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ARTICLE

Responses of Engelmann spruce to inoculation with *Leptographium abietinum*, a symbiotic fungus of the North American spruce beetle

Jane E. Stewart, Franklin L. Harris, Kristen Otto, and Thomas Seth Davis

Abstract: Symbiotic fungi associated with tree-killing bark beetles can alter host-tree physiology with consequences for tree survival, and symbiont genetic variation and environmental variability may impact these interactions. Here, we test whether multiple genetically distinct isolates of a symbiotic fungus (*Leptographium abietinum* (Peck) M.J. Wingf.) associated with North American spruce beetle (*Dendroctonus rufipennis* (Kirby, 1837)) vary in their ability to manifest defensive responses consistent with disease symptoms in seedlings of Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), a primary host-tree species for the beetle–fungus complex in North America. Our experiments incorporate variation in both host-tree water availability and host defenses (phloem monoterpene concentration). Three central findings emerged: (*i*) isolates varied considerably in their effects on host trees — inoculation with *L. abietinum* isolates from Colorado caused significantly larger phloem lesions than isolates from Wyoming, though all isolates caused phloem oxidation; (*ii*) neither water availability nor spruce phloem monoterpene concentrations impacted lesion formation; and (*iii*) both inoculation with *L. abietinum* and water deficit inhibited the formation of callus tissue at wound sites. We conclude that *L. abietinum* isolates vary in their virulence and that inoculation is not lethal but may benefit beetles by altering tree defensive responses.

Key words: Dendroctonus, ophiostomatoid fungi, phytopathogen, symbiosis.

Résumé : Les champignons symbiotiques associés aux scolytes qui causent la mort des arbres peuvent modifier la physiologie de l'arbre hôte, ce qui a des répercussions sur la survie des arbres, et la variation génétique des symbiotes ainsi que la variabilité environnementale peuvent avoir un impact sur ces interactions. Dans cette étude, nous avons testé si de multiples isolats génétiquement distincts du champignon symbiotique (*Leptographium abietinum* (Peck) M.J. Wingf.) associé au dendroctone nord-américain de l'épinette (*Dendroctonus rupipennis* (Kirby, 1837)) varient quant à leur capacité à susciter des réactions de défenses consistantes avec les symptômes de maladie chez des semis d'épinette d'Engelmann (*Picea engelmannii* Parry ex Engelm.), une des principales espèces d'arbre hôte du complexe scolyte-champignon en Amérique du Nord. Nos expériences incorporent à la fois la variation de la disponibilité de l'eau pour l'arbre hôte et celle des défenses de l'arbre hôte (concentration des monoterpènes dans le phloème). Trois résultats principaux ressortent : (*i*) les isolats variaient considérablement quant à leurs effets sur les arbres hôtes; l'inoculation avec des isolats de *L. abietinum* provenant du Colorado a causé des lésions significativement plus importantes dans le phloème que les isolats provenant du Wyoming, quoique tous les isolats aient causé l'oxydation du phloème, (*ii*) cependant, ni la disponibilité de l'eau, ni la concentration des monoterpènes dans le phloème de l'épinette n'ont eu d'impact sur la formation des lésions, (*iii*) tant l'inoculation avec *L. abietinum* qu'un déficit hydrique ont inhibé la formation de tissus calleux à l'endroit de la blessure. Nous concluons que les isolats de *L. abietinum* varient quant à leur virulence et que l'inoculation n'est pas létale mais peut être bénéfique pour les scolytes en altérant les réactions de défense de l'arbre. [Traduit par la Rédaction]

Mots-clés : Dendroctonus, champignons ophiostomatoïdes, phytopathogène, symbiose.

Introduction

Over the past decade, significant decline of spruce–fir forest has been documented across the western United States; much of this decline is attributed to North American spruce beetle (*Dendroctonus rufipennis* (Kirby, 1837)), a primary agent of mortality of New World spruces. Endemic spruce beetle populations typically colonize weakened or suppressed host trees, but epidemic "outbreak" populations can occur when there is a high availability of weakened hosts (e.g., from wind or drought events; Veblen et al. 1991; Hart et al. 2014) or climate conditions are otherwise favorable to the beetle (Bentz et al. 2010). During outbreaks, beetles rapidly overcome host defenses during pheromonally coordinated "mass attacks", and outbreak events often precipitate landscape-scale tree mortality with broad-ranging ecological, economic, and cultural effects on forest ecosystem dynamics (Morris et al. 2018).

Spruce beetles, like other *Dendroctonus* species, are associated with a consistent suite of fungal symbionts that appear to play an important role in beetle life history. The primary symbiont of spruce beetle is *Leptographium abietinum* (Peck) M.J. Wingf., a blue-staining species that is closely related to other well-known *Leptographium* fungi, including some that are plant pathogens (Solheim 1995; Solheim and Safranyik 1997; Six and Bentz 2003). *Leptographium abietinum* is transmitted to host trees by dispersing

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adult beetles and is inoculated into the phloem of host trees during mating and oviposition. The fungus subsequently grows throughout the subcortical egg-gallery environment, and beetle larvae feed and develop on phloem colonized by the fungus. Compared with other associations between bark beetle and fungus, relatively little is known about interactions among beetle, symbiont, and host tree in the spruce beetle system. However, a stronger understanding of these interactions can serve to both inform basic ecological theory and provide targets for the development of management applications for control of outbreak populations.

