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Pinus taeda L. response to differential inoculum density of *Leptographium terebrantis* colonized toothpicks

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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. Thus, we designed a series of stepwise experiments using *L. terebrantis* colonized toothpicks which focused on the inoculum transfer from the toothpicks to excised *Pinus taeda* stem segments and living saplings, respectively, at different inoculum densities. 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 blue-stain and sapwood occlusions in *P. taeda* stems and saplings, respectively. The volume of occluded, visually damaged sapwood increased by 1.96 cm^3 per radial inoculation point on average. Fungal colonized toothpicks can be used as a suitable alternative to agar discs for studying bark beetles vectored fungi and their host interactions.

KEYWORDS
blue-stain, *Leptographium*, mass inoculation, ophiostomatoid fungi, *Pinus taeda*

1 | INTRODUCTION

With their distinctive ability to cause sap-stain and tree disease, ophiostomatoid fungi are an ecologically and economically important group of fungi worldwide (Seifert, de Beer, & Wingfield, 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 defences, which can lead to host mortality under certain circumstances (Horntvedt, Christiansen, Solheim, & Wang, 1983; Jacobs & Wingfield, 2001). Developing a better understanding of how ophiostomatoid fungi affect water and nutritional status of a host is critical for predicting when trees may succumb to fungal invasion. To develop an improved understanding of this host–pathogen interaction, it is important to challenge the host with the level of fungal inoculum similar to that is likely to be encountered by trees grown under natural field conditions.

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 (Lee, Kim, & Breuil, 2006; Solheim, Långström, & Hellqvist, 1993; Yamaoka, Takahashi, & Iguchi, 2000) or with a sterile razor blade to create a bark flap in seedlings to insert a plug of agar containing mature fungal mycelia (Devkota, Enebak, &

Eckhardt, 2018a). 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 (Devkota, Nadel, & Eckhardt, 2018b; Devkota et al., 2018a; Kuroda, 2005; Parmeter, Slaughter, Chen, Wood, & Stubbs, 1989). Wounds caused by both the cork borer and bark flap methods may, however, contribute to local tissue necrosis and occlusion of vascular tissue (Matusick & Eckhardt, 2010; Yamaoka et al., 2000). 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. Development of a method that closely mimics natural inoculation by bark beetles is important so as to assess the relationship between inoculum load, tissue damage and symptom development.

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 (Fäldt, Solheim, Långström, & Borg-Karlson, 2006; Matusick, Nadel, Walker, Hossain, & Eckhardt, 2016; Yamaoka et al., 2000). 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, whereby only fungal spores are introduced by the bark beetle associates, and the results may correspondingly contrast with the host effects following natural inoculation. Hence, there is a need for a uniform 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 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, Yart, & Salle, 2009). For example, the threshold is 50–120 for *Dendroctonus ponderosae* Hopkins on *P. contorta* Dougl, (Raffa, 2001; Raffa & Berryman, 1983) and 850 for *Ips acuminatus* Gyllenhal on *P. sylvestris L.* (Guérard, Dreyer, & Lieutier, 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, Solheim, Hellqvist, & Gref, 1993; Solheim et al., 1993). These mass inoculation experiments represent a significant advancement in the understanding of host responses to the beetle–fungal complex. 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, Walsh, & Cole, 1982) and cause vascular tissue occlusion (Oliva, Stenlid, & Martínez-Vilalta, 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 (Davydenko, Vasaitis, & Menkis, 2017; Fäldt et al., 2006; Yamaoka et al., 2000), 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, we designed a series of stepwise experiments using *L. terebrantis*, a common ophiostomatoid species in eastern North America, to determine the operability of using fungal colonized toothpicks for point inoculation studies of mature *P. taeda* hosts. The initial experiment focused on determining how toothpicks should be prepared for successful fungal culture, while the second and third experiments focused on inoculum transfer in laboratory and field studies, respectively. Collectively, the specific aims of these experiments were (a) to determine whether toothpicks soaked with fungal growth media can act as a better surface for vectoring fungus than toothpicks without growth media (i.e., un-soaked), (b) to determine the effectiveness of toothpicks in transferring *L. terebrantis* to recently excised stem segments and living tree stem and whether they cause blue-stain and sapwood occlusions, respectively, and (c) to determine the effects of radial inoculation densities in *P. taeda* saplings.

