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

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ABSTRACT

Leptographium terebrantis has been implicated as a contributing factor of *P. taeda* decline and mortality over the past several decades. We examined the potential of *L. terebrantis* to cause decline symptoms and determined the relationship between pathogen spread and the formation of new sapwood. The study was undertaken in a 13-y-old *P. taeda* plantation using artificial inoculations of fungal-colonized, sterilized toothpicks. We found that *L. terebrantis* was not only re-isolated from dying inoculated trees but caused decline symptomology and mortality at a high inoculum density. It was found that 20% mortality and severe growth loss among surviving trees occurred with *L. terebrantis* infection at the high density. At lower inoculum densities, trees produced a complete ring of new sapwood that appeared to sustain tree physiology. This suggests that management practices in *P. taeda* plantations which minimize bark beetle infestation and pathogen inoculum densities allow adequate sapwood function for sustained growth.

1. Introduction

Pinus taeda (loblolly pine) is the dominant tree species in commercial forest plantations across the southeastern United States. In certain 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 basidiomycetes (Agaricomycetes) in the genera: *Armillaria*, *Heterobasidion*, *Phaeolus*, and *Phellinus* (Shaw and Kile, 1991; Worall and Harrington, 1992). Other tree root pathogens, namely in the genera *Leptographium* and *Phytophthora* belong to the ascomycetes (Pezizomycetes) and Oomycota, 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*) is the most destructive pest of *P. taeda*, and accounts for over 80 percent of the insect-related economic loss associated with this tree species (Price et al., 1998). Nonetheless, the ability of SPB to kill trees is partly attributed to mutualistic and 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 are generally not noted for killing host trees (Horntvedt et al., 1983; Lieutier et al., 2009; Six and Wingfield, 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*, an ophiostomatoid fungus vectored by *Ips typographus*, to kill mature Norway spruce trees (*Picea abies*) but

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did not observe mortality in artificially inoculated trees. In mature lodgepole pines (*Pinus contorta*), Lee et al. (2006) observed the development of chlorotic crowns but no mortality when trees were inoculated with *Leptographium longiclavatum*. These symptoms only became apparent 9 months following artificial inoculation of trees with a high inoculum density (800 points m^{-2}) (Lee et al., 2006).

The role of *Leptographium* species span from being a saprotroph, to being a weak pathogen, to being a primary pathogen (Hansen, 1997). Despite the low incidence of tree mortality caused by *Leptographium* species, these fungi have contributed to mortality in *Pinus* species in localized settings. For example, *Leptographium wageneri* causes considerable mortality in Douglas fir (*Pseudotsuga menziesii*), lodgepole pine, and ponderosa pine (*Pinus ponderosa*) in the U.S.A. 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 saprotrophs or weak pathogens and thus, are considered unlikely to cause either disease or mortality (Hansen, 1997).

In the southeastern U.S.A., several *Leptographium* species such as *Leptographium terebrantis*, *Leptographium serpens*, and *Leptographium procereum* have frequently been isolated from woody roots of declining *P. taeda* trees (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* and *Hylastes tenuis*, the weevil species, *Hylobius pales* and *Pachylobius picivorus* (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 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*) seedlings following artificial inoculation with *Leptographium* species (Wingfield, 1986; Rane and Tattar, 1987).

Complete sapwood colonization by *L. terebrantis* can occur within a few weeks after infection of pine seedlings (Devkota and Eckhardt, 2018). However, in 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 stem hydraulic conductivity but the current-year sapwood produced after inoculation remained uninfected with *L. terebrantis* and sustained water transport to the crown (Mensah et al., 2020). The present study was conducted to assess the potential of *L. terebrantis* to cause crown symptomology and mortality in mature, plantation *P. taeda* trees and determine the relationship between pathogen lesion, sapwood occlusion, and post-inoculation sapwood growth. We hypothesized that *L. terebrantis* infection would cause sapwood occlusion and loss of hydraulic function, and induce the development of sparse crowns. Furthermore, we hypothesized that sparse crowns would limit carbon fixation and allocation to new sapwood and cause tree mortality.

2. Materials and methods

2.1. Study organism

This study was undertaken with mature loblolly pine trees (*Pinus taeda*) using the fungus *Leptographium terebrantis*. 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).

2.2. Study site and experimental design

The study was located in a loblolly pine plantation near Eufaula, Alabama, U.S.A. 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 in the humid subtropical climatic zone. Soil series identified within the study area included Annemaine and Wahee. Their taxonomic classifications are fine, mixed, semi-active, thermic Aquic Hapludult and fine, mixed, semi-active, thermic Aeric Endoaquult, respectively. Annemaine is the predominant soil series, consisting of a fine sandy loam surface and clayey subsoil that is moderately well-drained. Wahee contains a clay-loam subsoil overlain by a fine sandy loam surface and is poorly drained (Trayvick, 2005; Ditzler et al., 2017). Average annual precipitation and air temperature of the area are 1407 mm and 18.1 °C, respectively (NOAA, 2020). The plantation was established in 2003 at a 1.2 m * 3.0 m spacing using open-pollinated seedlings and was third-row thinned at an age of 12 y in 2014. The study site received nitrogen and phosphorus fertilization but no herbicide or pesticide control at planting and no fertilization or other chemical treatments after planting. The study location has a site index of 22 m at 25 y.

