06
	Al	350.1±15.2	336.5±43.6	393.6±47.9	373.2±19.4	350.5±39.5	354.5±11.7
2019	Macronutrient (%)						
	N	1.02±0.03	1.05±0.05	1.03±0.04	1.08±0.05	0.91±0.03	1.01±0.01
	S	0.07±0.002	0.06±0.005	0.07±0.003	0.08±0.005	0.06±0.003	0.07±0.001
	P	0.07±0.002	0.07±0.003	0.07±0.002	0.08±0.004	0.08±0.002	0.07±0.001
	K	0.40±0.015	0.37±0.050	0.36±0.016	0.38±0.031	0.36±0.022	0.38±0.01
	Mg	0.09±0.003	0.09±0.007	0.09±0.008	0.09±0.012	0.09±0.009	0.09±0.003
	Ca	0.19±0.010	0.21±0.024	0.18±0.030	0.21±0.028	0.25±0.026	0.20±0.009
	Na	0.02±0.003	0.01±0.002	0.01±0.002	0.01±0.002	0.01±0.001	0.01±0.002

Micronutrient (mg/Kg)						
B	12.4±0.60	13.3±1.17	13.5±1.23	15.8±1.57	15.2±2.08	13.2±0.48
Zn	15.9±0.77	16.2±0.80	16.0±1.09	17.0±2.32	17.0±1.15	16.2±0.54
Mn	341.9±27.3	367.3±12.5	311.3±50.7	402.3±66.3	469.8±98.6	360.2±20.9
Fe	24.8±0.94	22.5±1.7	26.0±1.9	30.8±4.9	26.9±3.8	25.6±0.91
Cu	5.7±0.15	5.3±0.21	5.0±0.09	5.8±0.307	5.8±0.36	5.6±0.11
Al	450.9±23.3	443.0±21.7	476.2±42.0	464.7±38.7	432.5±43.3	451.8±15.6

Foliar nutrient sufficiency threshold for *Pinus taeda* according to Albaugh et al. (2010) and references cited therein: N: 1.2%, S: 0.10-0.12%, P: 0.12%, K: 0.35-0.40, Mg: 0.08%, Ca: 0.15%, B: 4-8mgkg^{-1}, Zn: 10-20 mgkg^{-1}, Mn: 20-40 mgkg^{-1}, Cu: 2-3 mgkg^{-1}. Na, Fe and Al sufficiency threshold not determined for *P. taeda*. Note that these values are for samples collected during the dormant season from December to February and cannot be directly compared to growing season data.

[1] Control, not wounded; Wound, wounded control; Low, Medium and High are increasing levels of *L. terebrantis* inoculum density assigned for treatment. [2] N, nitrogen; S, sulphur; P, phosphorus; K, potassium; Mg, magnesium; Ca, calcium; Na, sodium; B, boron; Zn, zinc; Mn, manganese; Fe, iron; Cu, copper; Al, aluminum.

Table 5. 6: Probabilities of a greater *F*-value (*P* > *F*) of four shoot morphological variables approximately three years (34 months) after stem inoculation of *P. taeda* with *L. terebrantis*. Shoots were the second flush produced in 2019 and were measured in January -February 2020.

Variable	Df[1]	F value	*P* > F
Shoot length	4	0.55	0.7010
Fascicle number	4	1.25	0.2971
Fascicle length	4	2.80	0.0327
Density	4	0.56	0.6895

[1] Df, degrees of freedom; *P* > *F*, probability of a greater *F*-value.

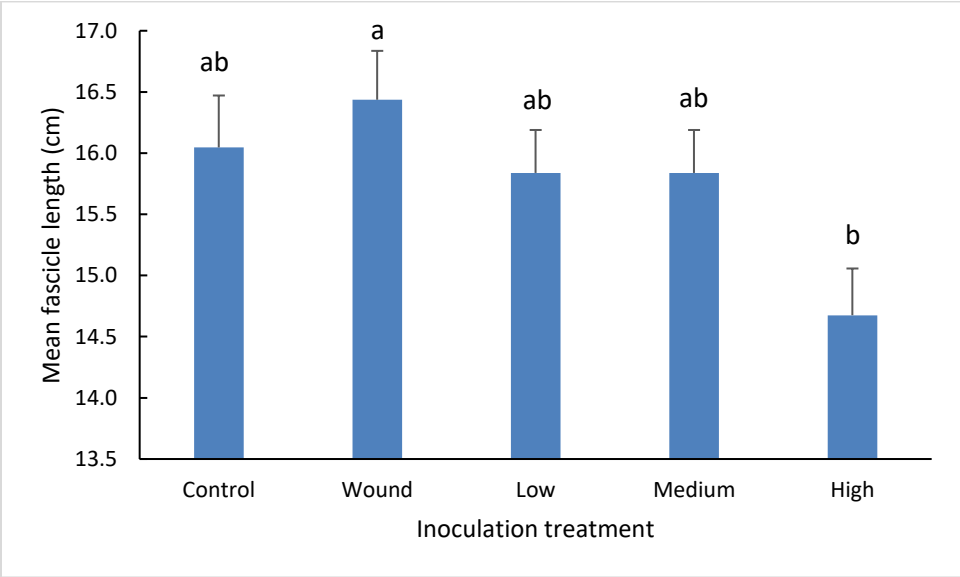


Figure 5. 1: Mean fascicle length of *P. taeda* approximately three years (34 months) after stem inoculation with *L. terebrantis*. Bars represent one standard error of the mean. Means associated with the same lower case letter are not significantly different by Tukey's Honest Significant Difference test at α = 0.05.

5.5 Discussion

Foliar nutrient responses of mature loblolly pine trees to stem inoculation with *L. terebrantis* were assessed over a 2-year period. The pathogen affected foliar N, Mn and Fe concentrations and reduced mean fascicle length. The foliar nutrients and fascicle length changes were most severe in the high inoculum density treatment which also showed symptoms of pine decline.

Soil pH is an important factor for optimum tree growth as it affects the solubility of mineral nutrients in the soil, and their availability to, and uptake by trees. Although soil pH differed significantly before treatments were applied, the range of plot soil pH was 5.0 - 5.4 which is within the optimum pH range (4.5- 6) for loblolly pine production (Schultz, 1997). Therefore, it is unlikely that soil pH influenced inoculation treatment effects in this study. Available soil P in the treatment plots was similar to the critical threshold of 3-5 mg kg^{-1} in loblolly pine stands (NCFS, 2012), thus, available soil P at the study site was at sufficient level for loblolly pine.

Foliar nutrient concentration is an indication of mineral nutrient availability in the soil and foliar macronutrients concentrations prior to treatment application showed less variability compared to foliar micronutrient concentrations. The high variability among foliar micronutrient levels may be due to high spatial variability within the plots. Similarly, Albaugh et al., (2010) noted higher variability among micronutrient levels relative to macronutrient levels when foliar nutrient concentrations were characterized in loblolly pine plantations across the southeastern U.S. The mean initial foliar macronutrient concentrations of N, P, K, S, Ca, and Mg are comparable to critical or adequate levels in loblolly pines (Albaugh et al., 2010; Jokela, 2004). Similarly, concentrations of the foliar micronutrients, Mn, B, and Cu were comparable to earlier

findings (Jokela, 2004; FNC, 2009). This indicates that, soil and foliar nutrient concentrations were present at sufficient levels prior to treatment application.

Leptographium terebrantis inoculation affected foliar N concentration and this effect was evident by 12 months after inoculation and continued until trees were harvested at 34 months post-inoculation. In both years foliar N concentration among the low, medium, wound and control treatments were higher than that of the high inoculum treatment. Nitrogen is one of the essential mineral nutrients for plant growth and its concentration in foliage is closely related to maximum net photosynthesis rate (Evans, 1989). The amount of N uptake from the soil is largely determined by plant growth rate (Gastal and Lemaire, 2002). Mensah et al. (Unpublished) found a decline in relative radial growth among the treatment trees which correlate with a reduction in foliar N concentration. Across treatments, the trajectory of foliar N concentration reduced by 7.3 % between 2018 and 2019.

The reduction in foliar N concentration could be ascribed to water deficit during the 2019 growth season (moderate drought). The water deficit caused less root uptake of N in 2019 compared to 2018 due to low soil water content and earlier fascicle senescence in 2019 than 2018. However, the level of N reduction was most severe in high inoculum treatment (12.5%) as compared to control (4.6%). Foliar N concentrations also suggest that the high inoculum density treatment was subjected to additional water stress not experienced by the other treatments. This additional moisture stress experienced by the high inoculum treatment was due to a decrease in stem hydraulic conductance (HC) caused by the pathogen occlusion. Moreover, a significant reduction in the cumulative root length density (CRLD) of the high inoculum treatment reduced the absorbing root surface area. Collectively, the lower HC and CRLD among the high inoculum treatment may explain the lower foliar N concentration.

Foliar P concentration was significantly affected by treatment period. Across treatments, foliar P concentration was 22% less in 2019 compared to 2018. However, foliar P concentration at pre- and post-treatment with *L. terebrantis* was below sufficiency level of 0.12% (Albaugh et al. 2010; Jokela 2004). This is a direct contrast to soil P concentration which was at sufficiency level. The limited P concentrations in the foliage perhaps may be due to several factors including moisture deficit, soil compaction and soil pH. Soil pH values below 5.5 and between 7.5 and 8.5 have been shown to limits P availability to plants (Penn and Camberato, 2019). The average pH across treatments plot was 5.2, which is below 5.5 and may possibly explain the limited P uptake from the soil.

While N and P are mobile, and exhibited a decrease in foliar concentration between 2018 and 2019, Mn, Fe, Cu, and Al which are plant-immobile, increased in foliar concentration between the same period. A similar, marginally significant trend (P= 0.0688) was observed for Ca which is also plant immobile. Across 2018 and 2019, the high inoculum density treatment yielded higher foliar Mn and Fe concentrations compared to those of the control, wound and low treatments. By 2019, the high inoculum density treatment caused greater sapwood occlusion compared to other treatments (Chapter 6). Sapwood occlusion results in the loss of stem hydraulic conductivity (Butnor et al., 1999; Lee et al., 2006; Mensah et al., 2020). Thus, compromised sapwood function as well as a decrease in new root growth (Chapter 4) suggests that the high treatment trees experienced greater water deficit compared to trees receiving other treatments. A concomitant water stress-induced decrease in crown leaf area by premature senescence (Chapter 4), and concentration of plant-immobile mineral nutrients in transpiring foliage is one explanation for elevated foliar Mn and Fe levels in the high treatment trees.

Although the observed levels of foliar Mn are within the realistic range observed by Albaugh et al. 2010 of 84 to 916 mg kg^{-1}, they are greater than the recommended level of 200 – 400 mg kg^{-1} (Jokela, 2004). Furthermore, elevation of Mn concentration in plant tissue has the potential to negatively alter physiological processes tied to growth. Van Lear and Smith (1990) showed that Mn was toxic to *Pinus elliottii* Engelm. (slash pine) seedlings when foliar levels reached 300mg kg^{-1}. Perhaps foliar Mn in the high treatment trees accumulated to a level that was detrimental to fascicle physiological processes. For example, Kitao et al., (1997) found that the net photosynthetic rate at saturating light and ambient CO_2 decreased with increasing foliar Mn concentrations in white birch (*Betula platyphylla* var. *japonica*). They noted that the carboxylation efficiency, decreased with greater leaf Mn accumulation.

Iron is an essential nutrient for plants but can be toxic when it accumulates at high levels (Connolly and Guerinot, 2002; Santana et al. 2014). For example, Santana et al., (2014) noted anatomical damage, such as protoplast retraction, changes in cell volume, and cell collapse, when *Setaria parviflora* (Poir.) Kerguélen and *Paspalum urvillei* Steudel plants were exposed to excess iron.

Observations of deficient foliar N, elevated foliar Mn and Fe levels were an indirect response to water limitation caused by the simultaneous occurrence of moderate drought and pathogen interference in sapwood function at the high inoculation level. As pathogen spread worsened water stress, carbon limitations led to a drop in new root growth and a loss of whole-tree leaf area (Chapter 4). This leaf area response is attributed, in part, to a 11% decrease in mean fascicle length in response to the high inoculation treatment. It is hypothesized that foliar N deficiency represents an additional compromise to carbon fixation caused by the pathogen.

Similarly, elevated foliar Mn levels that adversely affect photosynthesis challenge the recovery of whole-tree carbon dynamics for normal growth.

5.4 Conclusion

Stem inoculation of *Pinus taeda* trees with colonized toothpicks of *L. terebrantis* affected foliar nutrient concentrations and mean fascicle length which was most severe at high inoculum density treatment. Pre-treatment foliar nutrient concentrations were at sufficiency level, but inoculation with the pathogen affected foliar N, Mn and Fe concentrations. The deficient foliar N, elevated foliar Mn and Fe levels particularly in the high inoculum treatment were an indirect response to water limitation caused by the simultaneous occurrence of moderate drought and *L. terebrantis* interference in sapwood function. As pathogen spread worsened water stress, carbon limitations led to a reduction in fascicle length.