2 | MATERIALS AND METHODS

2.1 | Excised *Pinus taeda* stem segment inoculation

To determine whether toothpick point inoculations can successfully transfer the fungus to plant tissue, 48 stem segments, each of 16 cm length was excised from seven-year-old naturally regenerating *P. taeda* saplings (6 ± 0.5 cm diameter) without any above-ground signs and symptoms of a disease. To prevent the moisture loss, the cut segments were wrapped with polyethylene bags and transported in an ice-filled box. Immediately following the transportation to the laboratory, paraffin wax was melted in glass containers, and exposed ends of the stem segments were sealed by dipping them into the melted wax and stored at 8°C until used.

Wooden toothpicks were used as a substrate for the inoculum production. Approximately 700 toothpicks were autoclaved in 500 ml Erlenmeyer flasks covered with aluminium foil at 121°C and 0.103 MPa for 60 min. To determine whether sterile toothpick soaked in malt extract broth (MEB) will facilitate the fungal transfer compared to un-soaked dry toothpicks, half of the sterilized wooden toothpicks were soaked in MEB overnight in a sterile environment and the other half were left un-soaked. 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 at equidistant in the MEA plates. Four mycelial agar plugs of 5-mm-diameter discs of actively growing *L. terebrantis* (ATCC accession no. MYA-3316) 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. The *L. terebrantis* isolate used for the study was isolated from the roots of *P. taeda* with damaged local tissue and deteriorated crowns in Talladega National Forest, Oakmulgee Ranger District, AL, USA (Eckhardt, Weber, Menard, Jones, & Hess, 2007). The fungal isolate utilized in the study was highly pathogenic to *Pinus taeda* host as compared to 41 other *L. terebrantis* isolates (Devkota & Eckhardt, 2018) isolated by Eckhardt et al. (2007).

In order to mimic the size and depth of feeding by an individual bark beetle during the natural feeding process, 1.5-mm-diameter drill bit was used to drill an approximately 5-mm-deep hole to reach to the phloem of the stem segments. To prevent any external contamination, bark was surface sterilized with 70% ethanol prior to inoculation. Each drilled point served as an inoculation point. The free end of a toothpick with sporulating fungus was inoculated in each drilled hole and the protruding end was cut with a sterile knife. The number (density) and the distance between (spacing) the holes determined the levels of inoculation treatment. To determine the impact of density and spacing of inoculation points, four different inoculation treatments were adopted: (a) vertically 2.4 cm apart (5 points per segment) (LV), (b) vertically and radially 2.4 cm apart (10 points per segment) (LVR), (c) vertically 1.2 cm apart (10 points per segment) (HV) and (d) vertically and radially 1.2 cm apart (20 points per segment) (HVR), (Figure 1). Each type of inoculation was treated to six randomly selected stem segments. To maintain high relative humidity, inoculated segments were kept in sterile bins filled with moist sterile sand and covered with a moist sterile cheesecloth.

After 8 weeks, the stem segments were cut transversely with a band saw into smaller segments to expose the cross-sectional area underneath each inoculation point in order to observe fungal growth. The area of the fungal staining on 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 each wood segment was calculated assuming a perfect cylinder ($\pi r^2 h$). The volume of the entire stained tissue in each segment was then calculated using the formula $S \times h$ (where S = average stained area of each cross-section and h = total length of the segment). Finally, the total percentage of stains in each segment was calculated using the formula $(Sh / \pi r^2 h) \times 100$. 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.