Fifteen plots containing two rows, 3.0 m apart, of 10 trees per row were established in the plantation at age 13 y in December 2015. All plot trees were permanently identified by numbered metal tags. Among dominant trees in one 10-tree row per plot, five were randomly chosen as measurement trees and were outfitted with a manual dendrometer band (D1, UMS GmbH, Munich, Germany) installed 1.4 m above the ground line (DBH). Five treatments were randomly assigned to five treatment plots in a completely randomized experimental design with three replications (5 trees * 5 treatments * 3 plots = 75 trees). A weather station (WatchDog 2000, Spectrum Technologies Inc., Aurora, IL, U.S.A.) 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, dominant measurement trees that were fitted with dendrometer bands in one of the two rows per plot. Treatments were 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 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. The initial series of inoculation points was 26.0 cm above ground line. Three additional series of inoculation points were 1.2 cm, 2.4 cm and 3.6 cm below the initial inoculum points (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 density treatment with 40–58 sterile toothpicks per tree.

2.3. 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, U.S.A.). The inoculation points, approximately 1.2 mm in diameter and 5 mm deep, were drilled into the tree 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, U.S.A.), were inoculated with *L. terrebranits* or not inoculated, and incubated in the dark at 23 °C for 24 d 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).

2.4. Post fungal inoculation monitoring and measurements

Post-treatment observation of the inoculation zone and tree appearance was undertaken monthly from April 2017 to December 2019. Observations of host response to treatment 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 occlusion assessments. One stem disc, 5 cm wide, was extracted above and below DBH. 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 °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.

2.5. 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 on opposite sides of the stem segment. Lesion lengths at four equidistant locations around the circumference of the stem was calculated by tree as the mean of four values.

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 darkly stained sapwood appearance (Solheim and Krokene, 1998; Lee et al., 2006). Occluded stem length by tree was calculated as the mean of two values, equidistant around the stem circumference, on opposite sides of the stem segment.

2.6. 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. The basal surface of the discs was traced onto a transparent plastic sheet and disc areas were determined by a planimeter (Lasico®, Los Angeles, CA, U.S.A.). 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 disc sapwood area in the 100 cm stem sections.

2.7. Classification of sapwood at the inoculation zone

Sapwood infection by *L. terebrantis* was used to classify the 5.0 cm wide stem discs cut transversely through the inoculation zone of the trees. Sapwood at the inoculation zone was given one of three classifications: continuous new sapwood around the circumference of the stem but non-uniform in width, discontinuous new sapwood around the circumference of the stem, or absence of newly grown sapwood. *Leptographium terebrantis* was re-isolated from each transverse section of the harvested trees except the control and wound treatments. Specifically, a 5 mm piece of stem tissue cut from around an 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 re-isolation of the introduced fungus from the host tissue.

2.8. Data analysis

Main effects of *L. terebrantis* inoculum density on stem lesion and occlusion length, sapwood occlusion length and area, and new sapwood area after treatment were analyzed by one-way analysis of variance (Proc GLM, SAS Inc., Cary, NC, U.S.A.). Relationships between occluded stem length and stem lesion length and new and occluded sapwood areas were analyzed using Proc Reg (SAS Inc., Cary, NC, U.S.A.). Prior to analyses, normally distributed and homogenous variance of experimental errors were verified using Shapiro-Wilk and Levene's tests, respectively (Snedecor and Cochran, 1980) Treatment effects and Tukey's differences in least square means were considered significant at an α level of 0.05. The control treatment was not included in these analyses because of a consistent absence of lesions and occlusions.

3. Results

By 5 months after inoculation with *L. terebrantis*, a relatively high amount of oleoresins exuded from the bottom of the duct taped edge of the inoculation zone of the high inoculum treatment trees (Fig. 1A), with less oleoresins exuded from the duct taped edge of inoculation zones of the low and medium inoculation treatment trees. The wound treatment trees did not produce a visible oleoresin response during the study period (Fig. 1B). Inoculated trees exhibited oleoresin exudation and black stain in the inoculation zone (Fig. 1C), and stem lesions 34 months after inoculation (Fig. 1D). Excavation of a limited number of lateral roots near the surface of the soil indicated that stem lesions extended vertically into the taproot and then horizontally from the taproot into lateral roots of some high inoculum treatment trees (Fig. 2A and B).

