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Research paper

Interspecific variation in spruce constitutive and induced defenses in response to a bark beetle–fungal symbiont provides insight into traits associated with resistance

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Differences in defensive traits of tree species may predict why some conifers are susceptible to bark beetle–fungal complexes and others are not. A symbiotic fungus (*Leptographium abietinum* (Peck) M.J. Wingf.) associated with the tree-killing bark beetle (*Dendroctonus rufipennis* Kirby) is phytopathogenic to host trees and may hasten tree decline during colonization by beetles, but defense responses of mature trees to the fungus have not been experimentally examined. To test the hypothesis that interspecific variation in spruce resistance is explained by defense traits we compared constitutive (bark thickness and constitutive resin ducts) and induced defenses (resin flow, monoterpene composition, concentration, phloem lesion formation and traumatic resin ducts) between two sympatric spruces: Engelmann spruce (*Picea engelmannii* Parry ex Engelm.—a susceptible host) and blue spruce (*Picea pungens* Engelm.—a resistant host) in response to fungal inoculation. Four central findings emerged: (i) blue spruce has thicker outer bark and thinner phloem than Engelmann spruce, which may restrict fungal access to phloem and result in less beetle-available resource overall; (ii) both spruce species induce monoterpenes in response to inoculation but blue spruce has higher constitutive monoterpene levels, induces monoterpenes more rapidly, and induces higher concentrations over a period of time consistent with spruce beetle attack duration; (iii) Engelmann and blue spruce differed in the monoterpenes they upregulated in response to fungal inoculation: blue spruce upregulated α-pinene, terpinolene and γ-terpinene, but Engelmann spruce upregulated 3-carene and linalool; and (iv) blue spruce has a higher frequency of constitutive resin ducts and produces more traumatic resin ducts in annual growth increments than Engelmann spruce, though Engelmann spruce produces more resin following aseptic wounding or fungal inoculation. These findings suggest that higher constitutive resin duct densities and monoterpene concentrations, as well as the ability to rapidly induce specific monoterpenes in response to *L. abietinum* inoculation, are phenotypic traits associated with hosts resistant to spruce beetle colonization.

Keywords: *Dendroctonus*, forest entomology, *Leptographium*, monoterpenes, phenotypic traits, tree defenses.

Introduction

Bark beetles in the genus *Dendroctonus* are major biotic drivers of coniferous tree mortality worldwide; some of the most 'aggressive' beetle species feed on phloem and must overcome host tree defenses in order to successfully colonize these tissues and reproduce (Raffa and Berryman 1983). Interactions between *Dendroctonus* beetles and host trees typically result in death for either the tree under attack or the colonizing beetles, and there is strong selection on both beetles and trees to develop mechanisms to overcome host defenses and to resist colonization, respectively (Lieutier 2002). Tree defense mechanisms used to resist colonization are both physical (e.g.,

outer bark thickness and bark texture) and chemical (e.g., production of toxic compounds such as terpenoids and phenolics); in the case of chemical defenses these may also be pre-formed constitutive traits or induced by beetle attack (Raffa and Smalley 1995, Franceschi et al. 2005). As a mechanism to exhaust inducible defenses, most *Dendroctonus* species have evolved pheromones to coordinate mass attacks on host trees. Pheromone signaling by beetles during the host tree attack process can recruit thousands of additional individuals and often results in rapid tree death after beetles colonize the phloem (Seybold et al. 2000). However, some *Dendroctonus* species are also associated with symbiotic fungi that provide beetles with nutrition but may also interact with tree defenses to hasten decline under some circumstances (Six 2003, Klepzig and Six 2004). During the colonization phase of their life cycle, beetles 'mass-inoculate' host trees with fungal propagules that are vectored either in specialized secretory structures (i.e., mycetangia; Vega and Biedermann 2020) or externally via phoresy. These interactions remain poorly studied for some of the most destructive beetle species; however, they are critically important for understanding tree physiology, susceptibility of different tree species to beetle attack and the factors that contribute to landscape-level tree mortality. For example, tree 'recognition' of bark beetle–fungal symbionts could be a mechanism by which trees mount appropriate defensive responses (Erb et al. 2012), and the rate and magnitude at which trees respond to fungal symbionts could predict their probability of survival during bark beetle attack (Krokene 2015).

Over the past two decades, populations of spruce beetle (*Dendroctonus rufipennis* Kirby), an oligophagous herbivore that colonizes most spruce species within its large geographic range, has undergone an extensive outbreak in the southern Rocky Mountain region that has resulted in the death of millions of Engelmann spruce trees (*Picea engelmannii* Parry ex Engelm.) (Jenkins et al. 2014, Colorado State Forest Service 2018). However, sympatric blue spruce (*Picea pungens* Engelm.) has experienced virtually no mortality during this event, indicating that it is likely this species is either resistant to or tolerant of spruce beetle attack. This observation is reported from previous outbreaks as well (Massey and Wygant 1954, Schmid and Frye 1977). The mechanisms underlying these differences in mortality between the two spruce species are not clear and have not yet been extensively tested but could be related to differences in their response to the fungi associated with spruce beetle (Figure 1). Previous studies indicate that the spruce beetle is consistently associated with the symbiotic fungus *Leptographium abietinum* (Peck) M.J. Wingf. (Six and Bentz 2003), which is highly tolerant to toxic terpenoids (Davis et al. 2018) that are common in spruce phloem tissues and likely metabolizes at least some of these compounds (Davis et al. 2019). Recent work suggests that the fungus is pathogenic to Engelmann spruce but not necessarily lethal (Stewart et al. 2020). However, this has not yet been examined in mature trees *in natura*, and potential phytopathogenic effects of exposure to *L. abietinum* have not been compared between putatively susceptible and resistant spruce species. Moreover, it is unknown whether either Engelmann or blue spruce produces an induced chemical defense in response to *L. abietinum* colonization, which could help to elucidate patterns of susceptibility within and between them (Ott et al. 2011, 2021).

To address these knowledge gaps and improve our understanding of the phenotypic factors that contribute to host tree resistance against the spruce beetle–*L. abietinum* complex, we tested the effects of fungal inoculation on two sympatric spruces species—one that is a common host of spruce beetle in western North America (Engelmann spruce) and one that is only rarely used as a host by the beetle and is apparently resistant (blue spruce) (Schmid and Frye 1977). Our motivations were to evaluate induced defensive responses in these two species following exposure to *L. abietinum* and to test the hypothesis that responses to the fungus correlate with observed patterns of resistance to spruce beetle of the two species. Using mass inoculation procedures, we tested how introduction of *L. abietinum* to vascular tissues of mature spruce trees in natura impacted (i) development of phloem necrotic lesions, (ii) formation of resin ducts and resin flow rates and (iii) induction of monoterpenes in the phloem of mature forest trees. Our results provide new insights into the tree defensive traits associated with resistance to the spruce beetle–*L. abietinum* complex in the southern Rocky Mountains and help to enhance our interpretation of complex interactions in insect–host–symbiont tritrophic systems.