2.2 | *Pinus taeda* sapling stem inoculation

To determine whether the toothpick point inoculations can effectively be transferred to living tissue, an experimental plot was

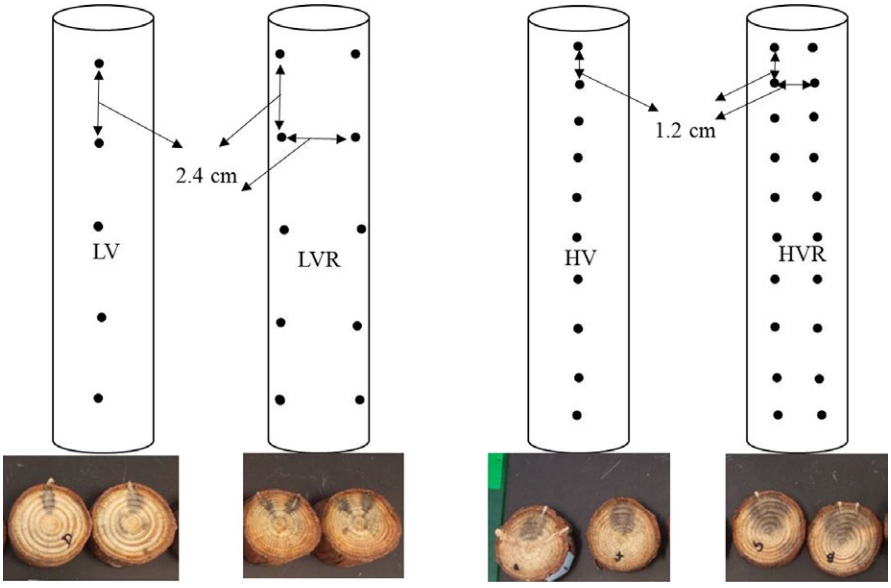

FIGURE 1 Excised *Pinus taeda* stem segments showing different inoculation points for *Leptographium terebrantis* inoculation followed by associated blue staining in discs from each inoculation point at the bottom. (Note: LV: Vertically 2.4 cm apart, LVR: Vertically and radially 2.4 cm apart, HV: Vertically 1.2 cm apart, and HVR: Vertically and radially 1.2 cm apart)

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* trees were selected and then distributed across three replicate groups. 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 (Figure 2 and Figure 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 the stem segment inoculation experiment. Fungal cultured toothpicks presoaked in MEB were used for inoculation. 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. 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, and the inoculation was conducted in similar manner as described for the excised *P. taeda* stem segments. After the inoculation, the protruding ends of the toothpicks were clipped, and inoculation zone was sealed with duct tape to prevent contamination due to external contaminants. 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.

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. Each treated stem segment was sectioned into four small segments using a band saw. The stem segments were painted with FastGreen dye (0.25 g L^{-1} water) and the tissue that remained unstained was regarded as occluded tissue. Occluded areas were traced onto transparent sheets and measured with a planimeter as described above. Area of occluded tissue at the cross-section of each segment was measured, and the total volume of the occluded tissue per sapling segment was calculated. The occlusion length was determined by scraping the bark of the stem segment. From each tree, 5 mm section of the stem tissue around the inoculation point was plated on MEA as described earlier to confirm re-isolation of the *L. terebrantis*.

2.3 | Data analysis

Data were analysed using a general linear model (GLM) in SAS statistical software (SAS Institute, 9.4 versions, Cary, NC). Data were first checked for normality and equal variance. Data for stain volume from stem segments and occlusion length and volume from the saplings were log transformed. Stem segment diameter was used as a covariate in the model. Pairwise comparisons were undertaken using the Post Hoc Tukey's test on the four fungal treatments at $\alpha = 0.05$. Graphs were generated using STATISTICA 10.

3 | RESULTS

3.1 | Excised *Pinus taeda* stem segment inoculation

The toothpick served as an effective substrate for *L. terebrantis* inoculum development and inoculation. Close examination of the toothpicks indicated that the fungus had completely colonized the toothpicks. Fungal sporulation was greatly favoured towards the free end of the toothpick. The tissue in the free end of excised wooden segments was blue-stained 2 weeks following *L. terebrantis* inoculation. Upon sectioning the stem segments after 8 weeks, pie-shaped blue staining at cross-sections was observed.