CHAPTER VI

Leptographium terebrantis inoculation and associated crown symptoms and tree mortality in *Pinus taeda*

6.1 Abstract

Leptographium terebrantis S.J. Barras and T.J. Perry, is a bark beetle vectored fungus, commonly isolated from roots of *Pinus taeda* L. undergoing tree decline. Over the past several decades, the root pathogen has been implicated as a contributing factor of *P. taeda* decline and mortality. We examined the potential of *L. terebrantis* to cause decline symptoms and determine the relationship between pathogen spread and the formation new sapwood growth. The study was undertaken in 13-year-old *P. taeda* plantation near Eufaula, Alabama, U.S.A. using artificial inoculations of fungal-colonized, sterilized toothpicks. We found that *L. terebrantis* was not only associated with dying trees but caused decline symptomology and mortality when trees were inoculated at a high density. The highest of three inoculum densities inoculated at a rate of one *L. terebrantis* colonized toothpick per 1.2 cm of bark circumference was the threshold of inoculum density required to produce decline symptomology 19 months following inoculation. It was found that 20% mortality and severe growth loss among surviving trees occurred with *L. terebrantis* infection at the high inoculum density. At the two lower inoculum densities, despite pathogen spread, trees were capable of producing a complete ring of new sapwood that sustained physiological activities. The results of this study suggest that forest management practices which

minimize bark beetle infestation in *P. taeda* plantations will result in reduced pathogen inoculum densities.

6.2 Introduction

Pinus taeda L. (loblolly pine) is the dominant tree species in commercial forest plantations across the southeastern United States. In some settings this tree species may be susceptible to several fungal root pathogens that reduce tree growth and result in mortality (Hansen and Goheen, 2000; Chavarriaga et al., 2007; Gori et al., 2013). The majority of fungal tree root pathogens are members of the Basidiomycetes in the genera: *Armillaria*, *Heterobasidion*, *Phaeolus*, and *Phellinus* (Shaw and Kile, 1991; Worall and Harrington, 1992). Other tree root pathogens, namely *Leptographium* and *Phytophthora* belong to the Ascomycetes and Oomycetes, respectively, and are known to infect *P. taeda* roots and result in tree decline symptomology (Harrington and Cobb, 1988; Hansen, 2015).

Southern pine beetle (SPB) (*Dendroctonus frontalis* Zimm.), is the most destructive pest of *P. taeda*, and accounts for over 80 percent of the insect-related economic loss of these forests estimated at $1.5 billion (Price et al.,1998). Nonetheless, the ability of SPB to kill trees is partly attributed to symbiotic fungal associates that are vectored during insect infestation (Schultz, 1999; Repe and Jurc, 2010). The beetles carry fungi in mycangia or on the exoskeleton (Six, 2003) and infect host trees through feeding activities. *Leptographium* species are among the root pathogens vectored by bark beetles (Harrington and Cobb, 1988).

Fungi associated with bark beetles play a key role in the interaction between the host and infesting beetles (Berryman, 1972; Goheen and Hansen, 1993; Six and Wingfield, 2011). For instance, once established, these fungi alter tree carbon metabolism by stimulating host tree

defenses (Six and Wingfield, 2011). Also, as the pathogen spreads, it may provide supplemental nutrition for the developing bark beetle larvae (Paine et al., 1997). The fungi vectored by bark beetles, however, are not noted for killing host trees (Horntvedt et al., 1983; Lieutier et al., 2009; Six andWingfield, 2011; Krokene, 2015), but are mostly regarded as a contributory factor to tree mortality caused by bark beetle damage to the vascular cambium and phloem (Berryman, 1972; Six and Wingfield, 2011). For example, Horntvedt et al., (1983) described the potential of *Ceratocystis polonica* (Siem.) C. Moreau, an ophiostomatoid fungus vectored by *Ips typographus* L., to kill mature Norway spruce trees (*Picea abies* L. Karsten), but did not observe mortality in artificially inoculated trees. They also noted that a large portion of the sapwood must be infected to inhibit water transport and alter tree physiological processes. In mature lodgepole pines (*Pinus contorta* Dougl. ex Loud. var *latifolia* Engelm. ex S. Wats), Lee et al., (2006) observed the development of chlorotic crowns but no mortality when trees were inoculated with *Leptographium longiclavatum* Lee, Kim & Brueuil. These symptoms only became apparent nine months following artificial inoculation for trees inoculated with a high inoculum density (800 points/m^2) (Lee et al., 2006).

Despite the low incidence of tree mortality caused by *Leptographium* species, in localized settings, bark beetle-associated *Leptographium* fungi have been reported to contribute to mortality in *Pinus* species. For example, *Leptographium wageneri* Kendrick is known to cause considerable mortality in Douglas fir (*Pseudotsuga menziesii* Mirb. Franco), lodgepole pine (*Pinus contorta* Dougl. ex Loud.), and ponderosa pine (*Pinus ponderosa* Dougl. ex P. & C. Laws.), in the U.S. and Canada (Jacobs and Wingfield, 2001). This fungus is vectored by *Hylastes* and *Pissodes* species and once the tree is infected by the pathogen, mortality is attributed to sapwood occlusion caused by the fungus. Other *Leptographium* species are regarded

as either weak pathogens or saprophytes and thus are considered unlikely to either cause disease or mortality (Hansen, 1997).

In the southeastern U.S., several *Leptographium* species such as *L. terebrantis* Barras & Perry, *L. serpens* (Goid.), and *L. procereum* (Kendrick) Wingfield have frequently been isolated from woody roots of declining *P. taeda* trees (Klepzig et al., 1991, Eckhardt et al., 2004; 2007). Particularly, in localized areas across several counties in central Alabama and Georgia in the United States, *Leptographium* species have been isolated from *P. taeda* plantations exhibiting symptoms of decline (Eckhardt et al., 2007; 2010). These fungi are vectored by root-feeding bark beetles such as *Hylastes salebrosus* Eichhoff and *H. tenuis* Eichhoff, the weevil species, *Hylobius pales (*Herbst) and *Pachylobius picivorus* (Germar) (Eckhardt et al., 2004), and lower stem bark beetles such as *Dendroctonus* species (Barras and Perry, 1971; Klepzig et al., 1991). Characteristically, trees attacked by the beetle-fungal complex develop sparse and chlorotic crowns, short needles, reduced radial growth and deterioration of fine roots (Ostrosina et al., 1999; Eckhardt et al., 2007; 2010).

Although *L. terebrantis* infection of woody roots has been associated with declining, mature pine trees, the ability of this fungus to independently cause tree growth decline and/or mortality has not yet been established. To date, studies on the pathogenicity of *L. terebrantis* have primarily focused on the virulence of various isolates at different stages of host development (Devkota and Eckhardt, 2018). Several species of *Leptographium* are pathogenic to seedlings, saplings, and the woody roots of mature pines (Wingfield, 1986; Matusick et al., 2016; Devkota and Eckhardt, 2018; Devkota et al., 2019). Mortality, however, has only been reported for eastern white pine (*Pinus strobus* L.) seedlings following artificial inoculation (Wingfield, 1986; Rane and Tattar, 1987).

Complete sapwood colonization by *L. terebrantis* can occur within weeks after infection of pine seedlings (Devkota and Eckhardt, 2018). However, for mature trees, the period for complete sapwood colonization may take several months to years (Horntvedt et al., 1983; Lee et al., 2006). In a stand of young *P. taeda* trees, Mensah et al., (2020) noted thorough occlusion of sapwood that existed at the time of stem inoculation with *L. terebrantis*. Despite high sapwood occlusion, neither foliage symptoms nor mortality occurred by 24 weeks after inoculation. Furthermore, occluded sapwood-imposed limitations to hydraulic conductivity but the current-year sapwood produced after inoculation remained uninfected with *L. terebrantis* and sustained water transport (Mensah et al., 2020). The study was conducted to assess the potential of *L. terebrantis* to cause crown symptomology and mortality in plantation *P. taeda* trees and determine the relationship between pathogen lesion, occlusion and post-inoculation sapwood growth. We hypothesized that *L. terebrantis* infection will cause sapwood occlusion and loss of hydraulic function and induce formation of sparse crown. Furthermore, crown thinning would limit carbon fixation and allocation for new sapwood growth and cause tree mortality.

6.3 Materials and methods

6.3.1 Study organism

This study was undertaken on mature loblolly pine trees (*Pinus taeda* L.) using the fungus *Leptographium terebrantis* S.J. Barras and T.J. Perry. The fungal isolate (LOB-R-00-805) used for the study was originally isolated from woody roots of declining *P. taeda* trees at Talladega National Forest, Oakmulgee Ranger District, AL, U.S.A. (Eckhardt et al., 2007; Devkota and Eckhardt, 2018) and cultured on sterile toothpicks as described by Devkota et al., (2019). Previous studies found this fungal isolate to be the most virulent among 42 *L. terebrantis* isolates (Devkota and Eckhardt, 2018).

6.3.2 Study site and experimental design

The study was located in a loblolly pine plantation near Eufaula, Alabama, U.S. in Barbour County (32°1'13.10"N, 85°12'31.76"W). The plantation was situated on the East Gulf Coastal Plain physiographic region and the humid subtropical climatic zone. Soil series identified within the study area included Annemaine and Wahee. Their taxonomic classification is a fine, mixed, semi-active, thermic Aquic Hapludult and fine, mixed, semi-active, thermic Aeric Endoaquult, repectively. Annemaine is the predominant soil series, consisting of a fine sandy loam surface and clayey subsoil, and moderately well drained. Wahee contains a clay loam subsoil overlain by fine sandy loam surface and poorly drained (Trayvick, 2005; Ditzler et al., 2017). Average annual precipitation and air temperature of the area are 1407 mm and 18.1 ^{o}C, respectively (NOAA, 2020). The plantation was established in 2003 at1.2 m x 3.0 m spacing using open-pollinated seedlings and third-row thin at 12 years age in 2014. The study site received nitrogen and phosphorus fertilization at planting but no herbicide or pesticide control after planting and has a site index of 22 m at 25 years.

Fifteen plots containing two rows, 3.0 m apart, of 10 trees per each row were established in the plantation at age 13 years in December 2015 in a completely random experimental design with three replications and five inoculation treatments. All plot trees were permanently identified by numbered metal tags and outfitted with a manual dendrometer band (D1, UMS GmbH, Munich, Germany) installed at 1.4 m above the ground line (DBH) on five randomly chosen trees in each row per plot. A weather station (WatchDog 2000, Spectrum Technologies Inc., Aurora, IL, U.S.) was installed adjacent to the study site to monitor local precipitation, air temperature, solar radiation, relative humidity, and wind speed.

Inoculation treatments were applied to the five randomly chosen measurement trees that were fitted with dendrometer bands in one of the two rows per plot. Treatments of the study included a no inoculation or wounding (control), no inoculation but sterile toothpick wounding (wound), and three levels of increasing fungal inoculum density (low, medium, high). Inoculum densities were selected based on earlier studies that established the relationship between number of *L. terrebrantis* toothpick inoculum points, occluded radial area of the stem (Devkota et al., 2019), and stem hydraulic conductivity in loblolly pine (Mensah et al., 2020). The treatments were applied by a procedure similar to that described by Devkota et al., (2019) with modification due to differences in tree size. For each tree, the number of inoculation points was marked on a stencil sheet adhered to the inoculation zone to ensure proper inoculum placement around the stem circumference. Three series of inoculation points were identified at 1.2 cm, 2.4 cm and 3.6 cm below the initial inoculum point (Devkota et al., 2019). The low, medium, and high inoculum densities received three series of 5-8, 20-28, or 40-58 *L. terebrantis*-colonized toothpicks, respectively, around the circumference of the lower stem in March 2017. The wound treatment was applied similar to the high inoculum treatment.

6.3.3 Inoculation method

Prior to treatment application, the dead cork of the bark was scraped around the circumference of the lower stem between 20 cm and 30 cm above the ground line with a 20.3 cm long ironton straight draw shave (Northern Tool + Equipment, Burnsville, MN, USA). The inoculation points, approximately 1.2 mm in diameter and 5 mm deep, were drilled into the trees stems through the identified points on the stencil sheet placed between 23 cm and 27 cm above ground level.

To prepare for treatment application, wooden toothpicks, sterilized at 121 °C for 30 min and soaked overnight in malt extract broth (MEB) (BD Bacto ™ Malt Extract, BD Biosciences, San Jose, CA), were inoculated with *L. terrebranits* or not inoculated and incubated in the dark at 23 ^{o}C for 24 days as described by Devkota et al., (2019). Trees were inoculated in March 2017 by inserting toothpicks containing *L. terebrantis* inoculum (mycelium and spores) 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 to prevent contamination (Devkota et al., 2019; Mensah et al., 2020).