Materials and methods

Site selection

In 2019, we established two study sites, one in which Engelmann spruce was a dominant component of the overstory (Willow Creek Pass; coordinates: 40°04′19″ N, 105°55′55″ W, elevation: 2926 m, stand basal area: 33.74 m^2 ha^{-1}) and another where blue spruce was dominant (Jack's Gulch; coordinates: 40°39′60″ N 105°12′29″ W, elevation: 2392 m, stand basal area: 36.85 m^2 ha^{-1}). It was necessary to choose different study sites for focal species because each species varies somewhat in habitat requirements: in Colorado, Engelmann spruce is often found at the alpine tree line in well-drained soils where heavy snowfall occurs, whereas blue spruce is more commonly found at slightly lower elevations in drainage basins and in more mesic soils (Schmid and Frye 1977). At each site, 20 trees of each species were selected based on the following criteria:

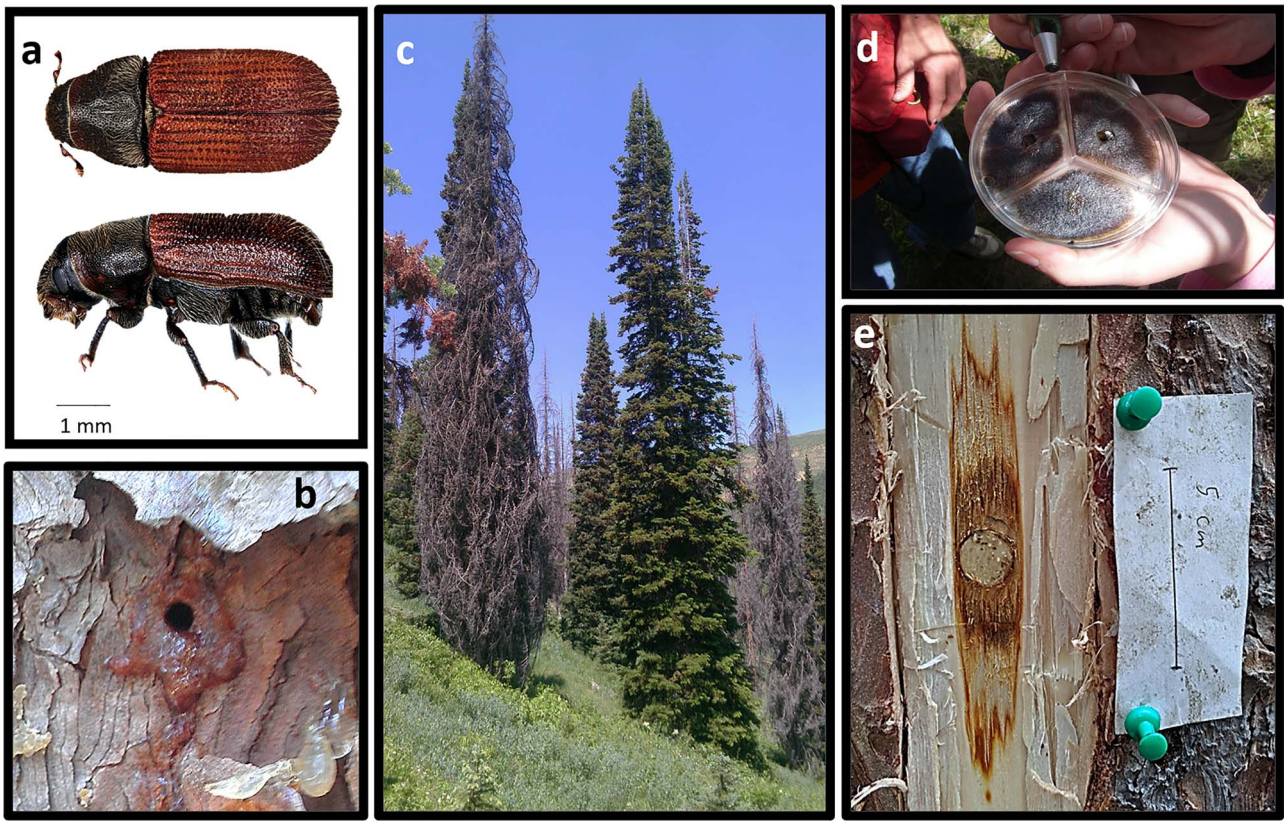

Figure 1. Image series of the study system, including: (a) the North American spruce bark beetle (*D. rufipennis* Kirby); (b) entry holes made by spruce beetles into Engelmann spruce bark, with resinosis at the entry site; (c) mature Engelmann spruce that succumbed to spruce beetle attack and sympatric surviving trees; (d) the spruce beetle–symbiotic fungus *L. abietinum* growing in culture; and (e) phloem necrotic lesions that developed in response to inoculation with the fungal symbiont.

(i) diameter at breast height of 33–38 cm and (ii) at least 50 m from roads.

Phloem inoculation

Following selection of study trees ($n = 20$ of each species), half the trees at each site were randomly selected to receive one of two treatments: (i) a mass inoculation treatment with *L. abietinum* designed to approximate the density of attacking spruce beetle (one inoculation per dm^2) or (ii) a control treatment (one sham inoculation per dm^2) (Raffa and Berryman 1983). Treatments were applied on the bole of trees using a grid template between 1 and 2 m above the root collar; depending on tree size, total inoculations ranged from 54 to 72 per tree. The inoculation treatment consisted of *L. abietinum* strain 'CO-7' (previously identified as a pathogenic isolate; Stewart et al. 2020) mycelia grown for 7 days (at 23 °C in the dark) on 2% malt extract agar; the control treatment consisted of only the growth media. Trees were inoculated by removing 10-mm circular punches of bark and placing media containing either *L. abietinum* mycelia or growth media only (control treatment) into the wound, with the mycelia facing the phloem in the case of *L. abietinum* treatments. Bark pieces were placed back over the wound.

Sampling of phloem and resin

On the day of treatment (Day 0) and at two time periods after (7- and 49-days post-inoculation), phloem samples were collected from randomly selected wound sites for analysis of phloem monoterpene concentration. To collect phloem, bark was gently removed from selected wound sites using a draw blade, and the presence or absence of phloem lesions was noted. If lesions were present, they were excavated and photographed alongside a ruler for scale, and phloem samples for monoterpene analysis were collected from within lesions. Photographs of lesions were later analyzed using ImageJ (Schneider et al. 2012) to calculate lesion length (mm). For most sham inoculations (and at Day 0), no lesions were present, therefore phloem samples were collected immediately adjacent to wound sites. Phloem samples were collected using a 10-mm circular punch, placed into labelled plastic bags, and immediately returned to the laboratory in dry ice. Additional phloem samples were collected at 49-days post-inoculation and were cultured in growth media (2% malt extract agar) for 7 days in the dark to verify that the inoculum (*L. abietinum*) was recoverable from sampled tissues.

In addition to making phloem collections for analysis of monoterpenes and re-isolation of fungi, resin mass was also

measured for each study tree at two time periods. Prior to inoculation treatments at Day 0, bark and phloem was removed from each study tree at either a 0 or 180° bearing (randomly selected) at the bottom of the inoculation grid using a 10-mm circular punch, and bark and phloem thickness were measured with a sliding stage incremental micrometer. An aluminum funnel with a 15-ml centrifuge vial was attached to the hole where the bark and phloem was removed to collect resin exudates, and duct tape was fixed over the funnel to prevent precipitation from entering collection vials. The vials were capped and labeled 7 days later, and collected resin mass was weighed in the lab to the nearest 0.1 g. At 42 days following inoculation, this process was repeated at the opposite bearing (i.e., 0 or 180°), so that mass of 7-day resin flow was measured at Day 7 and Day 49 of the study.

Quantifying phloem monoterpene concentrations

Methods and instrument parameters for the analysis of monoterpene concentrations in spruce phloem have been previously described (Erbilgin and Colgan 2012, Davis et al. 2018). Fresh phloem samples were pulverized in liquid N, and 100 mg of the resulting phloem powder was transferred into a 1.5-ml centrifuge tube for extraction. A 500-µl aliquot of hexane (>99% purity, Sigma-Aldrich, St. Louis, Missouri, USA, lot STBG5019V) 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. Tissues were re-extracted and the second extract was added into the first for a total extract volume of ∼1 ml. Multiple extraction times were tested (2 min, and 1 and 24 h) and it was confirmed that monoterpene concentrations in extracts of phloem samples were similar regardless of extraction time. Extracts were stored at −40 °C until analyzed by coupled gas chromatography/mass spectrometry (GC/MS).