The volume of the stain in broth-soaked (105.56 ± 14.87 cm^3) toothpicks inoculated stem segments was higher compared to those inoculated with the un-soaked (53.33 ± 14.87 cm^3) toothpicks ($p = 0.0027$) (Tables 1 and 2; Figure 4). *Leptographium terebrantis* was successfully re-isolated from all inoculated segments.

The distance between the inoculation points had a significant effect on total percentage of the fungal staining ($F_{(3, 44)} = 5.92$, $p = 0.0009$) (Table 1 and Figure 1). There was nearly 100% staining in some of the stem segments, which were inoculated vertically and radially 1.2 cm apart. The stem segments inoculated both radially and vertically had more coverage of fungal stain compared to the stem segments only inoculated radially or vertically (Table 2).

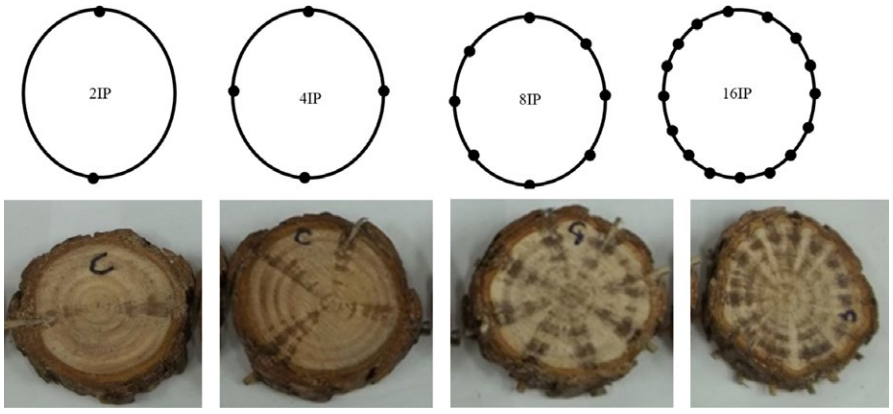

FIGURE 2 Radial position of inoculation points of four different inoculum densities of *Leptographium terebrantis* in young *Pinus taeda* trees followed by tissue occlusions caused by those inoculum densities (2, 4, 8 and 16 inoculation points from left to right) in the bottom (Note: 2IP: two inoculation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

3.2 | *Pinus taeda* tree stem inoculation

The average groundline diameter of *P. taeda* saplings was 6.4 (± 1.3) cm. 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 and 5). 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.

FIGURE 3 *Pinus taeda* sapling inoculated at 16 radial points × 4 vertical points with sterilized toothpicks (control)

TABLE 1 The effects of various variables on percentage of tissue staining (*N* = 48)

Source	DF	*F* value	*p* > F
Diameter	1	1.06	0.3103
Media	1	10.24	0.0027
Treatment	3	5.92	0.0009
Media*Treatment	3	0.24	0.8707

Note. Treatment: different inoculum load; media: soaked or un-soaked.

Percentage of tissue occlusion area, occlusion length and volume increased with increasing radial inoculum density (Table 3 and Figure 2). Occluded area was significantly different ($F_{(3, 68)}$ = 22.84, p = <0.0001) among treatments (Table 3). 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).

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 3) 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 4). 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 4. The occlusion length showed a weak association with the inoculum density and accounted only for 32% of the variation (Table 4).

TABLE 2 Estimates of the difference in staining caused by various *Leptographium terebrantis* inoculum loads in excised *Pinus taeda* stem segments

Parameter	Log (Estimate) percentage stain	Standard error	*T*-value	*p* > |t|
So vs Un-so	−0.281	0.088	−3.2	0.0027
HVR vs HV	−0.465	0.125	−3.71	0.0006
HVR vs LVR	−0.252	0.132	−1.91	0.0639
HVR vs LV	−0.473	0.120	−3.95	0.0003
HV vs LVR	0.212	0.126	1.68	0.1003
HV vs LV	−0.009	0.123	−0.07	0.9447
LVR vs LV	−0.221	0.130	−1.7	0.0963

Note. So: Toothpicks soaked in broth; Un-so: toothpicks un-soaked in broth, HVR: vertically and radially 1.2 cm apart, HV: vertically 1.2 cm apart, LVR: vertically and radially 2.4 cm apart and LV: vertically 2.4 cm apart.

