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Effect of *Leptographium terebrantis* on Foliage, New Root Dynamics, and Stemwood Growth in a Loblolly Pine (*Pinus taeda* L.) Plantation

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Abstract: The course of the bark beetle-vectored fungus, *Leptographium terebrantis* S. J. Barras and T. J. Perry, in stemwood growth loss of declining pines in the southeastern United States was assessed in a 13-year-old loblolly pine (*Pinus taeda* L.) plantation near Eufaula, Alabama, U.S.A. Using stem inoculation as a surrogate for root infection, we hypothesized that *L. terebrantis* infection impairs sapwood function and thus limits the tree leaf area (A_L), new root production, and stemwood growth. Sterile toothpicks colonized by *L. terebrantis* at varying inoculum densities was used to elicit host growth responses. In the third year after inoculation, the root pathogen reduced the foliage moisture content, whole-tree leaf area (A_L), the ratio of A_L to tree sapwood area (A_S), and stemwood growth in trees receiving the high inoculation treatment relative to those receiving the low or medium inoculation treatments, or the wound or control treatments after seven months of water deficit. The absence of a similar response to water deficit among trees that were noninoculated, wounded, or inoculated at the low or medium densities suggests that, in the loblolly pine–*L. terebrantis* pathosystem at our study site, the physiological stress caused by water deficit and the high inoculum density was required for the pathogen to elicit a stemwood growth loss. Thus, in loblolly pine forests of the southeastern United States, where climate and soil conditions yield prolonged periods of physiological stress, the presence of *L. terebrantis* has the potential to reduce stand volume and widen the gap between the predicted and actual stemwood production.

Keywords: decline; drought; hydraulic adjustment; inoculation; leaf area; physiological stress

1. Introduction

Loblolly pine (*Pinus taeda* L.) is the principal tree species grown in forest plantations across the southeastern U.S. [1]. To sustain economic and ecological contributions of this tree species in the region, about one billion loblolly pine seedlings are planted each year [2,3]. The dominance of this tree species in the region is projected to increase for decades as a result of improved genetics and silvicultural treatments that enhance plantation productivity [4]. Over the past six decades, loblolly pine growth loss and mortality associated with *Leptographium* root disease have been reported in localized areas of central Alabama and Georgia [5].

These localized areas of forest decline appear to be caused by complex interactions between pests and abiotic factors [6,7]. Several theories have been formulated to explain the forest decline concept [6,8–11]. Common among these theories is the supposition that no single factor causes forest decline, but rather it is a multiplicity of factors that act sequentially for a decline outcome. The decline spiral model (DSM) proposed by Manion [6] is the most common among the forest decline theories. The DSM reclassified the cause of

forest decline from chain reaction theory [8] to the systematic occurrence of predisposing, inciting, and contributing factors.

According to the DSM, predisposing factors are long-term, underlying conditions related to genotype, regional climate, and soil quality that may put permanent physiological stress on trees. Inciting factors are relatively short-term conditions, such as drought and insect-induced defoliation that worsen the stress imposed by predisposing factors [6,12,13]. For example, Bigler et al. [14], noted in the Rhône Valley and other dry areas of the European Alps during the early 20th century that the high mortality of dense, mature stands of Scots pine (*Pinus sylvestris* L.) 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 [6,15] and may accelerate growth loss and mortality. For example, insects such as the 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 Rhône Valley Scots pine mortality event during drought reported by Bigler et al. [14].

Forest declines threaten sustainable timber production in many areas across North America. Over 121,000 hectares of *Quercus* spp. (oak) forests were negatively affected by decline in the Ozark Mountains of northern Arkansas and southern Missouri in 1999 and 2000 [16,17]. 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 [18], whitebark pine (*Pinus albicaulis* Engelm.) in western Canada [19], pinyon pine (*Pinus edulis* Engelm.) in the western U.S. [20], and loblolly pine [21,22] in the southeastern U.S.

Since the 1950s, loblolly pine decline (LPD) has been reported in several Alabama counties [5,21,22]. 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 [22,23]. Root-feeding bark beetles are commonly observed in woody roots of declining loblolly pines exhibiting these symptoms [22,24].

In accordance with the DSM [6], the bark beetle-fungal complex of LPD acts as a contributing factor of decline after trees have been predisposed by other factors. The role of stem-feeding bark beetles in pine mortality is well known, with damage caused by insect tunneling beneath the bark, subsequent larvae production, and the spread of associated pathogenic fungi. These processes girdle the tree, disrupt the transport of photosynthate from foliage to roots, and reduce water translocation in the outer sapwood [25–27]. Similarly, root-feeding bark beetles damage the vascular cambium and conducting tissues near the point of woody root entry [28]. Further disruption of the xylem and phloem occurs by the spread of vectored fungal associates, and the net effect may lead to sapwood occlusion, radial growth loss, and tree mortality [29]. These fungal associates are not considered to be aggressive pathogens unless they overcome host defenses leading to an advancement of sapwood occlusion [30,31]. *Leptographium terebrantis* S. J. Barras and T. J. Perry is one of several bark-beetle-vectored fungi commonly isolated from woody roots of loblolly pine exhibiting the symptoms of LPD [22,32,33].

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 [34–36]. At the same time, tree growth and physiological responses to *L. terebrantis* are variable despite distinct signs of pathogenesis [36]. Thus, the contribution of *L. terebrantis* infection to LPD when vectored by root-feeding bark beetles is unknown. *Leptographium* spp. may grow into xylem tissue and disrupt water and mineral nutrient transport from the soil to the tree crown [36]. This has the potential to compromise xylem function by limiting water conductance and reducing both stomatal function and carbon

fixation [37]. For example, Hossain et al. [38] demonstrated a loss of leaf-level stomatal conductance due to sapwood infection by a canker pathogen in *Corymbia calophylla* (Lindl.) K.D. Hill and L.A.S. Johnson (marri). Poor xylem function risks inadequate water to sustain the carbon supply for normal foliage, stem, and root system growth, which may be attributed to carbon limitation or a carbon allocation shift favoring chemical defenses [39]. The latter was demonstrated by Viiri et al. [40], who noted a reduction in total soluble carbohydrates near the site of fungal infection when Norway spruce (*Picea abies* L.) was inoculated with the bark-beetle-associated fungus, *Ceratocystis polonica* (Siemaszko) C. This loss of carbohydrate near the infection site was attributed, in part, to the induced production of terpenes [40,41].

