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Water-deficit and fungal infection can differentially affect the production of different classes of defense compounds in two host pines of mountain pine beetle

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Running head: Production of defense chemicals vary by induction treatments

Summary – Bark beetles are important agents of tree mortality in conifer forests and their interaction with trees is influenced by host defense chemicals, such as monoterpenes and phenolics. Since mountain pine beetle (*Dendroctonus ponderosae*) has expanded its host range from lodgepole pine (*Pinus contorta*)-dominated forests to the novel jack pine (*Pinus banksiana*) forests in western Canada, studies investigating the jack pine suitability as a host for this beetle have exclusively focused on monoterpenes and whether phenolics affect jack pine suitability to mountain pine beetle and its symbiotic fungus *Grosmannia clavigera* is unknown. We investigated the phenolic and monoterpene composition in phloem and foliage of jack and lodgepole pines, and their subsequent change in response to water-deficit and *G. clavigera* inoculation treatments. In lodgepole pine phloem, water-deficit treatment inhibited the accumulation of both the total and richness of phenolics, but had no effect on total monoterpene production or richness. Fungal infection also inhibited the total phenolic production and had no effect on phenolic or monoterpene richness, but increased total monoterpene synthesis by 71%. In jack pine phloem, water-deficit treatment reduced phenolic production, but had no effect on phenolic or monoterpene richness or total monoterpenes. Fungal infection did not affect phenolic or monoterpene production. Lesions of both species contained lower phenolics but higher monoterpenes than non-infected phloem in the same tree. In both species, richness of monoterpenes and phenolics was greater in non-infected phloem than in lesions. We conclude that monoterpenes seem to be a critical component of induced defenses against *G. clavigera* in both jack and lodgepole pines; however, a lack of increased monoterpene response to fungal infection is an important evolutionary factor defining jack pine suitability to the mountain pine beetle invasion in western Canada.

Keywords: Chemical defenses, Canadian boreal forest, invasion dynamics, constitutive and induced plant defenses, secondary compounds of phloem and foliage

Introduction

Coniferous trees face numerous biotic (e.g., insect and pathogen attacks) and abiotic (e.g., drought) stressors during their long life span. A complex defense system involving anatomical, physical, and chemical defenses is employed by conifers in response to stress and are mediated through signaling pathways that ultimately lead to the production of several classes of defense compounds such as monoterpenes and phenolics (Franceschi et al. 2005, Keeling and Bohlmann 2006, Eyles et al. 2010). These compounds are constitutively present in plant tissues, providing immediate resistance to insect or pathogen attack. If the attack persists and the organism is not deterred, a "second tier of defence" in the form of induction responses is triggered to further protect the plant (Franceschi et al. 2005). Induced defenses qualitatively and/or quantitatively differ from constitutive defenses, and induction effects may persist for several hours to seasons, depending upon the types of chemicals induced (Franceschi et al. 2005, Eyles et al. 2010). Collectively, constitutive and inducible responses can form the basis of conifer defenses to insect or pathogen attacks (Franceschi et al. 2005, Erbilgin et al. 2006, Keeling and Bohlmann 2006, Witzell and Martin 2008, Eyles et al. 2010).

Bark beetles (Coleoptera: Curculionidae, Scolytinae) are among the most important agents of tree mortality in North American coniferous forests (Bentz et al. 2010). Several aspects of bark beetle-host tree interactions, particularly those involving the attack of living trees, are primarily influenced by host defensive chemicals (Raffa and Berryman 1987). First, some bark beetles must ensure tree mortality in order reproduce and complete their development within tree phloem. Unsuccessful reproduction, and thus failed brood production, can occur due to toxic defense-related compounds that kill or reduce the activities of attacking beetles and their phytopathogenic fungal symbionts. Second, after emerging from natal host trees, beetles must

undergo a series of host colonization stages (host selection, host entry, aggregation, reproduction, and emergence) in order to breed (Erbilgin et al. 2006). While some species utilize volatile chemicals such as monoterpenes as long-distance cues to locate potential hosts (i.e. Erbilgin and Raffa 2000), the role of host compounds in host location by other species is less clear. For example, female mountain pine beetles (*Dendroctonus ponderosae* Hopkins; MPB) utilize a combination of random landing and visual orientation for host location (Safranyik et al. 2010). Third, beetle aggregation pheromones—some are converted from host monoterpenes while others are produced *de novo* following exposure to host monoterpenes—emitted along with host volatiles from beetle entrance holes can ensure successful host colonization and mating (Raffa et al. 2005, Blomquist et al. 2010).