6.3.4 Post fungal inoculation monitoring and measurements

Post-treatment observation of the inoculation zone and tree appearance were undertaken monthly from April 2017 to December 2019. Observations of host response to treatments included oleoresin exudates near the edge of the inoculation zone, presence of chlorotic foliage, and sparse crowns. In January and February 2020 which was 34 months post-inoculation, treated trees were cut at ground-level with a chainsaw and examined for insect attack along the stem of the tree; although none was found. For each tree, the stem section between ground level and 100 cm above ground level that contained the inoculation zone was extracted by a chain saw and transported to the laboratory for lesion and occlusions assessments. One stem disc, 5 cm wide, was extracted above and below 1.3 m (DBH) above ground level. The two stem discs from each tree were sealed in a plastic bag and transported to the laboratory where moisture content was determined by disc weights before and after discs were oven-dried at 70^{o}C to a constant weight. Disc moisture content was expressed as a percentage of oven-dried weight, and stem moisture content was expressed as the mean of stem disc moisture content above and below DBH.

6.3.5 Vertical stem lesion and occlusion assessment

The extent of lesion spread was determined for each tree by assessing the inoculation zone of the extracted 100 cm long stem sections. Duct tape was removed and bark above and below the inoculation zone was gently shaved with an ironton straight draw shave to expose the phloem-cambium interface. Discoloration due to resinosis in the phloem indicated the vertical extent of the lesion caused by pathogen spread. Shaving continued in both upward and downward directions, and around the entire stem section until the distal end of lesions was detected. Lesion length by tree was calculated as the mean of the four values.

In order to assess the extent of stem sapwood occlusion, a chain saw was used to sequentially cut stem discs, 5.0 cm wide, from terminal and basal ends of the extracted stem sections until occluded sapwood was observed. Occlusion was identified by a darkened sapwood appearance (Solheim and Krokene 1998; Lee et al., 2006). Occluded stem length by tree was calculated as the mean of two values on opposite sides of the stem segment.

6.3.6 Radial stem occlusion and sapwood assessment

Areas of occluded sapwood and sapwood growth after inoculation were measured on the basal side of each disc cut from the occluded length of the stem sections. These areas were traced onto a transparent plastic sheet and their areas were determined by a planimeter (Lasico®, Los Angeles, CA, USA). Areas of total sapwood, occluded sapwood, and sapwood growth since inoculation among all 5 cm wide stem discs per 100 cm long stem section were determined by tree. Subsequently, areas of sapwood occlusion and new sapwood growth after inoculation by tree were expressed as a percentage of total sapwood area in 100 cm stem sections.

6.3.7 Classification of sapwood at the inoculation zone

Sapwood infection by *L. terebrantis* was used to classify the transverse sections that were taken through the inoculated zone of the trees. Sapwood at the inoculation zone was classified as continuous new sapwood but non-uniform in width, discontinuous new sapwood around the circumference of the tree, or absence of new sapwood growth. *Leptographium terebrantis* was re-isolated from each transverse section of the harvested trees. From each section a 5 mm of the stem tissue around the inoculation point was plated on selective media (MEA containing 800 mg L^{-1} of cycloheximide and 200 mg L^{-1} of streptomycin sulphate) to confirm the re-isolation of the inoculated fungus from the host tissue. Plates were incubated at 23°C for 14 days and cultures resulting from plating were morphologically identified.

6.3.8 Data analysis

The main effect of *L. terebrantis* inoculum density on stem lesions, sapwood occlusion and new sapwood area formed were analyzed by one-way analysis of variance (Proc GLM, SAS Inc., Cary, NC, USA). The relationship among occluded sapwood, lesions and new sapwood area was analyzed using Proc Reg (SAS Inc., Cary, NC, USA). Prior to analysis, each dependent variable was checked for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests respectively. The treatment effects were considered significant at $\alpha=0.05$ and Tukey's adjustment for differences in least square means. Linear (occlusion vs lesions) and non-linear (new sapwood area vs occlusion area) relationships were also examined. The control treatment was not included in the analysis because of a consistent absence of lesions and occlusions.

6.4 Results

Visibly, a high volume of oleoresins exuded from the inoculation zone of the high inoculum treatment trees with less oleoresins visibly produced by the low and medium inoculation treatment trees by five months after inoculation with *L. terebrantis* (Figure 6.1). The wound treatment trees did not visibly produce oleoresins during the study period. Oleoresin exudation from the high inoculation treatment trees continued but no crown symptoms were evident 12 months post-inoculation. Foliage chlorosis was initially detected among some high inoculum treatment trees 19 months (Figure 6.2) following inoculation after a summer drought in 2018. Foliage chlorosis and the development of sparse crowns continued and were associated with 20% mortality among the high inoculum treatment trees. Lateral roots of some high inoculum treatment trees showed resinosis (Figures 6.3a and 6.3c).

Inoculation treatment significantly affected occlusion lesion length ($P<0.0001$). Lesion length among the low, medium and high treatments were significantly different at 16.0, 21.5 and 27.6 cm, respectively (Figure 6.6a). A significant ($P<.0001$, $r^2=0.95$) linear positive correlation was found between lesion and occlusion lengths caused by the pathogen.

Inoculation treatment significantly affected occlusion length ($P<0.0001$). The trend in occlusion length in response to inoculum density was similar to, but less distinct than that of occluded sapwood area. Occlusion lengths of the high and medium inoculation densities were not significantly different and averaged 38.4 cm (Figure 6.6b). Occlusion lengths associated with both high and medium inoculation densities were significantly greater than that of the low inoculation density (21.7%) and control wound treatment (1.2%). Occluded length of the low inoculation density was significantly greater than that of the control wound treatment.

Inoculation treatment effect significantly (P<0.0001) affected sapwood occlusion area (Table 6.1). The high (83.1%) and medium (36.2%) inoculum treatments were significantly different from each other and from the low and control wound treatments. The low and wound treatments were not significantly different and averaged 8.5% (Figure 6.6c).

Fungal inoculation treatment had no significant effect on stem moisture content (MC) at breast height (P=0.2862) (Table 6.1). However, a non-significant trend in tissue MC was evident as MC decreased between the wound control (89.2%) and inoculum density (low: 86.9%, medium: 84.7%, high: 77.8%) treatments. The pathogen was re-isolated from 100% of the sapwood disks of harvested trees that were inoculated at the low, medium or high inoculum densities.

Post-treatment sapwood growth was not significantly different among the wound control, and low and medium inoculum densities and averaged 31.5%. Post-treatment sapwood growth of the high inoculum density (15.2%) was significantly less than those of the wound control, and low and medium inoculum densities. The pattern of post-treatment sapwood growth in response to an increase in inoculum density is opposite to that of lesion and occlusion lengths, and occluded sapwood cross-section area (Figure 6. 4).

A significant (P<0.0001, r^2=0.89) but non-linear relationship was observed between post-treatment sapwood growth and occlusion area (Figure 6.7). The inflection point of this function (40, 33.4) represents the threshold of occluded sapwood area that was tolerated while post-inoculation sapwood growth continued at a normal level. The low and medium inoculum treatments were 97.5% and 9.2% below the inflection point, respectively and high inoculum treatment was 107.5% above the point. The post-inoculation sapwood of the low and medium

inoculum and control wound trees appeared as a uniform and continuous ring (Figure 6.4a, b, c). The post-inoculation sapwood of the high inoculum density trees was, however, non-uniform in width (Figure 6.5a), discontinuous around the circumference of the tree (Figure 6.5b and 5c), or absent (Figure 6.5d). Among trees treated with the high inoculum density, 27% formed a continuous ring of sapwood and 53% formed a discontinuous ring of sapwood around the periphery of occluded sapwood, and 20% failed to produce sapwood after stem inoculation with *L. terebrantis*.

Table 6. 1: Probabilities of a greater F value (*P>F*) for lesion, occlusion length, occlusion area, new growth and stem moisture content of *P.taeda* inoculated with *L. terebrantis*.

Variable	**Df**	**F value**	***P > F***
Lesion	3	64.29	<0.0001
Occlusion length	3	39.31	<0.0001
Occlusion area	3	146.18	<0.0001
New growth	3	14.16	<0.0001
Moisture content	3	1.31	0.2862

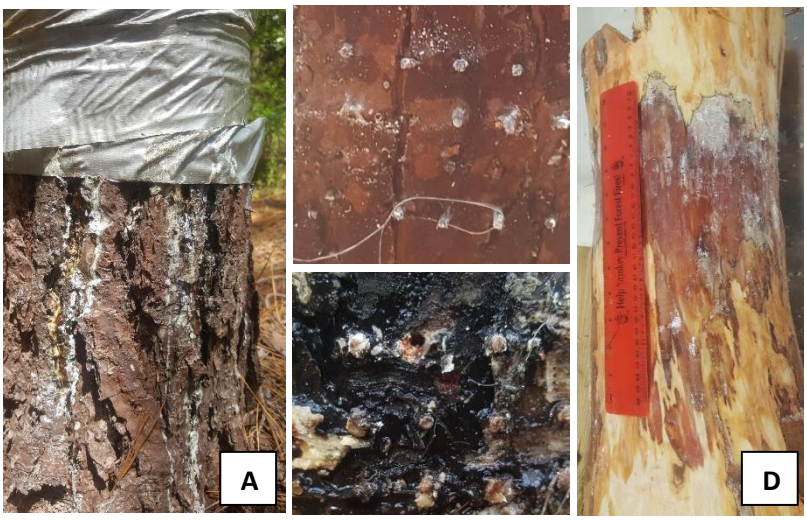


Figure 6. 1: Inoculation zone symptoms of high inoculum treatment trees of *P. taeda* (a) oleoresin exudates (b) wound treatment without oleoresin exudates (c) black stains (d) occlusions extending from the inoculation zone.

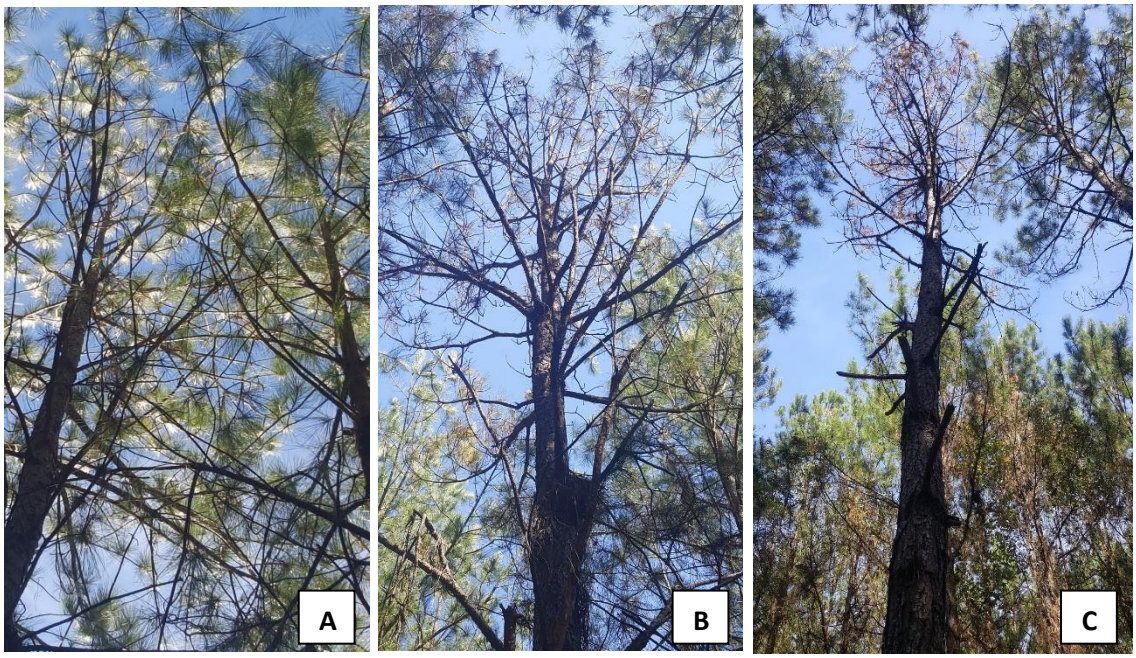


Figure 6. 2: *P. taeda* showing crown symptoms after inoculation with *L. terebrantis* at high inoculum density (a) two trees showing symptomatic crown (b) chlorotic and thin crown (c) crown of dying tree.