Crown symptoms were not evident among the trees of any treatment for 12 months post-inoculation. Foliage chlorosis was initially detected among four (26.7%) trees of the high inoculation treatment 19 months following inoculation. Foliage chlorosis and the development of sparse crowns continued to progress (Fig. 3A and B), ultimately resulting in 20% mortality among the high inoculum density trees (Fig. 3C).

Inoculation treatment significantly affected stem lesion length 34 months after treatment ($P < 0.0001$) (Table 1). Stem lesion length among the low, medium, and high treatments were significantly different with mean values of 16.0, 21.5, and 27.6 cm, respectively (Fig. 4A).

At 34 months post-inoculation, occluded stem lengths of the high and medium inoculation treatments were not significantly different and averaged 38.4 cm (Table 1). Occluded stem lengths associated with both high and medium inoculation treatments were significantly greater than that of the low inoculation and wound treatments. Occluded stem length of the low inoculation treatment was significantly greater than that of the wound treatment (Fig. 4B). Among the stem sections receiving the low, medium, and high inoculation treatments, a significant ($P < 0.0001$, $r^2 = 0.95$) linear relationship was found between stem lesion length and occluded stem length 34 months after inoculation.

Inoculation treatment significantly ($P < 0.0001$) affected occluded radial stem area (Table 1). Occluded radial stem area of the high (83.1%) and medium (36.2%) inoculation treatments were significantly different from each other and from those of the low and wound treatments. Occluded radial stem areas of the low and wound treatments were not significantly different and averaged 8.5% (Fig. 4C).

New radial stem area was significantly affected by inoculation treatment (Table 1). At 34-months post-treatment, new radial stem area was not significantly different among the wound, and low and medium inoculum density treatments and averaged 31.5%. New radial stem area of the high inoculum density treatment (15.2%), however, was significantly less than those of the wound, and low and medium inoculum density treatments (Fig. 4D). Opposing patterns of post-treatment radial areas of new and occluded sapwood were observed in response to an increase in inoculum density (Fig. 5).

Inoculation treatment had no significant effect on stem moisture content (MC) at breast height ($P = 0.2862$) (Table 1). However, a non-significant trend of decreased MC was observed between the wound treatment (89.2%) and the three inoculation treatments as inoculum density increased (low: 86.9%, medium: 84.7%, high: 77.8%). The pathogen was re-isolated from 100% of the sapwood discs of harvested trees that were inoculated at the low, medium, or high inoculum densities. The control trees had no occlusions or lesions and upon re-isolation, no fungal isolates were found.

A significant ($P < 0.0001$, $r^2 = 0.89$) non-linear relationship was observed between post-treatment new radial stem area and occluded radial stem area (Fig. 6). The inflection point of this function (40.0%, 33.4%) represents the threshold (40%) of occluded sapwood area beyond which a decline in sapwood growth was attributed to post-inoculation pathogen spread. Among trees treated with the high inoculum density, 27% formed a continuous ring of new sapwood around the periphery of sapwood inoculated 34 months earlier (Fig. 7A), 53% formed a discontinuous ring of sapwood around the periphery of sapwood inoculated 34 months earlier (Fig. 7B and C), and 20% failed to produce sapwood after stem inoculation with *L. terebrantis* (Fig. 7D).