In this study, we assessed the annual stemwood growth of loblolly pine trees grown in a commercial plantation using stem inoculation as a surrogate for woody root inoculation [34]. These trees were either noninoculated or inoculated at one of three inoculum 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 the leaf area and stem sapwood area 34 months after inoculation provide knowledge about the mechanism of tree decline as *L. terebrantis* infection progresses in LPD. We hypothesized that *L. terebrantis* infection impairs sapwood function and thus limits tree leaf area, new root production, and stemwood growth.

2. Materials and Methods

2.1. Experimental Location and 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) with a site index of 22 m at 25 years, as described by Mensah et al. [29]. Mean annual precipitation and air temperature at the site are 1407 mm and 18.1 °C, respectively [42]. Loblolly pine seedlings from an open-pollinated seed source were used to establish the plantation at a 1.2 m × 3.0 m spacing in 2003. The soil is a complex of Annemaine and Wahee fine sandy loams. Both series consist of fine sandy loam surface soil. Annemaine, the predominant soil series, is moderately well-drained and Wahee is poorly drained due to its clay–loam subsoil [43,44].

Fifteen plots containing two rows, 3.0 m apart, of 10 trees per 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. The treatments were no inoculation or wounding (control), no inoculation but sterile toothpick wounding (wound), and three levels of increasing fungal inoculum density [29].

All plot trees were permanently identified by numbered metal tags. Inoculation treatments were applied to five dominant trees in each ten-tree row, randomly identified as measurement trees and outfitted with a manual dendrometer band (D1, UMS GmbH, Munich, Germany) installed at 1.4 m above the ground-line (diameter at breast height, DBH). A weather station (WatchDog 2000, Spectrum Technologies Inc., Aurora, IL, USA) was installed adjacent to the study site to monitor local precipitation and air temperature.

2.2. Minirhizotron Tube Installation

Four clear, acrylic minirhizotron root tubes, 82 cm in length and with a 3.81 cm inner diameter, were installed around each of two dominants, treated trees per plot. Trees were chosen among those with a DBH that approximated mean plot DBH. Each tube was installed at a 152.4 cm distance from the base of the bole and at a 45° 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 [45,46]. The 10 cm length of the upper end of the tube was covered with black tape to prevent light from entering the tube, and the tube was plugged with a rubber stopper to prevent entry by insects or water. Each tube was then covered with a small plastic pot, as described by Duwadi [46].

2.3. Inoculation Method

The inoculation procedure was performed as described by Mensah et al. [29]. The inoculation treatment was applied in March 2017 by inserting *L. terebrantis*-colonized or sterile toothpicks into drilled holes in the lower stem of treated trees [29,36,47]. Trees treated with the low, medium, or high inoculum densities received one inoculated toothpick per 10.0, 2.4, or 1.2 cm over the bark, respectively. As a result, trees treated with the low, medium, or high inoculum densities received 5 to 8, 20 to 28, or from 40 to 58 *L. terebrantis*-colonized toothpicks, respectively. Wound-treated trees received sterile toothpicks at the high inoculum density level.

2.4. Stem and Root Growth Measurement

Annual tree DBH, expressed as cm, was determined with measurement tree dendrometer bands in January 2016, 2017, 2018, and 2019. Total measurement tree height was quantified and expressed as m by a TruPulse 200 Rangefinder-Hypsometer (Laser Technology Inc., Centennial, CO, USA) in January of each year between 2016 and 2020. Measurement tree basal area (BA), expressed as cm^2, was determined by Equation (1),

$$BA = (\pi D^2)/4 \tag{1}$$

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

Relative radial stem growth (RG) of the measurement trees was determined annually between 2016 and 2019 as the ratio of BAI and BA_1, expressed as $mm^2 \, cm^{-2}$ [48]. Annual outside-bark stem volume of the measurement trees was estimated as described by Burkhart [49], using the combined-variable function [50] for plantation loblolly pine, and expressed as m^3.

New root growth was assessed with the aid of an optical root periscope (JRD Merrill Specialty Equipment, Logan, UT, USA) with a fiber optic light powered by a battery. New root ($\leq$2 mm diameter) growth measurements started six months after minirhizotron root tube installation which was one-month postinoculation in April 2017 and were repeated at three-month intervals [46]. Numbers of root intersections by depth increment and minirhizotron tube were counted. Pine roots of inoculated and control trees were identified by color and diameter. New root growth, reported as root length density (RLD) according to Newman [51], was expressed as $cm \, cm^{-2}$. Cumulative root length density (CRLD) was summed by minirhizotron tube and averaged by tree. Presently, we report mean CRLD in October 2019, when a significant treatment effect on CRLD was detected prior to tree harvest.

2.5. Leaf Area, Sapwood Area, and Tissue Moisture Content Measurements

In January and February 2020, the five treated measurement trees 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 the live foliage fresh weight per tree (W_1). Green foliage was transported to the laboratory. Three subsamples, each containing between 15 and 25 fascicles (3 needles per fascicle) 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 °C to a constant weight, and total foliage dry weight was calculated (W_2) expressed as g. The moisture content (MC) of the foliage at the time of tree harvest was expressed as percentage of oven-dried weight by the equation, $MC = [(W_1 - W_2)/W_2] \times 100$.