Since the invasion of novel jack pine (*Pinus banksiana*) forests by MPB in western Canada and the threat of beetle expansion into more eastern portions of the pine's geographical range (Cullingham et al. 2011), understanding the factors underlying jack pine suitability to the beetle is of critically important research direction. To date, such research has exclusively focused on the activities of monoterpenes at different stages of host colonization in jack pine and reported that: (1) jack pine appeared to have less pronounced chemical defenses than a historical host of MPB, lodgepole pine (*Pinus contorta*) (Lusebrink et al. 2011, 2016, Erbilgin and Colgan 2012). Particularly, jack pine not only quantitatively lacks important defense chemicals (e.g., limonene), but also contains large amounts of chemicals (e.g., α-pinene) that can facilitate beetle colonization (Erbilgin et al. 2014, Taft et al. 2015). (2) Prior to its expansion into jack pine forests, MPB invaded a zone of jack-lodgepole pine hybrids in Alberta. This has likely facilitated a host shift and improved beetle success on jack pine trees because hybrids show chemical characteristics of jack and lodgepole pines (Lusebrink et al. 2013). (3) Similarities in the

composition of monoterpenes between jack and lodgepole pines have likely allowed MPB to successfully colonize jack pine because its chemicals are compatible for beetle pheromone production, aggregation on host trees, and larval development (Erbilgin et al. 2014). (4) Changes in jack pine chemistry due to prior insect (Colgan and Erbilgin 2011) and pathogen (Klutsch et al. 2016) attacks can affect the successful colonization of jack pine by altering its suitability to MPB. Taken together, these studies highlight a critical, research question in MPB–host interactions: Do lodgepole and jack pines differ in their defense responses to MPB and its fungal symbionts?

The potential roles that other classes of defensive compounds, particularly phenolics, play in defining jack pine suitability to MPB and its symbiotic fungi remain an uninvestigated component of this question. In fact, phenolic composition in the phloem and foliage of mature jack pine has never been reported. In other bark beetle-host tree systems, phenolics can be a major component of induced defenses (Brignolas et al. 1995, Evensen et al. 2000, Faccoli and Schlyter 2007, Schiebe et al. 2012, Sherwood and Bonello 2013). For example, Schiebe et al. (2012) reported that trees surviving from attacks by *Ips typographus* were characterized by greater amounts of some phenolic compounds relative to those trees attacked and killed by this beetle. In addition, jack pine occupies drought-prone sandy soils of limited fertility in western Canada (Burns and Honkala 1990, Kenkel et al. 1997). Trees growing in such soils can experience periodic water deficit, which may hinder the production of defense chemicals, thus increasing the likelihood of tree death from low-density bark beetle attacks (Berryman 1982, Arango-Velez et al. 2014, 2016). Under such conditions, even the low densities of MPB currently present in jack pine forests could effectively exploit trees with weakened defensive capabilities. Therefore, a clear understanding of how water-deficit alters jack pine's defensive induction to

MPB can be critical to predicting the beetle's geographical expansion into jack pine forests under predicted increases in drought frequency in western Canada (Arango-Velez et al. 2016).

