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Rapid monoterpene induction promotes the susceptibility of a novel host pine to mountain pine beetle colonization but not to beetle-vectored fungi.

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Running head: Pine susceptibility to beetle and fungal attack.

Abstract

Chemical induction can drive tree susceptibility to and host range expansions of attacking insects and fungi. Recently, mountain pine beetle (*Dendroctonus ponderosae*; MPB) has expanded its host range from its historic host lodgepole pine (*Pinus contorta* var. *latifolia*) to jack pine (*P. banksiana*) in western Canada. Beetle success in jack pine forests likely depends upon the suitability of tree chemistry to MPB and its symbiotic phytopathogenic fungi. In particular, how rapid induced defenses of jack pine affect MPB colonization and the beetle's symbionts is unknown. In the field, we characterized and compared differences in rapid induced phloem monoterpenes between lodgepole and jack pines in response to various densities of *Grosmannia clavigera*—a MPB symbiotic fungus used to simulate beetle attack—inoculations. Overall, lodgepole pine had higher limonene and myrcene, but lower α-pinene, concentrations than jack pine. However, myrcene concentrations in jack pine increased with inoculation density, while that in lodgepole pine did not respond to density treatments. We compared the growth and reproduction of MPB's symbiotic fungi, *G. clavigera*, *Ophiostoma montium*, and *Leptographium longiclavatum*, grown on media amended with myrcene, α-pinene, and limonene at concentrations reflecting two induction levels from each pine species. Myrcene and α-pinene amendments inhibited the growth but stimulated the reproduction of *G. clavigera*, whereas limonene stimulated its growth while inhibiting its reproduction. However, the growth and reproduction of the other fungi were generally stimulated by monoterpene amendments. Overall, our results suggest that jack pine rapid induction could promote MPB aggregation due to high levels of α-pinene (pheromone precursor), a positive feedback of myrcene (pheromone synergist), and low levels of limonene (resistance). Jack pine is likely as susceptible to MPB-

vectored fungi as lodgepole pine, indicating that jack pine induction will likely not adversely affect symbiont activities enough to inhibit the invasion of MPB into jack pine forests.

Introduction

Conifer trees possess an array of complex physical and chemical defenses (either constitutive or induced) that can combine to resist pathogen attack and insect herbivory (Franceschi et al. 2005, Raffa et al. 2005, Erbilgin et al. 2006, Wallis et al. 2008, Eyles et al. 2010). For example, pine inner bark exudes pressurized oleoresin as a physical impediment to insect invasion of vascular tissues (Raffa and Berryman 1983b, Phillips and Croteau 1999, Keeling and Bohlmann 2006, Raffa et al. 2008). Oleoresins also represent a chemical defense strategy as they contain a cocktail of toxic terpenoid compounds (e.g., sesquiterpenes, diterpenes, and monoterpenes), whose concentrations can rapidly increase (i.e., are induced) in response to attack (Paine and Hanlon 1994, Raffa et al. 2005, 2008, Keeling and Bohlmann 2006). Concentrations exceeding the biological tolerance of invaders can persist for hours to seasons in order to confer prolonged resistance to additional attack (Erbilgin et al. 2006, Eyles et al. 2010). However, invading insects and pathogens specialized to attack a group of related species (e.g., pines [*Pinus* spp.]) often have adapted ways to circumvent, tolerate, or exploit host defenses (Jermy 1984, Brenebaum 1995, Becerra 1997, Futuyma 2008). For example, bark beetles (Coleoptera: Curculionidae, Scolytinae) can attack host pines *en masse* and utilize the phytopathogenicity of their symbiotic fungi to overwhelm tree defenses, ultimately resulting in tree death (Wood 1982, Franceschi et al. 2005, Raffa et al. 2005, 2008). Therefore, effective early-onset (rapid) and localized defensive induction is critical to halting beetle colonization and tree mortality (Keefover-Ring et al. 2016). Thus, the intra- and interspecific variability of defense induction helps define gradients of host susceptibility to specialized invaders such as bark beetles and their

symbiotic fungi (Byers and Birgersson 1990, Keeling and Bohlmann 2006, Eyles et al. 2010, Lusebrink et al. 2011, Raffa et al. 2013, Taft et al. 2015b).

Mountain pine beetle (*Dendroctonus ponderosae* Hopkins; MPB) has killed at least 28 million hectares during outbreaks in primarily lodgepole pine (*Pinus contorta* var. *latifolia* Douglas ex Loudon) forest over the past two decades and is one of the most destructive forest pests in North America (Bentz et al. 2009, 2010, Safranyik et al. 2010, Man 2012). In Canada, the recent MPB outbreak in lodgepole pine forests of British Columbia and Alberta has spread into naïve jack pine (*Pinus banksiana* Lamb) forests after passing through a dividing zone of lodgepole-jack pine hybrids (Cullingham et al. 2011, Lusebrink et al. 2013). Whether MPB will expand through the corridor of jack pine to attack eastern pine forests is unclear. However, predicting the likelihood of this threat requires a clear understanding of the factors underlying jack pine susceptibility to MPB colonization.

The host and geographical range expansion into jack pine forests of Alberta was potentially facilitated in part by the less pronounced defenses of jack pine as well as the use of jack pine secondary compounds by MPB for conspecific aggregation (Erbilgin and Colgan 2012, Clark et al. 2014, Erbilgin et al. 2014, Taft et al. 2015a). If constitutive defense-related chemicals do not halt MPB ingress, beetle attack can induce the production of defense-related monoterpenes that are toxic to invading beetles and are a part of oleoresin-based defenses (Raffa et al. 2005). In lodgepole pine, the rate at which these compounds are induced is a critical factor in colonization success as beetle aggregation is likely to fail when monoterpenes that rapidly accumulate at entrance sites kill beetles invading at low densities (Raffa and Berryman 1983b, Raffa et al. 2008, Boone et al. 2011). Thus, the success of MPB attacks may be higher in trees slower to deploy or with a lower production capacity of effective defense-related monoterpenes.