The total leaf area of each subsample of fascicles was determined by volume displacement of bundles of two fascicles, as described by Johnson [52]. Values by subsample were summed and projected leaf area by fascicle subsample was expressed by dividing total subsample leaf area by 3.142 [53]. 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 and expressed as cm^2 g^{-1}. Tree SLA was estimated as the mean of three SLA values by tree. Projected leaf area by tree (A_L) was calculated as the product of mean tree SLA and W_2 and expressed as m^2.

A wood disc, approximately 5 cm thick, was cut with a chainsaw at DBH. The circumference of sapwood area (no heartwood) was traced on a transparent sheet and sapwood area (A_S) expressed as cm^2 was determined with a planimeter (Lasico®, Los Angeles, CA, USA). Aboveground hydraulic structure at the time of tree harvest was characterized as the ratio of tree projected leaf area and sapwood area (A_L:A_S) and was expressed by tree as m^2 cm^{-2}.

2.6. Data Analysis

Before data analyses, mean plot DBH, total tree height, stemwood volume, relative stem radial growth (RG), tree leaf area (A_L), cumulative root length density (CRLD), foliage moisture content (MC), and tree A_L:A_S were assessed for normality and equal variance assumptions, and data were transformed as needed. 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 procedure of SAS statistical software (SAS Institute, Version 9.4, Cary, NC, USA) with compound symmetry as the covariance structure. Tree A_L, CRLD, MC, and A_L:A_S were analyzed by one-way analyses of variance and the GLM procedure of SAS statistical software (SAS Institute, Version 9.4, Cary, NC, USA). Treatments were control, wound, or low, medium, or high inoculum densities. Significant main and interaction effects were further evaluated by a pair-wise comparison among treatment means using the post-hoc Tukey's Honest Significant 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 [54]. Probabilities of a greater *F*-value associated with analyses of variance and linear regressions and mean comparisons were considered significant at an α-level of 0.05, and marginally significant at an α-level of 0.10 when a biologically related difference was significant.

3. Results

3.1. Precipitation and Temperature at the Study Site

Annual precipitation during the 4-year study was 992, 1311, 1260, and 955 mm in 2016, 2017, 2018, and 2019, respectively (Figure 1). Prior to tree inoculation in March 2017, water deficit occurred in September through November 2016 with no precipitation in October. Mean weekly Palmer Drought Severity Indices (PDSI) in September, October, and November 2016 were -1.5, -1.9, and -2.7, respectively, indicating that drought severity increased from mild to moderate during this period [42]. Water deficit also occurred in 2019, with 45% less precipitation than the 30-year average between March and September. Mean weekly PDSI between March and September 2019 decreased from -0.2 to -3.0, indicating that drought severity increased from mild to severe during this period [42]. Overall average monthly air temperature for the measurement period was 18.2, 18.0, 18.1, and 18.4 °C in 2016, 2017, 2018, and 2019, respectively. The average monthly air temperature during the 7-month drought period in 2019 was 22.8 °C relative to the 30-year average of 22.4 °C within the same period.
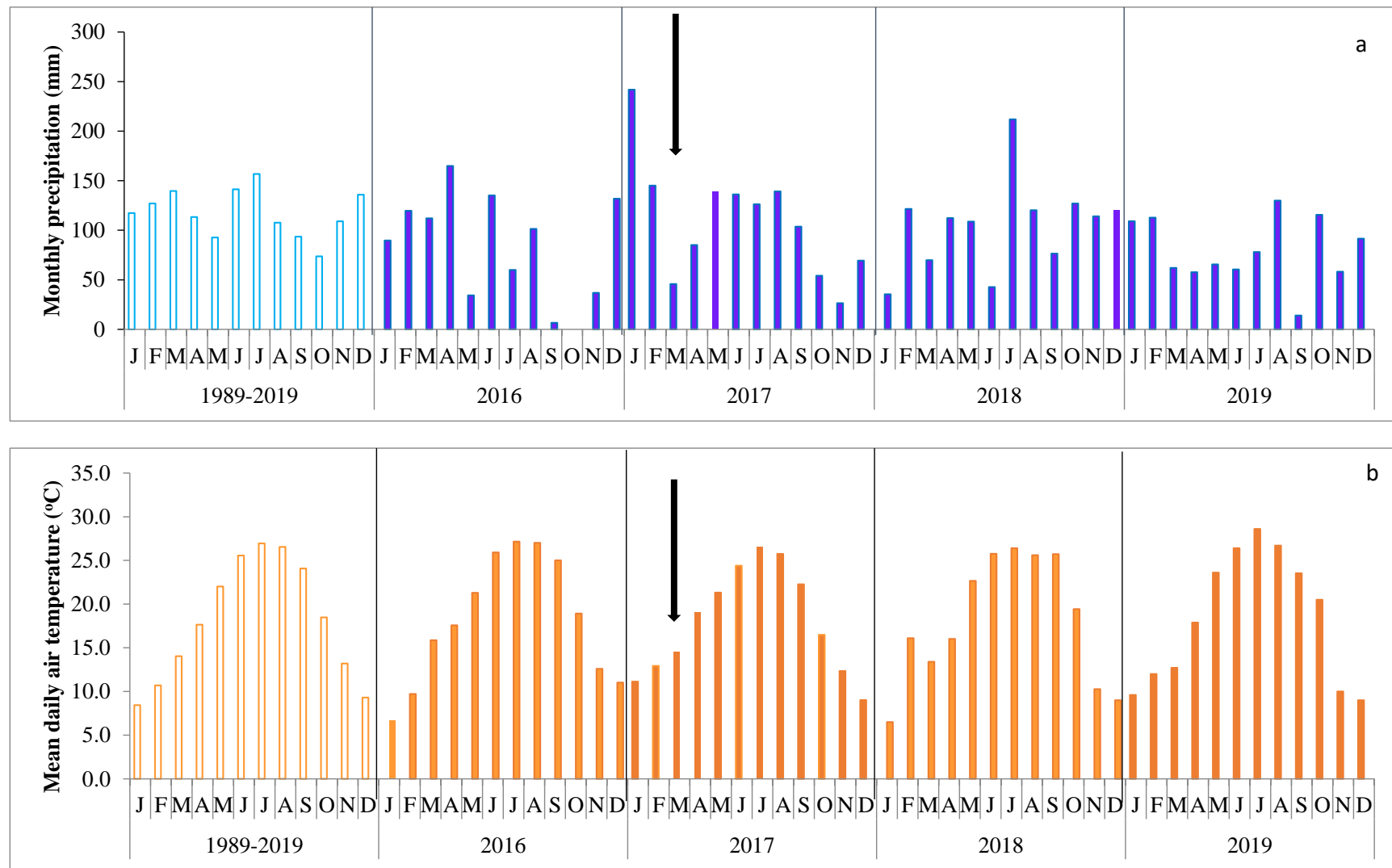
Figure 1. Monthly precipitation (**a**) and mean daily air temperature (**b**) at the study site near Eufaula, AL, from 2016 to 2019 (filled bars) and 30-year averages of these climatic variables (unfilled bars) between 1989 and 2019 [42]. Arrows indicate the time of inoculation treatment.