Recent research indicates that *L. abietinum* can both tolerate and metabolize some host defensive compounds (monoterpenes) (Davis et al. 2018*a*), outcompete pathogenic microbes that can kill adult beetles (Davis et al. 2018*b*), and bioconcentrate a suite of primary and secondary compounds, including nitrogen, phosphorus, and fatty acids, that may be nutritionally important to the spruce beetle (Bentz and Six 2006; Davis et al. 2019). These collective effects indicate that the fungus likely benefits the beetle during colonization of host trees. In addition, some species of spruce exhibit induced reaction zones (lesion formation) when inoculated with *L. abietinum*, including Lutz spruce (*Picea* × *lutzii* Little), white spruce (*Picea glauca* (Moench) Voss), and Sitka spruce (*Picea sitchensis* (Bong.) Carrière) (Werner and Illman 1994), suggesting that host trees recognize and respond to the presence of *L. abietinum* in phloem tissues. Other studies have shown that *L. abietinum* is pathogenic on multiple hosts, including Sitka spruce and white spruce (Solheim and Safranyik 1997; Ohsawa et al. 2000), and isolates of *L. abietinum* caused mortality to white spruce but not to Sitka spruce seedlings. However, across most of the contiguous United States, the primary host for spruce beetle is Engelmann spruce (*Picea engelmannii* Parry ex Engelm.) (Alexander 1987), and no work has yet investigated whether *L. abietinum* causes a physiological response in Engelmann spruce. It is possible that introduction of *L. abietinum* into the phloem of Engelmann spruce during colonization by spruce beetle has phytopathogenic effects, and the present study provides a first description of these interactions.

Earlier investigations have indicated that *L. abietinum* is genetically variable even within regional beetle populations (Davis et al. 2019), but the consequences of this variation for host trees are not yet understood. Here, we test whether *L. abietinum* manifests symptoms in Engelmann spruce that are consistent with phytopathogenicity, interpreting our tests within the context of both genetic and environmental variation. Our objectives are to (*i*) evaluate the effects of multiple genetically distinct isolates of *L. abietinum* on lesion formation, a symptom consistent with tree defensive induction in response to a biotic challenge, in Engelmann spruce; (*ii*) assess concentrations of host-tree defensive chemicals, specifically monoterpene hydrocarbons, as resistance factors that limit development of lesions in spruce phloem; and (*iii*) determine whether trees with reduced water availability are more susceptible to *L. abietinum* than well-watered trees. We address these objectives using greenhouse studies and experimental inoculations to control for fungal symbiont identity and water availability. Our results provide new information about the effects of *L. abietinum* on a widespread host tree of the spruce beetle, with consequences for the interpretation of interactions among spruce beetles, their symbionts, and host trees.

Methods and materials

Fungal symbionts of spruce beetle

Isolates were collected and identified as described by Davis et al. (2018*a*). Briefly, isolates were collected from spruce beetle galleries that were excavated from colonized Engelmann spruce from infested sites in Colorado and Wyoming, United States. Fungi were isolated from surface washes of live adults. Six isolates of *L. abietinum* were selected for the present study and genotyped using four loci (internal transcribed spacer (ITS) regions): the larger subunit (LSU) of the 28S region (ITS-LSU), calmodulin, β-tubulin, and translation elongation factor 1α (Davis et al. 2019). GenBank numbers for Colorado isolates CF1, CF7, CF11, and CF12 at the ITS-LSU are MH084773, MH084764, MH084768, and MH084776, respectively. In this study, Wyoming isolates WY5 and WY7 were sequenced at the ITS-LSU regions using primers ITS3-LR5 (White et al. 1990) for species identification as described by Romón et al. (2014). GenBank numbers for WY5 and WY7 are MN837534 and MN837535, respectively. Selected isolates were examined further for virulence to Engelmann spruce seedlings.

Greenhouse conditions and watering treatments

A total of 110 Engelmann spruce saplings were obtained from the Colorado State Forest Service Nursery as 3-year-old seedlings and transferred to 3.5 L pots. Seedlings were watered to saturation while allowing for drainage every other day for 90 days to ensure that trees were healthy before treatments began. Seedlings were separated into two blocks reflecting two greenhouse benches, allowing the experiment to be repeated twice concurrently, and experimental watering was applied. Each individual seedling was weighed at full pot capacity prior to application of treatments. Trees in each block were then randomly selected to receive an ample-water treatment (1.0 g $H_2O \cdot g^{-1}$ soil) or a low-water treatment (0.5 g $H_2O \cdot g^{-1}$ soil). Twice per week, trees were weighed on a scale (CBK35, Adam Equipment, Oxford, Conn., USA) and given the appropriate amount of water to maintain the ample- or low-water treatments. This watering regime was applied to the seedlings for a period of 90 days prior to inoculation with *L. abietinum* to ensure adequate time for trees to adjust to watering treatments. Greenhouse conditions during the experiment were as follows (mean ± standard error (SE)): temperature, 16.8 ± 0.3 °C; relative humidity, 22.3% ± 0.3%; and photoperiod, 16 h light : 8 h dark.