Upon arrival, female beetles synthesize the aggregation pheromone *trans*-verbenol, from α-pinene derived from the host tree, which then synergizes with host myrcene and initiates a mass attack that overwhelms pine defenses (Raffa and Berryman 1983a, Pureswaran et al. 2000, Erbilgin et al. 2014, Taft et al. 2015b). Thus, tree defensive responses can vary with beetle attack density, and the rapid deployment of chemical defenses at high concentrations is critical to beetle colonization success and mass attack occurrence (Raffa et al. 2005, Boone et al. 2011). While more delayed induction responses (e.g., six weeks post-attack) have been examined in lodgepole and jack pines (Lusebrink et al. 2011, 2016, Erbilgin and Colgan 2012, Clark et al. 2012), whether rapid induction responses (e.g., seven days post-attack) differ between these species is unknown. If rapid induction, and thus the susceptibility to MPB, differs between jack and lodgepole pines, then this response may be a strong indicator of beetle colonization success and thus outbreak potential in jack pine.

Mountain pine beetle success in jack pine will depend on the growth and reproduction of the beetle's symbiotic fungi in this novel host environment. Three symbiotic, phytopathogenic fungi (Ascomycota: Ophiostomataceae) are vectored by MPB in western Canada: *Grosmannia clavigera* (Robinson-Jeffery and Davidson) Zipfel, de Beer, and Wing., *Ophiostoma montium* (Rumford) von Arx, and *Leptographium longiclavatum* Lee, Kim and Breuil (Whitney and Farris 1970, Six 2003, Lee et al. 2005, Roe et al. 2011). These fungi weaken host pines by infecting and necrotizing phloem and sapwood tissues, reducing pine health and resistance to MPB attack (Raffa et al. 2008, Six 2013). The successful development of beetle larvae depends upon the presence of fungal hyphae, a preferred food source rich in essential nitrogen and ergosterol (Bentz and Six 2006, Adams and Six 2007, Bleiker and Six 2007, Goodsman et al. 2012). Further, these fungi metabolize certain host monoterpenes that can be toxic to adult MPB

(DiGuistini et al. 2011, Wang et al. 2013, 2014). Adult beetles fill their mycangia with fungal spores prior to emergence in order to facilitate the colonization of new host trees (Bleiker et al. 2009). How the growth and reproduction of MPB-vectored fungi respond to rapid monoterpene induction in host pines is largely unknown. However, the dependency of MPB on its symbiotic fungi makes elucidating these responses critical to predicting beetle success in jack pine forests.

Here, the fungal symbionts of MPB were used in field and laboratory experiments to examine potential differences in lodgepole and jack pine susceptibility to beetle colonization and fungal infection. In a field experiment, rapid monoterpene induction was compared between these pine species and in response to increasing densities of *G. clavigera* inoculations used to simulate changing MPB attack-pressure. Based on the results of this experiment, low (1st quartile) and high (3rd quartile) concentrations of myrcene, α-pinene, and limonene were used to amend artificial media to determine their effects on the growth and reproduction (as conidia density) of *G. clavigera*, *O. montium* and *L. longiclavatum*. These methods were used to investigate several research questions. (1) Does rapid monoterpene induction differ between jack and lodgepole pines? (2) Does this induction respond to increasing densities of simulated MPB attack? (3) Do rapid induction levels in lodgepole and jack pine phloem differentially affect the growth and reproduction of MPB-vectored fungi? (4) How do these responses relate to the relative susceptibility of jack pine to MPB colonization and fungal infection?

Materials and methods

Field experiment

Seventy-seven study trees were selected from lodgepole (N=39) and jack pine (N=38) stands located near Hinton (N53°45.925', W118°22.298') and Lac La Biche (N55°07.054',

W111°59.360'), Alberta, respectively, in July 2013. For each species, trees with 25.0 – 30.0 cm diameter at breast height (DBH; 1.4 m above the ground) were randomly assigned one of five wound-inoculation treatment groups (seven to eight replications per treatment). Treatments consisted of several densities of *G. clavigera* inoculations following the methods of Raffa and Berryman (1983b) to reflect MPB densities (i.e., attack pressure) during the colonization of host trees: 2, 4, 8, 16, or 32 inoculations per 0.3 m^2 of bole area. *Grosmannia clavigera* was chosen for inoculation over the other fungi for several reasons. (1) This fungus is the most aggressive pathogen vectored by MPB (Raffa and Berryman 1983b, Solheim 1995, Solheim and Krokene 1998). (2) *Grosmannia clavigera* often has the highest relative abundance compared to *O. montium* and/or *L. longiclavatum* in MPB-colonized/killed trees and in the mycangia of dispersing beetles (Six 2003, Roe et al. 2010). Each inoculation was applied by first boring a hole through the outer bark to the sapwood with a 4-mm-diameter cork borer and into which fungal inoculum (a 4-mm-dia plug of a ten day-old *G. clavigera* cultures grown on malt extract agar [MEA]) was inserted such that mycelium was in contact with tree sapwood. Inoculations were equally spaced for treatments with more than two inoculations, and were applied to the north side of tree boles at 1.4 m above the ground. A single inoculation was also administered on the south side of each study tree as a paired control against which to compare treatment responses. A pre-inoculation control representing constitutive conditions was not collected because others have demonstrated pine monoterpenes rapidly quantitatively and qualitatively respond to *G. clavigera* inoculations (Raffa and Berryman 1983b, Keefover-Ring et al. 2016). For example, Raffa and Berryman (1983b) showed pine induction responses begin at least as early as three days post-inoculation, with induced monoterpene levels in lesions/phloem exceeding constitutive levels by 4.5 times seven days post-inoculation. Monoterpene induction

in response to *G. clavigera* inoculation is also evident in both lodgepole and jack pine after longer periods of time (Erbilgin and Colgan 2012, Erbilgin et al. 2017). However, including controls representing constitutive conditions may be appropriate for studies investigating questions operating at large spatial scales where there is a high chance of sampling individuals or populations with wide chemotypic variation, or when investigating the effects of factors that limit or alter tree induction responses.