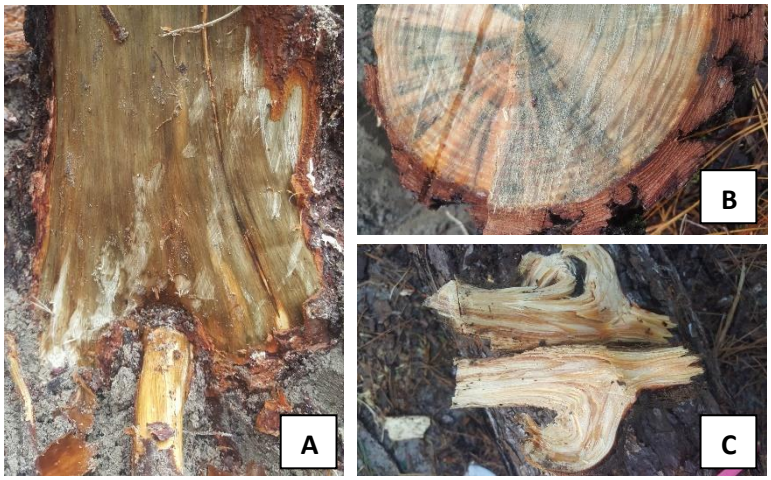


Figure 6. 3: Below ground and stump surface symptoms of high inoculum treatment trees of *P. taeda* (a) lateral root resinosis (b) blue black stains at the surface of a stump (c) split lateral root showing resinosis.

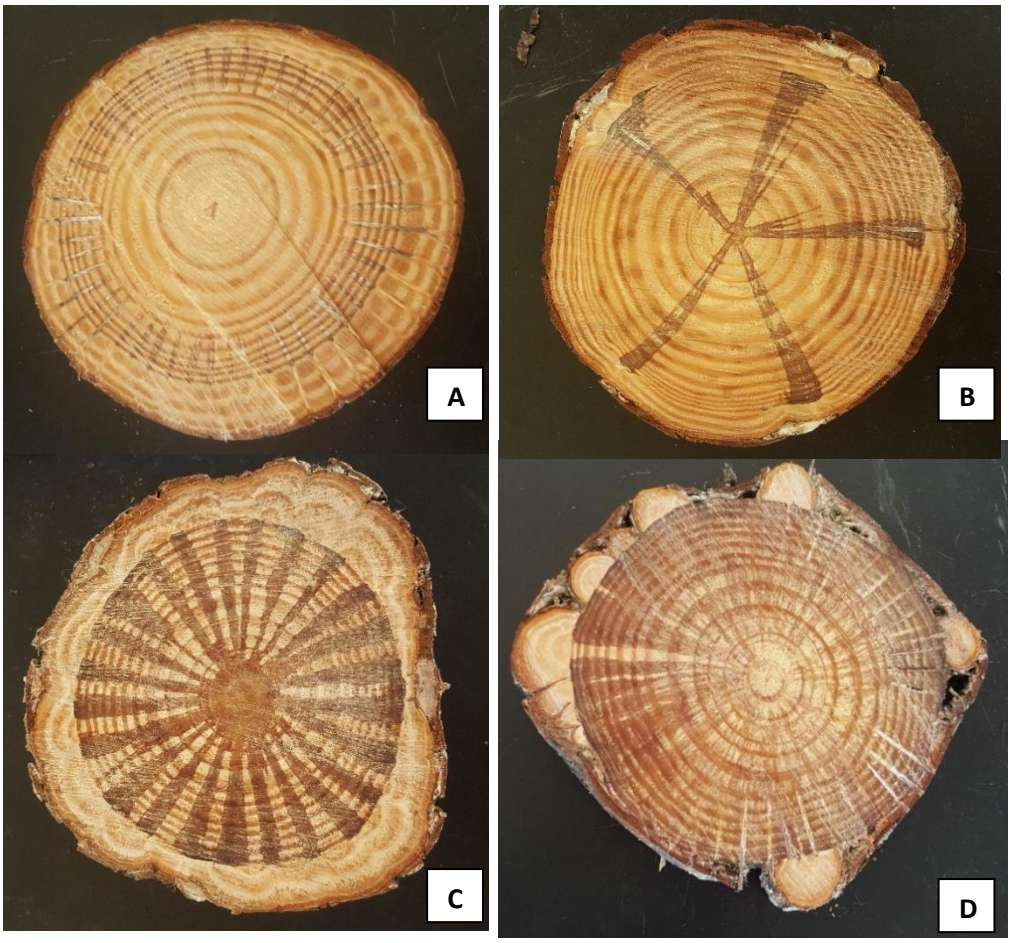


Figure 6. 4: Cross-sections at the inoculation zone of *P. taeda* trees showing infection levels of the different treatment (a) wound treatment with sterile toothpicks (b) occlusions at low inoculum density (c) occlusions at medium inoculum density (d) occlusions at high inoculum density.

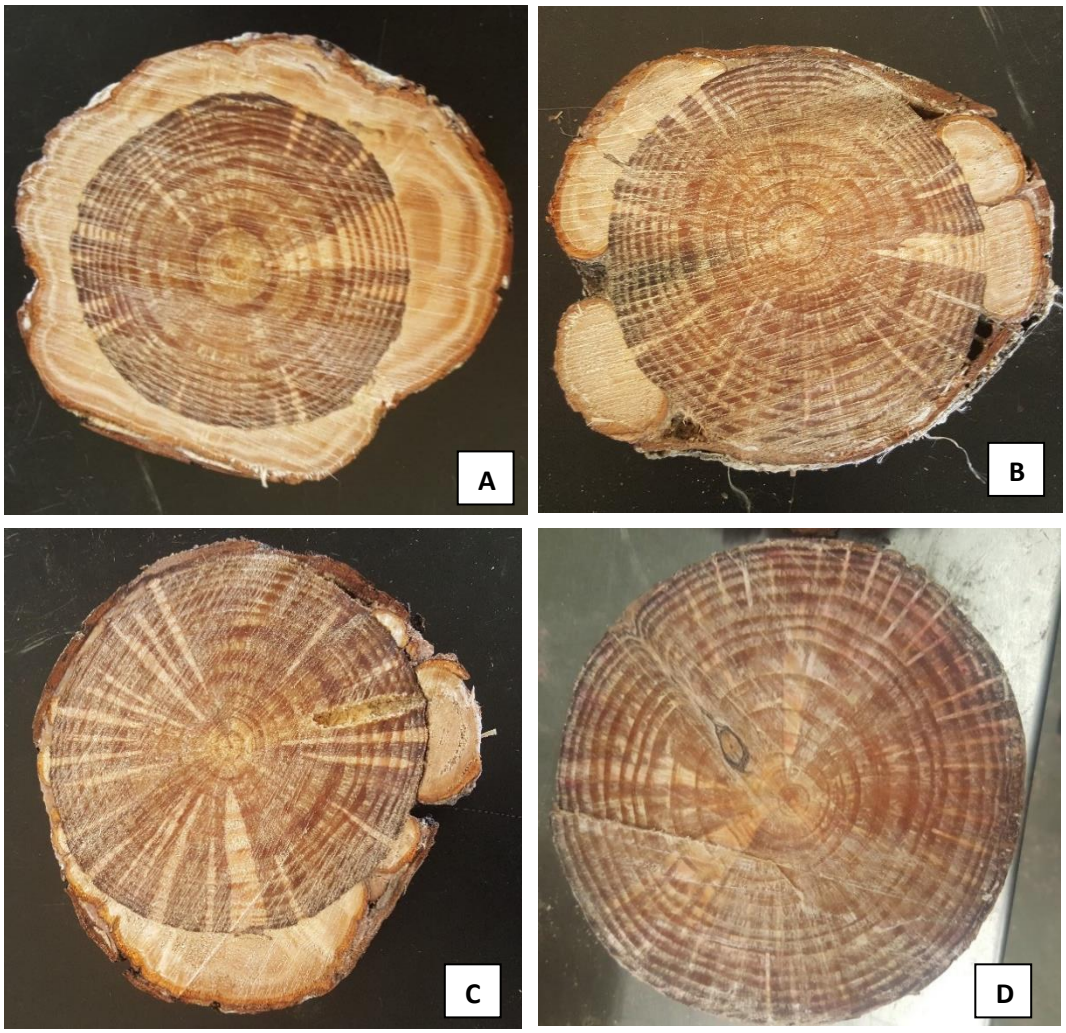


Figure 6. 5: Cross-sections of high inoculum *P. taeda* trees showing new sapwood (a) trees with continuous new sapwood growth (b) discontinuous new sapwood with approximately 50% of new growth ring (c) discontinuous new sapwood with less than 50% of new growth ring (d) trees without new sapwood around occluded sapwood.

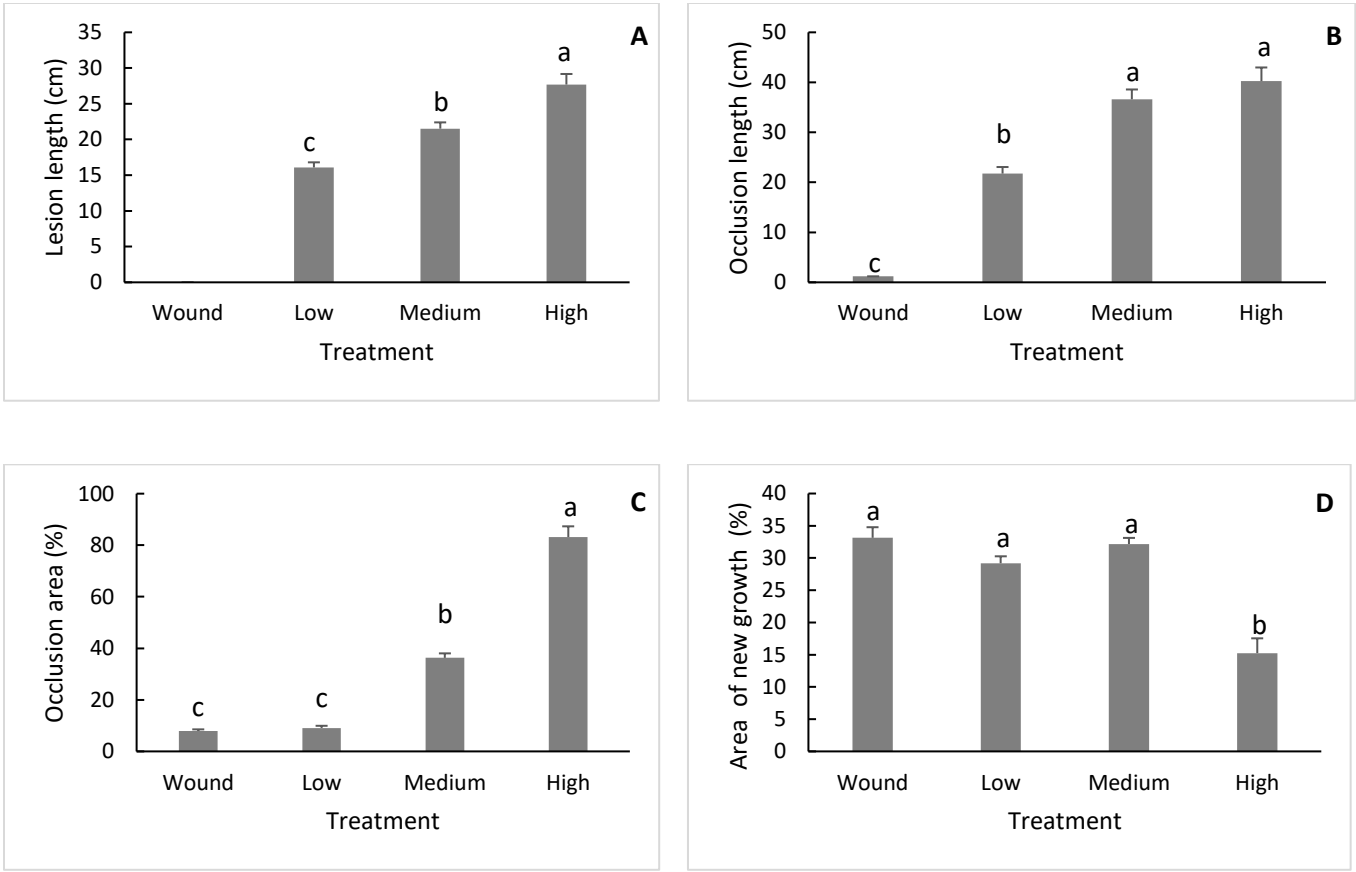


Figure 6. 6: *Pinus taeda* response to *L. terebrantis* infestation following lower stem inoculation: (a) lesions produced in phloem (b) occlusion length (c) occlusion area) and (d) area of new sapwood growth formed following destructive sampling in January and February 2020.