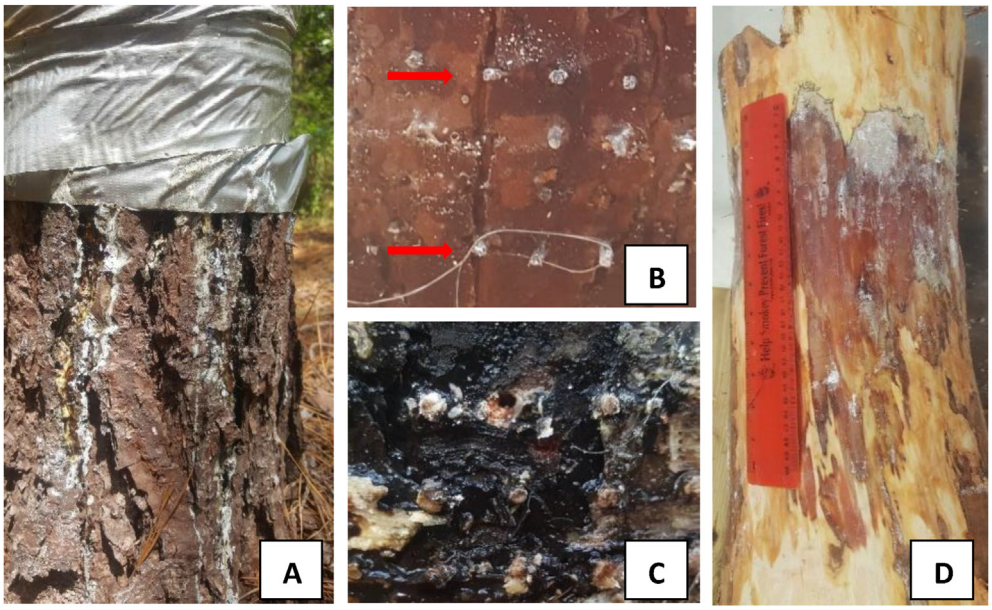

Fig. 1. Inoculation zone symptoms of high inoculum treatment *P. taeda* trees: (A) oleoresin exudates at 5-months post-inoculation, (B) inoculation zone of the wound treatment 34 months after wounding with an absence of oleoresin exudates, (C) inoculation zone of a high inoculum density tree 34 months after treatment exhibiting oleoresin exudates and black stains, and (D) occluded stem length extending above and below the inoculation zone. Arrows identify the top and bottom series of inoculation points in the inoculation zone.

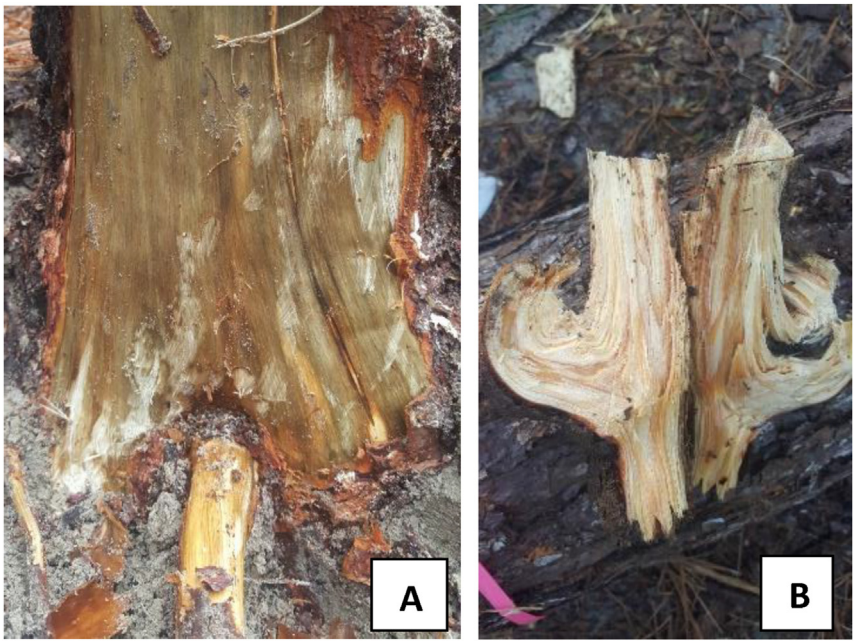

Fig. 2. Extension of lesions from the stem to woody roots near the surface of the soil 34 months after *P. taeda* trees received the high inoculum density treatment: (A) lateral root lesion and resinosis, (B) split lateral root showing occlusion.

Fig. 3. *P. taeda* crown symptoms after *L. terebrantis* inoculation treatment: (A) two high inoculation treatment trees showing symptomatic crowns 19 months after treatment, (B) chlorotic and thin crown 34 months after treatment with the high inoculum density, (C) crown of a dying tree 34 months after treatment with the high inoculum density.

Table 1
Probabilities of a greater *F*-value (*P*>*F*) for lengths of lesion and occluded stem, radial areas of occluded and new sapwood, and stem moisture content of *P. taeda* trees 34 months after inoculation with *L. terebrantis* in the lower stem.

Variable	degrees of freedom	*F*-value	*P* > *F*
Stem lesion length	3	64.29	<0.0001
Occluded stem length	3	39.31	<0.0001
Occluded radial stem area	3	146.18	<0.0001
New radial stem area	3	14.16	<0.0001
Stem moisture content	3	1.31	0.2862

4. Discussion

This study represents the first attempt to assess the potential of the root pathogen, *L. terebrantis,* to contribute to crown symptomology and tree mortality in mature loblolly pine. Using artificial stem inoculation as a surrogate for woody roots infected by an insect-vectored pathogen, the mechanism and process of *L. terebrantis* infection in pine decline was studied. We found that stem infection at a high fungal inoculum density caused progressive crown decline associated with eventual mortality in some trees. It is important to note that this crown deterioration and associated tree mortality coincided with a moderate drought at the