Inoculation with *L. abietinum* and tree measurements

Isolates of *L. abietinum* (CF1, CF7, CF11, CF12, WY5, and WY7) were selected for pathogenicity testing based on our previous genetic studies (Davis et al. 2019). Isolates were grown on 2% malt extract agar (MEA; Sigma–Aldrich, St. Louis, Miss., USA) plates for 20 days prior to inoculations. Saplings were inoculated by removing the outer bark 100 mm above the soil surface with a 5 mm diameter cork borer, placing an agar plug containing mycelium on the wound, and then covering the wound with Parafilm (Bemis Company, Neenah, Wis., USA). As a control for the effects of inoculation treatment, a subset of seedlings received "control" inoculation that contained only agar with no *L. abietinum* mycelia. In each block, each possible inoculation treatment (n = 7; six *L. abietinum* isolates and one control treatment) by water treatment (n = 2; ample and low water) combination consisted of seven replicates. Following inoculation, experimental watering of study seedlings continued for 90 days, after which point the experiment was terminated and study seedlings were destructively sampled for measurements of seedling condition.

Bark was removed from study seedlings around the inoculation site using a razor blade, and lesion length (in millimetres) and circumference were measured using a digital caliper to quantify variation in tree responses to the different *L. abietinum* isolates. The formation of callus tissue was scored on a binary scale (no callus tissue formed, 0; callus tissue formed, 1). Phloem discoloration (relative degree of oxidation of phloem tissues) at the inoculation site was also scored using a rating scale that ranged from 1 to 5, consistent with a range of no phloem staining to severe phloem staining. A rating of 1 indicated that only callus material was observed at the inoculation site; callus tissue appeared in situations in which lesion development did not occur. A rating of 2 indicated some callus tissue and discoloration, but only at the inoculation site. A rating of 3 indicated callus tissue and discolor-

ation outside of the inoculation site. A rating of 4 indicated moderate discoloration outside of the inoculation site with no callus tissue. A rating of 5 was assigned when there was considerable discoloration extending in all directions around the inoculation site (above and below) and no formation of callus tissue. Similarly, relative tree vigor was scored at the end of the experiment using a visual rating scale that ranged from 1 to 5, consistent with a range of dead to vigorous individuals. A rating of 1 indicated that the tree was dead. A rating of 2 was assigned when dead needles were present on ≥50% of the crown and resinosis was observable on the stem. A rating of 3 was assigned when yellow or dead needles were present on ≤50% of the crown and resinosis was observable. A rating of 4 was assigned when there was either evidence of resinosis or yellow or dead needles but not both. A rating of 5 was assigned when there were no visual symptoms of tree stress.

Analysis of monoterpenes in spruce phloem

Phloem tissue was collected at the inoculation site using cork borers and was used to quantify constitutive phloem monoterpene concentrations at the start of the experiment. Phloem tissue was separated from xylem and bark using a scalpel, which was cleaned with ethanol between uses, and placed in a 2 mL vial. Following excision, monoterpenes were immediately extracted from phloem using methods described by Erbilgin and Colgan (2012), with slight modifications. Phloem samples were pulverized in liquid nitrogen, and 100 mg of pulverized phloem was transferred into a 1.5 mL centrifuge tube (part No. 022364111, Eppendorf, Hamburg, Germany). A 500 μL aliquot of hexane (>99% purity; lot STBG5019V, Sigma–Aldrich) was added, and sample tubes were vortexed for 1 min at maximum speed. The resulting extract was centrifuged at 10 000*g* for 2 min, and the hexane extract was decanted into a 2 mL borosilicate autosampler vial (part No. 5182-0715, Agilent Technologies, Santa Clara, Calif., USA). Tissues were re-extracted, and the second extract was added into the first for a total extract volume of ~1 mL. Extracts were stored at –40 °C until analyzed by coupled gas chromatography – mass spectrometry (GC–MS).

To analyze monoterpene content in extracts, we injected a 1 μL aliquot of each extract onto a 7820A gas chromatograph (Agilent Technologies) coupled to a 5977B mass-selective detector (Agilent Technologies) equipped with an HP-5 Ultra Inert column (dimensions: 30 m × 250 μm external diameter × 0.25 μm internal diameter; part No. 19091S-433UI, Agilent Technologies). Injections were automated, and the GC–MS was operated in splitless mode with a front inlet temperature of 250 °C; the carrier gas was helium at a flow rate of 1.2 mL·min^{-1} and 63 kPa. The temperature program was as follows: initial temperature of 40 °C for 5 min, increased by 10 °C·min^{-1} until 250 °C, and then held for 5 min. Total ion chromatograms were acquired using the ChemStation data analysis software (version F.01.03.2357, Agilent Technologies); chromatograms were integrated using the MassHunter software (version B.07.00, Agilent Technologies). Monoterpenes in extracts were putatively identified by comparing electron mass spectra of integrated peaks with those in the National Institute of Standards and Technology (NIST) mass spectral library, and identifications were confirmed by comparing electron mass spectra and retention times against authentic standards. A total of seven monoterpenes were reliably identified from phloem seedling extracts: (+)-α-pinene, (–)-β-pinene, (+)-3-carene, myrcene, terpinolene, sabinene, and β-phellandrene. Compound quantities (in micrograms per millilitre) in extracts were determined by comparing peak areas of authentic standards at known concentrations with those of samples and correcting for initial sample mass. The terpene standards included (+)-α-pinene (98% purity; Sigma–Aldrich), (–)-β-pinene (99% purity; Sigma–Aldrich), (+)-3-carene (>90% purity; Sigma–Aldrich), myrcene (95% purity; Sigma–Aldrich), terpinolene (90% purity; Sigma–Aldrich), sabinene (75% purity; Sigma–Aldrich), and β-phellandrene (>80% purity, custom order; Synergy Semiochemicals, Delta, B.C., Canada). Previous studies have indicated that monoterpene concentration has larger effects on the growth of *L. abietinum* than monoterpene composition (Davis et al. 2018*a*); therefore, in addition to examining monoterpenes individually, we summed quantities in mass and treated total phloem monoterpene concentration (in micrograms of monoterpenes per gram of phloem) as the variable of interest for analysis.