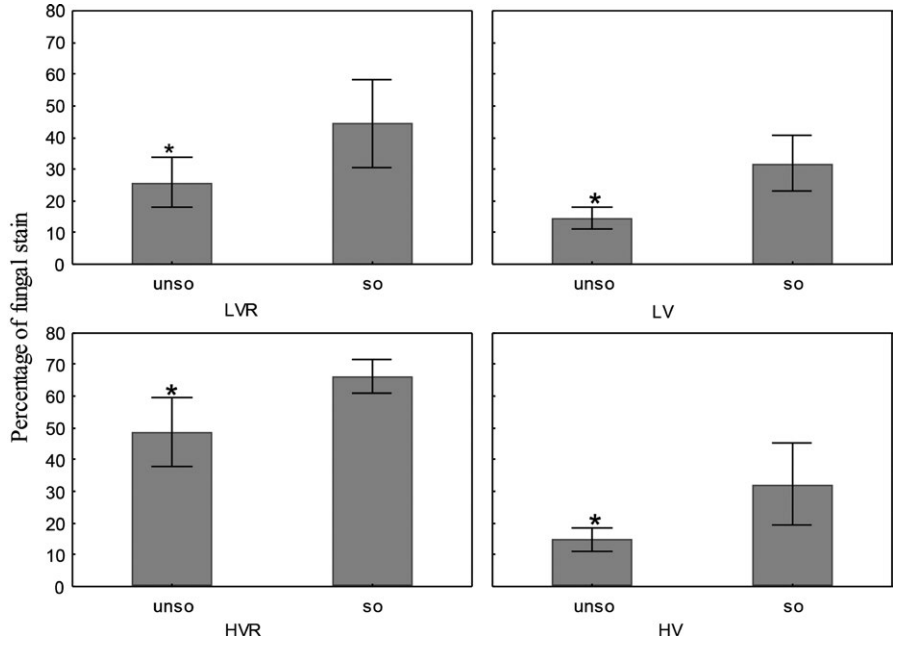

FIGURE 4 The percentage of the total tissue stained by *Leptographium terebrantis* cultured in broth-soaked and un-soaked toothpicks at different inoculum loads in excised *Pinus taeda* stem sections. Bars represent standard errors. Asterisk (*) represents statistically significant differences between percentage of stain caused by fungus cultured on malt extract soaked and un-soaked toothpick at α = 0.05. (Note: LVR: Vertically and radially 2.4 cm apart, LV: Vertically 2.4 cm apart, HVR: Vertically and radially 1.2 cm apart and HV: Vertically 1.2 cm apart