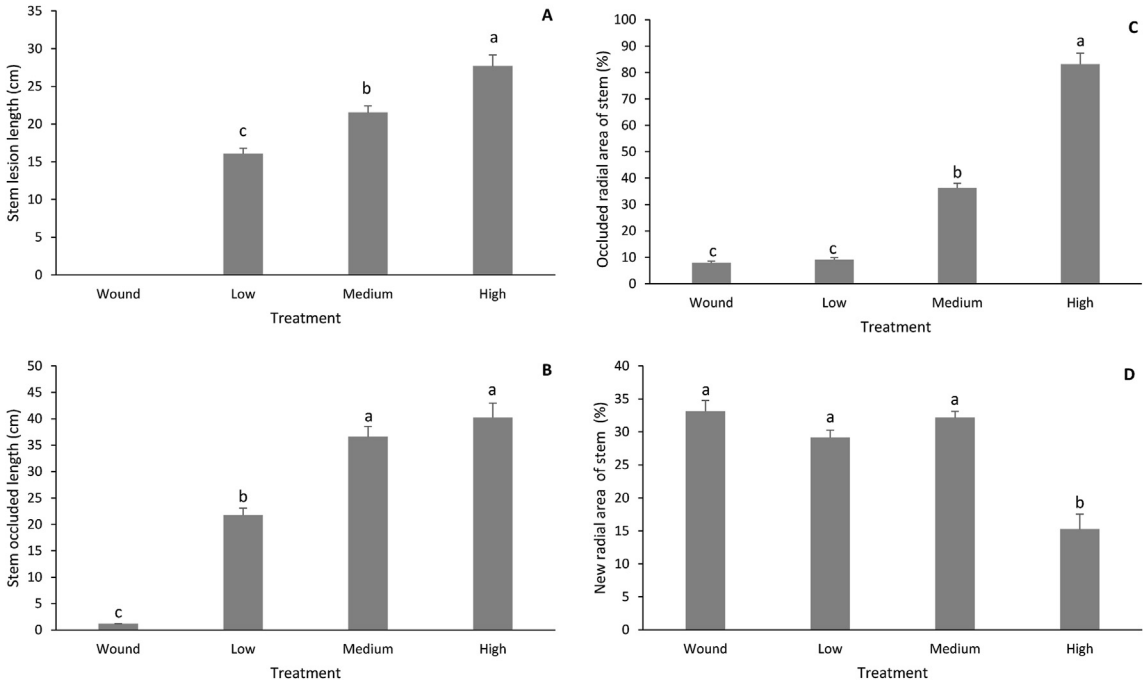

Fig. 4. *Pinus taeda* response to *L. terebrantis* infestation 34 months following lower stem inoculation: (A) stem lesion length observed at the interface between the phloem and vascular cambium, (B) stem occluded length, (C) occluded radial area of the stem section, and (D) new radial area of the stem section. Means by variable associated with different lower-case letters are significantly different by Tukey's Honestly Significant Difference test at an α-level of 0.05. Error bars are standard errors of the mean.

study site. Nevertheless, below the threshold of fungal infection of one colonized toothpick per 1.2 cm over the bark circumference, there was no manifestation of crown symptomology, despite local tissue damage. This infection threshold applies to artificially inoculated stems, and it may differ for woody roots of loblolly pines undergoing similar decline symptoms.

Fungal inoculum density must reach a critical threshold to cause symptom development in the host (Baker, 1971; Mitchell, 1978; Gilligan, 1983). Among the three *L. terebrantis* inoculum densities, only the high inoculum density contributed to tree mortality. Previously, pine seedling mortality involving *L. terebrantis* was reported (Wingfield, 1986), but knowledge of mature tree mortality due to *L. terebrantis* infection was unknown. Other *Leptographium* species have been shown to cause tree mortality at low inoculum levels when additional tree stresses were imposed. For instance, Solheim et al. (1993) found that *Leptographium wingfieldii* and *Ophiostoma minus* fungi killed *Pinus sylvestris* (Scots pine) when inoculated at 800 points m^{-2}. Additionally, they noted that both fungi caused mortality at lower inoculum densities when tree vigor was reduced by other factors. Lee et al. (2006) found that in mature lodgepole pine, artificial inoculations with *L. longiclavatum* caused the formation of chlorotic crowns. However, symptoms were only apparent 9 months post-inoculation, no mortality was observed, and symptomology was restricted to a high density (800 points m^{-2}) treatment.

In the present study, damage caused by *L. terebrantis* infection occurred in two phases. The first phase was direct damage to sapwood caused by *L. terebrantis*, including tissue necrosis (i.e., lesions) and vascular tissue occlusion. Transverse stem sections at the inoculation zone of the high inoculum trees indicated that over 80% of the sapwood was occluded. The occlusion was likely due to the combination of pathogen infestation and a hypersensitive response by the host, including the deposition of oleoresin and other secondary metabolites in an attempt to stop invasion by the pathogen (Viiri et al., 2001; Arango-Velez et al., 2018). Blockage of sapwood causes disruption of the water transport system (Butnor et al., 1999; Mensah et al., 2020). Additionally, the lesions which were a direct response to pathogen invasion, blocked phloem tissue and inhibit translocation of photosynthate from the crown to the root system. The growth of new roots is largely dependent on current photosynthate (Lynch et al., 2013). Thus, it is likely that root system expansion was compromised among trees with severe stem occlusion.