To quantify monoterpene concentrations in extracts, a 1-µl aliquot of each extract was injected onto a 7820A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a 5977B mass selective detector (Agilent Technologies) equipped with an HP 5 ultra-inert column (Agilent Technologies, part no. 19091S-433UI; dimensions: 30 m × 250 µm external diameter × 0.25 µm internal diameter). Injections of phloem extracts onto the column were automated, and the GC/MS was operated in splitless mode with a front inlet temperature of 250 °C; 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 was 40 °C for 5 min, then increased by 10 °C per min and held constant at 250 °C for 5 min. Total ion chromatograms were acquired using the Chemstation data analysis software (Agilent Technologies, version F.01.03.2357), and chromatograms were integrated using the MassHunter software (Agilent Technologies, version B.07.00). Monoterpenes in extracts were confirmed by comparing electron mass spectra and retention times against authentic standards. A total of eight monoterpenes were reliably identified from phloem extracts including (+)-α-pinene, (−)-β-pinene, (+)-3-carene, β-myrcene, terpinolene, γ-terpinene, β-phellandrene and (+)-linalool.

The concentration of each monoterpene (mg monoterpene per g phloem, fresh weight) in extracts was determined by relativizing monoterpene peak areas in samples to peak areas of authentic monoterpene standards at known quantities (1 mg) to solve for mg monoterpene per 100 mg phloem. A correction factor of 10× was applied to convert concentrations to mg monoterpenes per g phloem. The monoterpene standards used included:(+)-α-pinene (98% purity, Sigma-Aldrich), (−)-β-pinene (99% purity, Sigma-Aldrich), β-myrcene (95% purity, Sigma-Aldrich), (+)-3-carene (>90% purity, Sigma-Aldrich), γ-terpinene (95% purity, Sigma-Aldrich), terpinolene (90% purity, Sigma-Aldrich), β-phellandrene (>80% purity, Synergy Semiochemicals, Delta, British Columbia, Canada) and (+)-linalool (97% purity, Sigma-Aldrich). Total phloem monoterpene concentration was determined by summing the mass (mg) of individual monoterpenes in each sample.

Quantifying resin ducts in growth increments

Xylem cores were also collected from study trees in order to compare constitutive resin ducts and the formation of resin ducts following inoculation treatments. At the bottom of the inoculation grid at a randomly selected bearing, xylem cores were collected using a 5-mm increment borer. This process was repeated in randomly selected inoculated lesions at Day 7 and Day 49 with the goal of determining whether resin duct densities varied between the two species or following inoculation treatments. In the laboratory, tree cores were air-dried, mounted, and then progressively sanded following standard dendrochronological techniques (Fritts 1976). We scored cores for resin ducts following methods described by DeRose et al. (2017). Briefly, ducts were scored in each growth increment between 2009 and 2019 as containing no resin ducts, constitutive resin ducts or tangential resin ducts. Increments that contained tangential ducts were further separated into 'Class 1', 'Class 2' and 'Class 3' traumatic ducts. 'Constitutive' refers to annual growth rings in which a single resin duct or non-tangential resin ducts were recorded; 'Class 1' refers to annual growth rings in which two or more tangentially aligned resin ducts were observed; 'Class 2' refers to annual growth rings in which tangentially aligned resin ducts comprised between 20 and 90% of the growth increment; 'Class 3' refers to annual growth rings in which tangentially aligned resin ducts comprised > 90% of the growth increment (Figure 2). Radial growth rate

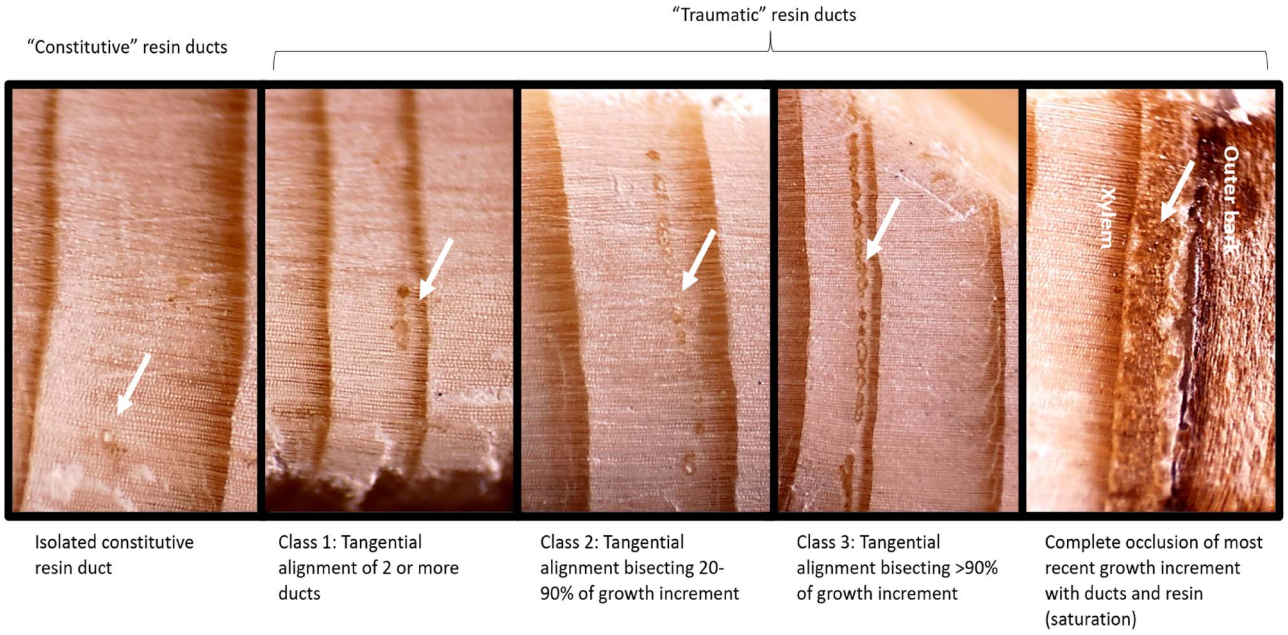

Figure 2. Increment cores collected from Engelmann spruce that show isolated constitutive resin ducts, traumatic resin ducts (Class 1–3) formed in response to pressure from the symbiotic fungus *L. abietinum*, and complete occlusion of the outer-most growth increment in response to *L. abietinum* inoculation.

(mm year^{-1}) for all increments in each core was also measured using a sliding stage micrometer.

Data analysis

Unless otherwise stated, all statistical analyses were performed using the R programming language (R Development Core Team 2020) and incorporate a Type I error rate of $\alpha = 0.05$ for assigning statistical significance; however, we interpreted effects significant at the $\alpha = 0.10$ level as 'marginally significant'.

To evaluate basic differences between tree species that might impact interpretation of defensive responses following inoculation with spruce beetle–symbiotic fungi, mean values of certain tree life-history traits were compared using two-tailed, two-sample Student's *t*-tests. These traits included mean tree size (diameter at breast height), estimated tree age (years), radial growth rates (mm year^{-1}) and bark and phloem thickness (mm).