For each tree, phloem sections (2 x 2 cm), containing both non-infected phloem and fungal lesion tissues, were excised from two randomly-chosen inoculation points (treatments with more than two inoculations) or both points (treatment with two-inoculation points) at least 3 cm apart seven days after treatment application. Thus, samples possessed both *G. clavigera*-infected (lesion) and non-infected phloem, with the former representing at least 75% of the sample area. Both tissue types were collected in combination in order to ensure enough material was available for monoterpene extraction. These samples were wrapped in tin foil, with samples from inoculation treatments wrapped separately from the controls, and flash frozen in the field using dry ice and stored at -40°C in the laboratory.

Chemical analysis

To extract monoterpenes from phloem, samples were ground in liquid nitrogen, and 100 mg of ground tissue were extracted twice with 0.5 mL methyl tert-butyl ether containing a surrogate standard of 0.004% tridecane at room temperature. Samples were vortexed for 30s at 3,000 rpm before being sonicated for 15 min. After this, each sample was centrifuged for 15 min at 0°C and 13,000 rpm. Extracts were transferred into glass chromatography vials and stored at −40°C until analysis. Extracts (0.2 µL) were injected in splitless mode into a coupled gas

chromatograph-mass spectrometer (7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with an enantioselective column (HPChiral 20β; ID 0.25 mm, length 30 m; Product ID: 9091GB233; Agilent Tech.). Extracts were analyzed with hydrogen as the carrier gas at a flow of 1.2 mL min^{-1} and with a temperature program of 75° C for 6.8 min, then 15°C min^{-1} to 130° C (held for 5 min), then 120°C min^{-1} to 235°C.

Peaks were identified using the following standards: (–)-α-pinene, (+)-α-pinene, (–)-β-pinene, (+)-β-pinene, (–)-camphene, (+)-camphene, myrcene, (*S*)-(–)-limonene, (*R*)-(+)-limonene, 3-carene, terpineol (chemical purity > 90%), γ-terpinene (>97%), (+)-cymene, sabinene, β-thujone (enantiomeric ratio of 92.5/7.5), pulegone (>97%), terpinolene (>90%), borneol, α-terpinene (>95%) (Sigma-Aldrich), *cis*-ocimene (>90%, SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (>74%, Glidco Inc., Jacksonville, FL, USA). Chemical purities were 99%, unless noted otherwise above. Compounds were identified by comparing retention times and mass spectra to those of the standard chemicals. Chemical quantities were calculated using response curves generated from analyses of a dilution sequence of known quantities of standards and reported as concentration (μg/mg fresh weight of tissue).

Bioassays of MPB-associated fungi

Because the results of the field experiment identified myrcene, limonene, and α-pinene to differ between jack and lodgepole pine induction responses and/or respond to the inoculation density treatments, artificial media was amended with these compounds to test their effects on the growth (as culture area) and reproduction (as conidia density) of *G. clavigera*, *L. longiclavatum,* and *O. montium*. The responses of these three fungi to pine induction levels resulting from *G. clavigera* infection were tested because *in planta* this fungus infects, necrotizes, and elicits an

induction response in pine phloem in advance of at least *O. montium,* which invades the resulting lesions (Solheim 1995). Thus, pine induced defenses are experienced by the primary invading *G. clavigera* as well as the slower invading *O. montium* and presumably *L. longiclavatum*.

Cultures of the fungi were grown on the same MEA formulation used in the field experiment except with individual amendments of limonene, myrcene, or α-pinene from the above standards. For limonene and α-pinene amendments, a racemic mix of enantiomers was used because both positive and negative enantiomers of these compounds differed between pine species, with the -/+ enantiomeric ratio of both compounds being greater in lodgepole compared to jack pine. Fungal responses to four amendment concentrations (Table 1)—representing low (first quartile) or high (third quartile) induction levels of each monoterpene separately for lodgepole and jack pine responses pooled across inoculation treatments of the field experiment—of each monoterpene and a non-amended MEA control (Table 1) were compared, resulting in 13 treatments/control per fungus. These concentrations used in limonene and α-pinene amendments represent the sum concentration of positive and negative enantiomers (i.e., total detected limonene or α-pinene). The media was amended by mixing a pure (99%) chromatography standard of each monoterpene into autoclaved MEA (cooled for 10 minutes) prior to pouring into plates. Each treatment was replicated 15 times.

Culture area (mm^2) was measured by image analysis using ImageJ (National Institutes of Health, Bethesda, MD, USA) (Abramoff et al. 2004) after a growth period of four days in permanent darkness at 22ºC. Although the change in monoterpene concentration in or emitted from the media was not quantified, the scent of compounds was detectable at the end of the experiment indicating the fungi were exposed to the monoterpenes throughout the duration of the experiment. A 1 mm-tall (5 mm in diameter) section of the plug originally used to inoculate the

culture plates was used to assess conidia production (as a proxy for fungal reproduction) as described in Cale et al. (2016). Conidia density was quantified by vortexing the 1-mm (5-mm diameter) section in a microtube with 1 mL 0.5% Tween20 for 30 sec. This spore suspension was pipetted into a hemocytometer to quantify conidia concentration (number per mL). Conidia concentrations were standardized using culture area (plus the plug section area) prior to data analysis.

Data analysis

We calculated descriptive statistics for concentrations of all detected monoterpenes, which were summed to get total monoterpenes. Descriptive statistics for compounds not used in the below analyses are listed by pine species and inoculation treatment in Supplementary Table 1. Total monoterpenes as well as a subset of nine individual (chiral and non-chiral) monoterpenes with known bioactivities in the MPB-pine system and occurred in both lodgepole and jack pine were used in the statistical analyses described below. The overall effect of inoculation on monoterpene profiles (all nine individual compounds) was examined by comparing the profiles of single inoculation controls to those of all inoculation treatments pooled. These comparisons were analyzed using permutational multivariate analysis of variance (PerMANOVA; 10,000 permutations), and separate tests were performed for lodgepole and jack pines. Variation in monoterpene profiles among inoculation treatments (excluding controls) and between species as well as inoculation-species interactions were tested using two-way PerMANOVA.