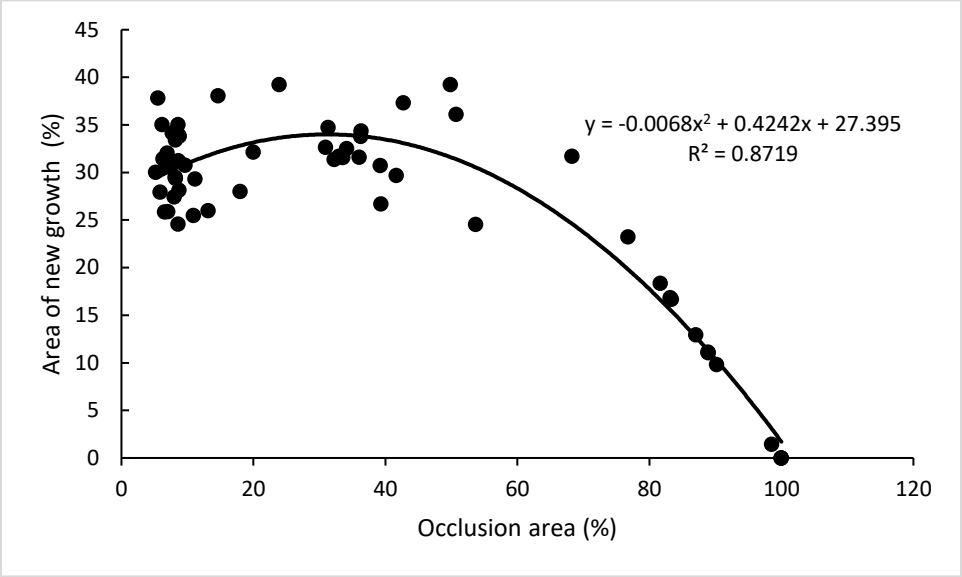


Figure 6. 7: A significant non-linear relationship between area of new sapwood growth devoid of infestation and sapwood occlusion area caused by *L. terebrantis* infestation.

6.5 Discussion

We assessed the potential of the root pathogen *L. terebrantis* to contribute to loblolly pine crown symptomology and tree mortality. Using artificial stem inoculation as a surrogate for woody roots to study the mechanism and process of *L. terebrantis* infection in pine decline, we found that stem infection at high fungal inoculum density caused the formation of sparse crown. The crown deterioration and associated tree mortality, however, occurred during moderate drought at the study site. Nevertheless, below this threshold of fungal infection of one colonized toothpick per 1.2 cm over the bark circumference, there was no manifestation of crown symptomology. It is noteworthy that this threshold applies to artificially inoculated stems, and that the threshold of inoculum density may differ for woody roots of loblolly pine undergoing growth decline.

Fungal inoculum density must reach a critical threshold to cause symptoms development in the host. Among the three *L. terebrantis* inoculum densities, only the high inoculum density

contributed to tree mortality. Previously, pine seedling mortality involving *L. terebrantis* has been reported (Wingfield, 1986) but mortality in mature trees was unknown. Other *Leptographium* species have been shown to cause tree mortality at low inoculum levels when additional tree stresses was imposed. For instance, Solheim et al., (1993) found that *Leptographium wingfieldii* Morelet and *Ophiostoma minus* (Hedge.) H. et P. Syd. fungi killed *Pinus sylvestris* L (Scots pine) when inoculated at 800 points/m^2. Additionally, they noted that both fungi also cause mortality at lower inoculum density when the tree vigor was reduced through pruning. Lee et al., (2006) found that in mature lodgepole pine (*Pinus contorta* Dougl. ex Loud. var *latifolia* Engelm. ex S. Wats), artificial inoculations with *Leptographium longiclavatum* sp. nov., vectored by the mountain pine beetle caused the formation of chlorotic crowns. However, symptoms were only apparent 9 months post-inoculation and only occurred at a high inoculum density (800 points/m^2) without mortality.

The damage caused by *L. terebrantis* infection occurred in two phases. The first phase was direct damage to sapwood caused by *L. terebrantis* which includes lesions and occlusions. Transverse sections of the high inoculum trees at the inoculation zone indicate that over 80% of the sapwood was occluded. The occlusion was due to the deposition of oleoresins and other secondary metabolites in response to pathogen spread (Viiri et al., 2001; Arango-Velez et al., 2018). The comparatively higher lesions and sapwood occlusions produced in the high inoculum treatment trees may explain the apparent whole-tree physiological disorders associated with the high inoculum trees. Additionally, the lesions, a direct cause of pathogen invasion, block phloem tissues and inhibit translocation of photosynthates from the crown to below ground tissues. The growth of new fine roots is largely dependent on new photosynthates (Lynch et al., 2013), which affect fine root turnover.

The second phase of damage was the loss of sapwood and whole-tree hydraulic function when significant *L. terebrantis* spread as a contributing factor, coincided with drought. This indirect response to *L. terebrantis* infection occurred when together, reduced sapwood function caused by the high inoculum density and drought as an inciting factor created water limitations to C-fixation. Moisture stress due to xylem tissue occlusion compromises stomatal conductance leading to a decrease in photosynthesis rate and whole-crown carbon fixation (Wertin et al., 2010; Oliva et al., 2014). By the time trees were destructively harvested, it was evident that post-inoculation sapwood growth was C-limited among trees treated with the high inoculum density. This reiterate the assertion of *Leptographium* sp. being a weak pathogen (Hansen, 1997) and unlikely to independently cause crown symptoms. Specifically, as long as predisposing and inciting factors are not present, the mechanism of *L. terebrantis* in loblolly pine decline may not be manifested.

Stem moisture content was affected beyond the lesion and occlusion areas suggesting a similar effect on foliage water stress. The relatively low MC of the high treatment at DBH suggests that the effect of the sapwood occlusion transcend the inoculation zone and can have whole-tree effects, including foliage MC (Mensah et al., unpublished). The reduction in MC content of the high treatment trees relative to the low and medium treatments, perhaps imposed water and nutrient stress on the foliage, leading to chlorosis, needle shedding, and lack of needle replacement (i.e. thinning crowns) (Butnor, et al., 1999; Lee et al., 2006; Mensah et al., 2020). Collectively, the pathogen inoculum density, moisture stress due to pathogen spread and the drought condition play a crucial role in *L. terebrantis* and *P. taeda* interactions, leading to whole-tree symptomology. Under drought conditions or moisture stress, tree vigor is reduced and pathogen-induced sapwood occlusion and lesions may hasten crown symptoms and tree

mortality, as has been observed in natural pine stands with high pathogen infection (Eckhardt et al., 2007; Kolb et al., 2019). Elsewhere, reductions in tree vigor increases pathogen activity at lower inoculum levels in ophiostomatoid fungi (Solheim et al., 1993).

Tree response to *L. terebrantis* inoculum density could be classified into three levels, continuous new sapwood but non-uniform in width, discontinuous new sapwood around the circumference of the tree, or lack of new sapwood. Continuous new sapwood growth after inoculation was observed in trees that were wounded or received the low or medium inoculum density. In addition, four of the 15 trees receiving the high inoculum density grew a continuous but non-uniform band of sapwood around their stem circumference. Despite the greater occluded sapwood area compared to the wound, low, and medium trees, the "superior" high inoculum trees sustained growth after inoculation. This suggests that the physiological conditions required for adequate C-fixation and allocation to the stem were met in these four "superior" trees. Again, a positive genetic effect on hydraulic function perhaps sustained whole-crown gas exchange at higher rate during the day instead of shutting down. More than half (8 trees) of the high inoculated trees formed a discontinuous sapwood growth around the circumference of the trees. The absence of continuous sapwood growth perhaps may be attributed partly to pathogen damage to, or occlusion of vascular cambium and/or phloem cells. Without active phloem in the vicinity of the vascular cambium, transport to the vascular cambium cells would die.

Three high inoculum trees failed to produce new sapwood growth around the occluded sapwood. Absence of new sapwood formation could be explained by the tree's genetics which did not allow production of oleoresin or other defenses to isolate the pathogen. Again, it may also be attributed to a low ratio of root to shoot, and under the stress of massive sapwood occlusion and a smallish root system. These trees could not keep water supply for C-fixation to

grow adequate sapwood after inoculation to compensate for the massive occlusion of older sapwood. Devkota et al., (2018) demonstrated intraspecies variation in relative susceptibility of *P. taeda* trees to *L. terebrantis* and *Grosmannia huntii* after 8 weeks of infestation. The variation in susceptibility of loblolly pine to the pathogen may be due to the seed source used for the establishment of the stand plantation. Open pollinated stands carry different male genotypes and the differences in genes perhaps may explain the difference in susceptibility of loblolly pine to *L. terebrantis* infection.

Loblolly pine trees and saplings differ in their post-inoculation sapwood response to similar high inoculum *L. terebrantis* inoculation. Susceptibility to decline (inability to sustain sapwood growth) might be greater for older trees compared to younger trees at the same inoculum level. Larger trees have higher maintenance respiration costs and thus will have less fixed C available for growth or even defense at the vascular cambium/phloem location. Again large trees in plantation settings near or at crown closure may have less leaf area/stem basal area and lower light levels than open-grown young trees. Therefore the larger trees may not be able to supply the C as readily for post-inoculation sapwood growth and defense compared to the younger trees. Finally, the hydraulic conductivity of inner sapwood is lower than that of outer sapwood and larger trees may not be able to sustain water supply to their crowns as easily as younger trees. This limits whole-crown C-fixation in larger trees compared to younger trees. For example, Mensah et al., (2020) found that young *P. taeda* trees inoculated at similar inoculation densities could tolerate *L. terebrantis* in naturally regenerated stands approximately 5-7 years old. The young trees produced a complete ring of new growth around the occluded sapwood regardless of the inoculum density (Mensah et al., 2020). In contrast, three high inoculum trees in this study failed to produce new sapwood to sustain hydraulic function culminating in

mortality. This suggests that the chances of *P. taeda* trees survival following attack by bark beetle associated *L. terebrantis* may be determined by the size of new sapwood growth formed.

6.6 Conclusions

The study shows that *L. terebrantis* can contribute to the formation of sparse crown symptomology and tree mortality in plantation *P. taeda*. Crown deterioration occurred at high fungal inoculum density coupled with drought during the study period. Below this inoculum threshold of one colonized toothpick per 1.2 cm over the stem circumference, the trees survived by forming post-inoculation sapwood devoid of fungal infection to sustain hydraulic function. Post inoculation sapwood formation among the high inoculum treatment was either continuous with non-uniform width or discontinuous around the occluded sapwood. High inoculum trees that failed to produce sapwood around the occluded sapwood culminated in tree mortality. This indicates that the chances of *P. taeda* trees survival following attack by bark beetle associated *L. terebrantis* may be determined by the new sapwood growth formed.

CHAPTER VII

Effect of *L. terebrantis* on the production of defensive chemical compounds in loblolly pine

7.1 Abstract

The effect of *L. terebrantis* infected loblolly pine trees on the production of oleoresins and total phenolic (catechin) was assessed. It was hypothesized that *L. terebrantis* infestation would induce oleoresin and catechin production, and cause growth decline as carbon was reallocated for the synthesis of defensive compounds. In addition it was further hypothesized that a positive linear relationship would exist between induced oleoresins and catechins. Inoculation of loblolly pine trees with *L. terebrantis* induced oleoresins production and the quantity produced by the fungal treatments was higher than the wound (induced) and control (constitutive) treatments. In contrast, total phenolics did not differ prior to and post-treatment application, but the quantity of phenolics produced decreased with fungal inoculum density. A significant inverse linear relationship was found between the induced oleoresins and catechin. *L. terebrantis* also grew on MEA amended with different catechol concentrations, suggesting the potential of the fungus to utilize catechol as a source of carbon for growth.

7.2 Introduction

Pinus taeda L. (Loblolly pine) is commercially one of the most important pine species grown throughout the Southern United States. There is a degree of susceptibility of this species to attack by several pests and pathogens, however, there are several structural defense barriers and mechanisms that offer a degree of protection against such attacks (Paine et al., 1987; Seybold et al., 2006; Metsämuuronen and Sirén, 2019; Turner et al., 2019)

The defense barriers are both mechanical and chemical in nature that act sequentially and collectively to ward-off attack by invading pathogens and pests (Nebeker et al., 1992; Ruel et al., 1998; Franceschi et al., 2005). Mechanically, the periderm and secondary phloem (bark) form the first line of defense, which may be strengthened by solid chemical compounds such as calcium oxalate. Calcium oxalate ($C_2H_2CaO_5$) crystals are embedded in the phloem (Hudgins et al., 2003) to provide extra support to the bark, but this inert compound has no effect on pathogens. However, its physical toughness inhibits bark-boring and chewing insects from boring into the tree (Hudgins et al., 2003). Upon breaching the mechanical barriers, chemical compounds are activated and enter the attacking sites.

For pines, several secondary metabolites including terpenes, phenolics and alkaloids, possess antimicrobial properties (Franceschi et al., 2005). These compounds exert defensive pressures against the invaders (pest, pathogens and pest-pathogen complexes) (Lewinsohn et al., 1991; Lieutier, 1993). Based on the quantity and quality of the activated chemical, the invading agent may succumb or be overcome by the chemical barrier. Among the secondary metabolites, oleoresins (terpenes) are the major defensive chemical compound in pines, synthesized by resin ducts and stored in the resin cells (Ruel et al., 1998; Turner et al., 2019). During attack by pests, the resin cells are disrupted and the oleoresins flush out or kill the invaders due to its toxicity

(Franceschi et al., 2005). Ultimately, the wounds created by the invaders are sealed when the volatile component of these exudates (metabolites) evaporates with the non-volatile component of the oleoresins remaining.