3.2. DBH, Total Tree Height, Stemwood Volume, and Relative Radial Stem Growth

At the time of plot establishment in December 2015, mean and standard error values of measurement tree DBH, total tree height, and stemwood volume were 16.9 ± 0.5 cm, 14.0 ± 0.2 m, and 0.32 ± 0.02 m^3, respectively. Measurement tree appearance was monitored for 15 months between January 2016 and the time of treatment application in March 2017, and during this period, no symptoms or signs of disease were observed. At the time of treatment application, measurement tree DBH, total tree height, and stemwood volume were not significantly different among the assigned treatments and averaged 17.40 ± 0.52 cm (mean $\pm$ standard error), 14.82 ± 0.21 m, and 0.36 ± 0.02 m^3, respectively, across the treatment plots.

As a main effect, stem inoculation of loblolly pine trees with *L. terebrantis* did not significantly affect tree DBH, total tree height, or stemwood volume (Table 1). After treatment application in March 2017, tree DBH and stemwood volume were significantly affected by year. Mean DBH was 18.4 ± 0.5 cm, 19.1 ± 0.4 cm, and 19.7 ± 0.5 cm, and mean stemwood volume was 0.43 ± 0.02 m^3, 0.49 ± 0.02 m^3, and 0.54 ± 0.03 m^3 in 2017, 2018, and 2019, respectively. Interaction between fungal treatment and year did not significantly affect tree DBH or stemwood volume.

Table 1. Probabilities of a greater *F*-value ($p > F$) from two-way analyses of variance of diameter at breast height (DBH), total tree height, stemwood volume, and relative stem radial growth (RG) 34 months following stem inoculation of *P. taeda* trees with *L. terebrantis* near Eufaula, AL, USA.

Variation	Source of Variation	df [1]	Mean Square	$p > F$
DBH	Treatment (T)	4	0.1	0.9826
	Year (Y)	2	283.07	<0.0001
	T × Y	8	1.12	0.3515
Total tree height	T	4	2.22	0.0697
	Y	2	443.26	<0.0001
	T × Y	8	2.75	0.0062
Stemwood volume	T	4	0.99	0.415
	Y	2	227.63	<0.0001
	T × Y	8	1.73	0.0915
RG	T	4	2.82	0.0277
	Y	2	84.02	<0.0001
	T × Y	8	4.94	<0.0001

[1] df, degrees of freedom; year (Y); treatment (T).

Total tree height was significantly affected by the interaction between inoculation treatment and year (Table 1). Total tree height did not differ by inoculation treatment in 2017 (Figure 2a). In 2018, however, total tree height of the low inoculum density trees was significantly greater than those of trees receiving the wound or high inoculum density treatments. In 2019, total tree heights by inoculation treatment were significantly greater than those in 2017 but statistically similar to those in 2018. Also in 2019, total tree height was significantly lower among the high inoculum density trees compared to those receiving the low or medium inoculum density treatments but not different from the control and wound treatments. By the end of the study in 2019, the total tree height of the high inoculum density trees (16.7 ± 0.22 m) was 9.7% less than the mean total tree height of the wound and low inoculum density (18.5 ± 0.24 m) trees.

