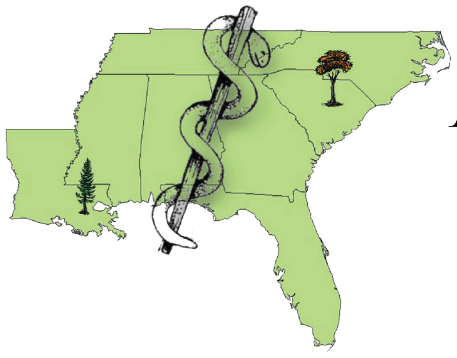

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PINUS TAEDA SAPLINGS RESPONSE TO
LEPTOGRAPHIUM TEREBRANTIS DIFFERENTIAL INOCULUM DENSITY

by
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2.1. ABSTRACT

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

2.2. INTRODUCTION

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

Host–pathogen interactions are commonly explored by introducing fungal mycelia or spores in the healthy host tissue under controlled (laboratory) or semi-controlled (glasshouse or field-grown trees) conditions. A variety of methods and protocols have been used. The most commonly used

techniques to explore ophiostomatoid fungi and their host interaction include creating a wound using a cork borer in field-grown trees (Solheim et al., 1993; Yamaoka et al., 2000; Lee et al., 2006) or with a sterile razor blade to create a bark flap in seedlings to insert a plug of agar containing mature fungal mycelia (Devkota et al., 2018b).

These artificial inoculation techniques have consistently been used to successfully infect host tissue and to determine the resulting tissue necrosis and occlusion in the host (Parmeter et al., 1989; Kuroda, 2005; Devkota et al., 2018a; Devkota et al., 2018b). Wounds caused by both the cork borer and bark flap methods may, however, contribute to local tissue necrosis and occlusion of vascular tissue (Yamaoka et al., 2000; Matusick and Eckhardt, 2010). Additionally, the common wounding methods are inadequate for examining the effects of mass inoculation of fungi, which occurs when several beetles attack and inoculate the host with the associated ophiostomatoid fungi. In addition to causing localized wounds that are more extensive than what occurs naturally, the commonly used inoculation methods introduce unrealistically large quantities of actively growing fungal mycelium into the host tissue. For example, in artificial inoculation experiments involving *Leptographium* spp., mycelial plugs of 5–12 mm diameter are commonly used to inoculate the host tree (Yamaoka et al., 2000; Fäldt et al., 2006; Matusick et al., 2016). The beetle vector, *Dendroctonus frontalis* Zimmermann (southern pine beetle), of *L. terebrantis* is, however, about 2–4 mm long and 1–1.5 mm wide (Thatcher, 1981). Due to practical concerns, including the experimental length (commonly 8 weeks), these inoculation methods have been developed to ensure timely results. Inoculation methods may, however, greatly contrast with the natural inoculation process. Hence, there is a need for a alternative inoculation approach that can more closely mimic pathogen introduction in the host during bark beetle attack.

Host damage resulting from the beetle–fungi complex is a function of fungal virulence, inoculation density (the number of the fungal inoculation points per unit of bark) and inoculum load (Horntvedt et al., 1983; Solheim, 1992), among other factors. The attack density of the bark beetles above which tree mortality may occur is expressed as a critical attack threshold (number of attacks per m^2 of bark) (Lieutier et al., 2009). For example, the threshold is 50–120 for *Dendroctonus ponderosae* Hopkins on *P. contorta* Dougl, (Raffa and Berryman, 1983; Raffa, 2001) and 850 for *Ips acuminatus* Gyllenhal on *P. sylvestris L.* (Guérard et al., 2000). Critical thresholds of inoculation density have been determined as 300–400 for *L. wingfieldii*, and 400–800 for *Ophiostoma minus* (Hedgcock) H. & P. Sydow on *P. sylvestris* L. (Långström et al., 1993; Solheim et al., 1993).

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

Thus, consideration of inoculation at different radial densities may be important for examining the response of conifers to bark beetle-vectored ophiostomatoid fungal inoculation. Inoculations using fungal cultured toothpicks have been used in the past, including pathogenicity test of *L. terebrantis* in *P. strobus* L. seedlings (Wingfield, 1986). The toothpicks may serve as a reliable means for inoculum transfer as the width of the beetle (1–1.5 mm) is comparable to that of the toothpick (1–2 mm). Toothpick or point inoculations may be a useful method for understanding the response of

Pinus species to multiple or mass fungal inoculations. Despite numerous studies that have been conducted involving the inoculation of ophiostomatoid fungi in conifers (Yamaoka et al., 2000; Fäldt et al., 2006; Davydenko et al., 2017), the intricate relationship between these fungi and their host may not have been adequately understood due to the lack of an efficient inoculation technique which can simulate the natural fungal inoculation by bark beetles (Guérard et al., 2000).