To determine if the induction of all or certain monoterpenes responded to inoculation treatments (at least two inoculations), we separately tested treatment and species main effects and interaction for total and individual monoterpenes using two-way ANOVA. This procedure

also tested inoculation-species interactions. Tukey's honest significant differences tests were used to examine pairwise differences for significant main effects or simple effects for significant interactions. These analyses were further used for two additional derived response variables for chiral compounds: total concentration (sum of negative and positive enantiomer concentrations) and enantiomeric ratio (negative divided by positive enantiomer concentrations).

One-way ANOVA was used to test the statistical significance of differences in fungal growth and reproduction responses to amendment treatments for a given monoterpene-fungus combination. Pairwise comparisons using Tukey's honest significant difference tests were performed following significant omnibus tests. Overall, study data were either log- or rank-transformed prior to analysis to satisfy assumptions of normality and heteroscedasticity, as needed. Figures were constructed using non-transformed data.

All analyses were performed within the R software environment version 3.3.1 (R Core Team 2016). PerMANOVAs were performed using the "Adonis" function of R package Vegan version 2.0-10 (Oksanen et al. 2013). All study data are freely available through the University of Alberta Libraries' Dataverse network (doi: 10.7939/DVN/10850).

Results

Monoterpene profile responses to Grosmannia clavigera *inoculation density*

One-way PerMANOVA indicated monoterpene profiles did not significantly differ (i.e., $P>0.05$) between controls and treated (inoculation treatments pooled) phloem for either lodgepole or jack pine. However, lodgepole and jack pines differed in monoterpene profiles as indicated by a significant species main effect from a two-way PerMANOVA ($F_{1,67}=42.99$, $P=0.001$). No significant inoculation main effect or species-inoculation interactions were detected.

Total and individual induced monoterpenes

Variation in total induced monoterpenes among treatments and between pine species is shown in Table 2. Although total monoterpenes did not significantly differ among treatments, they did significantly differ between pine species ($F_{1,67}$=6.74, P=0.012). Overall, total monoterpenes were 73% more concentrated in jack pine (mean=32.8 (± 4.6 SE) µg/mg fresh weight of tissue) compared to lodgepole pine (mean=19.0 (± 2.3 SE) µg/mg). No significant species-treatment interactions were detected.

The nine individual (chiral and non-chiral) monoterpenes were detected in lodgepole and jack pine phloem and among inoculation treatments (Table 2). Five of these compounds responded to inoculation treatments and/or differed between pine species: myrcene as well as (+) and (–) enantiomers of limonene and α-pinene. These compounds together represented the majority of monoterpenes detected among inoculation treatments and between species (Table 2). For myrcene, induction significantly interacted with inoculation treatment and pine species (Fig. 1, $F_{4,67}$=3.49, P=0.012). Simple effects of the pine species-inoculation density interaction indicated myrcene concentrations increased with inoculation density in jack pine, with concentrations increasing 500% when inoculation density increased from 2 to 32 per 0.3 m^2 (Fig. 1, Table 2). Myrcene concentrations in lodgepole pine did not respond to inoculation treatments. Further, simple effects indicated that concentrations were lower in jack pine as compared to lodgepole pine by 83% for the 2 inoculations treatment, and by 77% for the 4 inoculation treatment (Fig. 1). Myrcene concentrations were comparable between species at greater inoculation densities (Fig. 1).

Two-way ANOVA detected significant species main effects for total limonene (Fig. 2A), limonene enantiomeric ratio (Fig. 2B). Overall, limonene was less concentrated in jack than in lodgepole pine, with total limonene being 80% lower (Fig. 2A, $F_{1,67}$=25.21, P<0.001), and the ratio of (–)- to (+)-limonene being 44% lower (Fig. 2B, $F_{1,67}$=16.16, P<0.001). Similarly, (–)-limonene was 81% lower ($F_{1,67}$=32.12, P<0.001) in jack pine (mean=0.2 (± 0.1 SE) µg/mg) compared to lodgepole pine (mean=1.0 (± 0.2 SE) µg/mg), whereas (+)-limonene was 79% lower ($F_{1,67}$=13.13, P<0.001) in jack pine (mean=0.1 (± 0.1 SE) µg/mg) compared to lodgepole pine (mean=0.5 (± 0.1 SE) µg/mg). Species-inoculation interactions were non-significant for total limonene, limonene enantiomeric ratio, and individual limonene enantiomers.

Overall, α-pinene concentrations did not respond to inoculation treatments but differed between lodgepole and jack pines. The magnitude of these differences varied by α-pinene form, with total α-pinene being 3,000% higher (Fig. 2C, $F_{1,67}$=292.75, P<0.001) and the α-pinene enantiomeric ratio being 87% lower (Fig. 2D, $F_{1,67}$=281.29, P<0.001) in jack pine compared to lodgepole pine. Similarly, (–)-α-pinene was 1,675% more concentrated ($F_{1,67}$=64.63, P<0.001) in jack pine (mean=7.1 (± 2.3 SE) µg/mg) compared to lodgepole pine (mean=0.4 (± 0.1 SE) µg/mg), whereas (+)-α-pinene concentrations were 7,350% higher ($F_{1,67}$=514.68, P<0.001) in jack pine (mean=14.9 (± 1.9 SE) µg/mg) compared to lodgepole pine (mean=0.2 (± 0.1 SE) µg/mg). No significant species-inoculation interactions were detected for these compounds.

Fungal growth and reproduction on monoterpene- amended media

Monoterpene levels reflecting lodgepole and jack pine rapid defensive responses to *G. clavigera* inoculations affected the growth and reproduction of *G. clavigera*, *L. longiclavatum*, and *O. montium*. However, the magnitude and directionality of these responses varied by fungal

species, amendment concentration (low and high levels detected in lodgepole or jack pine phloem), and individual monoterpene (myrcene, limonene, and α-pinene).