The second phase of damage manifested itself as the loss of whole-tree hydraulic function when significant *L. terebrantis* spread coincided with drought. Based on our observations, we hypothesize that reduced sapwood function resulting from the high inoculum density combined with soil moisture limitations from drought led to a reduction in 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. These trees were characterized by sparse crowns, chlorotic needles, and reduced and irregular radial growth. This suggests that a threshold of fungal inoculum density and pathogen spread must be attained before growth decline occurs. However, from our study, it remains unclear whether *L. terebrantis* can independently cause progressive crown deterioration and tree mortality in the absence of additional stressors such as drought. Our observations after 34 months suggest that severe *L. terebrantis* infection acted in combination with other stress factors such as drought to initiate loblolly pine decline. This reiterates the assertion of *Leptographium* species being weak pathogens (Hansen, 1997) that are unlikely to independently cause crown symptoms and tree mortality.

Past research has documented the co-occurrence of severe sapwood occlusion and moisture loss in the vicinity of stem inoculation with a wilt pathogen (Butnor et al., 1999; Mensah et al., 2020). For example, Mensah et al. (2020) reported a 20% loss of

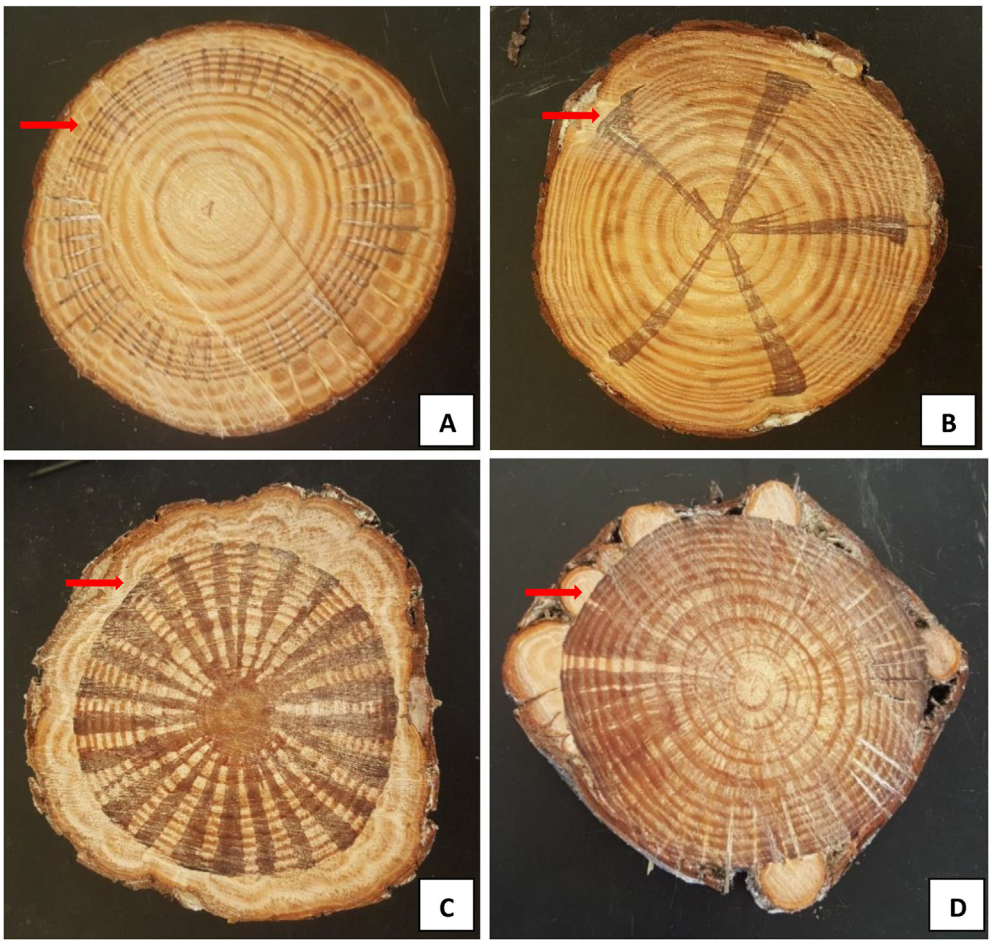

Fig. 5. Cross-sections at the inoculation zone of *P. taeda* tree stems showing occluded and new sapwood areas by treatment 34 months after inoculation: (A) wound treatment with sterile toothpicks, (B) low inoculum density treatment, (C) medium inoculum density treatment, (D) high inoculum density treatment. Arrows mark the outside edge of the inoculation zone.