Data analysis

A least squares linear model was used to analyze the effects of inoculation treatment (n = 7; six *L. abietnum* isolates and one control inoculation), water treatment (ample water (1.0 mg H_2O·g^{-1} soil) versus low water (0.5 mg H_2O·g^{-1} soil)), the interaction of inoculation treatment × water treatment, seedling phloem monoterpene concentration (log-transformed; Fig. 1), and variance due to blocking (bench position, b = 2) on the responses of (*i*) mean Engelmann spruce phloem lesion length (in millimetres), (*ii*) mean phloem oxidation (scored from 1 (none) to 5 (severe)), and (*iii*) mean relative health of seedlings (scored from 1 (dead) to 5 (vigorous)). A χ^2 test was used to analyze the probability of forming callus tissue at the inoculation site (0 or 1) due to the effects of inoculation and watering treatments, and logistic regression was used to analyze the probability of forming callus tissue due to the effect of varying monoterpene concentrations. Statistical significance for model effects was assigned using a type I error rate of α = 0.05. When significant variation was detected in responses due to modeled effects, Tukey's honestly significant difference (HSD) test was applied to make pairwise comparisons of means. All analyses were implemented using the R programming language base package version 3.5.1 ("Feather Spray"; R Core Team 2018).

Results

There was a significant effect of inoculation treatment on mean phloem lesion length ($F_{[6,159]}$ = 8.577, P < 0.001), and seedlings that received the control inoculation exhibited smaller mean lesion sizes than all Colorado isolates of *L. abietinum*, but Wyoming isolates were not different from the control treatment (Fig. 2). There was no evidence for a significant effect of the water treatment ($F_{[1,159]}$ = 2.438, P = 0.120), inoculation treatment × water treatment ($F_{[6,159]}$ = 0.906, P = 0.491), or monoterpene concentration ($F_{[1,159]}$ = 0.410, P = 0.522) on mean lesion sizes. However, monoterpene concentrations varied considerably among study trees and ranged from 1 to 58 μg monoterpenes·g^{-1} phloem, with a mean ± SE of 7.3 ± 0.6 μg·g^{-1}.

The oxidation of phloem varied significantly because of the main effect of inoculation treatment ($F_{[6,159]}$ = 15.212, P < 0.001), but there was no evidence for a significant effect of water treatment ($F_{[1,159]}$ = 1.469, P = 0.227), inoculation treatment × water treatment ($F_{[6,159]}$ = 0.855, P = 0.529), or monoterpene concentration ($F_{[1,159]}$ = 1.325, P = 0.251) on phloem discoloration. Inoculation with all *L. abietinum* isolates resulted in significantly more phloem staining than the control inoculation, and Colorado isolates generally caused more discoloration than Wyoming isolates. Similarly, mean visual estimates of seedling vigor varied significantly because of the main effect of inoculation treatment ($F_{[6,159]}$ = 2.511, P = 0.023), but there was no evidence for an effect of water treatment ($F_{[1,159]}$ = 0.156, P = 0.692), inoculation treatment × water treatment ($F_{[6,159]}$ = 1.490, P = 0.184), or monoterpene concentration separately or in mass ($F_{[1,159]}$ = 0.428, P = 0.513) on seedling vigor (Supplementary Table S3[1]). Although no trees died during

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjfr-2019-0362.

Fig. 1. Distribution of (*a*) total and (*b*) log-transformed concentration of six phloem monoterpenes (in micrograms of monoterpene per gram of phloem) in Engelmann spruce seedlings at the inoculation site at the beginning of the experiment, shown relative to (*c*) water treatment and (*d*) experimental block. D, dry; W, wet; B1, block 1; B2, block 2.