Relative stem radial growth (RG) was also significantly affected by interaction between inoculation treatment and year (Table 1). Values of RG did not differ among inoculation treatments in 2017 or 2018 but were significantly affected by inoculation treatment in 2019, with 47% lower RG among the high inoculum density trees compared to the control, wound, and low inoculum density trees (Figure 2b). Among the high inoculum density trees, RG declined significantly in 2019 compared to RG in 2017 and 2018. Additional decreases in RG between 2017 and 2019 were observed in the control and medium inoculum density trees.
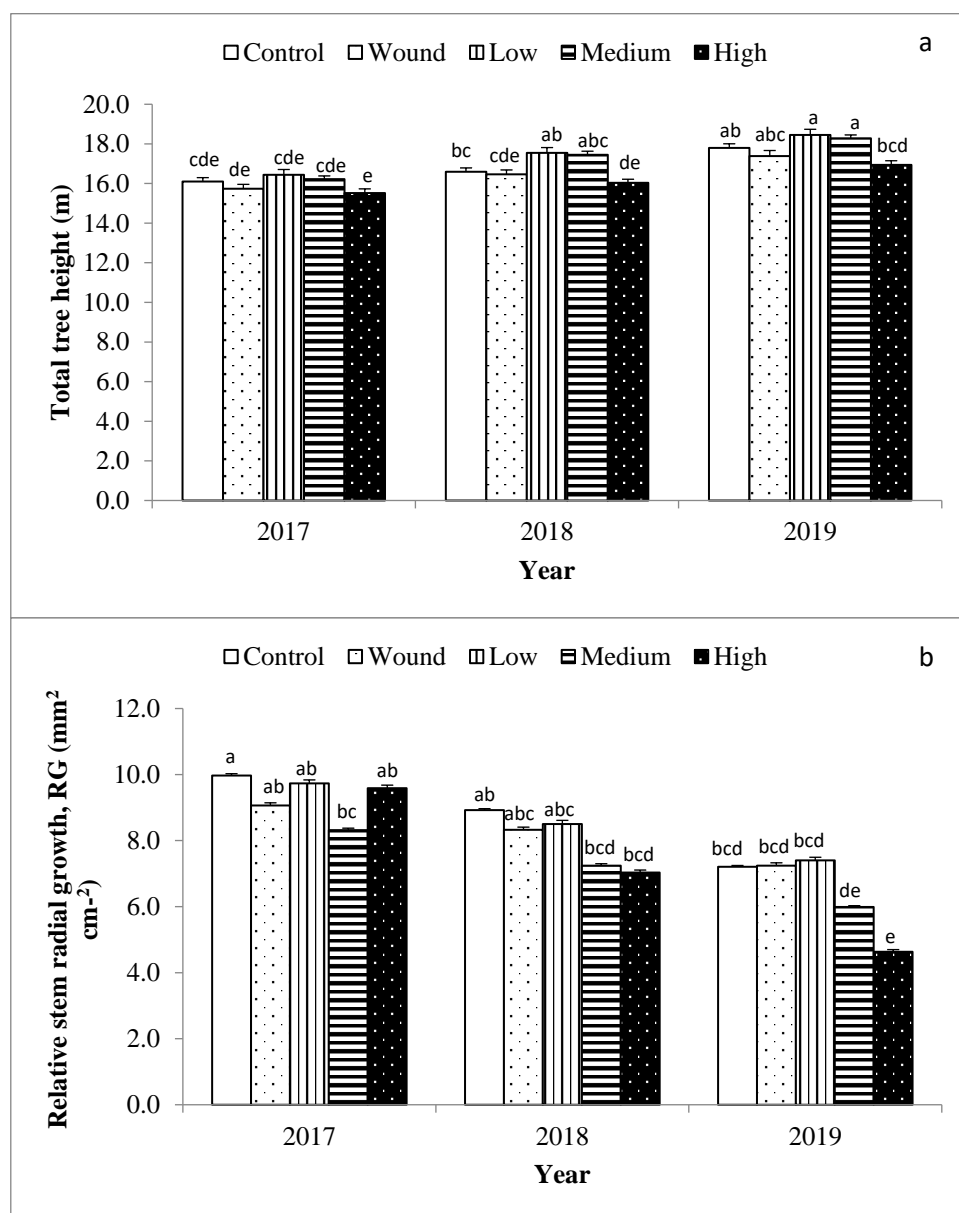
Figure 2. Mean *P. taeda* total height (**a**) and relative stem radial growth (**b**) 34 months following lower stem inoculation with *L. terebrantis* in March 2017. Means associated with different lowercase letters are significantly different at an *α*-level of 0.05 by the Tukey HSD test for multiple comparisons. Bars represent one standard error of the mean.

At the end of 2017, decline symptoms such as chlorotic and thin crowns 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.

3.3. Leaf and Sapwood Areas and Foliage Moisture Content

Estimates of A_L and $A_L:A_S$ were significantly affected by the inoculation treatment at the end of the study (Table 2). Values of A_L were significantly lower among trees receiving the high inoculum density treatment compared to those receiving the control treatment (Figure 3), but did not differ significantly from the wound, low, or medium inoculum density treatments. Similarly, $A_L:A_S$ was significantly reduced by the high inoculation treatment compared to the control and wound treatments (Table 3). A marginally significant

effect of the inoculation treatment on MC was observed at the end of the study ($p = 0.0904$), with 15% lower MC among trees treated with the high inoculum density compared to the wound trees (Table 3).

Table 2. Probabilities of a greater *F*-value ($p > F$) from one-way analyses of variance of tree projected leaf area (A_L), ratio of tree projected leaf area and sapwood area (A_L:A_S), and foliage moisture content (MC) 34 months following stem inoculation of *P. taeda* trees with *L. terebrantis* near Eufaula, AL.

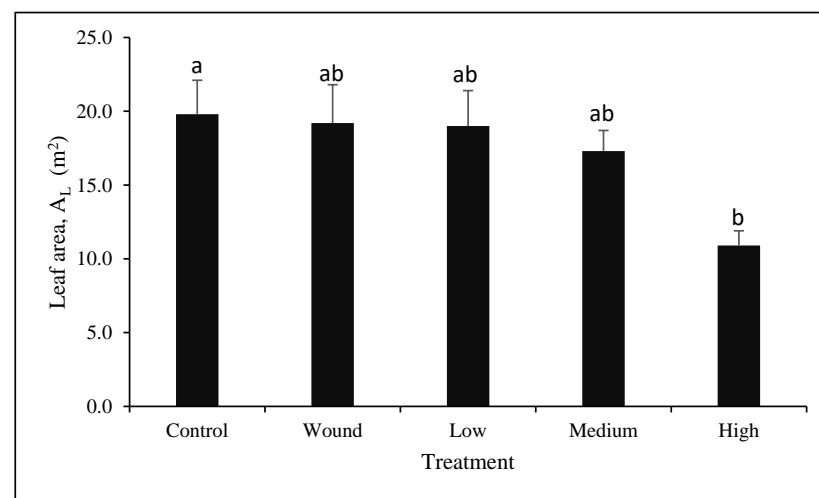| Variable	df [1]	*F*-Value	$p > F$
A_L	4	3.07	<0.0001
A_L:A_s	4	2.81	<0.0001
MC	4	2.11	0.0904

[1] df: degrees of freedom.

