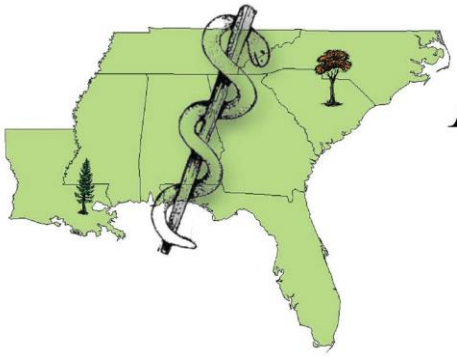


AUBURN UNIVERSITY
FOREST HEALTH COOPERATIVE

# RESEARCH REPORT 20-06

EFFECT OF *L. TEREBRANTIS* ON THE PRODUCTION OF DEFENSIVE CHEMICAL COMPOUNDS IN LOBLOLLY PINE

by
John Mensah and Lori Eckhardt

## 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 23ºC 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 $\alpha$=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).

## 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.

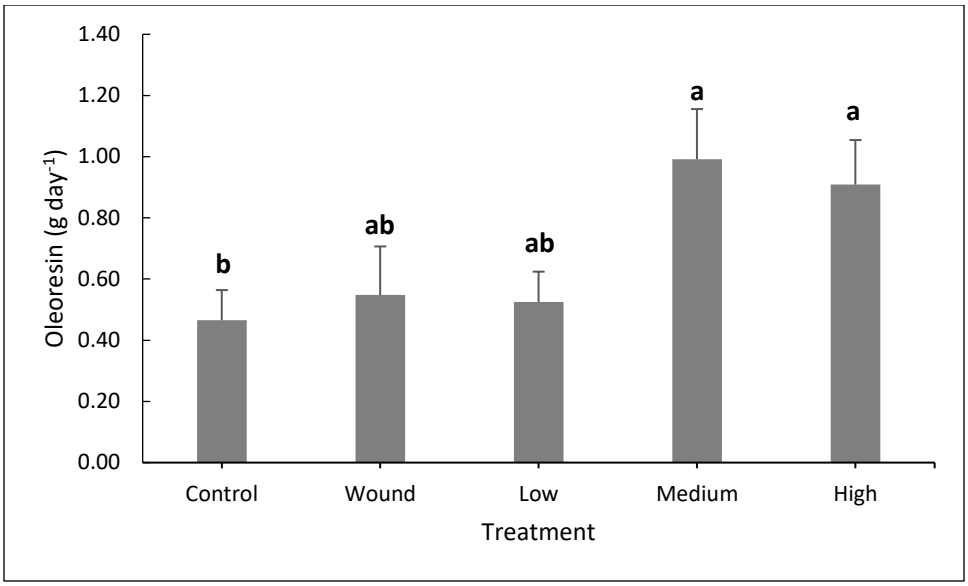



**Figure 7.1.** Amounts of oleoresins produced by *P. taeda* following inoculation with *L. terebrantis* colonized or sterile toothpick and non-inoculated control treatment.

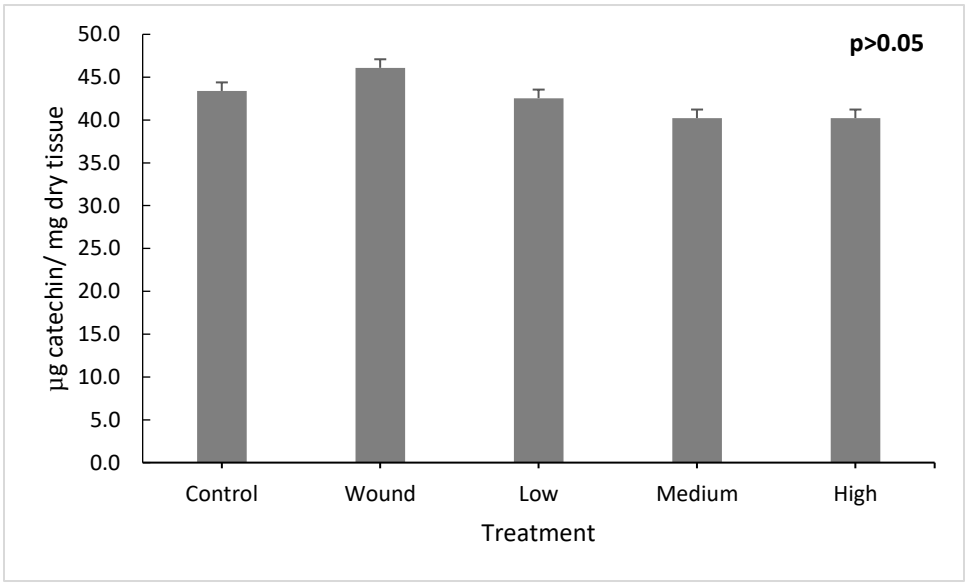



**Figure 7.2.** Amounts of total phenolic produced following inoculation with *L. terebrantis* colonized or sterile toothpick and non-inoculated control treatment. Note the reduction in total phenolic content among the low, medium and high inoculum treatments relative to the control and wound treatments.