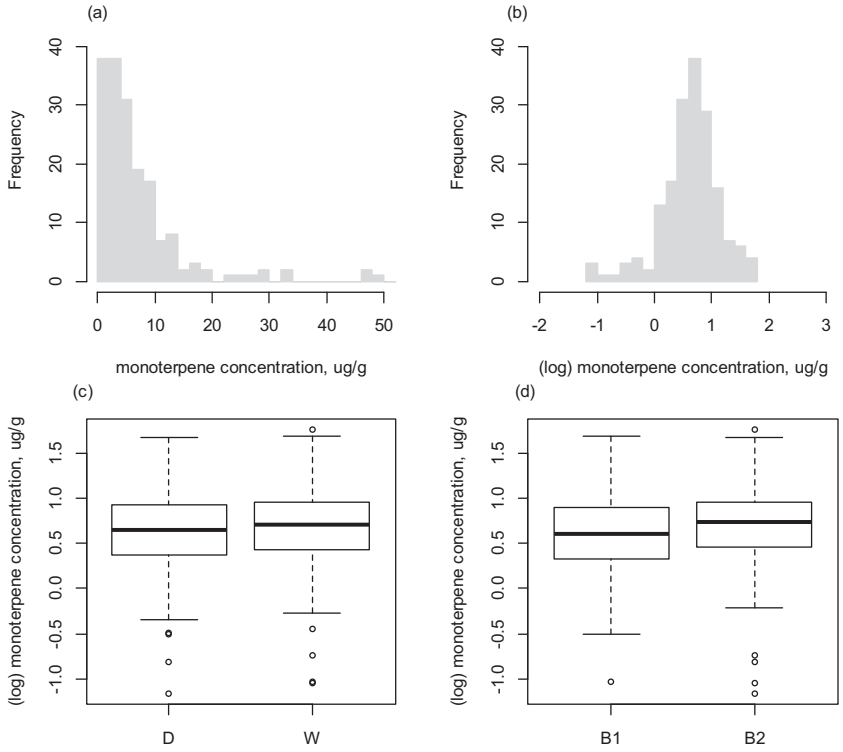
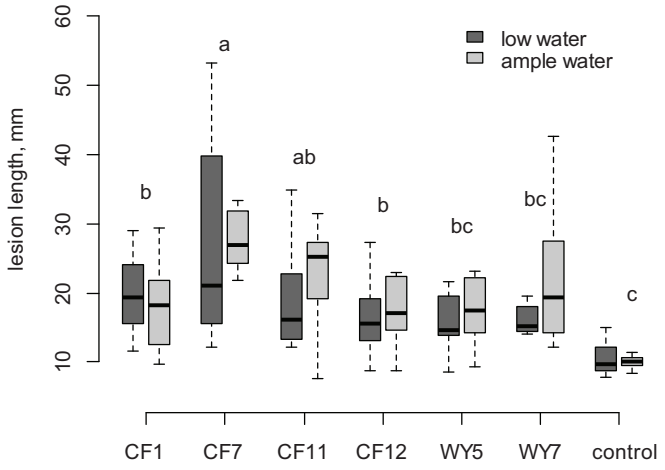


Fig. 2. The effects of inoculation with malt extract agar (MEA) media containing *L. abietinum* mycelia or control inoculation (MEA media with no microbial growth) and watering treatment on the distribution of necrotic lesion length in Engelmann spruce seedlings after 90 days. Lettering shows Tukey's honestly significant difference (HSD) test relative to the main effect of inoculation treatment; inoculation treatments not connected by the same letter are significantly different ($P < 0.05$). CF, Colorado isolate; WY, Wyoming isolate.



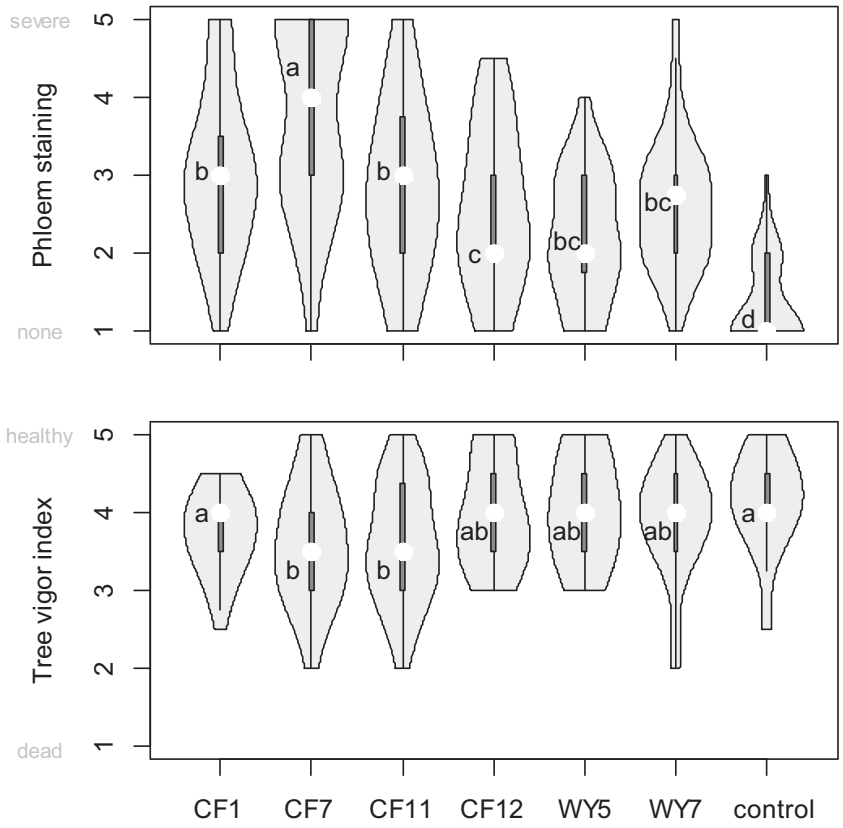
the experiment, seedlings that were control-inoculated generally had the highest visual indices of vigor, and seedlings inoculated with Colorado isolates of *L. abietinum* generally had the lowest visual indices of vigor (Fig. 3). A summary of linear model analysis, including proportion of variance explained for each modeled effect, is provided in Supplementary Table S1.[1]