A nested two-way analysis of variance (ANOVA) model was used to evaluate the effects of inoculation treatment ($n = 2$; inoculation with growth media containing actively growing *L. abietinum* mycelia vs sham inoculation with blank media) and tree species ($n = 2$; Engelmann spruce vs blue spruce), as well as their interaction, on 7-day resin flow mass (g) and necrotic lesion length (cm). The model was nested by time-since-inoculation (0-, 7- and 49-days post-inoculation). No measurements were taken at Day 0 since there was no expectancy of immediate lesion formation and no possibility of collecting a 7-day resin flow measurement. Prior to analysis, resin flow mass was log-transformed to adhere to assumptions of normality. A nested design was chosen over a repeat-measures design because we were specifically interested in the effect of time-since-inoculation to test whether *L. abietinum* inoculation induces resin flow at wound sites and formation of necrotic lesions in phloem of host tree species, rather than modeling variance due to time effects as a random effect. A post-hoc test (Tukey's HSD) was used to make all pairwise comparisons of means.

A similar model was used to evaluate the effects of inoculation treatment, tree species, as well as their interaction on the concentrations (mg/g) of eight monoterpenes (α-pinene, β-pinene, Δ-3-carene, myrcene, β-phellandrene, γ-terpinene, terpinolene and linalool) that are abundant in spruce phloem (Davis et al. 2018) and the sum of these monoterpenes. The model was nested by time-since-inoculation ($n = 3$; 0-, 7- and 49-days post-inoculation). A two-factor ANOVA was used to analyze variation in the frequency of resin duct formation in annual growth increments ($n = 1177$ total growth increments scored) of blue spruce and Engelmann spruce, treating 'species' as a fixed effect and 'year' (2010–19) as a random effect.

In addition, we used linear models (i.e., regression analysis) to quantify associations between lesion size, resin flow and total monoterpene composition for study trees at 7- and 49-days post-inoculation. Since no lesions or resin flow data were available immediately following inoculation treatments, no comparisons were made for the 0-day time step. These analyses treated lesion size and resin flow as independent variables (predictors) and total phloem monoterpene concentration as the dependent variable (response).

Results

Morphological comparison between blue and Engelmann spruce

Mean ± SE age of blue spruce was 95.5 ± 4.0 years, whereas mean age of Engelmann spruce used in this study was 131.1 ± 3.7 years. The mean ± SE lifetime radial growth rate of blue spruce was 1.601 mm year^{-1} ± 0.075 mm whereas mean lifetime radial growth rate of Engelmann spruce was 1.124 mm year^{-1} ± 0.049 mm, and this difference was statistically significant ($t_{38} = -5.276$, $P < 0.001$). The radial growth for the 2010–19 period had a mean ± SE of 1.445 mm year^{-1} ± 0.138 mm for blue spruce whereas that of Engelmann spruce was 1.097 mm year^{-1} ± 0.079 mm ($t_{38} = -2.068$, $P = 0.045$), indicating that in recent years blue spruce grew on average 25% faster than Engelmann spruce. On average, blue spruce outer bark thickness was 9.4 ± 0.5 mm, whereas Engelmann spruce outer bark thickness was 3.9 ± 0.2 mm, and this difference was statistically significant ($t_{38} = 10.746$, $P < 0.001$). In contrast, mean ± SE phloem thickness in blue spruce was 3.3 ± 0.3 mm but mean phloem thickness in Engelmann spruce was 5.7 ± 0.2 mm, and this difference was also statistically significant ($t_{38} = -6.703$, $P < 0.001$). In addition, there was a strong negative linear relationship between outer bark thickness and phloem thickness, and this was evident both within and across study species ($F_{1, 38} = 33.860$, $P < 0.001$, Figure 3).

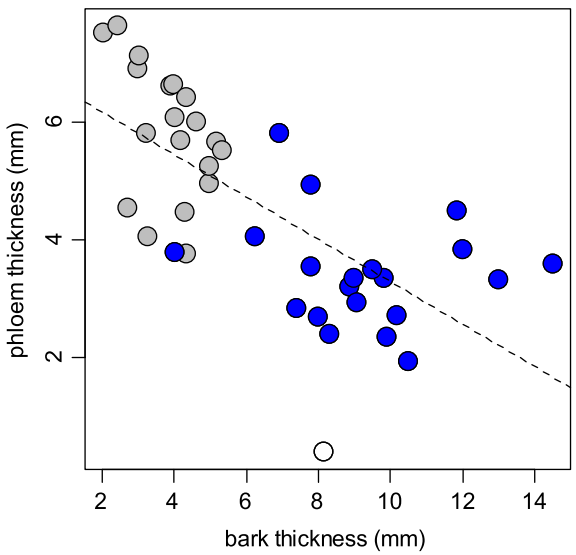

Figure 3. The relationship between outer bark and phloem thickness across blue spruce (blue symbols) and Engelmann spruce (gray symbols). The dashed line shows the least-squares linear regression model of phloem thickness against outer bark thickness (phloem thickness mm = 6.884–0.359 * (bark thickness mm), $R^2 = 0.457$). Number of replicates is $n = 20$ for each species.

Phloem lesion formation and resin flow in response to fungal inoculation

There was a significant effect of tree species (blue vs Engelmann spruce) on the response of mean 7-day resin flow mass (Table 1a); at 7-day post-inoculation blue spruce produced considerably more resin than Engelmann spruce, but mean quantities were low (0.320 g and 0.001 g for blue and Engelmann spruce, respectively). However, by 49-day post-inoculation this pattern switched, and Engelmann spruce produced ∼61% more resin on average. The effects of inoculation treatment on mean resin mass bordered between 'significant' and 'marginally significant' (Table 1a), indicating relatively small differences between the effects of sham inoculation and inoculation with *L. abietinum* on resin flow in both species; trees that were sham-inoculated produced ∼25% more resin on average, suggesting that fungal inoculation may occlude resin exudation. There was no evidence of a species × inoculation treatment interaction. However, as expected, there were strong effects of time-since-inoculation on resin flow. When averaged across both species, mean 7-day resin flow was ∼13-fold higher at 49-day post-inoculation than at 7-day post-inoculation (Figure 4a).

All modeled parameters had significant effects on mean lesion length (Table 1b). At 7-days post-inoculation, mean lesion length differed between the two tree species, and lesions in blue spruce were ∼11% longer. By 49-days post-inoculation, this pattern was reversed, and lesions in Engelmann spruce were ∼16% longer on average. Lesion lengths in Engelmann spruce did not differ between sham- and *L. abietinum*-inoculations at 7-days post-inoculations. In contrast, at 49-days post-inoculation, mean lesion lengths were 61% longer in trees inoculated with *L. abietinum*. The same pattern was not true for blue spruce; at 7- and 49-days post-inoculation, lesion lengths were 39 and 87% longer, respectively, in trees inoculated with *L. abietinum*. This

Table 1. Summary of nested two-way ANOVA testing the effects of inoculation treatment (sham inoculation vs inoculation with *L. abietinum*) on mean 7-day resin flow mass and necrotic lesion formation in blue spruce (*P. pungens*) and Engelmann spruce (*P. engelmannii*) at 7- and 49-days post-inoculation.