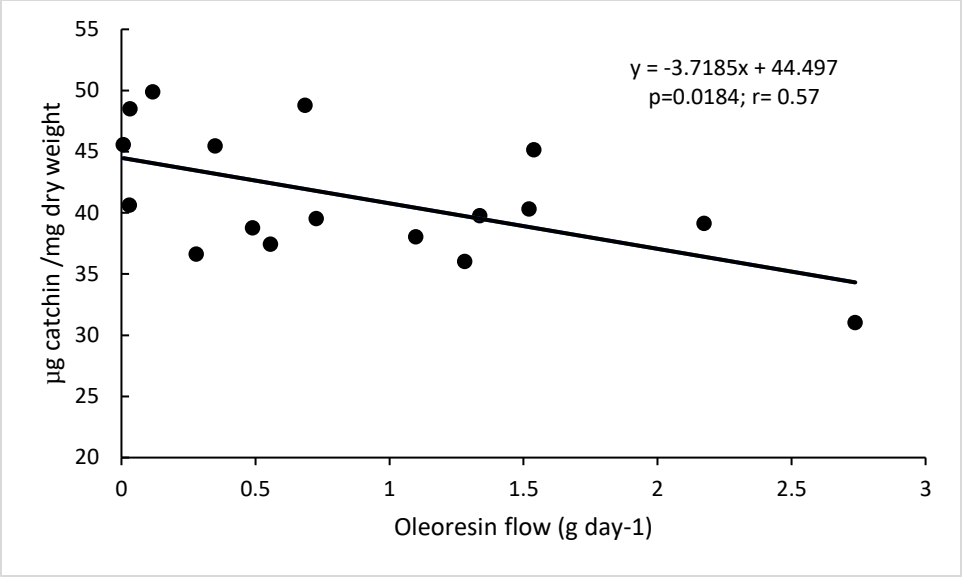
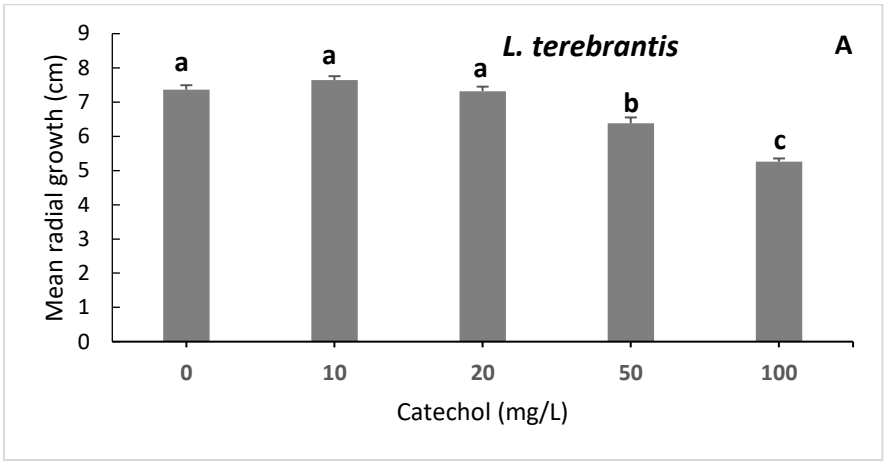
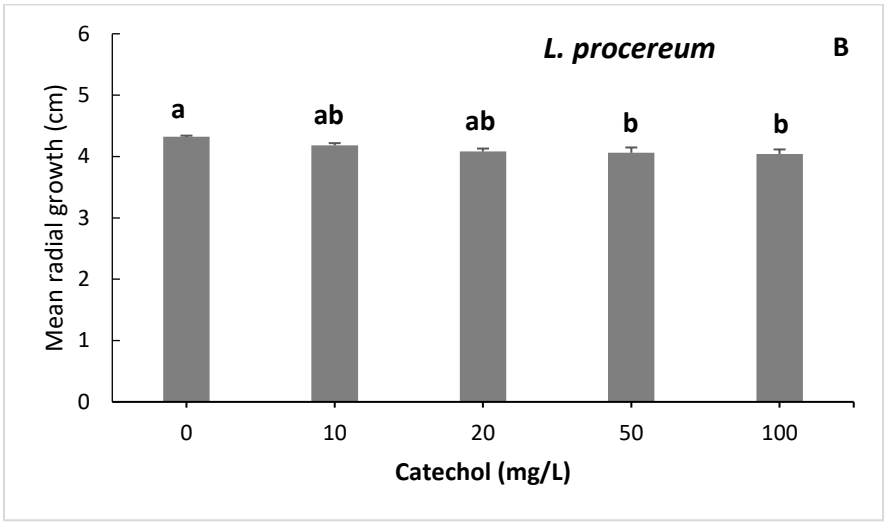