The probability of seedlings forming callus tissue at the inoculation site varied significantly because of the effect of inoculation treatment ($\chi^2_{[6]} = 30.169$, $n = 176$, $P < 0.001$), and seedlings that were control-inoculated had a higher probability of forming callus tissue at the wound site (92% formed callus tissue) than seedlings that were inoculated with *L. abietinum* (when averaged across all six isolates, 43% of seedlings formed callus tissue). The probability of seedlings forming callus tissue also varied significantly because of the effect of watering treatment ($\chi^2_{[1]} = 9.176$, $n = 176$, $P = 0.002$); 39% of seedlings that received the low-water treatment formed callus tissue, whereas 62% of seedlings that received the ample-water treatment formed callus tissue. However, seedling phloem monoterpene concentration had no effect on the probability of forming callus tissue at the inoculation site ($\chi^2_{[1]} = 0.084$, $n = 176$, $P = 0.771$) (Fig. 4).

Discussion

Here, we report a first test of the effects of inoculation with *L. abietinum*, the primary fungal symbiont of the spruce beetle, on Engelmann spruce. Our results suggest that inoculation of Engelmann spruce with *L. abietinum* caused formation of phloem lesions and oxidation while inhibiting formation of callus tissue. There was no evidence that constitutive phloem monoterpene concentrations nor short-term host-tree water stress impacted lesion development. These collective results not only indicate that *L. abietinum* can cause disease symptoms in Engelmann spruce phloem, but also demonstrate that there are differences in virulence among the *L. abietinum* isolates tested. One isolate, *L. abietinum* CF7, from an outbreak population of spruce beetle in the southern Rocky Mountains, was the most virulent, and other isolates collected from Colorado were more virulent than those collected from Wyoming (central Rocky Mountain region). Our data indicate that symbiotic fungi of spruce beetle may have different phytopathogenic properties depending on provenance,

Fig. 3. Violin plots summarizing the effects of inoculation with MEA media containing *L. abietinum* mycelia or control treatment (MEA media alone) on phloem oxidation (upper panel) and tree vigor (lower panel) after 90 days. Phloem oxidation and tree vigor were rated for each individual seedling on a scale from 1 to 5. Seedlings with little phloem oxidation were rated as 1, and seedlings with severe phloem oxidation were rated as 5. Seedlings with low tree vigor were rated as 1, and healthy seedlings were given a score of 5. Lettering shows Tukey's HSD test relative to the main effect of inoculation treatment; inoculation treatments not connected by the same letter are significantly different ($P < 0.05$).