Variable	Source	df	*F*	*P*
(a) Resin flow mass (g)	Treatment (day)	2	3.042	0.053
	Species (day)	2	14.658	<0.001
	Treatment × species (day)	2	0.895	0.412
	Day	1	144.1266	<0.001
	Error	79	–	–
(b) Lesion length (cm)	Treatment (day)	2	4.388	0.015
	Species (day)	2	190.043	<0.001
	Treatment × species (day)	2	4.314	0.017
	Day	1	142.238	<0.001
	Error	79	–	–

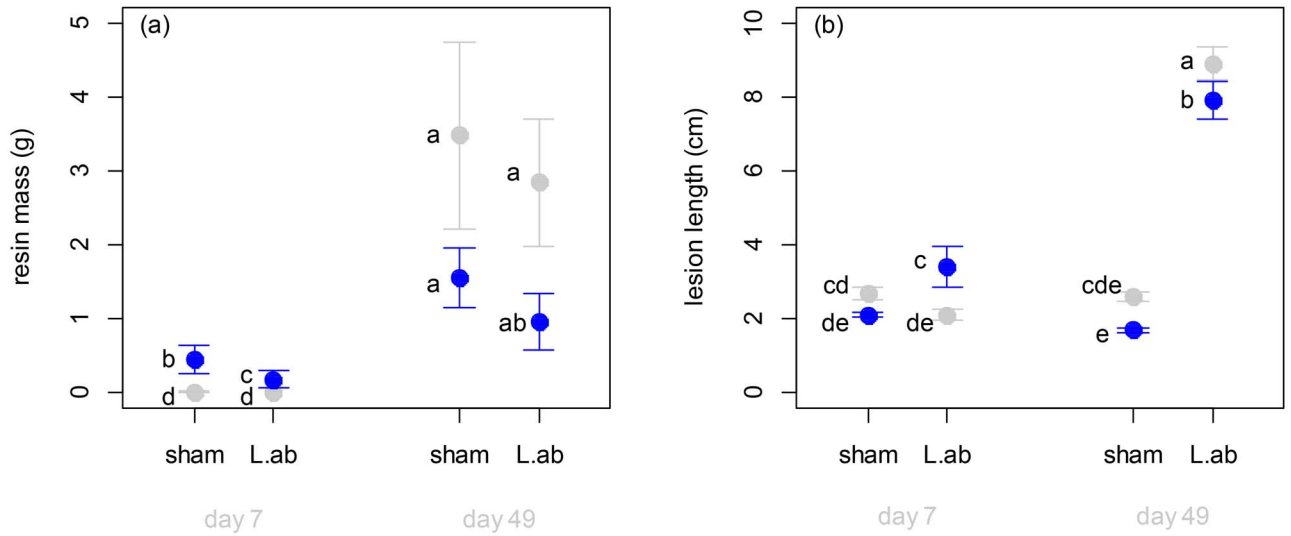

Figure 4. The effects of mass inoculation treatment (sham inoculation vs inoculation with *L. abietinum* (abbreviated 'L.ab.' on the *x*-axis)) on mean (a) 7-day resin flow mass (g) and (b) necrotic lesion length (cm) at 7-and 49-days post-inoculation. Bars show plus or minus one standard error of the mean, and lettering denotes Tukey's HSD test on all pairwise comparisons of means ($n = 10$ replicates per mean). Means not connected by the same letter are significantly different at $P < 0.05$.

indicates that blue spruce develops lesion earlier, but Engelmann spruce develops more lesion tissue over time (Figure 4b). In re-isolation tests sampling phloem from lesion areas at Day 49, *L. abietinum* was recovered from Engelmann spruce phloem lesions in 100% of samples, whereas the fungus was only recovered from 60% of blue spruce samples. This difference was statistically significant ($\chi^2 = 47.531$, df $= 1$, $N = 20$, $P < 0.001$).

Monoterpene induction in response to fungal inoculation

Mean concentrations of all monoterpenes as well as total (summed) monoterpene concentration varied due to the main effect of inoculation treatment (Table 2); by 49-days post-inoculation, mean concentrations of all monoterpenes in trees inoculated with *L. abietinum* significantly exceeded that of trees treated with sham-inoculation (Table 3), indicating that introduction of *L. abietinum* into subcortical tissues causes upregulation of phloem monoterpenes in comparison with mechanical wounding alone. In addition, for blue spruce treated with *L. abietinum*, mean concentrations of α-pinene, β-pinene and myrcene (as well as total monoterpene concentrations) exceeded that of sham-inoculated trees by 7-days post-inoculation, indicating rapid upregulation of some but not all monoterpenes in blue spruce phloem following challenge with *L. abietinum* (Figure 5). Although both blue spruce and Engelmann spruce upregulated concentrations of all measured monoterpenes in response to *L. abietinum* inoculation, the two species differed in their composition. Specifically, mean concentrations of Δ-3-carene and linalool in the phloem of Engelmann spruce considerably exceeded concentrations found in blue spruce phloem; conversely, mean concentrations of α-pinene, γ-terpinene and terpinolene in the phloem of blue spruce considerably exceeded concentrations found in Engelmann spruce phloem. Overall monoterpene concentrations were higher in blue spruce phloem than in Engelmann spruce phloem at all three sample periods and across both treatments with the exception of sham inoculation by 49 days, indicating that blue spruce has higher constitutive phloem monoterpene concentrations than Engelmann spruce and induces more phloem monoterpenes in response to *L. abietinum* inoculation, but not in response to mechanical damage (Table 3).

Analysis of annual growth increments and comparison of resin ducts

The expression and formation of resin duct defenses, both constitutive and induced, varied between the two studied species. On average, Engelmann spruce was 1.6 times less likely to form resin ducts in annual growth increments than blue spruce ($F_{10,9} = 10.746$, $P < 0.001$), and, blue spruce formed constitutive resin ducts nearly twice as often as Engelmann spruce ($F_{10,9} = 17.052$, $P < 0.001$). The formation of Class 1 traumatic resin ducts differed marginally due to the effect of species: on average, blue spruce formed Class 1 resin ducts slightly more frequently than Engelmann spruce ($F_{10,9} = 2.689$, $P = 0.076$). In contrast, neither Class 2 traumatic resin ducts ($F_{10,9} = 0.508$, $P = 0.846$) nor Class 3 traumatic resin ducts ($F_{10,9} = 1.000$, $P = 0.504$) differed in their abundances in growth increments of the two species; only a single growth

Table 2. Summary of nested two-way ANOVA testing the effects of inoculation treatment (sham inoculation vs inoculation with *L. abietinum*) on mean monoterpene concentrations (mg monoterpene per g phloem) in the phloem of mature blue spruce (*P. pungens*) and Engelmann spruce (*P. engelmannii*) at 0-, 7-, and 49-days post-inoculation.

Monoterpene	Source	df	*F*	*P*
(a) α-pinene	Treatment (day)	3	87.950	<0.001
	Species (day)	3	36.722	<0.001
	Treatment × species (day)	3	32.462	<0.001
	Day	2	77.538	<0.001
	Error	108		
(b) β-pinene	Treatment (day)	3	31.902	<0.001
	Species (day)	3	0.487	0.692
	Treatment × species (day)	3	1.622	0.188
	Day	2	36.698	<0.001
	Error	108		
(c) Δ-3-carene	Treatment (day)	3	12.661	<0.001
	Species (day)	3	8.490	<0.001
	Treatment × species (day)	3	7.732	<0.001
	Day	2	12.829	<0.001
	Error	108		
(d) β-phellandrene	Treatment (day)	3	20.638	<0.001
	Species (day)	3	1.188	0.317
	Treatment × species (day)	3	0526	0.672
	Day	2	23.721	<0.001
	Error	108		
(e) β-myrcene	Treatment (day)	3	30.004	<0.001
	Species (day)	3	0.228	0.876
	Treatment × species (day)	3	0.158	0.924
	Day	2	31.979	<0.001
	Error	108		
(f) Terpinolene	Treatment (day)	3	195.913	<0.001
	Species (day)	3	69.064	<0.001
	Treatment × species (day)	3	71.218	<0.001
	Day	2	220.665	<0.001
	Error	108		
(g) γ-terpinene	Treatment (day)	3	94.226	<0.001
	Species (day)	3	7.404	<0.001
	Treatment × species (day)	3	7.994	<0.001
	Day	2	104.878	<0.001
	Error	108		
(h) Linalool	Treatment (day)	3	13.263	<0.001
	Species (day)	3	6.9433	<0.001
	Treatment × species (day)	3	2.140	0.099
	Day	2	22.910	<0.001
	Error	108		
(i) Total monoterpenes	Treatment (day)	3	70.667	<0.001
	Species (day)	3	3.212	0.025
	Treatment × species (day)	3	4.015	0.009
	Day	2	73.052	<0.001
	Error	108		

increment in a single blue spruce tree was scored as containing Class 3 traumatic resin ducts, whereas no Engelmann spruce increments were scored as containing Class 3 traumatic resin ducts (Table 4). In 35% of Engelmann spruce, resin completely filled growth increments in the year of fungal inoculation (2019), whereas this was true for 60% of blue spruce; however, this difference was not statistically significant ($\chi^2 = 2.506$, $N = 40$, df $= 1$, $P = 0.111$).