Figure 3. Mean projected tree leaf area of *P. taeda* in the third year after lower stem inoculation with *L. terebrantis*-colonized or sterile toothpicks or no inoculation. Means associated with different lowercase 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.

Table 3. Means and standard errors of the ratio of tree projected leaf area and sapwood area (A_L:A_S) and foliage moisture content expressed on dry weight basis (MC) 34 months following stem inoculation of *P. taeda* trees with *L. terebrantis* near Eufaula, AL, USA.

Treatment	A_L:A_S	MC (%)
Control	0.072 ± 0.06 a [1]	116.3 ± 3.4 ab
Wound	0.068 ± 0.06 a	117.8 ± 5.8 a
Low	0.064 ± 0.05 ab	114.0 ± 3.6 ab
Medium	0.063 ± 0.04 ab	110.3 ± 3.0 ab
High	0.039 ± 0.04 b	100.5 ± 3.5 b

[1] Means associated with different lowercase letters are significantly different at an *α*-level of 0.05 and 0.10 for A_L:A_S and MC, respectively, by the Tukey HSD test for multiple comparisons.

A significant ($p < 0.0001$) positive linear relationship was found between measurement tree leaf area (A_L) and DBH for all inoculation treatments except the high inoculum density (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: $p = 0.0011$, $r^2 = 0.57$) (Figure 4). By the end of the study, four high inoculum density trees had died, and the relationship between A_L and DBH among the remaining 11 high inoculum density trees was not significant. Slope and y-intercepts associated with the control, wound, and low, and medium inoculum density treatments were not significantly different from each other.
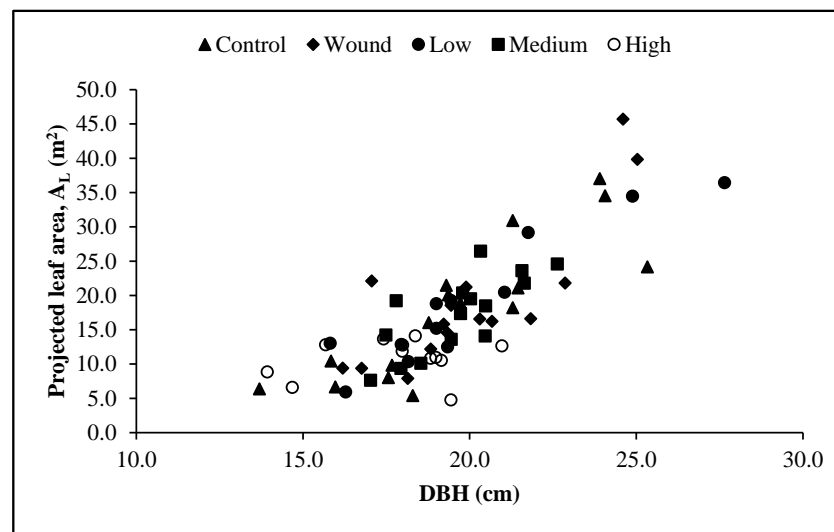
Figure 4. Relationship between diameter at breast height (DBH) and projected tree leaf area of *P. taeda* 34 months following lower stem inoculation with *L. terebrantis*-colonized toothpicks. Significant linear relationships were observed among the 15 trees receiving the control ($p = 0.0005$, $r^2 = 0.84$), wound ($p = 0.0013$, $r^2 = 0.67$), low ($p = 0.0001$, $r^2 = 0.87$), or medium ($p = 0.0011$, $r^2 = 0.57$) inoculation treatment. Among the high inoculum density trees, four died during the study, and the relationship between DBH and projected tree leaf area for the remaining 11 trees was not significant.

3.4. Root Growth

Cumulative root length density (CRLD) was not significantly affected by the inoculation treatment in 2017, 2018, or in February 2019 (Duwadi 2019), but in October 2019, which was 31 months after inoculation, CRLD was significantly affected by the inoculation treatment ($p = 0.0361$). The mean CRLD of trees treated with the high inoculum density was 60% lower than that of trees receiving the wound treatment, but did not differ significantly from mean CRLD of the control, low, or medium treatment (Figure 5).
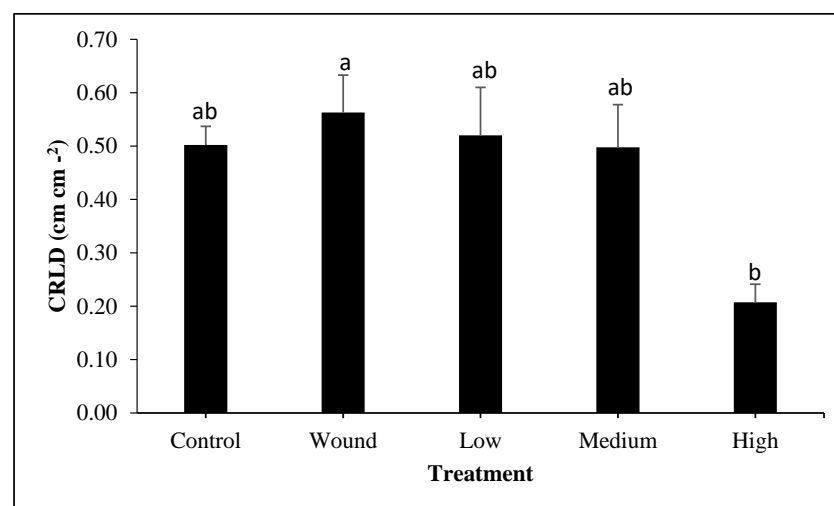

Figure 5. Cumulative root length density (CRLD) in October 2019 at the 0 to 50 cm soil depth 31 months following stem inoculation of *P. taeda* trees with *L. terebrantis*. Means associated with different lowercase letters are significantly different at an α-level of 0.05 by the Tukey HSD test for multiple comparisons. Bars represent one standard error of the mean.