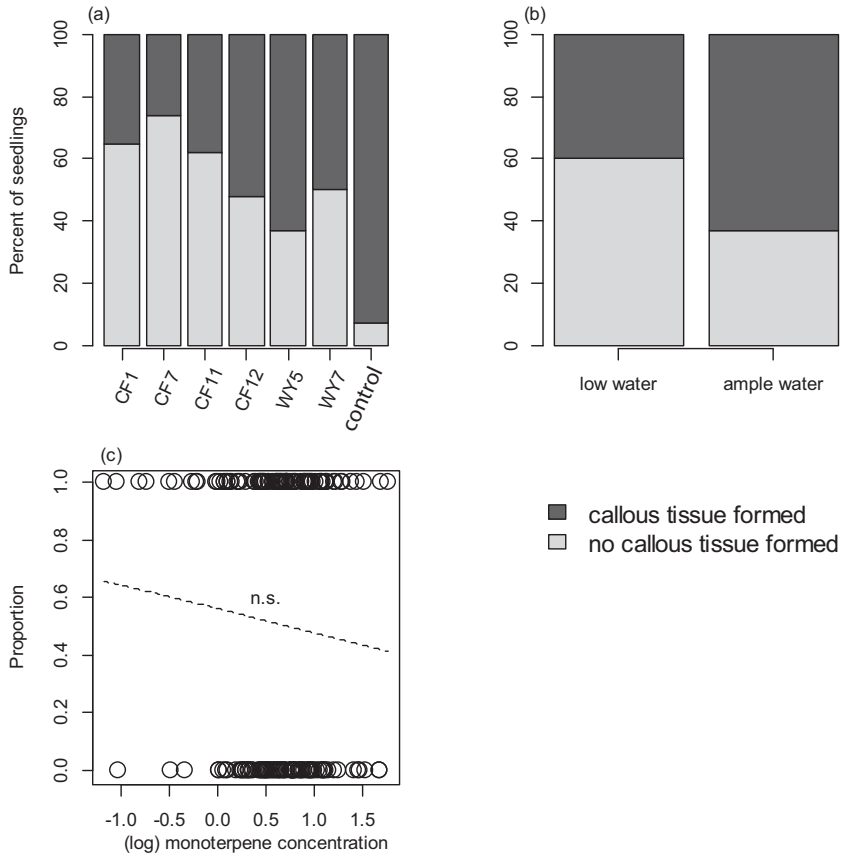


with consequences for patterns of bark beetle colonization of host trees and tree mortality on the landscape. At present, little is known about genetic diversity in *L. abietinum* aside from a single previous study that examined a relatively small sample of isolates (Davis et al. 2019). A description of population genetic variation in this species could help to elucidate which beetle populations are associated with phytopathogenic strains of *L. abietinum* and whether this is correlated with a higher probability of beetle outbreaks.

There is some controversy on whether *Leptographium* species are true pathogens or are primarily beneficial to their vectors, whereby fungal lesion development enhances beetle success but does not result in tree mortality (Jacobs and Wingfield 2001; Six and Wingfield 2011). Our data are consistent with the latter — we did not observe mortality of any study trees in our experiment, but inoculation with *L. abietinum* did cause phloem damage. This is in contrast to findings from other similar associations between bark beetle and fungus; for instance, *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., a fungal symbiont of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins, 1902), is pathogenic to host trees and causes significant phloem occlusion and sometimes mortality in both seedlings and mature hosts (Shrimpton 1973; Rice et al. 2007; Wyka et al. 2016). Similarly, a fungal symbiont (*Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf.) associated with the European spruce bark beetle (*Ips typographus* (Linnaeus, 1758)) is highly virulent to Norway spruce (*Picea abies* (L.) Karst.), and mass inoculations of the fungus can kill well-defended, mature trees outright (Krokene and Solheim 1998; Jankowiak 2005).

By comparison, the role that *Leptographium* species play in tree mortality after colonization by bark beetles is nuanced, and previous research has suggested that virulence varies considerably depending on fungal species, host species, and even isolates within a fungal species (as demonstrated in the present study). Multiple *Leptographium* species are pathogenic, and several studies have documented mortality of trees at the seedling or sapling stage. For instance, *Leptographium terebrantis* S.J. Barras & T.J. Perry caused mortality of ponderosa pine (*Pinus ponderosa* Douglas ex. P. Lawson & C. Lawson) seedlings (Owen et al. 1987) and eastern white pine (*Pinus strobus* L.) seedlings (Wingfield 1986). Of the eight isolates of *Leptographium procerum* (W.B. Kendr.) M.J. Wingf. inoculated onto eastern white pine, only two caused mortality in pine seedling and at low frequencies (~10%), compared with *L. terebrantis*, which caused 80% mortality within 5 months (Wingfield 1986). All *L. procerum* isolates caused lesion development in phloem, but these were significantly smaller than those caused by both *L. terebrantis* and *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf. However, in a multispecies study of *Leptographium* virulence that included *Leptographium lundbergii* Lagerb. & Melin, *Leptographium serpens* (Goid.) Siemaszko, *L. procerum*, and *L. terebrantis*, Eckhardt et al. (2004) found that *L. lundbergii* was the most virulent and *L. procerum* was the least virulent. These previous studies, coupled with our findings, highlight the fact that it is difficult to draw conclusions about the pathogenic effects of beetle-associated fungal symbionts from a single study and that results are not likely to be broadly transferrable across systems, symbiotic species, or even locations.

Fig. 4. The proportion of study trees that formed callus tissue because of the effects of (*a*) isolate identity and (*b*) watering treatment. (*c*) A logistic regression fitting proportion of study trees forming callus tissue to phloem monoterpene concentration. n.s., not significant.



Recent studies have indicated that tree drought stress plays a role in driving the emergence of outbreak populations of spruce beetle (e.g., Hart et al. 2014), but the physiological mechanisms underlying this phenomenon are not yet understood. One possibility is that drought stress may impede tree responses to microbes introduced by beetles, effectively reducing the ability of challenged trees to mount an appropriate defensive response. This hypothesis has garnered some support from recent studies; for example, inducible defenses of lodgepole pine (*Pinus contorta* Douglas ex Loudon) in response to inoculation with *G. clavigera* are reduced in trees treated with experimental water deficit (Arango-Velez et al. 2016). Similarly, Matusick et al. (2008) showed that mortality of longleaf pine (*Pinus palustris* Mill.) seedlings inoculated with *L. serpens* was significantly higher when seedlings were exposed to drought; however, lesion sizes of *L. serpens* did not vary between ample-water and drought-stress treatments. In another study examining loblolly pine (*Pinus taeda* L.) seedlings exposed to *L. terebrantis* and *Grosmannia huntii* (Rob.-Jeffr.) Zipfel, Z.W. de Beer & M.J. Wingf., Devkota et al. (2018) found that lesion sizes were significantly longer for *L. terebrantis*, but not for *G. huntii*, under drought conditions. In the present study, we did not find any statistical support for the hypothesis that drought stress was associated with phloem occlusion following inoculation of Engelmann spruce with *L. abietinum*. However, variability in lesion sizes was high, and study trees trended towards developing larger lesions on average under drought conditions (Fig. 2); accordingly, the effects of water stress on Engelmann spruce responses to *L. abietinum* may nonetheless be ecologically significant under field conditions in which evapotranspirative demands are greater than those in the greenhouse and water is more limiting.