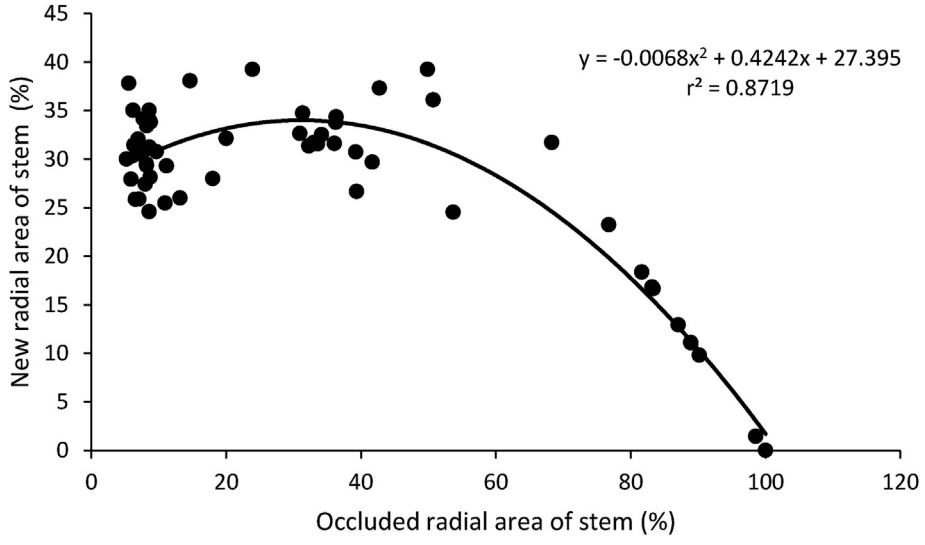

Fig. 6. A significant ($P < 0.0001$, $r^2 = 0.89$) non-linear relationship between new radial stem area without infestation and occluded radial stem area 34 months after lower stem inoculation with *L. terebrantis*.

stem moisture content 16 weeks after stem inoculation of young loblolly pine trees with *L. terebrantis*. In the present study, we quantified stem moisture content at DBH which was more than 1 m above the location of stem inoculation and observed a non-significant trend of moisture loss as inoculum density increased. Specifically, wound, low, medium, and high inoculation treatments