Overall, myrcene concentrations either did not affect or negatively affected fungal growth while simultaneously stimulating fungal reproduction. For amendments simulating lodgepole pine induction levels of myrcene, fungal growth did not respond to myrcene amendments, except *G. clavigera* whose growth was inhibited in the low amendment treatment (Table 3, Fig. 3A, C, E). However, low and high myrcene concentrations from this pine stimulated the reproduction of each fungus (Table 3, Fig. 3B, D, F). For amendments simulating jack pine induction levels of myrcene, the growth of *G. clavigera* and *L. longiclavatum* were inhibited by the low and high concentration treatments (Table 3, Fig. 3A, E). However, *O. montium* growth was only slightly inhibited and did not respond to low and high treatments, respectively (Table 3, Fig. 3C). These treatments simulating jack pine induction stimulated the reproduction of each fungus, except *G. clavigera* did not respond to the high myrcene concentration amendment (Table 3, Fig. 3B, D, F).

Media amended with low and high limonene concentrations from lodgepole and jack pines tended to differentially affect the fungi, with the effects of limonene being more consistent between amendment concentrations from jack and lodgepole pine than was observed for myrcene. For amendments simulating limonene induction in lodgepole pine, *G. clavigera* growth was stimulated and inhibited by low and high concentration treatments, respectively (Table 3, Fig. 4A). However, these treatments inhibited the reproduction of this fungus (Table 3, Fig. 4B). Conversely, *O. montium* and *L. longiclavatum* growth and reproduction were stimulated by limonene concentration treatments simulating lodgepole pine induction (Table 3, Fig. 4C-F). For amendments simulating limonene induction in jack pine, *G. clavigera* growth

was stimulated while reproduction was inhibited by low and high concentration amendments (Table 3, Fig. 4A, B). However, *O. montium* and *L. longiclavatum* growth and reproduction were stimulated by these amendments (Table 3, Fig. 4C-F).

Fungal growth and reproduction responded to most α-pinene amendments simulating lodgepole and jack pine induction concentrations. For amendments simulating lodgepole pine induction, *G. clavigera* growth did not respond to either low or high concentration amendments, whereas the reproduction of this fungus was stimulated by the low concentration but did not respond to the high concentration (Table 3, Fig. 5A, B). However, *O. montium* and *L. longiclavatum* growth and reproduction was stimulated by these amendments (Table 3, Fig. 5C-F). For amendments simulating α-pinene induction in jack pine, *G. clavigera* growth was inhibited by low and high concentration amendments, whereas *G. clavigera* reproduction did not respond to the low concentration and was stimulated by the high concentration (Table 3, Fig. 5A, B). *Ophiostoma montium* growth and reproduction were stimulated by these amendments. Although *L. longiclavatum* growth was stimulated by low and high α-pinene concentrations simulating jack pine induction, its reproduction did not respond to either amendment (Table 3, Fig. 5C-F).

Discussion

On the basis of rapidly induced monoterpenes, jack pine is likely more susceptible to MPB colonization than lodgepole pine due to differences in the levels of monoterpenes that promote beetle aggregation behavior (e.g., myrcene and α-pinene) and inhibit beetle attack (e.g., limonene). Between tree species, myrcene concentrations only in jack pine phloem responded to inoculation density, such that concentrations of this compound increased 500% between the

lowest and highest density treatments. While α-pinene induction did not respond to inoculation density in either pine, induction of this compound in jack pine was 18 – 77 times greater than that in lodgepole pine. Our findings support those of others (Clark et al. 2012), indicating that mountain pine beetle attack increases myrcene concentration in lodgepole pine phloem. Such changes likely coincide with an increase in the emission concentration of this compound from attacked trees, as phloem and emission monoterpene concentrations can be positively associated (Taft et al. 2015a). Volatile myrcene synergizes with beetle aggregation pheromones, and thus is important to beetle mate finding and reproduction as well as overwhelming host tree defenses (Pureswaran et al. 2000, Raffa et al. 2005, Borden et al. 2008). Further, mass attack of host trees is invariably linked to α-pinene as newly arrived females hydroxylate this compound to synthesize *trans*-verbenol (an aggregation pheromone attractive to both sexes), whose emission from beetles increases with α-pinene levels in lodgepole and jack pine phloem (Pitman et al. 1968, Pitman and Vite 1969, Gries et al. 1990, Blomquist et al. 2010, Taft et al. 2015a). Thus, as long as α-pinene occurs at non-toxic concentrations, trees with high α-pinene levels could experience heavy beetle colonization (Pureswaran et al. 2000, Safranyik et al. 2010). Our results indicate that a positive feedback between MPB attack and myrcene induction could occur as beetles colonize jack pine. Such a feedback could likely synergize with the substantially higher levels of α-pinene to encourage rapid MPB colonization resulting in jack pine mortality from mass attack.

Low levels of limonene in the rapid induction response may limit jack pine resistance to MPB colonization. Although limonene concentrations did not respond to inoculation density, we found that overall limonene concentrations seven days after inoculations were 78 – 80% lower in jack pine compared to lodgepole pine phloem. This compound is toxic to MPB and thus is an

important component of pine defenses against beetle attack and colonization, and limonene levels can be a defining characteristic of beetle-resistant lodgepole pine (Raffa and Berryman 1983a, Raffa et al. 2005, Boone et al. 2011, Reid and Purcell 2011, Manning and Reid 2013). In lodgepole pine, MPB-attacked trees can have phloem limonene concentrations greater than those of non-attacked trees (Clark et al. 2010, 2014, Boone et al. 2011, Goodsman et al. 2013). For example, limonene concentrations can increase by 95% following initial MPB attack (Raffa and Berryman 1987). Although we used *G. clavigera* inoculations as a surrogate for MPB attack, pines can respond to ophiostomatoid fungi by accumulating monoterpenes such as limonene to high concentrations that negatively affect beetle vectors (Raffa and Smalley 1995). Thus, lower levels of limonene in jack pine phloem indicate a greater susceptibility to MPB colonization which may compound with that of myrcene and α-pinene to hasten mass attack onset and tree death relative to that of lodgepole pine.