In addition to lesion development, the formation of callus tissues in response to wounding, insect damage, and pathogen exposure is a dimension of host resistance that may be important for limiting tissue damage by closing wound sites. As a response to biotic challenge, formation of callus tissue appears to be relevant and observable for both seedlings and mature trees. For instance, both mature trees and seedlings of lodgepole pine formed callus tissues in response to inoculation with *Europhium clavigerum* Rob.-Jeffr. & R.W. Davidson (syn. *Ophiostoma clavigerum* (Rob.-Jeffr. & R.W. Davidson) T.C. Harr) (Shrimpton and Watson 1971). Callus tissue is associated with resistance to some fungal infections, including those of *Cronartium fusiforme* Hedgc. & N.R. Hunt ex Cummins (Jacobi 1982) and *Phytophthora cinnamomi* Rands (McComb et al. 1987), in which spore germination and mycelial growth was suppressed in callus tissues. In the present study, formation of callused tissue was observed surrounding lesions and was correlated with both isolate pathogenicity and water stress conditions. For example, we observed that seedlings inoculated with less virulent isolates from Wyoming (i.e., WY5 and WY7) formed callus tissue more often than seedlings inoculated with isolates from Colorado (e.g., CF7). This suggests that virulent isolates inhibit formation of callus tissues (Fig. 4*a*), which may correlate to the probability of host-tree survival following exposure to beetle-vectored fungal symbionts. Furthermore, trees that were well watered were more likely to form callus tissue (Fig. 4*b*), which indicates that inhibition of callus tissue formation may be a mechanism by which water stress reduces tolerance or resistance of host trees to biotic challenges, including fungal symbionts of bark beetles.

Secondary metabolites such as terpenoids are also important resistance factors that can impact the progression and severity of disease symptoms in conifers following exposure to bark beetle fungal symbionts, and terpenoids can inhibit or promote the growth of fungal symbionts, depending on concentration (Keeling

and Bohlmann 2006). Constitutive phloem monoterpene concentrations were measured for all spruce seedlings at the outset of the experiment and analyzed as a potential factor influencing the development of necrotic lesions, phloem oxidation, tree vigor following inoculation, and callus tissues. Although study seedlings varied considerably in total phloem monoterpene concentration (Fig. 1*a*), there was no clear evidence that this variation had any effect on measured tree responses to *L. abietinum*. Other studies have shown that monoterpene concentrations in conifer phloem tend to increase in response to inoculation with bark beetle fungal symbionts, consistent with an induced response, which may provide some resistance to biotic challenge (Raffa and Smalley 1995). From the present study, we conclude that there was little effect of constitutive phloem monoterpene concentration on lesion formation associated with *L. abietinum* in Engelmann spruce, but we did not measure induced responses, and the effects of induced monoterpene concentrations on lesion development merits further attention. In addition, constitutive monoterpene concentrations were approximately one to two orders of magnitude lower in seedlings measured in the present study than those in mature Engelmann spruce phloem (Davis et al. 2018*a*), indicating fundamental differences between constitutive terpenoid-based defenses in seedlings versus mature trees. Mature Engelmann spruce form traumatic resin ducts (i.e., induce resin production) in stands that experience spruce beetle outbreaks (DeRose et al. 2017), but it is presently unknown whether seedlings are capable of the same response.

In the present study, test trees were inoculated at a single location on the bole; however, in a forest, *L. abietinum* would ostensibly be mass-inoculated into host trees during spruce beetle aggregation and colonization of phloem tissues. Our results provide a baseline understanding of the virulence of *L. abietinum* to well-watered and drought-stressed seedlings, but additional progress will be made if subsequent tests focus on assessing induced responses in mature Engelmann spruce following mass inoculation with *L. abietinum* in a field setting. We conclude from the present work that (*i*) genetically distinct *L. abietinum* isolates vary in their putative virulence to Engelmann spruce; (*ii*) inoculation with *L. abietinum* is sufficient to cause disease symptoms in immature Engelmann spruce phloem but is not lethal; (*iii*) water availability is associated with the formation of callus tissues, an "appropriate" defensive response (Erb et al. 2012) following tissue damage, and trees with greater access to water are more likely to form callus tissues; and (*iv*) constitutive phloem monoterpene concentrations have little effect on tree responses to *L. abietinum* inoculation, but this should be further examined under more realistic conditions. Although *L. abietinum* is reported as potentially pathogenic to several spruce species, there remains relatively little understanding of the effects of this species on bark beetle success and tree survival in the field, especially in Engelmann spruce forest, which is the primary forest cover type affected by spruce beetle in much of North America. A better understanding of the ecology of interactions among spruce beetle, *L. abietinum*, and Engelmann spruce is needed to elucidate potential drivers of bark beetle population dynamics and factors associated with patterns of forest mortality across large landscapes.

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