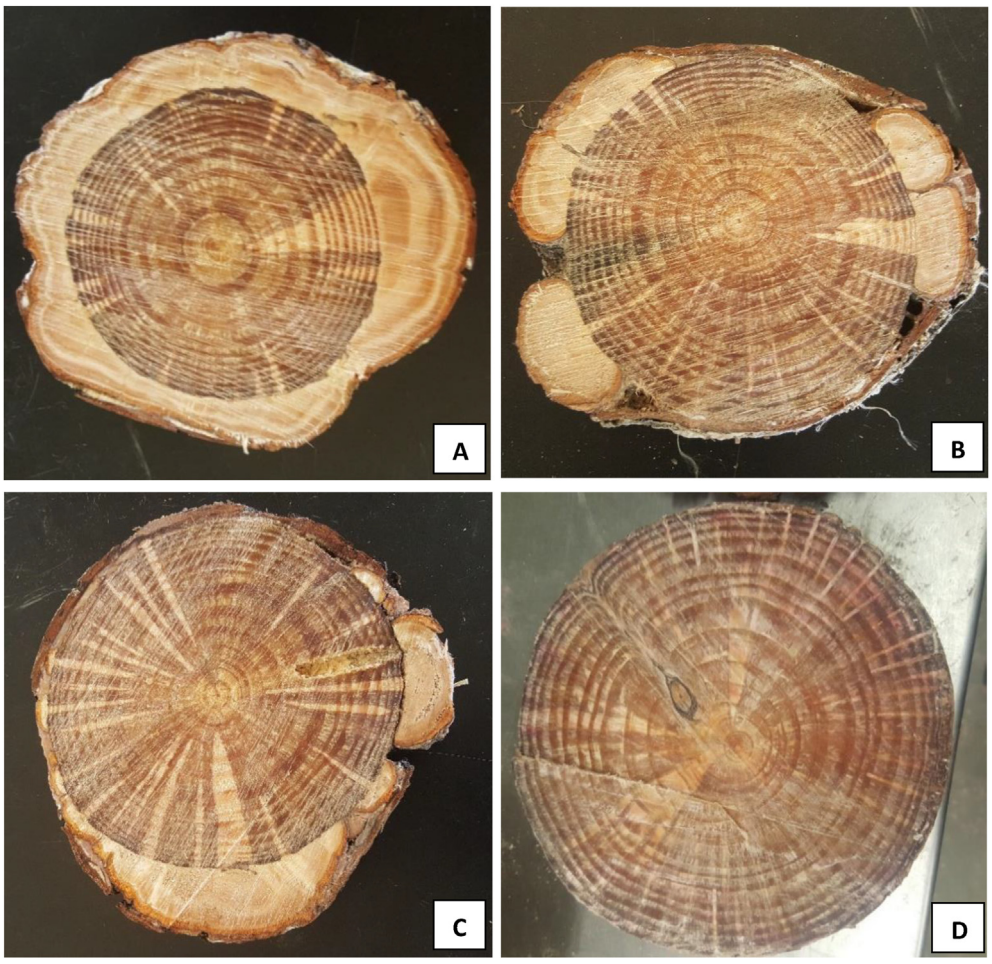

Fig. 7. Stem cross-sections of *P. taeda* trees 34 months after receiving the high inoculum density treatment: (A) tree with a continuous ring of new sapwood, (B) tree with a discontinuous ring of new sapwood that is approximately 50% of normal, (C) tree with a discontinuous ring of new sapwood that is less than 50% of normal, (D) tree with no new sapwood.

yielded 89.2%, 86.9%, 84.7%, and 77.8% stem moisture content, respectively. This trend suggests that the effect of the sapwood occlusion on infected trees transcends the inoculation zone to have whole-tree effects. Low tissue moisture content may exacerbate existing water limitations to photosynthesis caused by drought leading to needle chlorosis, fascicle senescence, and lack of foliage replacement (i.e., thinning crowns) (Butnor et al., 1999; Lee et al., 2006; Mensah et al., 2020). Collectively, pathogen inoculum density, moisture stress due to pathogen spread, and drought play a crucial role in *L. terebrantis* and *P. taeda* interactions that lead to whole-tree symptomology. Under moisture stress, tree vigor is reduced and pathogen-induced lesions and sapwood occlusions may hasten crown symptoms and tree mortality which has been observed in natural pine stands with high pathogen infection (Eckhardt et al., 2007; Kolb et al., 2019).

We classified tree responses to *L. terebrantis* inoculation into three categories of stem growth response that reflect tolerance: continuous but non-uniform sapwood growth around the circumference of the stem after inoculation, discontinuous sapwood growth around the circumference of the stem after inoculation, and absence of sapwood growth after inoculation. Continuous sapwood growth after inoculation was observed in trees that were wounded or received the low or medium inoculum density treatments. In addition, four of the 15 trees receiving the high inoculum density treatment grew a continuous but non-uniform ring of sapwood around their stem circumference. Sapwood growth was discontinuous or absent in the remaining 11 trees treated with the high inoculum density. Despite the relatively high sapwood occlusion of the four high inoculum density trees with continuous sapwood growth, they sustained physiological function for the appearance of normal carbon allocation to the stem.

Genetic variation in the open-pollinated seed source of this study may, in part, explain differences in the observed susceptibility of loblolly pine to high *L. terebrantis* infection. Past research shows that the fascicle-level gas exchange of loblolly pine is affected by the interaction between genotype and environment (King et al., 2008; Blazier et al., 2018). Furthermore, the pattern of carbon allocation among the shoot, stem, and root system of loblolly pine is under genetic control (Bongarten and Teskey, 1987; Stovall et al., 2013).

Formation of a discontinuous sapwood ring around the circumference of more than half (8 trees) of the high inoculum density trees may be attributed to two factors. First, pathogen damage to, or occlusion of the vascular cambium and phloem may have occurred soon after inoculation. Second, adequate allocation of carbon to the unaffected vascular cambium may have allowed the formation of a partial, discontinuous ring of sapwood after inoculation.

In addition to genotype effects on carbon fixation and allocation, tree size and its effect on carbon partitioning may have played a

role in sapwood growth after the high inoculation treatment. Demands for fixed carbon to produce foliage and sustain the respiration of all tissues are first met, followed by carbon allocation to other endpoints (Litton et al., 2007). Furthermore, the demand for carbon to support respiration increases by tree size (Ryan et al., 1994; Maier, 2001). Together, drought and compromised hydraulic function due to pathogen spread may have limited whole-tree carbon fixation among all high inoculum density trees. In the smaller of these trees, sapwood demand for carbon may have been partially or fully met after adequate carbon was allocated to foliage growth and respiration. In contrast, a sapwood carbon deficit may have been experienced in larger trees if inadequate carbon remained after foliar and respiratory needs were met.

5. Conclusions

This 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 a 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, trees survived by forming post-inoculation sapwood without fungal infection and were able to sustain hydraulic function. Post-inoculation sapwood formation in the high inoculation treatment was either continuous with a non-uniform width or discontinuous around occluded sapwood. High inoculum density trees that failed to produce sapwood around the occluded sapwood culminated in tree mortality. These observations indicate *P. taeda* survival following attack by bark beetles that vector a wilt pathogen may depend on the formation of new sapwood after infection. Furthermore, additional research is warranted to determine whether in a similar manner, sustained radial root growth of *P. taeda* despite *L. terebrantis* root disease, represents a mechanism of deterring tree decline.

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