To advance the adoption of artificial inoculation method that closely mimics natural inoculation by bark beetles, we utilized *L. terebrantis* colonized toothpicks to determine the pathogenicity of the pathogen on *P. taeda* saplings and identify potential inoculum densities for further studies in mature trees.

2.3. MATERIALS AND METHODS

2.3.1. Fungal isolate culturing

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

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

2.3.2 Pinus taeda sapling stem inoculation

To determine whether the toothpick point inoculations can effectively be transferred to living tissue, an experimental plot was established in naturally regenerating *P. taeda* stand (approximately 7 years old) with mean annual precipitation of 1523.74 mm near Andalusia, Alabama (31.1427° N, 86.6963° W). In May 2016, a total of 108 healthy *P. taeda* saplings were selected and then distributed across three replicate groups. The average groundline diameter of *P. taeda* saplings was 6.4 (±1.3) cm.

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

To determine the tissue damage caused by the inoculation method and toothpick alone, three trees per treatment within each replicate were inoculated with sterile toothpicks to serve as

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

2.3.3. Post inoculation assessment

Eight weeks after inoculation, the trees were cut at ground level and 15 cm above the inoculation zone and transported to the laboratory on ice. To determine the presence of infection, the percentage of the radial area of tissue occlusion, the length and volume of occluded sapwood tissue, and the degree of the infection across various levels of inoculation density were assessed. The occlusion length was determined by scraping the bark of the stem segment and cutting both ends of the stem until occluded tissue was identified.

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

2.3.4. Re-isolation

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

2.3.5 Data analysis

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

2.4. RESULTS

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

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

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

2.5. DISCUSSION

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

The increase in radial inoculation points increased occlusion length, area and volume. This is consistent with the earlier finding of Fernandez et al., (2004) who reported unstained sapwood area of *Pinus sylvestris* to decrease with increasing inoculum density of *Ophiostoma ips* (Rumbold) Nannfeldt. Furthermore, they reported that higher inoculum densities resulted in yellow-green coloration in needles as opposed to no symptoms at lower densities. *Leptographium wingfieldii* Morelet was also found to cause more sapwood occlusion in *P. sylvestris* trees relative to *Ophiostoma canum* (Münch) Syd. & P. Syd. and *Ophiostoma minus* (Hedgcock) H. & P. Sydow (Solheim et al., 2001) but did not cause mortality in the inoculated trees. Nonetheless, Solheim et al., (1993) found that *L. wingfieldii* and *O. minus* were able to kill *P. sylvestris* trees when the

inoculation points were 800 per m^2 on whole tree basis. They also found mortality to occur at a lower inoculum density (400 inoculations per m^2) when the trees were subjected to pruning stress. Mass inoculation loads have been found to cause the sudden decline of tree health (Horntvedt et al., 1983; Christiansen, 1985; Solheim and Krokene, 1998).

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

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

Table 2.1. Mean and standard errors of occlusion length and volume associated with different inoculation points.

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

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

Table 2.2. Associations between tissue occlusions and *Leptographium terebrantis* inoculation points in *Pinus taeda* saplings.

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

(Note: IP: Inoculation points.)

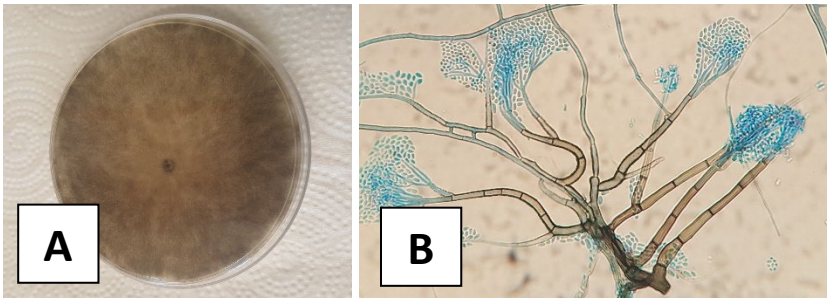

Figure 2.1. *L. terebrantis* (a) Pure culture on MEA and (b) Conidiophore bearing conidia cultured on MEA