The oleoresins are a complex mixture of monoterpenes, diterpenes and sesquiterpenes (Keeling and Bohlmann, 2006), with monoterpenes shown to inhibit fungal growth and development. For instance, Raffa and Smalley (1995) found that mature *Pinus resinosa* Ait and *P. banksiana* Lamb responded to fungal inoculation of bark beetle associated fungi, *Ophiostoma ips* (Rumb.) Nannf., *O. nigrocarpa* (R.W. Davidson) De Hoog and *L. terebrantis* by rapidly increasing monoterpene concentrations at the inoculation site thereby inhibiting fungal growth. Klepzig (1994) also demonstrated the ability of monoterpenes to inhibit spore germination, growth and beetle tunneling in addition to the phenolic inhibition of fungal mycelia growth.

Phenolic compounds are the most studied plant secondary metabolites with antimicrobial properties (Maddox et al., 2010; Daayf et al., 2012). Synthesized by the chloroplast and compartmentalized by storage in vacuoles, the release of phenolics is usually triggered by wounding, pathogen and pest infestation (Wink, 1997). Other environmental factors such as light, temperature, drought and pollutants that impose stress on plant also induce the synthesis of phenolic and other secondary metabolites (Isah, 2019; Berini et al., 2018). Phenolic compounds have several functions but resistance to pathogens and deterrence to pest are predominant (Beckman, 2000). Among the phenolics, the stilbenes, flavonoids and hydroxycinnamic have been demonstrated to possess antifungal properties (Witzell and Martín, 2008; Viiri et al., 2001). Within the conifers, the stilbenes and flavonoids have been extensively studied (Lieutier et al., 1996, Bois and Lieutier, 1997; Hammerbacher et al., 2013). For instance, stilbenes such as pinosylvin (PS) and pinosylvin monomethylether (PSME) and flavonoids like catechin have

been found to accumulate at the zone of fungal infection in *Pinus sylvestris* (Lieutier et al., 1996; Bois and Lieutier, 1997) and inhibit fungal spread.

The quantity and quality of induced secondary metabolites produced by plants may differ based on the stress level, which could affect other biochemical processes of the host (Burke and Carroll, 2016; Yang et al., 2018). The changes in concentration of these metabolites following pathogen invasion, may provide an indication as to the level of susceptibility of the host. This study was undertaken to determine the effect of *L. terebrantis* inoculum density on the induction of oleoresins and total phenolic compound (catechin) in mature loblolly pine trees. Furthermore, we undertook a lab study to assess the fungistatic effect of catechol on *L. terebrantis*, *L. procereum* and *Grosmannia alacris* growth.

It was hypothesized that *L. terebrantis* infestation will induce oleoresin and catechin production, and result in tree growth decline as a result of carbon reallocated for synthesis of defensive compounds. In addition, it was suspected that a positive linear relationship would exist between induced oleoresins and total phenolics, and MEA amended with catechol will suppress radial fungal growth.

7.3 Methods

7.3.1 Study site and experimental design

The study was located in a loblolly pine plantation near Eufaula, Alabama, U.S. in Barbour County (32°1'13.10"N, 85°12'31.76"W). The plantation was situated on the East Gulf Coastal Plain physiographic region and the humid subtropical climatic zone. Soil series identified within the study area included Annemaine and Wahee. Their taxonomic classification is a fine, mixed, semi-active, thermic Aquic Hapludult and fine, mixed, semi-active, thermic Aeric Endoaquult,

repectively. Annemaine is the predominant soil series, consisting of a fine sandy loam surface and clayey subsoil, and moderately well drained. Wahee contains a clay loam subsoil overlain by fine sandy loam surface and poorly drained (Trayvick, 2005; Ditzler et al., 2017). Average annual precipitation and air temperature of the area are 1407 mm and 18.1 ^{o}C, respectively (NOAA, 2020). The plantation was established in 2003 at1.2 m x 3.0 m spacing using open-pollinated seedlings and third-row thin at 12 years age in 2014. The study site received nitrogen and phosphorus fertilization at planting but no herbicide or pesticide control after planting and has a site index of 22 m at 25 years.

Fifteen plots containing two rows, 3.0 m apart, of 10 trees per each row were established in the plantation at age 13 years in December 2015 in a completely random experimental design with three replications and five inoculation treatments. All plot trees were permanently identified by numbered metal tags and outfitted with a manual dendrometer band (D1, UMS GmbH, Munich, Germany) installed at 1.4 m above the ground line (DBH) on five randomly chosen trees in each row per plot. A weather station (WatchDog 2000, Spectrum Technologies Inc., Aurora, IL, U.S.) was installed adjacent to the study site to monitor local precipitation, air temperature, solar radiation, relative humidity, and wind speed.

Inoculation treatments were applied to the five randomly chosen measurement trees that were fitted with dendrometer bands in one of the two rows per plot. Treatments of the study included a no inoculation or wounding (control), no inoculation but sterile toothpick wounding (wound), and three levels of increasing fungal inoculum density (low, medium, high). Inoculum densities were selected based on earlier studies that established the relationship between number of *L. terrebrantis* toothpick inoculum points, occluded radial area of the stem (Devkota et al., 2019), and stem hydraulic conductivity in loblolly pine (Mensah et al., 2020). The treatments

were applied by a procedure similar to that described by Devkota et al., (2019) with modification due to differences in tree size. For each tree, the number of inoculation points was marked on a stencil sheet adhered to the inoculation zone to ensure proper inoculum placement around the stem circumference. Three series of inoculation points were identified at 1.2 cm, 2.4 cm and 3.6 cm below the initial inoculum point (Devkota et al., 2019). The low, medium, and high inoculum densities received three series of 5-8, 20-28, or 40-58 *L. terebrantis*-colonized toothpicks, respectively, around the circumference of the lower stem in March 2017. The wound treatment was applied similar to the high inoculum treatment.

7.3.2 Inoculation method

Prior to treatment application, the dead cork of the bark was scraped around the circumference of the lower stem between 20 cm and 30 cm above the ground line with a 20.3 cm long ironton straight draw shave (Northern Tool + Equipment, Burnsville, MN, USA). The inoculation points, approximately 1.2 mm in diameter and 5 mm deep, were drilled into the trees stems through the identified points on the stencil sheet placed between 23 cm and 27 cm above ground level.

To prepare for treatment application, wooden toothpicks, sterilized at 121 °C for 30 min and soaked overnight in malt extract broth (MEB) (BD Bacto ™ Malt Extract, BD Biosciences, San Jose, CA), were inoculated with *L. terrebranits* or not inoculated and incubated in the dark at 23 ^{o}C for 24 days as described by Devkota et al., (2019). Trees were inoculated in March 2017 by inserting toothpicks containing *L. terebrantis* inoculum (mycelium and spores) 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 to prevent contamination (Devkota et al., 2019; Mensah et al., 2020).

7.3.3 Resin Sampling

Thirty trees within the stand were sampled prior to treatment application in March 2017. The north-south side of each tree was sampled by punching a hole with a 1.9cm diameter arch punch (No. 149) (Osbourne). A plastic connector was then screwed onto each hole to direct resin into a pre-weighed plastic tube attached to each connector (Figure 7.5a). The tubes were removed after 24hrs and transported to the laboratory on ice for analyzes. The average resin weight was measured and determined for each tree from the two installed tubes. A linear relationship among dbh, height and volume of resin was developed to estimate the amount of oleoresins produced by the non-sampled trees within the stand. In March 2019, 75 trees were sampled as described above to determine oleoresin flow rate.

7.3.4 Branch tissue sampling and phenolic assay

An upper crown shoot tissue was collected by shooting with a rifle and the foliage removed from the branch. The buds and foliage "candles" were cut-off and 10 cm of the main and lateral woody branches from the tip were cut and kept in paper bags. The bags were transported to the laboratory on dry ice and freeze dried for about 24 hrs. The dried tissue samples were ground into powder in a Wiley mill and allowed to pass through a 0.5 mm mesh screen. Fifty (50) mg of the powdered sample was extracted three times with 1 ml of 70% acetone with mixing for 30 min at 25 °C. Following each extraction, the insoluble material was pelleted by centrifugation (16,000 g, 2 min), and the supernatants were pooled by sample. Total phenolic concentration in the soluble fraction was determined by the Folin-Ciocalteu method as described by Booker et al., (1996). Extracted samples were diluted to 6.5 ml with 70% acetone, and 40 µl aliquots was mixed with 475 µl of 0.25 N Folin-Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO) followed three minutes later by 475 µl of 0.6 M Na_2CO_3. The sample

solution was incubated in darkness for 45 minutes and the absorbance was measured at 760 nm. Catechin was used to prepare a standard curve and the concentration of each sample was determined from the curve after measuring its absorbance, and results were expressed as catechin equivalents per mg dry mass.

7.3.5 Laboratory study

Malt extract agar (MEA) (BD Bacto ™ Malt Extract, BD Biosciences, San Jose, CA) media as described by Devkota et al., (2019) and amended with catechol at 10, 20, 50 and 100 mg/L was used for this study. Subsequently, 15 ml each of the amended media and control (un-amended media) was poured into Petri dishes (100 x15 mm) and replicated five times. Two perpendicular lines were drawn at the base of the Petri dish and inoculated with a 5 mm disc of actively growing pure cultures of *L. procereum* Kendrick M.J. Wingfield, *L. terebrantis* and *Grosmannia alacris* T.A. Duong, Z.W. de Beer and M.J Wingfield. The Petri dishes were incubated at 23^{o}C in the dark for 8 days and the mean radial growth measured along the lines.

7.3.6 Data analysis

Pre- and post-treatment total oleoresins and phenolics, and mean radial growth of the fungi were analyzed by one way analysis of variance (ANOVA) (Proc GLM, SAS Inc., Cary, NC, USA). The relationship among total phenolic content and oleoresin was analyzed using Proc Reg (SAS Inc., Cary, NC, USA). Prior to analysis, each dependent variable was checked for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests respectively. The main and interactive treatments effects were considered significant at α=0.05 and were further evaluated by a pair-wise comparison among means using the post-hoc Tukey honest significant difference (HSD) test for multiple comparisons.

7.4 Results

The pre-treatment constitutive oleoresins did not differ significantly (P=0.5315) among the trees and the average oleoresin flow rate was approximately 1.0 g per day. Maximum (1.11 g per day) and minimum (1.04 g per day) oleoresins were produced by the low and high treatments respectively (Figure 7.1a, Figure 7.5a). In contrast, pathogen inoculation significantly (P=0.0007) affected oleoresin flow rate. The oleoresin flow rate of the wound, low, medium and high inoculum treatments (induced) were not significantly different and averaged 0.75 g per day. The control treatment (constitutive) had a significantly lower oleoresin flow rate of 0.47 g per day as compared to the average of the medium and high inoculum treatment flow rate of 0.95 g per day (Figure 7.1).

The pre-treatment total phenolic content did not differ significantly (P=0.5105) among the treatment trees and the average total phenolic content was 43.1 µg catechin/mg dry tissue. The trees selected for wound and medium treatment produced the maximum (45.7 µg catechin/mg dry tissue) and minimum (40.7 µg catechin/mg dry tissue) total phenolic content respectively. Similarly, post-treatment total phenolic content was not significantly different (P=0.5605) and averaged 42.5 µg catechin/mg dry tissue. Nonetheless, a reduction trend in total phenolic content was obvious among the low, medium and high inoculum treatments. The total phenolic content decreased with increasing fungal inoculum density and the low treatment had the highest total phenolic content of 42.5 µg catechin/mg dry tissue (Figure 7. 2).

A negative trend was found between post-treatment total phenolics and oleoresins among the low, medium and high treatment levels which was absent in the control and wound treatments. When pooled together, a significant linear negative relationship (P=0.0184; r^2=0.32)

was found between total phenolics and oleoresins among the three levels of fungal inoculum (Figure 7.3).

The in-vitro assay demonstrated that *L. terebrantis* can grow on MEA amended with different concentrations of catechol. At 10 and 20 mg/L catechol amendment levels, mean radial growth of the pathogen was not significantly different from the unamended media, but at higher catechol levels growth was found to be significantly lower than the control (Figure 4a). A similar growth trend was found among other ophiostomatoid fungal species such as *Leptographium procerum* and *Grosmannia alacris*. However, *L. procereum* and *G. alacris* had the minimum and maximum growth on the catechol amended MEA after 8 days of incubation (Figure 7.4b, c and Figure 7.6).