Associations between phloem lesions, resin flow rate and total phloem monoterpene concentrations

There was no evidence of a linear relationship between resin flow rates and total phloem monoterpene concentrations for Engelmann spruce at 7-day post-inoculation treatment ($F_{1,18} = 0.060$, $P = 0.808$) or 49-day post-inoculation treatment ($F_{1,18} = 0.082$, $P = 0.777$). Similarly, there was no evidence of a linear relationship between resin flow rates and

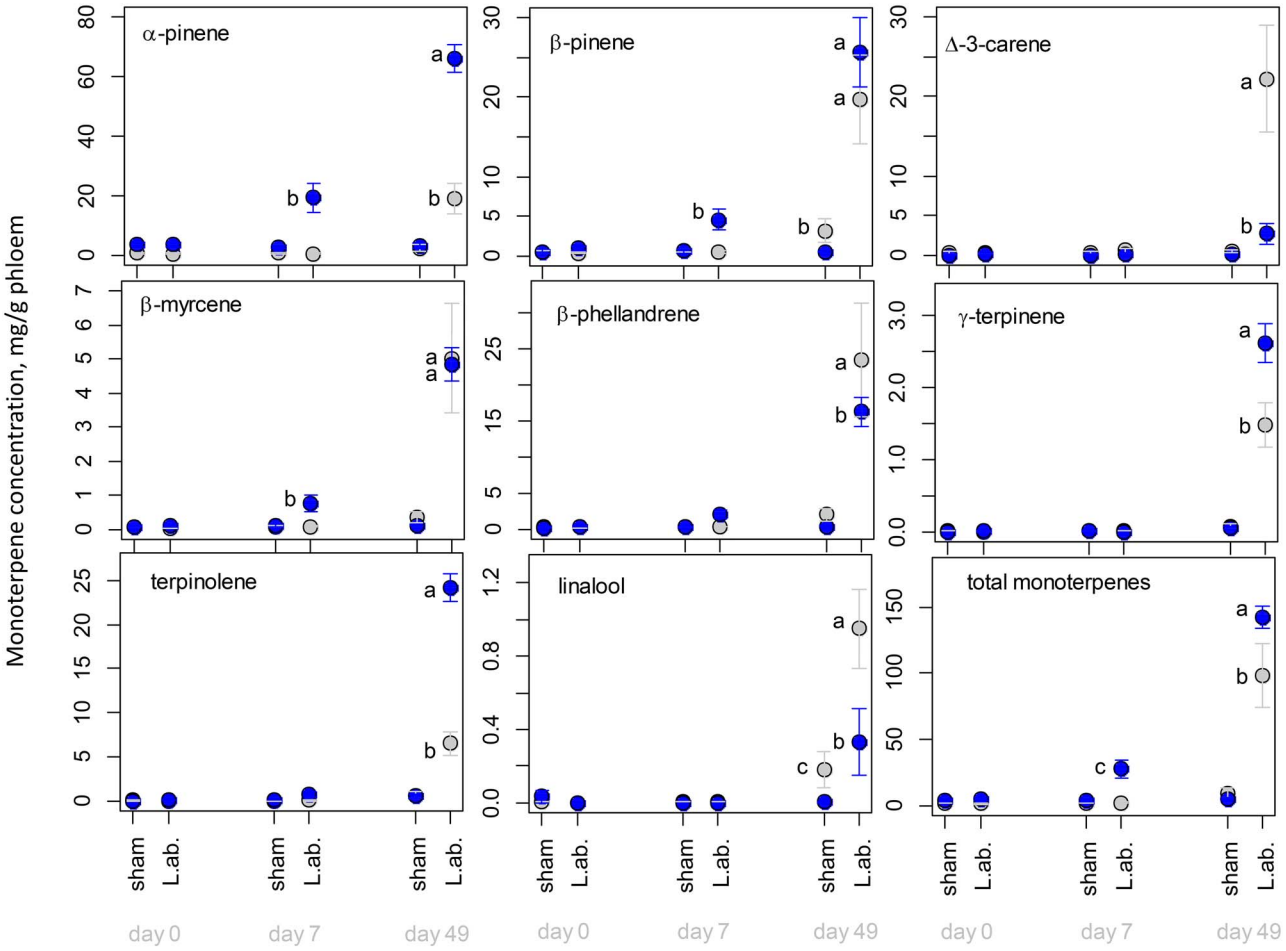

Figure 5. The effects of mass inoculation treatment (sham inoculation vs inoculation with *L. abietinum* (abbreviated 'L.ab.' on the *x*-axis)) on mean monoterpene concentrations (mg g_{-1}) in Engelmann spruce (gray) and blue spruce (blue) phloem tissue at 0-, 7-, and 49-days post-inoculation. Bars show plus or minus one standard error of the mean ($n = 10$ replicates per mean). Lettering denotes Tukey's HSD test; means not connected by the same letter are significantly different at $P < 0.05$.

Table 3. Comparison of mean (±SE) total phloem monoterpene concentrations (mg monoterpene per g phloem) in blue spruce (*P. pungens*) and Engelmann spruce (*P. engelmannii*) relative to inoculation treatment and time-since-treatment. Lettering denotes Tukey's HSD test on the species × inoculation treatment interaction, nested within day. Means not connected by the same letter (within day) are significantly different at $P < 0.05$.

Day	Inoculation treatment	Species	
		P. engelmannii	*P. pungens*
0	Sham	1.850 ± 0.405 b	4.362 ± 0.694 ab
	L. abietinum	1.395 ± 0.255 b	5.108 ± 1.337 a
7	Sham	1.955 ± 0.388 b	3.676 ± 1.047 b
	L. abietinum	2.086 ± 0.393 b	27.603 ± 6.623 a
49	Sham	9.337 ± 3.747 c	4.803 ± 1.367 c
	L. abietinum	98.309 ± 24.587 b	142.646 ± 8.241 a

total phloem monoterpene concentrations for blue spruce at 7-days post-inoculation treatment ($F_{1,18} = 0.283$, $P = 0.601$) or 49-days post-inoculation treatment ($F_{1,18} = 0.440$, $P = 0.515$), indicating that resin flow rates and monoterpene concentrations are uncorrelated for both species.