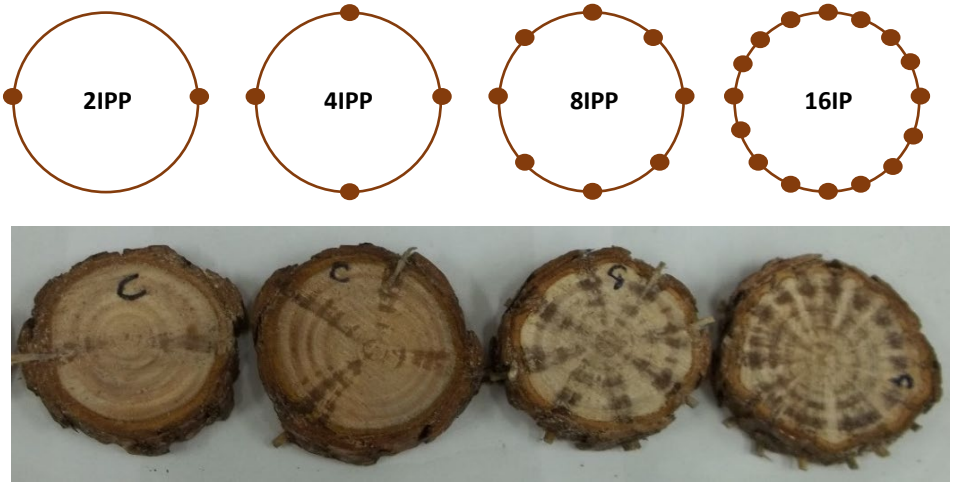

Figure 2.2. Radial position of inoculation points of four different inoculum densities of *Leptographium terebrantis* in young *Pinus taeda* trees followed by tissue occlusions caused by those inoculum densities (2, 4, 8, and 16 inoculation points from left to right) in the bottom.

(Note: 2IP: two inoculation points, 4IP: four inoculation points, 8IP: eight inoculation points, and 16IP: sixteen inoculation points).

Figure 2.3. *Pinus taeda* sapling inoculated at 16 radial points x 4 vertical points with sterilized toothpicks (control).

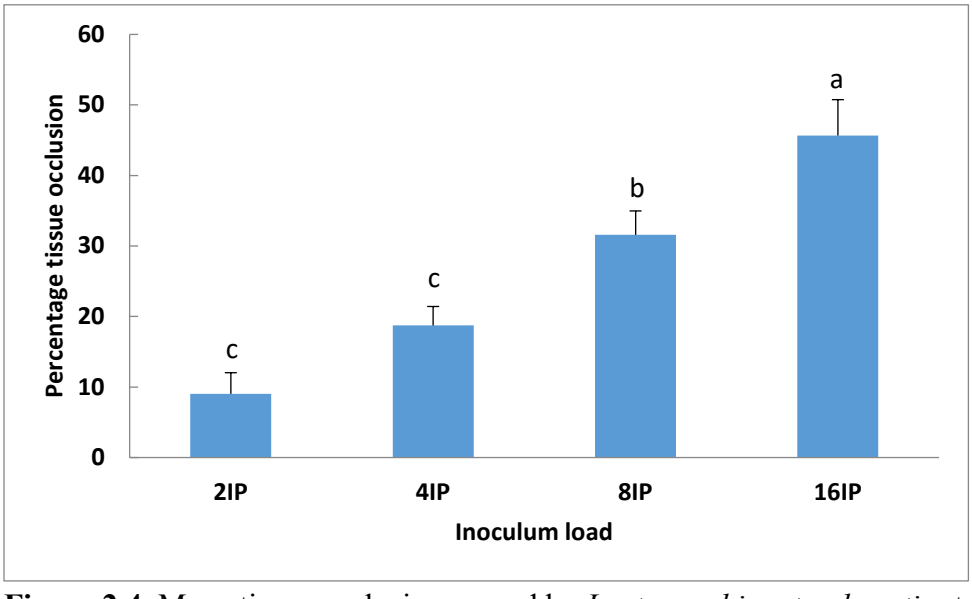

Figure 2.4. Mean tissue occlusion caused by *Leptographium terebrantis* at different inoculum loads in filed trees. Different letters indicate significant differences in percentage of tissue occlusion caused by different inoculation points at $\alpha = 0.05$. Error bars represent 95 % confidence intervals.

(Note: 2IP: 2 inoculation points, 4IP: four inoculation points, 8IP: eight inoculation points, 16IP: sixteen inoculation points).