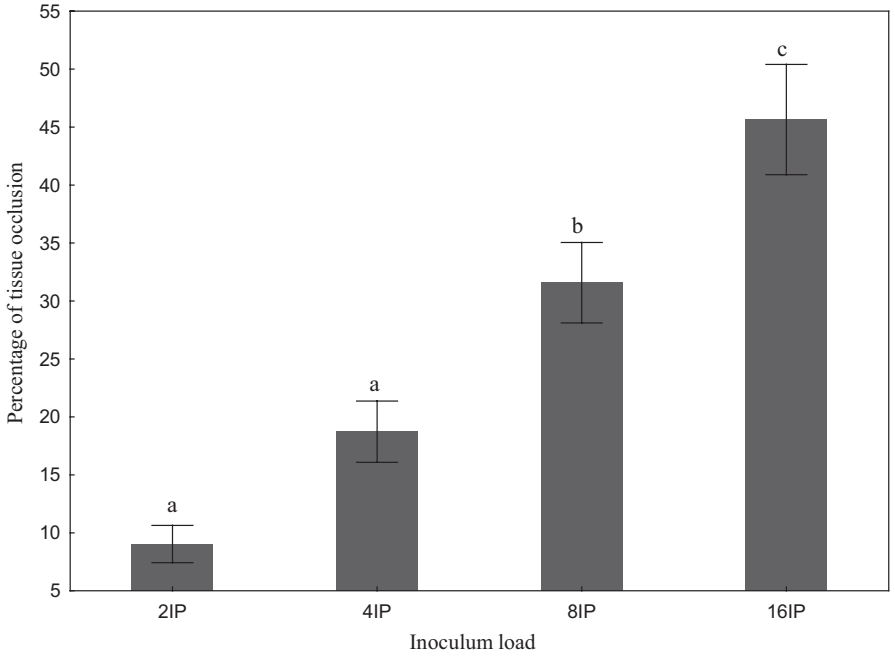

FIGURE 5 Mean tissue occlusion caused by *Leptographium terebrantis* at different inoculum loads in filed trees. Error bars represent standard errors. Different letters indicate significant differences in percentage of tissue occlusion caused by different inoculation points at α = 0.05. (Note: 2IP: 2 inoculation points, 4IP: four inoculation points, 8IP: eight inoculation points and 16IP: sixteen inoculation points)

4 | DISCUSSION

Toothpicks are suitable as a substrate for ophiostomatoid fungal inoculum production and inoculation. Ophiostomatoid fungi can be cultured on MEB soaked or un-soaked toothpicks. The relative performance of the broth-soaked toothpicks was, however, higher with regard to the fungal staining in the wood sections. Kusumoto, Masuya, Ohmura, and Kamata (2012) utilized toothpicks to inoculate a beetle-vectored fungus, *Raffaelea quercivora* Kubono et Shin., into logs of *Quercus crispula* Blume and *Q. serrata* Thunb. ex Murray. Similarly, *Pinus strobus* L. seedlings were successfully inoculated with *L. procerum* (Kendriek) Wingfield (= *Verticicladiella procera* Kendrick) (Wingfield (1986) and *L. terebrantis* (Wingfield,1985) colonized toothpicks.

Variation in the density of inoculation points played a significant role in determining the growth and spread of *L. terebrantis* into the stem segments of *P. taeda*. The higher number of inoculation points, and closer relative position to one another, clearly contributed to greater coalescence and expansion of blue-stain. The fungal spread in the cross-section was favoured by increasing the radial point of inoculation as compared to the vertical inoculation point. Results presented here are in agreement with the proposed pattern of movement of ophiostomatoid fungi within plant tissue, which includes more prominent radial spread through the ray parenchyma

TABLE 3 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 4 Associations between tissue occlusions and *Leptographium terebrantis* inoculation points in *Pinus taeda* saplings

Linear regression equations	Sample size	$p > F$	R^2
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.

cells followed by movement into tracheids (Ballard et al., 1982). Further, tracheid to tracheid movement of fungus occurs via bordered pits which leads to disturbance in the host water-conducting system (Ballard, Walsh, & Cole, 1984; Ballard et al., 1982). Moreover, after mass inoculation of ophiostomatoid fungi, the percentage of the functional sapwood decreases causing an increase in the loss of sapwood hydraulic conductivity (Encina, Valbuena, Acebes, Lieutier, & Fernandez, 2012). Single point and multiple point fungal inoculations utilizing toothpicks may be an important approach to evaluate the relative impact of various inoculation densities on water-status and overall health of the tree.

The fungus colonized toothpicks caused sapwood occlusions in the inoculated saplings. The colonized wooden toothpicks have been successfully used as an artificial vector for use in insect-vectored ophiostomatoid fungi inoculation studies under laboratory and field conditions as reported in few other toothpick inoculation studies (Takahashi, Matsushita, & Hogetsu, 2010; Wingfield, 1986). 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, Six, De Beer, Wingfield, & Roux, 2016), saplings of *Quercus crispula* Blume (Kusumoto et al., 2012), 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, Garcia, and Lieutier (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, Krokene, & Långström, 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 (Christiansen, 1985; Horntvedt et al., 1983; Solheim & 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, Gallego, Sánchez-García, & Pajares, 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 longer-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 greater period to allow for the development of symptoms and mortality, if they are to occur.

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