Rapid induction of myrcene, α-pinene, and limonene in lodgepole and jack pine to simulated MPB attack can cause shifts in the growth-reproduction balance of *G. clavigera*, but may not arrest both biological functions. In general, myrcene amendments reflecting pine induction levels favored *G. clavigera* reproduction over growth. α-Pinene elicited similar responses as myrcene concentrations present in jack pine, but did not affect *G. clavigera* growth at concentrations reflecting lodgepole pine induction. Although the growth and reproduction of this fungus was inhibited by the most concentrated limonene amendment treatment, less concentrated treatments favored fungal growth over reproduction. Our results indicate that myrcene and α-pinene can be fungistatic to *G. clavigera*, as they inhibited but not halted mycelial growth. Furthermore, these compounds may act as stressors or environmental cues to shift *G. clavigera* development from assimilative to reproductive growth thereby increasing

propagule availability to vectoring MPB (Kendrick 2000). Limonene amendments likely stimulated assimilative growth because *G. clavigera* can detoxify and metabolize this compound (DiGuistini et al. 2011, Wang et al. 2013, 2014). However, our results indicate that the capacity of this fungus to utilize limonene likely has a concentration threshold above which fungal growth and reproduction are inhibited. The presence of α-pinene levels in jack pine that are fungistatic to *G. clavaigera* may suggest that this host is at least in part less susceptible than lodgepole pine. However, considering the overall effects of myrcene, α-pinene, and limonene on fungal growth, the susceptibility of jack pine to *G. clavigera* may in fact be similar to that of lodgepole pine. Because monoterpenes are simultaneously induced *in planta*, additional work using media amended with a combination of these compounds is needed in order to reveal potential chemical synergisms or additive effects that influence the biology and activity of ophiostomatoid fungi in host pines. Elucidating such effects may allow us to integrate our understanding of how MPB and its vectored fungi respond to host chemical induction in order to help clarify MPB holobiont-pine interactions and in turn the phytochemical factors underlying pine resistance to the holobiont (Six 2013).

Inter-pine differences in rapid monoterpene responses to simulated MPB attack similarly facilitate *O. montium* and *L. longiclavatum* growth and reproduction. We demonstrated that the growth and reproduction of *O. montium* and *L. longiclavatum* was generally stimulated by low and high levels of induced monoterpenes elicited by *G. clavigera*. Although where *L. longiclavatum* occurs in the invasion sequence of pine phloem/sapwood is unknown, *G. clavigera* is the primary invader, whose lesions are later colonized by *O. montium* (Solheim 1995). *Ophiostoma montium* can maintain positive growth and is not nutrient-limited in these lesions despite their high monoterpene and low carbohydrate concentrations (Bleiker and Six

2009, Goodsman et al. 2012, 2013, Lusebrink et al. 2016). Such facilitation is likely explained by a capacity to detoxify and metabolize host terpenes, as has been demonstrated for limonene utilization by *O. montium* and *L. longiclavatum* (Wang et al. 2014). Thus, our results indicate that lodgepole and jack pine are likely similarly susceptible to *O. montium* and *L. longiclavatum* under the induction environment created by simulated MPB attack. However, whether monoterpene induction is similar among the MPB-associated fungi and potentially interacts with host pine species is unknown. Such an understanding could help more accurately predict the relative susceptibility of lodgepole and jack pine to these fungi.

Conclusions

Pine secondary compounds are a critical component underlying tree susceptibility to bark beetles and their symbiotic fungi. Here, we showed that the rapid induced monoterpene responses to simulated MPB attack may promote beetle aggregation and colonization of jack pine trees. The rapid monoterpene induction responses of jack pine have likely evolved in the absence of MPB pressure, potentially resulting in induction responses with a relatively low capacity to inhibit early stages of beetle colonization and in turn mass attacks. Thus, given beetle populations of a size conducive to mass attack, jack pine may be colonized and mass attacked by MPB faster than lodgepole pine. The susceptibility of jack pine to infection by the MPB-vectored phytopathogenic fungi is less clear. However, our results show that these fungi respond similarly to the rapid monoterpene induction levels of jack and lodgepole pines. Because these fungi are critical to successful MPB colonization and mass attack, this similarity indicates that the induction responses of jack pine will likely not adversely affect symbiont activities enough to inhibit the invasion of MPB into jack pine forests.

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Conflict of interest

None declared

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Figure captions

Figure 1. Interaction plot showing mean (±SE) myrcene concentration (µg/mg fresh weight of tissue) in lodgepole (*Pinus contorta* var. *latifolia*) and jack (*P. banksiana*) pine phloem by *Grosmannia clavigera* inoculation density treatments (2, 4, 8, 16, or 32 inoculations per 0.3 m^2 of bark; n=7 or 8).

Figure 2. Mean (±SE) total limonene concentration (µg/mg fresh weight of tissue; sum of enantiomer concentrations, A), limonene enantiomeric ratio (B, negative divided by positive enantiomer concentrations), total α-pinene concentration (µg/mg; C), and α-pinene enantiomeric ratio (D) in the rapid induction (seven days post-inoculation) responses of jack (*Pinus banksiana*, n=38) and lodgepole (*P. contorta* var. *latifolia*, n=39) pine phloem inoculated with *Grosmannia clavigera*, a fungal symbiont of mountain pine beetle (*Dendroctonus ponderosae*).

Figure 3. Mean percent differences in fungal growth (culture area; left column) and reproduction (conidia density; right column) between cultures of *Grosmannia clavigera* (A, B), *Ophiostoma montium* (C, D), and *Leptographium longiclavatum* (E, F) grown on myrcene-amended and non-amended (control) media. Myrcene amendments reflect induction levels detected in lodgepole (*Pinus contorta* var. *latifolia*; LP; Low concentration=0.25 µg/mg, High concentration=2.25 µg/mg) and jack (*P. banksiana*; JP; Low concentration=0.09 µg/mg, High concentration=0.36 µg/mg) pines. Control culture area means were 2,289.6 (±72.0) mm^2 for *G. clavigera*, 1,477.7 (±19.3) mm^2 for *O. montium*, and 2,474.0 (±63.7) mm^2 for *L. longiclavatum*. Control conidia density means were 726.3 (±87.0) conidia mm^{-2} for *G. clavigera*, 1,236.1

(±214.3) conidia mm^{-2} for *O. montium*, and 158.9 (±20.5) conidia mm^{-2} for *L. longiclavatum*. Each media treatment was replicated fifteen times. As indicated by Tukey's honest significant difference tests, non-significant differences between treatments are indicated by "NS" notation between bars, whereas significant differences are indicated with "*" (P<0.05 – 0.01), "**" (P<0.01 – 0.001), or "***" (P<0.001).