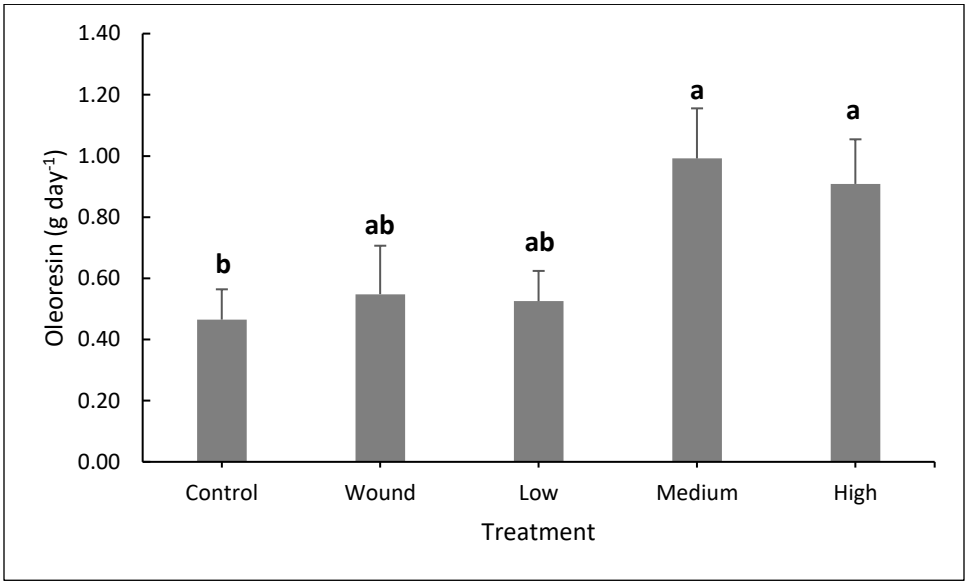


Figure 7. 1: Amounts of oleoresins produced by *P. taeda* following inoculation with *L. terebrantis* colonized or sterile toothpick and non-inoculated control treatment.

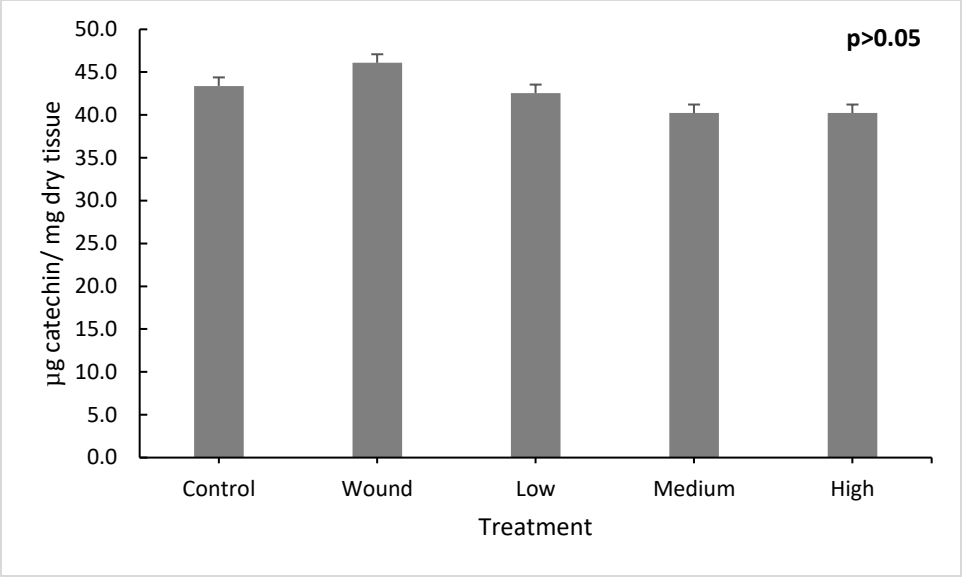


Figure 7. 2: Amounts of total phenolic produced following inoculation with *L. terebrantis* colonized or sterile toothpick and non-inoculated control treatment. Note the reduction in total phenolic content among the low, medium and high inoculum treatments relative to the control and wound treatments.

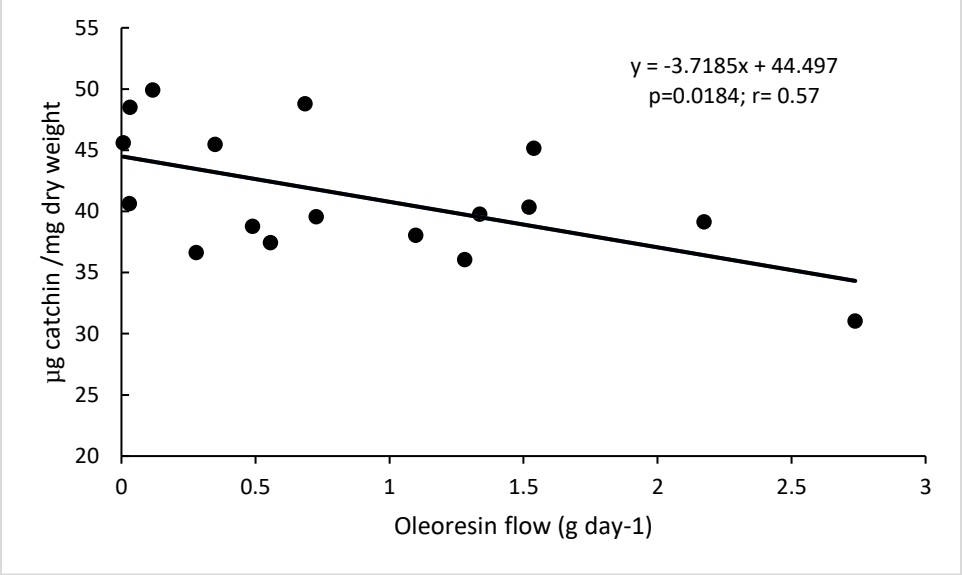
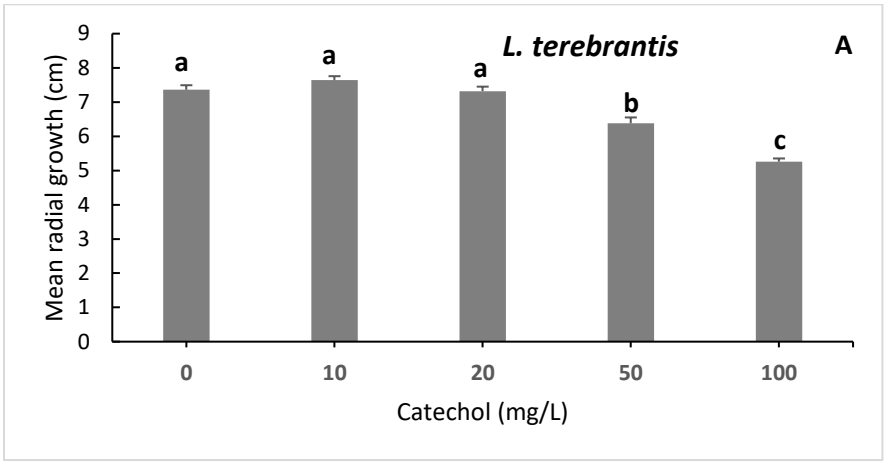
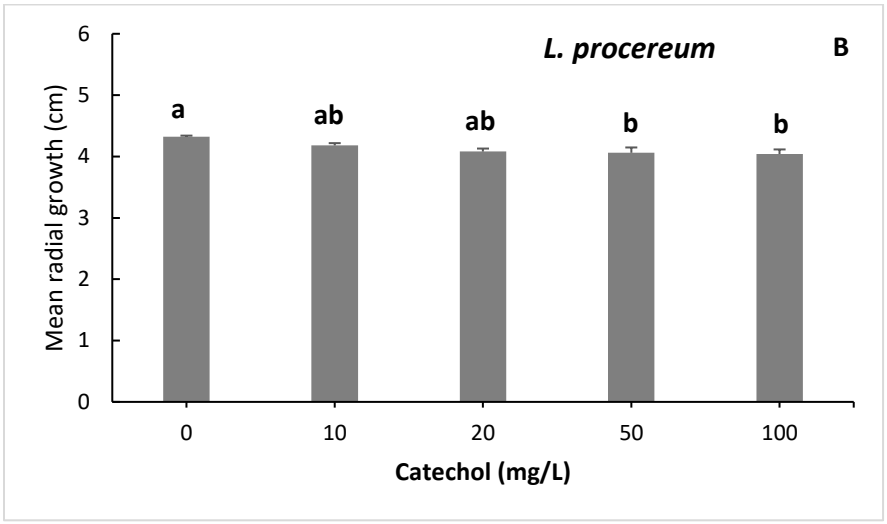


Figure 7. 3: Relationship between total phenolic content (catechin) and oleoresin flow rate after inoculation of the *L. terebrantis* inoculum densities.





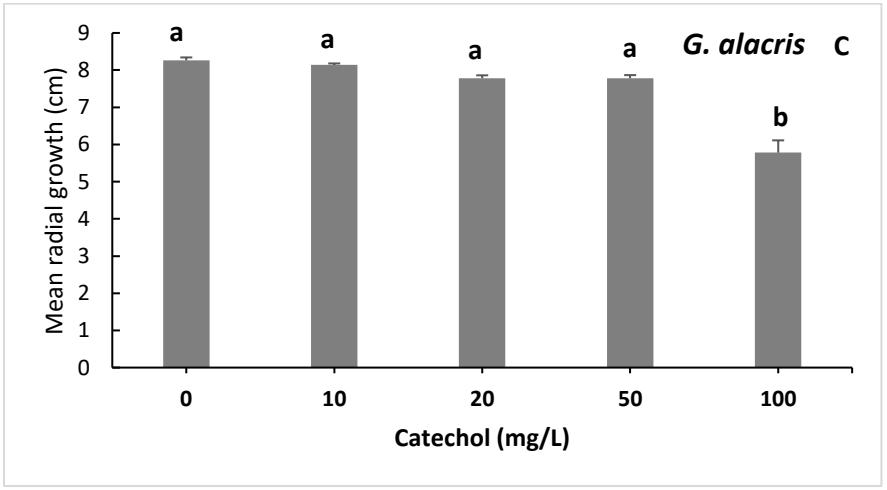


Figure 7. 4: Mean radial growth of bark beetle associated fungi on MEA amended with catechol solution and incubated at 23ºC for 8 days (a) *L. terebrantis* (b) *L. procereum* (c) *G. alacris*.

Figure 7. 5: (a) Oleoresins production from the north and south sides of loblolly pine tree prior to *L. terebrantis* inoculation (b) post-treatment oleoresins exudates from the high inoculum treatment trees (c) crystals of oleoresins in the phloem tissues of high inoculum treatment trees.

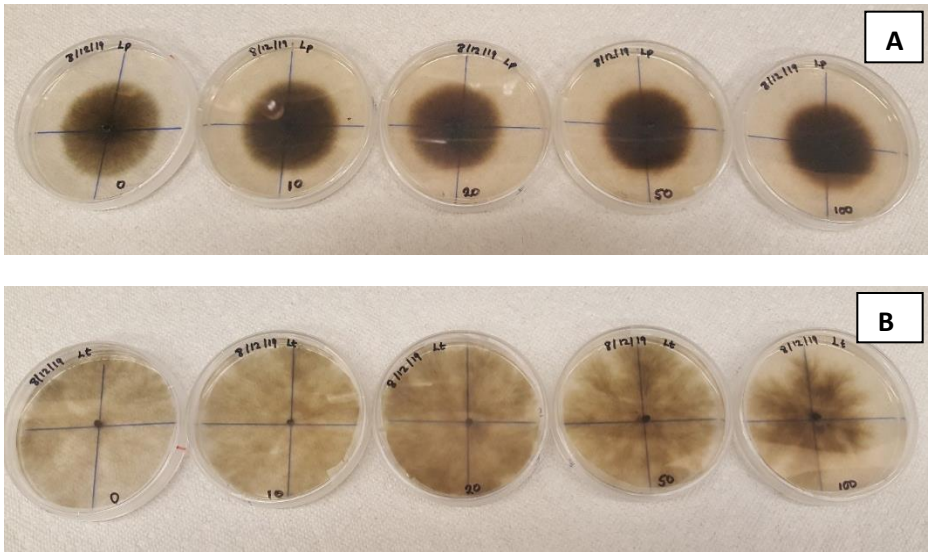


Figure 7. 6: (a) Growth of *L. procereum* on amended MEA after 5 days of incubation (b) and *L. terebrantis* on amended MEA after 10 days of incubation at 23^{o}C.

7.5 Discussion

The study assessed the production of defensive chemical compounds found in mature loblolly pine trees after inoculating them with sterile toothpicks colonized with *L. terebrantis* at different inoculum densities. The trees responded to *L. terebrantis* inoculation by inducing copious oleoresins production at high inoculum density, but total phenolic content decreased at high inoculum density. This suggests that the fungus can potentially metabolize phenolics and use it as a source of carbon for growth in contrast to the oleoresins.