Recently, Lusebrink et al. (2016) reported the effects of soil water deficit and simulated infection by a MPB-vectored fungus, *Grosmannia clavigera*, on monoterpene induction in the phloem of mature jack and lodgepole pines in Alberta, Canada. As a follow-up to this study, we investigated phenolic responses in the same trees. Specifically, we analyzed both constitutive and induced phenolics in lodgepole and jack pine phloem, foliage, and *G. clavigera*-induced lesions to answer the following questions: Does phenolic composition in jack and lodgepole pine tissues (phloem and foliage) change in response to water-deficit and simulated fungal infection treatments, and do phenolics play a role in jack pine defense against *G. clavigera*? Further, we investigated whether phenolics in *G. clavigera*-induced lesions and phloem differ and interact with water-deficit. A lesion represents host tissue with visible symptoms of a defense response to activities associated with live fungal hyphae infection. Because individual monoterpene results were reported in our accompanying paper (Lusebrink et al. 2016), here, we report test results for total monoterpenes and richness.

Although phloem is biologically more relevant as a food source to MPB than foliage (Seybold et al. 2006), we also report foliar phenolic responses of both pines because changes in the phloem chemistry due to insect or pathogen attacks can alter foliar chemistry or vice versa. Thus, understanding how water-deficit and simulated fungal infection affect foliar chemistry may be relevant to such organisms as defoliators (e.g. Colgan and Erbilgin 2011, Goodsman et al. 2015). Likewise, volatile chemicals emitted from conifer stems and foliage can be long-distance attractants of bark beetles and their natural enemies (e.g. Erbilgin et al. 2000, 2001).

Materials and methods

Experimental design and sampling

In the summer of 2010, we initiated a field study to investigate if soil water availability affects constitutive and induced defences of historical and novel hosts of MPB. The detailed methodology for the field component of this study was reported in Lusebrink et al. (2016). Briefly, we selected one lodgepole (Hinton, 53°45'55.5"N, 118°22'17.9"W) and one jack (Smoky Lake, 54°05'18.5"N, 112°14'48.6"W) pine sites in Alberta. At the lodgepole site, we randomly selected 40 mature lodgepole pine trees (diameter at breast height (DBH), 1.4 m above the root collar of 22.0 cm ± 1.63 SD) and 40 mature jack pine trees (DBH of 21.9 cm ± 2.35 SD) were selected at the jack pine site. At the time of selection, no trees had signs or symptoms of insect or pathogen attack. During the first week of May 2010, half of the trees at each site were subjected to one of two water treatments. The tree bases in the water-deficit treatment were surrounded and covered with a tarpaulin (3.66m × 4.27m) to reduce rain water infiltration to the subsoil while trees in the ambient treatment were left under ambient conditions. A time domain reflectometry was used to monitor soil water content around each tree and the results were reported in Lusebrink et al. (2016). Briefly, soil water content was significantly lower around the water-deficit trees than around the ambient trees at both jack pine and lodgepole pine sites (Fig. 2 in Lusebrink et al. 2016).

Approximately five weeks after the water treatments were applied; trees in each water treatment group were equally divided into two subsets, with one subset receiving wound inoculation of stems with *G. clavigera* and the other set remaining unwounded to act as a control. We did not include a mechanical wounding alone treatment because Lusebrink et al. (2013) reported that it did not cause any major change in the response of mature pine trees in Alberta. In inoculated trees, eight wounds were evenly spaced around the stem at breast height. Wounds

were made with a cork borer (1 cm dia.). The fungus (Northern Forestry Culture Collection, 2896) used in inoculations was isolated from adult MPB and beetle galleries in infested mature pine trees in Fox Creek, Alberta. For fungal inoculations, a plug of malt extract agar containing active fungal mycelium was placed into the wound site with the mycelium facing the sapwood. The inoculation point was covered with a layer of Parafilm M® and insect screen (Bemis Flexible Packaging, Oshkosh, WI, USA). Briefly, for each tree species, we had four treatments: water-deficit treatment with or without fungal infection and ambient water treatment with or without fungal infection.

In mid-August of 2010, 15 weeks after the water treatment was initiated, all 40 trees (n=10 for each of water-fungal treatment combinations per species) were felled. Foliage from the middle of crown and non-infected phloem tissue from between two lesions at breast height (here after referred to as phloem) were sampled from all harvested trees, including non-inoculated trees for which phloem samples were collected at breast height. Tissue inside the lesion was also sampled and kept distinct from that of non-infected phloem during all later analyses. All samples were frozen on dry ice in the field and stored at -40°C in the laboratory prior to extraction.