Figure 4. Mean percent differences in fungal growth (culture area; left column) and reproduction (conidia density; right column) between cultures of *Grosmannia clavigera* (A, B), *Ophiostoma montium* (C, D), and *Leptographium longiclavatum* (E, F) grown on limonene-amended and non-amended (control) media. Limonene amendments reflect induction levels detected in lodgepole (*Pinus contorta* var. *latifolia*; LP; Low concentration=0.17 µg/mg, High concentration=0.48 µg/mg) and jack (*P. banksiana*; JP; Low concentration=0.07 µg/mg, High concentration=0.26 µg/mg) pines. Control culture area means were 2,289.6 (±72.0) mm^2 for *G. clavigera*, 1,477.7 (±19.3) mm^2 for *O. montium*, and 2,474.0 (±63.7) mm^2 for *L. longiclavatum*. Control conidia density means were 726.3 (±87.0) conidia mm^{-2} for *G. clavigera*, 1,236.1 (±214.3) conidia mm^{-2} for *O. montium*, and 158.9 (±20.5) conidia mm^{-2} for *L. longiclavatum*. Each media treatment was replicated fifteen times. As indicated by Tukey's honest significant difference tests, non-significant differences between treatments are indicated by "NS" notation between bars, whereas significant differences are indicated with "*" (P<0.05 – 0.01), "**" (P<0.01 – 0.001), or "***" (P<0.001).

Figure 5. Mean percent differences in fungal growth (culture area; left column) and reproduction (conidia density; right column) between cultures of *Grosmannia clavigera* (A, B),

Ophiostoma montium (C, D), and *Leptographium longiclavatum* (E, F) grown on α-pinene-amended and non-amended (control) media. α-Pinene amendments reflect induction levels detected in lodgepole (*Pinus contorta* var. *latifolia*; LP; Low concentration=0.22 µg/mg, High concentration=0.69 µg/mg) and jack (*P. banksiana*; JP; Low concentration=9.28 µg/mg, High concentration=21.39 µg/mg) pines. Control culture area means were 2,289.6 (±72.0) mm^2 for *G. clavigera*, 1,477.7 (±19.3) mm^2 for *O. montium*, and 2,474.0 (±63.7) mm^2 for *L. longiclavatum*. Control conidia density means were 726.3 (±87.0) conidia mm^{-2} for *G. clavigera*, 1,236.1 (±214.3) conidia mm^{-2} for *O. montium*, and 158.9 (±20.5) conidia mm^{-2} for *L. longiclavatum*. Each media treatment was replicated fifteen times. As indicated by Tukey's honest significant difference tests, non-significant differences between treatments are indicated by "NS" notation between bars, whereas significant differences are indicated with "*" (P<0.05 – 0.01), "**" (P<0.01 – 0.001), or "***" (P<0.001).