4. Discussion

We investigated the potential of *L. terebrantis* to affect *P. taeda* growth and hypothesized that *L. terebrantis* infection impairs sapwood function and limits tree leaf area, new root production, and stemwood growth. *Leptographium terebrantis* compromised *P. taeda* xylem function by reducing stem MC and caused a decline in relative growth, leaf area, and new root growth, which became pronounced at high inoculum density. Although the effect of drought on the inoculated trees was not tested in the current study, the moderate drought recorded at the study site perhaps imposed additional stress on the inoculated trees and contributed to the negative effects of the pathogen on tree growth.

4.1. Stemwood Response to L. terebrantis

Relative stem radial growth (RG) of plantation loblolly pine was reduced after stem inoculation with *L. terebrantis*. Devkota et al. [55] attributed the loss of loblolly pine seedling shoot and root system biomass by *L. terebrantis* infection to sapwood occlusion. Other studies also attribute *Pinus* growth loss by wilt pathogen infection to sapwood occlusion followed by a direct decline in hydraulic conductivity that decreases carbon fixation [56–59].

Drought-induced water stress also has the potential to depress carbon fixation in loblolly pine [60–62]. These responses to drought are controlled, in part, by initial stomatal function and leaf area responses to water stress [63,64]. As water deficit continues, an increase or decrease in $A_S:A_L$ may evolve as an adjustment to sustain the whole-tree hydraulic function [65]. We attribute stem growth loss in the present study to physiological changes caused by the combined effects of the pathogen that include sapwood occlusion, decreased stem hydraulic conductivity, and interaction between the pathogen and moderate drought that reduced leaf area and disrupted hydraulic adjustment to water deficit.

The duration of physiological change leading to RG loss in plantation pine varies. A rapid decline in stem growth may occur when sapwood hydraulic function is aggressively compromised by pathogen invasion. Studies have shown that some ophiostomatoid fungal infections have immediate effects on stem sap flow, leading to foliage discoloration, impaired growth, and sudden tree mortality [66,67]. *Leptographium longiclavatum* sp. nov. caused the formation of chlorotic crowns, but no mortality was observed in mature *P. contorta* nine months postinoculation [67]. Mensah et al. [29] reported a delayed response to stem infection with *L. terebrantis* in a *P. taeda* plantation and found that crown symptoms occurred 19 months after inoculation, followed by significant tree decline or mortality 34 months after inoculation [29]. The timeframe between pathogen introduction and growth loss may depend on several factors, including intrinsic site variables, tree vigor at the time of and after infection, and other circumstances that affect carbon dynamics during the tree decline process.

In the present study, a significant decrease in RG was not observed until the third growing season after inoculation, and this occurred predominantly among trees treated with the high inoculum density. We attribute this lagged response to one or more means of compensating for the loss of sapwood function caused by occlusion. Variation in the length of time between wilt pathogen infection and tree mortality [29], and the challenge of landscape-scale efforts to monitor tree mortality related to pine decline [68] may be attributed, in part, to tree- and microsite-specific mechanisms of compensating for decreases in hydraulic function, and, therefore, delays in or avoidance of tree decline or mortality.

As water availability decreases, a downward adjustment in leaf area and a shift in carbon allocation that increases the depth or amount of rooting may occur in loblolly pine [65,69–71]. These conditions sustain moderate fascicle-level stomatal function and carbon fixation, which may or may not accompany a decrease in carbon allocation to stemwood [72–74]. At the same time, in localized areas, natural variation in edaphic conditions that control the plant-available water and root system depth could worsen existing water limitations and drive the trajectory of stemwood growth downward. In support, past research has shown that deep soil water acquisition is important to sustain tree vigor where soil texture or climate limit plant-available water [69,75]. As such, stemwood

growth loss and the occurrence of pine decline may be greater for individuals or patches of trees under extreme but isolated edaphic conditions compared to elsewhere in a forest stand. The localized nature of this scenario was described by Ryu et al. [76], with greater susceptibility to tree mortality where the site index was low and crown vigor was poor.

At the start of our study, we anticipated measurement trees were of relatively high vigor because of their dominant crown class and average site index of 22 m at 25 years [77]. We expected crown function of the dominant class to be high and effective in buffering pathogen impacts on carbon allocation to the stem. At the end of our study, the correlation between tree DBH and projected leaf area among all treatments except the high inoculation treatment indicated that the physiology of normal carbon allocation was compromised in the high inoculum density trees (Figure 4). In our study, stem infection with *L. terebrantis*, considered a weak to moderate pathogen [78], contributed to tree decline, while moderate drought in 2019 incited this process and accelerated its occurrence. Together, the pathogen and moderate drought overcame normal carbon relations, leading to stemwood growth loss. It is important to note that this course of loblolly pine decline is unique to our study.

4.2. Mechanism of Stemwood Growth Loss and Decline

The effects of pine decline on RG may have been direct or indirect. Direct effects included a loss of stem xylem function by occlusion that reduced the amount of water supplied to sustain stomatal conductance and photosynthesis, followed by less carbon allocation to the stem. Previous studies have shown that a loss of foliar moisture concentration occurs when stomatal conductance and photosynthesis are reduced by water stress [79,80]. By 34 months after inoculation, we observed a decrease in MC as the inoculum density increased, with 14% less MC in the high inoculum density trees compared to the noninoculated trees. Similarly, a reduction in sapwood MC and function has been shown when young *P. taeda* trees that were inoculated with *L. terebrantis* [36].