**Figure 7.3.** Relationship between total phenolic content (catechin) and oleoresin flow rate after inoculation of the *L. terebrantis* inoculum densities.



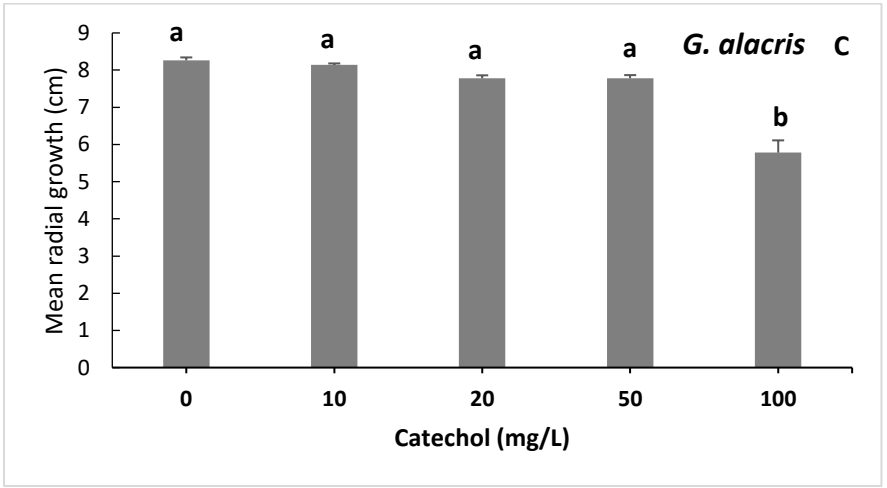




**Figure 7.4.** Mean radial growth of bark beetle associated fungi on MEA amended with catechol solution and incubated at 23°C for 8 days (a) *L. terebrantis* (b) *L. procereum* (c) *G. alacris*.


**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.

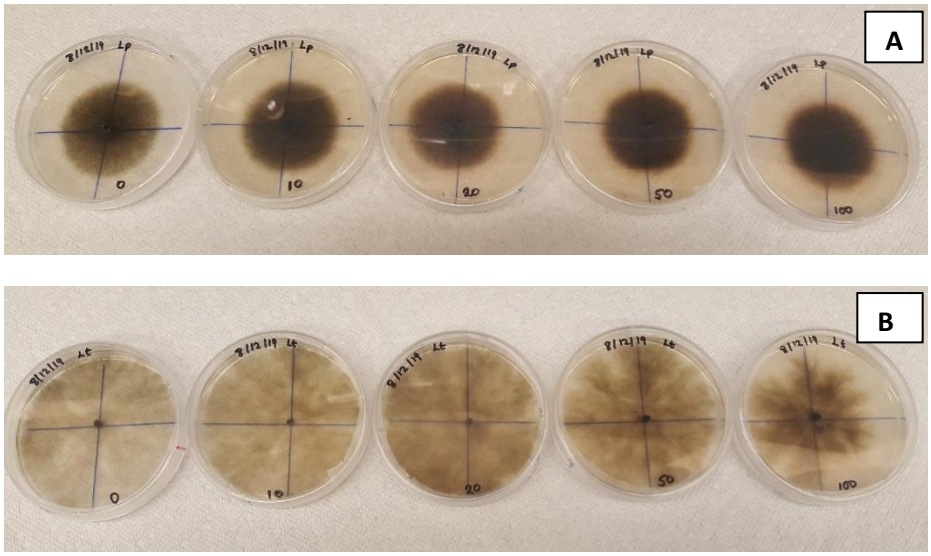



**Figure 7.6.** (a) Growth of *L. procereum* on amended MEA after 5 days of incubation (b) and *L. terebrantis* on amended MEA after 10 days of incubation at 23ºC.