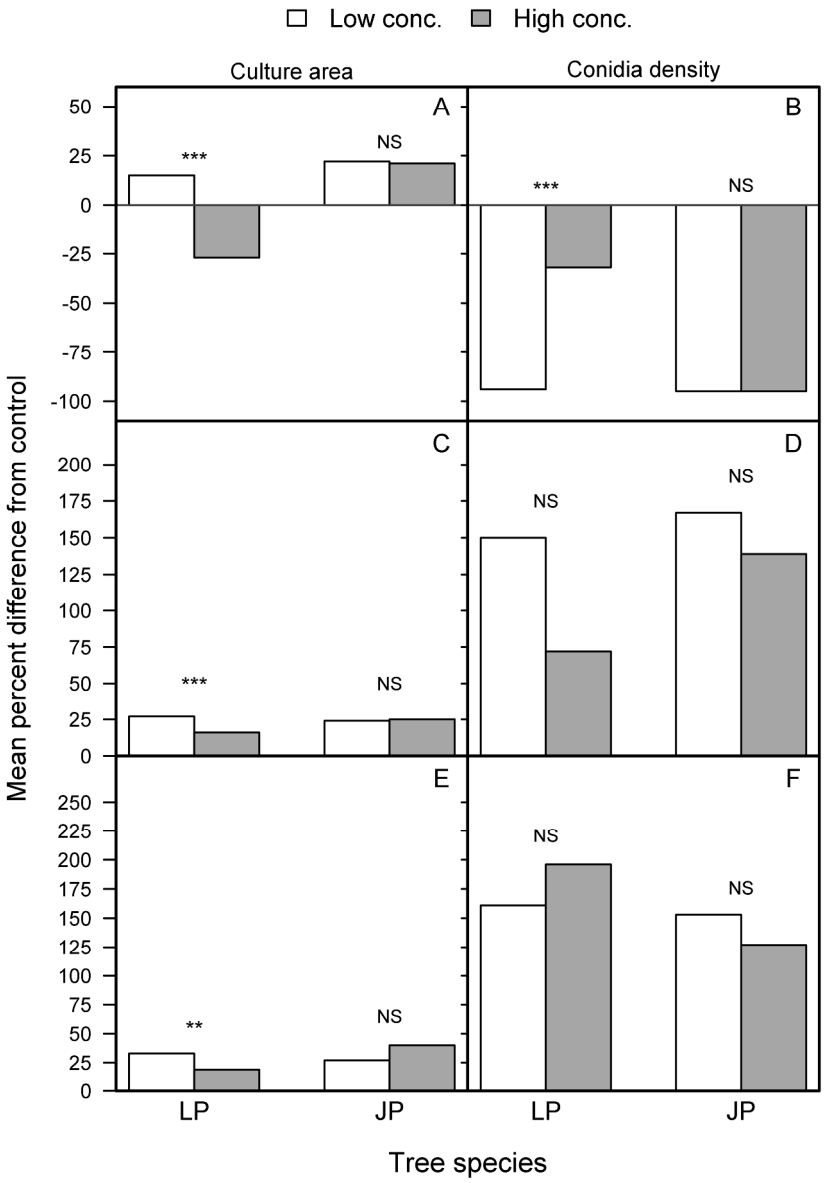

Figure 4. Mean percent differences in fungal growth (culture area; left column) and reproduction (conidia density; right column) between cultures of Grosmannia clavigera (A, B), Ophiostoma montium (C, D), and Leptographium longiclavatum (E, F) grown on limonene-amended and non-amended (control) media. Limonene amendments reflect induction levels detected in lodgepole (Pinus contorta var. latifolia; LP; Low concentration=0.17 µg/mg, High concentration=0.48 µg/mg) and jack (P. banksiana; JP; Low concentration=0.07 µg/mg, High concentration=0.26 µg/mg) pines. Control culture area means were 2,289.6 (±72.0) mm2 for G. clavigera, 1,477.7 (±19.3) mm2 for O. montium, and 2,474.0 (±63.7) mm2 for L. longiclavatum. Control conidia density means were 726.3 (±87.0) conidia mm-2 for G. clavigera, 1,236.1 (±214.3) conidia mm-2 for O. montium, and 158.9 (±20.5) conidia mm-2 for L. longiclavatum. Each media treatment was replicated fifteen times. As indicated by Tukey's honest significant difference tests, non-significant differences between treatments are indicated by "NS" notation between bars, whereas significant differences are indicated with "*" (P<0.05 – 0.01), "**" (P<0.01 – 0.001), or "***" (P<0.001).

164x236mm (600 x 600 DPI)

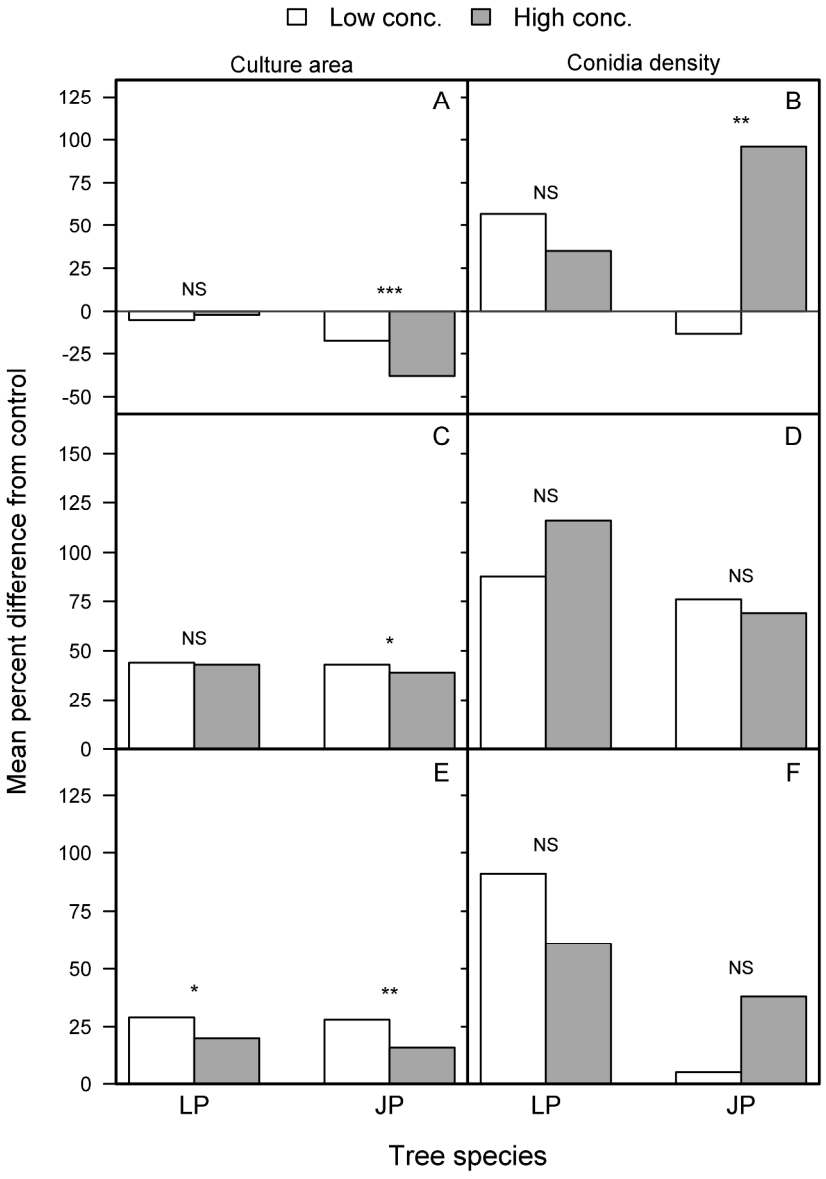

Figure 5. Mean percent differences in fungal growth (culture area; left column) and reproduction (conidia density; right column) between cultures of Grosmannia clavigera (A, B), Ophiostoma montium (C, D), and Leptographium longiclavatum (E, F) grown on α-pinene-amended and non-amended (control) media. α-Pinene amendments reflect induction levels detected in lodgepole (Pinus contorta var. latifolia; LP; Low concentration=0.22 µg/mg, High concentration=0.69 µg/mg) and jack (P. banksiana; JP; Low concentration=9.28 µg/mg, High concentration=21.39 µg/mg) pines. Control culture area means were 2,289.6 (±72.0) mm2 for G. clavigera, 1,477.7 (±19.3) mm2 for O. montium, and 2,474.0 (±63.7) mm2 for L. longiclavatum. Control conidia density means were 726.3 (±87.0) conidia mm-2 for G. clavigera, 1,236.1 (±214.3) conidia mm-2 for O. montium, and 158.9 (±20.5) conidia mm-2 for L. longiclavatum. Each media treatment was replicated fifteen times. As indicated by Tukey's honest significant difference tests, non-significant differences between treatments are indicated by "NS" notation between bars, whereas significant differences are indicated with "*" (P<0.05 – 0.01), "**" (P<0.01 – 0.001), or "***" (P<0.001).

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