Loblolly pine adjusts to water deficit, in part, by shifts in carbon allocation among the foliage, stem, and root system that sustain the supply of water for whole-tree stomatal function [69,81,82]. A downward adjustment in the ratio of the leaf area and conducting sapwood area (A_L:A_S) represents an adjustment in the hydraulic architecture that sustains the whole-crown gas exchange under conditions of increased vapor pressure deficit during drought [65,83,84]. These changes in A_L:A_S occur gradually over consecutive growing seasons [85]. A decrease in the ratio of the leaf and fine root surface areas (A_L:A_R) also reflects a hydraulic adjustment that sustains water supplied from the soil for gas exchange [69,86].

During prolonged periods of water stress, failure to adjust the hydraulic architecture by shifts in A_L:A_S and A_L:A_R may worsen stomatal limitations already established by pathogen-induced sapwood occlusion. At the end of our study, high inoculum density trees were characterized by a 46% decrease in A_L:A_S and 45% and 61% losses of tree leaf area and new root production, respectively, compared to the control trees. While a decrease in A_L:A_S was possible as an adaptation to water deficit, the magnitude of this response is well above that observed under normal circumstances. For example, only an 11% decrease in A_L:A_S in response to 5 years of simulated drought in plantation loblolly pine [65]. In addition to foliage loss, root deterioration has been observed in concert with pathogen-associated tree decline [22,87]. We propose that the combined effect of drought and stem occlusion on carbon fixation indirectly affected RG by decreases in leaf area and new root growth, as well as a failure to maintain hydraulic plasticity by favorable shifts in A_L:A_S and A_L:A_R.

Furthermore, it is possible that water stress due to the loss of the stem hydraulic function and sustained drought between March and September of 2019 caused the premature senescence of older foliage that would have normally remained in the crown as a source of fixed carbon until natural senescence in fall 2019 [88–90]. It has been shown that the maximum monthly needle-fall occurred earlier in dry years than in wet years in the 10-year-old plantation loblolly pine [88]. Therefore, both poor hydraulic function and moderate drought may have contributed to a loss of A_L among our high inoculum density

trees due to a reduction in new foliage growth as well as the premature senescence of mature foliage.

Regardless of the direct or indirect means of relative growth loss in declining pines, carbon deficit predisposes stands to attack by bark beetles and their associated fungal pathogens [91–94]. Pest infestation may further limit stem growth because fixed carbon that is normally allocated to the stem is redirected to induction of defense chemicals [39,95–97].

A gradual reduction in cumulative root length density at the 0 to 50 cm soil depth was also observed in response to the high inoculation treatment, and this effect became significant late in the third year after inoculation. Poor root system health is commonly observed in forest decline settings [23,98–100] and has been linked to carbon deficit [18] and pathogen spread in the root xylem [87].

As whole-crown carbon fixation decreases in response to stomatal limitations caused by water stress, carbon allocation may shift away from shoot growth to support root system maintenance and growth [81,101,102]. This was observed in *Pinus sylvestris* L. (Scots pine) seedlings acclimated to prolonged drought, with a decrease in foliage and stem biomass but no effect on root biomass [103]. A similar shift may have occurred across all inoculation treatments in our study in response to moderate drought in 2019. We propose that as the high inoculum density trees became carbon-limited by the combined effect of drought and *L. terebrantis* infection, adequate carbon allocation to root growth failed.

4.3. Importance to Forest Management

Comparison of tree leaf area and new root initiation between the control treatment and inoculation treatments with sustained stemwood growth validate the value of current-year sapwood in mitigating the progression of pine decline. Opportunities to slow decline-related growth loss and tree mortality may lie in management activities that sustain sapwood growth across a range of edaphic and climatic conditions. In essence, new sapwood assumes the dual purpose of stemwood production and continuous water supply when the hydraulic function of existing sapwood is compromised. In their regional survey of loblolly pine health, Coyle et al. [104] reported linkages between poor crown health and both stand basal area and hardwood presence which implicated competition for light and water as critical factors in poor pine health. Ongoing climatic shifts that increase evapotranspiration and plant water stress in the southeastern United States [105,106], combined with relatively high pine basal areas and levels of hardwood competition, could reduce historical carrying capacity. In this situation and without timely stand density management, water limitations may cause tree vigor to be compromised [5,107]. In stands receiving little density management, self-thinning could remedy soil resource limitations for sustained forest health. In this situation, however, the well-being of residual trees would be jeopardized by the decline factors that preceded self-thinning.

Woody root infestation with a wilt pathogen that exacerbates existing or developing water limitations serves as a contributing or inciting factor in the pine decline model [36]. It has been shown that new sapwood growth avoided detrimental water deficits despite the hydraulic limitation caused by the combined effect of old sapwood occlusion and drought [29]. Where water deficit is presently common or is predicted to worsen in the future, and infestation with root-feeding bark beetles is plausible, further research is warranted to determine stand density and thinning recommendations that sustain sapwood growth despite the hydraulic limitations caused by wilt pathogens associated with pine decline.

5. Conclusions

The present study used stem inoculation as a surrogate for woody root infection to demonstrate the progression of loblolly pine decline in plantation. We observed a decrease in RG only after stem inoculation with the high inoculation density treatment. It is important to note that this RG response is not analogous to that in natural pine decline settings where hydraulic malfunction begins in woody root sapwood. Furthermore, the

causal factors of pine decline are unique to each setting where it occurs. Thus, it is not possible to generalize about the underlying conditions and timeframe of pine decline based solely on observations in the present study. However, comparison of stemwood growth, tree leaf area, and root initiation between our high inoculum density trees and naturally declining loblolly pines provides insight about the trajectory of stand production where symptoms of pine decline have appeared. It is also apparent from our results that, despite the ongoing pine decline, sustained sapwood growth not only supports stemwood production but also ensures a supply of water for carbon fixation in the crowns of affected trees.

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