There was no evidence of a linear relationship between lesion size and total phloem monoterpene concentrations for Engelmann spruce at 7-days post-inoculation ($F_{1,18} = 1.151$, $P = 0.297$); however, phloem lesion size had a significant positive relationship with phloem monoterpene concentrations at 49-day post-inoculation ($F_{1,18} = 9.633$, $P = 0.006$, $R^2 = 0.348$). The same pattern was true for blue spruce; at 7-day post-inoculation there was no relationship between phloem lesion size and phloem monoterpene concentrations ($F_{1,18} = 0.420$, $P = 0.524$), but there was a significant positive association between lesion size and total phloem monoterpene concentrations at 49-days post-inoculation ($F_{1,18} = 233.241$, $P < 0.001$, $R^2 = 0.928$, Figure 6), indicating that larger phloem lesions are associated with upregulated phloem monoterpene concentrations for both species by 49-day s post-inoculation.

Table 4. A summary of the proportion of growth increments of each study species that formed no resin ducts, constitutive resin ducts, or traumatic resin ducts from 2010 to 2019 ($N = 1177$ annual growth increments examined). 'Constitutive' refers to annual growth rings in which a single resin duct or non-tangential resin ducts were recorded; 'Class 1' refers to annual growth rings in which two or more tangentially aligned resin ducts were observed; 'Class 2' refers to annual growth rings in which tangentially aligned resin ducts comprised between 20 and 90% of the growth increment, and 'Class 3' refers to annual growth rings in which tangentially aligned resin ducts comprised > 90% of the growth increment. Lettering denotes post-hoc comparison of means tests (Least-squares means test) between species.

Resin duct category	Species	
	P. pungens	*P. engelmannii*
No resin ducts	40.0% b	65.7% a
Constitutive resin ducts	38.9% a	20.9% b
Class 1 traumatic resin ducts	16.8%	10.9%
Class 2 traumatic resin ducts	4.2%	2.6%
Class 3 traumatic resin ducts	0.2%	0.0%

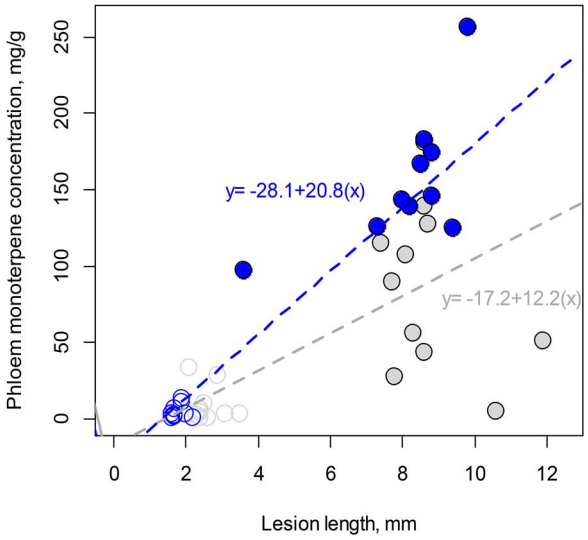

Figure 6. The relationship between phloem lesion size (cm) and total phloem monoterpene concentrations (mg g_{-1}) in Engelmann (gray symbols) and blue spruce (blue symbols) at 49-day post-inoculation. The dashed lines show linear models fit to each respective species, and both are significant at $P < 0.05$. Filled symbols denote trees inoculated with *L. abietinum* and open symbols denote trees which were treated with sham inoculations.

Discussion

Here we report that both Engelmann spruce, a susceptible host of the spruce beetle, and blue spruce, a resistant host, induce production of secondary metabolites (monoterpenes) and develop phloem lesions in response to mass inoculation with the spruce beetle–symbiotic fungus *L. abietinum*. The two tree species varied considerably in their defensive traits, and these differences were consistent with the respective susceptibility of each species to the spruce beetle–*L. abietinum* complex: (i) blue spruce has thicker outer bark and thinner phloem, potentially indicating that it may be more difficult to penetrate by colonizing beetles and that phloem resource volume itself is less abundant; (ii) necrotic lesion formation in response to *L. abietinum* inoculation is reduced in blue spruce phloem and fungal inoculum was recovered 40% less often from wound sites, suggesting that blue spruce may be more resistant to tissue damage from *L. abietinum* or that the fungus is unable to establish as effectively as in Engelmann spruce; and (iii) blue spruce has nearly twofold the constitutive resin duct density and induces ~31% higher phloem monoterpene concentrations than Engelmann spruce, indicating a more toxic phytochemical environment. These collective physiological differences help to explain observations that blue spruce is infrequently colonized by spruce beetle (Massey and Wygant 1954, Schmid and Frye 1977). Our study provides evidence that higher constitutive and inducible phloem monoterpene concentrations are tree traits that are associated with resistance to the primary symbiotic fungal associate of spruce beetle.

Fungal symbionts of bark beetles have a range of effects on the physiology of host trees (Six and Wingfield 2011, Jenkins et al. 2014). Some species are highly phytopathogenic and mass inoculations kill mature trees outright, as is the case of *Endoconidiophora polonica*, a symbiont of *Ips typographus* colonizing *P. abies* in northern Europe (Horntvedt et al. 1983). Other species are only moderately pathogenic and contribute to host decline but are otherwise not lethal to hosts (as is the case for *L. abietinum* isolated from *D. rufipennis*, Stewart et al. 2020), and still other bark beetle symbiotic fungi are completely non-pathogenic (e.g., *Entomocorticium* sp. A, a symbiont of the southern pine beetle (*Dendroctonus frontalis* Zimm., Hofstetter et al. 2005). In the present study, two sympatric spruce species responded differently in terms of the timing and chemical composition of monoterpene production in response to *L. abietinum* inoculation. Blue spruce phloem monoterpene concentrations increased significantly by 7-days post-inoculation, but Engelmann spruce phloem monoterpene concentrations did not differ until 49-days post-inoculation, indicating that blue spruce (a resistant host) recognizes and responds to *L. abietinum* more rapidly than Engelmann spruce (a susceptible host). Rapid local induced responses to fungal pathogens is an important dimension of host resistance in conifers (Wallis et al. 2008) and likely predicts performance and survival of the spruce beetle–*L. abietinum* complex. A limitation of our design is that additional intervals of time-since-inoculation were not evaluated, which would be useful for determining the precise timing of Engelmann spruce responses to inoculation. Nonetheless, for the first time we show that both species recognize and respond to *L. abietinum* presence in phloem tissues, even in the absence of vector beetles. It will be important to determine whether the monoterpene upregulation we report here is due to physical damage patterns or some pathogen-specific response (Erb et al. 2012), and this may provide further insight

into bark beetle resistance traits. For example, it is possible that the spruce species studied here induce monoterpenes in response to general components of fungal cell structures (such as glucans, chitins, sterols), or to compounds specific to *L. abietinum* and other ophiostomatoid fungi such as melanins or certain amino acids (Zink and Fengel 1990, Davis et al. 2019). Evaluating spruce responses to subcortical introduction of both general and specific compounds from fungal tissues will help determine selectivity of defense responses to bark beetle–fungal symbionts, for which there is previous evidence (Raffa and Smalley 1995, Zhao et al. 2019).

By 49-days post-inoculation, blue spruce had mounted an altogether larger monoterpene response than Engelmann spruce, but Engelmann spruce produced considerably more resin at wound sites than blue spruce. However, resin production in both species was similar in response to sham treatment and fungal inoculation (Figure 4a). This is consistent with Christiansen et al. (1999) and Krokene et al. (2000), who found that resin exudation was linked primarily to mechanical wounding rather than fungal inoculation. Although phloem monoterpene concentrations were higher in blue spruce following inoculation, overall 'monoterpene budget' may be higher in Engelmann spruce due to the 61% greater rate of resin exudation. However, this cannot be definitively determined without complementary measurements of resin monoterpene concentrations (here only phloem monoterpene concentrations were measured). Other authors have suggested that carbon starvation, due to a high cost of producing monoterpene defenses following a challenge by insects and their symbionts, is a potential mechanism of tree death during bark beetle colonization of host trees (Anderegg and Callaway 2012). Our results may be consistent with this hypothesis if overall carbon allocation to defenses is greater in Engelmann spruce than in blue spruce, and this merits further investigation.