Inducible oleoresins either by sterile toothpick (wound) or *L. terebrantis* colonized toothpicks (low, medium and high) was higher than the constitutive oleoresins in the control treatment. Several studies have reported a higher amount of oleoresins production following induction by wound, pathogens or pest attack (Klepzig et al., 1995; Knebel et al., 2008; Hood and Sala, 2015). For instance, Knebel et al., (2008) found that wounding loblolly pine with *Ophiostoma minus* (Hedgc.) Syd. & P. Syd inoculation caused an increased in resin flow relative

to wounding. However, increasing the inoculum density of *O. minus* did not yield higher amount of resin. Klepzig et al., (1995) also found that both phenolic and monoterpene production in the phloem increased following inoculation of 25-year-old red pine with *L. terebrantis* relative to non-inoculated (constitutive) trees. Nonetheless, differences in monoterpenes in wound inoculated and *L. terebrantis* inoculations were inconsistent. Qualitatively, they noted the *L. terebrantis* inoculated trees also contain higher amounts of monoterpenes constituents such as alpha and beta pinene compared to either wound inoculated or unwounded trees (Klepzig et al., 1995). But in this study, oleoresin produced by the wound inoculated trees was lower than either the low, medium or high fungal treatments.

Among the three inoculation densities of *L. terebrantis*, the medium treatments induced the highest amount of oleoresins at the time of sampling. It was anticipated that the high treatment would induce more oleoresins compared to the medium treatment. The relatively low oleoresin induced in the high treatment trees can be attributed to the initial visible oleoresin flow from the inoculated zone of the high inoculum treatment during summer of 2017, which was negligible in the low and medium treatments (Figure 7.6). Hood and Sala (2015) noted that vigorously growing trees tend to produce more resin than slower growing trees. It is therefore not surprising that in 2017, Mensah et al. (unpublished) found higher relative radial growth in the high treatment compared to low and medium treatments. This initial oleoresin flow perhaps contributed to the reduced oleoresin levels among the high inoculum treatment trees during sampling in 2019.

In addition, the visible oleoresin flow during the summer period may be due to higher temperature and the allocation of carbon for defense (Lorio, 1986; Arrango-Velez et al., 2018). During initial seasonal growth (in March), carbon is mostly allocated to the growing tissues, and

less carbon is available for the synthesis of defensive compounds in accordance with the growth differentiation hypothesis (Lorio, 1986). These tissues are preferential sinks for carbon during the early part of the growing season. However, as growth demand for carbon decreases during the later part of the season (May to August), more carbon is allocated for the synthesis of defense compounds (Lorio, 1986). The formation of oleoresin crystals may adversely affect carbon storage in roots and remobilization of carbon for growth. The non-volatile components of the oleoresin formed crystals in the phloem tissues and blocked the transport of photosynthates from the crown to the roots (Figure 7.5c). This blockage limits the supply of carbon for fine roots production.

The induction of oleoresin is influenced by the prevailing environmental conditions. In this study, the quantity of constitutive oleoresin produced prior to treatment application was higher than the constitutive (control) and inducible oleoresins (both wound and fungal treatment) after the pathogen inoculation. This variation may be attributed to temperature difference during the sampling period and precipitation. The daily average air temperature at pre- and post-treatment oleoresin sampling was 14.5 ^{o}C and 12.7 ^{o}C respectively (Mensah et al. Unpublished). Blanche et al., (1992) noted that environmental factors such as temperature and soil moisture also influence resin production. Additional, precipitation was highest (1311 mm) at the study area during 2017 at pre-treatment oleoresin sampling, whilst precipitation was lowest (955 mm) in 2019 during post-treatment oleoresin sampling. Since moisture is essential for the synthesis of biological compounds, the lower precipitation coupled with lower temperature may explain the lower levels of oleoresin induced following *L. terebrantis* inoculation.

In contrast to oleoresin induction, total phenolic production was less in *L. terebrantis* inoculated trees. The total phenolic content was, however, not affected by *L. terebrantis*

infection and the difference in phenolic content prior to treatment application and post-treatment was not significantly different. Irrespective of the level of fungal inoculum density (low, medium and high), the control and wound treatment had higher levels of phenolics compared to the fungal treatment. Moreover, among the fungal inoculum treatment, phenolic content decreased at high inoculum density. The reduction in phenolic content among the *L. terebrantis* inoculated trees suggest that either *L. terebrantis* utilized or degraded the phenolics by converting it into other metabolites, contrary to their role as antimicrobial compound (Witzell and Martín, 2008).

Some bark beetle associated fungi have been implicated in the decomposition of some phenolic compounds. For instance, *in-vitro* studies utilizing pure phenolic compounds such as flavonoids and stilbenes showed that the ophiostomatoid fungus, *Endoconidiophora polonica* (*Ceratocystis polonica*) vectored by *Ips typographus*, was able to transform the phenolics into muconoid-type ring-cleavage products (Wadke et al., 2016). Moreover, Hammerbacher et al., (2013) found that the stilbene concentration declined during infection of Norway spruce (*Picea abies*) with *E. polonica*. The reduction in stilbene concentration was attributed to the metabolism of the stilbene by the fungus. Although stilbenes are well known antifungal plant metabolites (Adrian et al., 1997), studies have shown that it can be degraded by some fungi.

In the laboratory study involving catechol (a derivative of catechin) in an amended MEA showed the capabilities of *L. terebrantis*, *L. procereum* and *G. alacris* to grow at different catechol concentrations. This suggests that *L. terebrantis* and other bark beetle vectored fungi can potentially degrade phenolic compounds (catechol) and utilize it as a source of carbon. It is therefore not surprising that in this study, the phenolic content declined following inoculation of loblolly pine trees with *L. terebrantis*.

7.6 Conclusion

Inoculation of loblolly pine trees with *L. terebrantis* induced oleoresins and catechin production. Among the fungal inoculum treatments, the medium produced more oleoresins relative to the high and low treatments. Nonetheless, the amounts oleoresins produced by the fungal treatments were higher than the wound (induced) and control treatments. Surprisingly, pre-treatment oleoresins were higher than post-treatment production and difference is attributed to differences in temperature and precipitation during the sampling periods. In contrast to the oleoresins, total phenolics did not differ prior to and post-treatment application, but the quantity of phenolics produced decreased with fungal inoculum density. *L. terebrantis* also grew on MEA amended with different catechol concentrations, suggesting the potential of the fungus to utilize catechol as a source of carbon for growth.

CHAPTER VIII

Summary and recommendations

8.1 Introduction

Pine decline (PD) is one of the problems that affect loblolly (*Pinus taeda* L.), slash (*Pinus elliottii* Engelm.) and shortleaf (*Pinus echinata* Mill.) pines in the southeastern U.S. but it is commonly associated with mature loblolly pine trees (Brown & McDowell, 1968; Eckhardt, 2004). Since the first documented case of PD in 1959, it has been reported in localized areas in several counties in central parts of Alabama and Georgia (Eckhardt et al., 2010; Forest Health Cooperative, 2017). Risk mapping analysis also indicates a risk of widespread PD across pine regions in the southeastern U.S. (Meyerpeter, 2012). This can potentially affect growth and productivity of southern pine forests.

As with growth declines, no single factor is responsible but several factors that act sequentially and collectively may cause PD in accordance with DSM as described by Manion (1991). Eckhardt et al., (2010) indicated that PD is caused by multiple stressors such as anthropogenic disturbance, site quality, climate variation, land use, insect and root pathogens, and mature stands. Various pathogens such as *Phytophthora cinnamomi*, *Heterobasidion annosum*, and *Leptographium* spp. have been implicated as contributing agents of PD. Nonetheless, root feeding bark beetles and their associated *Leptographium* spp are the most

dominant contributing agents associated with declining loblolly pine trees in the southeast (Brown & McDowell, 1968; Ostrosina et al., 1997; Eckhardt et al., 2007).

Among the *Leptographium* spp., *L. terebrantis* is frequently isolated from roots of loblolly pine trees undergoing decline in the southeast U.S. (Matusick et al., 2013; Eckhardt et al., 2007). Several studies have established the pathogenicity of *L. terebrantis* to loblolly pine seedlings and saplings (Matusick et al., 2016, Singh et al., 2014, Matusick & Eckhardt, 2010; Nevill et al., 1995). However, the influence of *L. terebrantis* on mature tree growth is not known. Again the ability of *L. terebrantis* to initiate and cause growth decline and or mortalities in field-grown loblolly pines trees is unknown. Hence, the study was initiated to assess the influence of *L. terebrantis* on *P. taeda* physiology, growth and productivity.

8.2 Results Summary

In an attempt to simulate the natural feeding habits of the bark beetles, toothpick colonized *L. terebrantis* was used to elicit *P. taeda* response. Sterilized toothpicks served as useful substrates for the production and transfer of *L. terebrantis* propagules onto the host. *L. terebrantis* colonized sterile toothpicks infected *P. taeda* saplings and compromised xylem function by causing sapwood occlusions. The occlusions significantly reduced stem hydraulic conductivity but did not impose moisture stress on the foliage of young loblolly pine trees.

Absence of foliage moisture stress is attributed to high predawn water potential and the new sapwood formed around the occluded area in the young trees. Again, no symptom of crown decline or mortality was observed among the young trees**.** Fascicle stomatal conductance was significantly affected by treatment period and inoculation density but not their interaction.

In mature loblolly pine stands, pre-treatment foliar macronutrients nitrogen (N), phosphorus (P) and potassium (K) exhibited least variability compared to micronutrients manganese (Mn) and boron (B) and were at adequate levels. Nitrogen, Mn and Iron (Fe) were significantly affected by *L. terebrantis* inoculation. The foliar N concentration decreased below the adequate levels and was most severe in the high inoculum treatment as compared to control treatment. In contrast, Mn concentration increased within the period and was most elevated in the high treatment trees coupled with elevated calcium (Ca). These modifications in foliar mineral nutrients especially N and P may have contributed to the significant reduction in fascicle length among the high treatment trees. Nonetheless, these alterations had no significant effect on fascicle number and density.

In contrast to the saplings, *L. terebranstis* infestation caused growth decline symptoms in mature *P. taeda* trees. Decline was due to pathogen occlusions and lesions, which compromixed xylem function, reduced water conduction and foliage moisture content. The physiological modification due to the pathogen infestation limited carbon fixation and caused a reduction in leaf area, ratio of leaf area to sapwood area and radial growth. The decline in radial growth was driven by moderate drought at the study site and was more pronounced in the high inoculum treatment trees. The decline was characterized by reduced radial growth, foliage chlorosis and resinosis of stem and lateral roots. Symptoms of growth decline resulted in 20% mortality among the high inoculum trees but no mortality occurred in the low and medium treatments.

The absence of crown symptoms in the low and medium treatments indicates that the inoculum levels were below the threshold of inoculum density necessary to cause decline symptoms. Moreover, the low and medium treatment trees formed a complete ring of new growth around the occluded sapwood. Among the high inoculum treatment, some trees also

failed to produce decline symptoms, indicating their tolerance to the pathogen. Tolerance is attributed to the formation of a complete or partial ring of new growth devoid of the pathogen infestation which sustained hydraulic function in sapwood and the trees physiological and biochemical processes.

Growth decline was driven by reduced precipitation as decline symptoms did not occur when localized precipitation was similar to the 30-year annual average precipitation for the area. This suggests that in addition to factors of decline (predisposing, inciting and contributing factors) as described by Manion (1991), another factor of reduced precipitation/drought , described in this dissertation as "activating factor" is required in *L. terebrantis* and *P. taeda* pathosystem to induce growth decline. Nonetheless, the induction of growth decline and mortality occurs when the inoculation density is at or above the critical inoculum threshold.

The amount of oleoresins induced by the fungal treatments was higher than the wound (induced) and control (constitutive) treatments. Surprisingly, pre-treatment quantitative oleoresins were higher than post-treatment oleoresins and the difference observed is attributed to temperature variation during the sampling periods. In contrast to the oleoresins, total phenolics (catechin) did not differ prior to and post-treatment application, but the quantity of phenolics produced decreased with fungal inoculum density. Moreover, In vitro studies also show that *L. terebrantis* can grow on MEA amended with different concentrations of catechol, suggesting that *L. terebrantis* may be capable of utilizing catechol as a source of carbon for growth.

8.3 Recommendations/Future Studies

It is recommended that the study must be repeated in diverse loblolly pine stands/families in different locations and site index at high inoculum density (one toothpick per 1.2 cm).

Additionally, the tree age or size class must also be varied, for instance trees in the following age classes 10, 15, 20, 25 etc may be considered. The size of the inoculation band can also be varied, for example 3.8 cm, 7.6cm, 15.2 cm instead of the single band (3.8 cm) size used in this dissertation. The genotypes of the tolerant trees in this study must be identified (cambium and fascicle samples are available in the Forest Health Dynamics Laboratory), characterized and incorporated into nursery and breeding programs to minimize the incidence of pine decline in southeastern U.S. Finally, the entire genome of the *L. terebrantis* isolate used must be sequenced to identify and characterize the specific genes conferring virulence to *L. terebrantis*.

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