Formation of phloem lesions in response to microorganisms is often interpreted as a manifestation of disease symptoms in conifer trees; lesion formation is consistent with a 'hypersensitive response' and indicates a localized zone of defensive induction, tissue callus and cellular necrosis that functions to compartmentalize damage from fungal hyphae (Franceschi et al. 2002). Previous studies indicate that smaller lesions generally indicate a better tree defense to fungal symbionts (Krokene et al. 2008, Erbilgin and Colgan 2012, Zhao et al. 2019), but lesion-formation responses are only seldom compared across multiple sympatric host tree species. We report that a resistant host species (blue spruce) developed phloem lesions more rapidly than a susceptible species (Engelmann spruce) and the susceptible species developed larger lesions over a period of time consistent with spruce beetle attack duration (Figure 4b). This result corroborates the expectation that smaller lesion sizes are associated with host tree resistance. We conclude that our data likely indicate that a rapid hypersensitive response (development of phloem necrotic lesions) is associated with overall reduced colonization by *L. abietinum*. In addition, we re-isolated *L. abietinum* significantly less frequently from blue spruce phloem than from Engelmann spruce phloem, further indicating that the fungus does not establish easily in the blue spruce subcortical environment. Phloem samples from within necrotic lesions provided evidence that differences in both monoterpene composition and concentration are associated with final lesion size.

Blue spruce and Engelmann spruce differed in the primary monoterpenes that were upregulated following inoculation (Figure 5). Blue spruce induced α-pinene and terpinolene, but Engelmann spruce induced 3-carene and β-pinene; linalool concentrations also increased in Engelmann spruce phloem following inoculation. In a recent study evaluating the toxicity of monoterpenes and monoterpene blends to *L. abietinum*, both terpinolene (mostly in blue spruce) and linalool (mostly in Engelmann spruce) were highly inhibitory to fungal growth: concentrations of 1 and 0.1%, respectively, were sufficient to totally inhibit *L. abietinum* in growth media (Davis et al. 2018). In blue spruce phloem, concentrations of terpinolene exceeded this threshold and were on average around 2%, consistent with the fungal inhibition observed in *in vitro* studies. However, the same was not true for Engelmann spruce, and phloem linalool concentrations averaged 0.08–0.09%, which is below the threshold previously described for inhibition (Davis et al. 2018). Consequently, even though most monoterpenes are toxic to *L. abietinum* in in vitro studies (depending on concentration), the present study shows that blue spruce upregulates concentrations of extremely toxic monoterpenes to a higher degree than Engelmann spruce in the field, which could be a mechanism that partially explains the differential resistance to spruce beetle between the two species. This is supported by recent evidence that variation in monoterpene composition and concentration significantly impacts spruce beetle survival in vitro (Davis 2020).

Although only monoterpenes were quantified in the present study, conifer induction responses to bark beetle–fungal symbionts can also include di- and sesqui-terpenes and other resin acids. For example, Keefover-Ring et al. (2016) found that di- and sesqui-terpenes increased 34- and 56-fold, respectively, in the phloem of ponderosa pines (*Pinus ponderosa* Dougl. ex. Laws.) challenged with the ophiostomatoid fungus *Grossmania clavigera* (a fungal symbiont of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins). Similarly, Mason et al. (2017) demonstrated that inoculation of red pine (*Pinus resinosa* Sol. ex. Aiton) phloem with *Ophiostoma ips* induces significant localized upregulation of palustric and abietic acids (diterpenes) and longifolene (a sesquiterpene) in phloem lesion tissues. The ecological importance of di- and sesqui-terpenes to bark beetles and their fungal symbionts is less represented in the literature than studies of monoterpenes,

potentially due to methodological challenges in identifying and quantifying these more complex terpenoids. Yet, complex terpenoids likely constitute an important component of conifer defense to beetles and their symbionts. For instance, Kopper et al. (2005) found that the diterpene abietic acid strongly inhibits mycelial growth and spore germination of *O. ips* but had little impact on the behaviors of the bark beetle *Ips pini* Say, a vector of *O. ips*. However, in studies of interactions between Norway spruce (*Picea abies* L.) and the European spruce bark beetle (*Ips typographus* L.), trees that survived mass-attack by beetles induced significantly higher quantities of both di- and sesqui-terpenes (Schiebe et al. 2012). Consequently, it will be important for future work in the Engelmann spruce–*D. rufipennis* system to examine responses of complex terpenoids to the beetle–fungal complex and elucidate their impacts on beetle colonization success and tree survival.

Blue spruce had higher frequencies of constitutive and traumatic resin ducts in growth increments than Engelmann spruce. The relationship between resin duct density and resin exudation is variable: in some species, there is no correlation (e.g., Kytö et al. 2001), whereas for other species resin duct density is a good predictor of resin flow (e.g., Blanche et al. 1992). Blue spruce had higher levels of resin flow over the short-term (7-day sample), but by 49-days post-treatment Engelmann spruce produced considerably more resin mass; suggesting that a more intense resin exudation response following either mechanical wounding or inoculation with spruce beetle–symbiotic fungi (Figure 4a) is not necessarily associated with resistance to spruce beetle. When only the current year's growth increment was examined, blue spruce exhibited complete saturation of cambial tissues with resin 25% more frequently than Engelmann spruce, potentially indicating that total resin mass exuded may be less important as a resistance trait than rapid localized traumatic resin duct production (DeRose et al. 2017, Vázquez–González et al. 2020). The frequencies of constitutive and traumatic resin duct production in both species are low in comparison to other conifers. For example, in ponderosa pine, lodgepole pine (*P. contorta* Douglas) and limber pine (*P. flexilis* E. James), all of which are sympatric with Engelmann spruce and blue spruce in Colorado, resin ducts are found in virtually all growth increments (Kane and Kolb 2010, Ferrenberg et al. 2014). In the present study 40–65% of spruce growth increments had no resin ducts at all over the 2010–19 period, and only 20–38% growth increments exhibited any constitutive resin ducts during the same period.

Here, we demonstrate that inoculation with a pathogenic isolate of the spruce beetle-symbiotic fungus (*L. abietinum*) manifests chemical responses from blue spruce, which is resistant to spruce beetle, and Engelmann spruce, which is susceptible to spruce beetle, in a region where both species are sympatric in the southern Rocky Mountains. The two species differed in the rate and magnitude of monoterpene induction in response to inoculation as well as the monoterpenes that were upregulated. Collective physiological differences that may explain patterns of spruce beetle colonization success in the two spruce species and potential resistance traits (i.e., those associated primarily with blue spruce) included reduced resource area (phloem) and thicker outer bark, rapid upregulation of total monoterpenes as well as induction of specific compounds (e.g., α-pinene), a limited hypersensitive response and rapid but conservative resin induction when challenged with a pathogenic spruce beetle-vectored fungus. A variety of other variables that were not examined here are also likely important for resistance to spruce beetle fungi including differences in water availability, stand density and thermal conditions. The interactions of these environmental conditions with spruce-induced responses should be further evaluated to develop a more complete understanding of the factors contributing to tree susceptibility in complex landscapes. Under projections of ongoing warming and increased drought in the Rocky Mountain region of the USA over the coming decades (Strzepek et al. 2010), these interactions may be particularly important as drought conditions are known to precipitate spruce beetle outbreaks (Hart et al. 2014).

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