Phenolic analysis

Tissues were freeze dried and ground with a TissueLyser II (Qiagen, Toronto, ON, Canada) using 0.5 mm diameter tungsten beads (Qiagen). The ground tissue (50 mg) was then weighed in 1.5mL micro centrifuge tubes and extracted according to Najar et al. (2014). Extracts were then diluted in a 1:1 ratio of extract and high-performance liquid chromatography (HPLC) grade water to remove excessive resin acids. Diluted samples were then centrifuged at 12,000 RCF for 5 min and the resulting supernatants were used for subsequent ultra-performance liquid chromatography (UPLC) coupled with a mass spectrophotometry (MS) analyses (described below).

We used a two-pronged method for peak separation and quantification of samples. We first quantified compounds in the extracts based on ultra violet (UV) peak area at 280 nm using UPLC and then each peak was assigned a tentative ID using HPLC-Mass Spectrometry (HPLC-MS). Since the UPLC provides a shorter run times and superior chromatographic separation and UV detection than the HPLC-MS, it was preferentially used for determining the peak areas with UV spectra. The areas for individual peaks were determined in Acquity H-Class UPLC (Waters, Milford MA, USA) equipped with a Waters Acquity Photodiode Array (PDA) detector, scanning all wavelengths between 230 and 400 nm and a Waters Acquity UPLC BEH C18 (2.1 x 100 mm, 1.7 µm particle size) column heated to 50^{o}C. Chromatographic separation was achieved using a binary solvent system with solvent A (water with 2% glacial acetic acid) and solvent B (methanol with 2% glacial acetic acid) with a flow rate of 0.42 ml min^{-1} using the following solvent gradient (percentages referring to solvent A only): 0-0.75 minutes hold at 97%; 0.75-9 minutes 97%-70%; 9-11 minutes 70%-10%; 11-13 minutes 10%-0%; 13-14.5 minutes hold at 0%; 14.5-15 minutes 0%-97%; 15.5-20.5 minutes hold at 97%. Using Waters Empower 3 software we quantified peaks at 280 nm using the ApexTrack integration algorithm for selecting peak apexes and the following processing method minimum criteria for determining what constitutes a peak: peak height = 2000; peak width = 10.0; peak area = 12500; peak threshold (used for determining baselines) = 2.00e+002. An injection volume of 0.7 µl of the diluted sample was used.

To identify peaks detected via the UPLC analysis above, pooled samples were run in an HPLC separation system coupled with a PDA and MS detector, henceforth dubbed HPLC-PDA-MS. To create the pooled samples, methanol extracts of the same tissue types in equal amounts (50 µl per sample) were combined together which were then diluted, as explained above, and used for HPLC-MS. For each sample, a 10 µl injection volume was used. The HPLC-PDA-MS

analyses were conducted using a Varian 212-LC pump system equipped with a Varian 410 Autosampler and a Waters XBridge BEH C18 (4.6 x 100 mm, 2.5 µl particle size) column at room temperature with post column flow split evenly between a Varian 500 IT Mass Spec, scanning for masses between 60-800 m/z, and a Varian ProStar 335 PDA detector, scanning at all wavelengths between 230-400 nm. This setup allowed for parallel detection of a peak's mass and UV profile. Using their UV profiles, relative retention times and elution orders, HPLC-PDA peaks were then matched to their corresponding UPLC peaks by hand. Because of differences in instrumentation and running conditions, the same sample run on the different instruments yielded similar but not identical UV chromatograms, so not all UPLC peaks could be reliably matched to their corresponding HPLC-MS peaks.

Chromatographic separation was achieved using a binary solvent system with solvent A (water with 0.1% glacial acetic acid) and solvent B (methanol with 0.1% glacial acetic acid) at a flow rate of 0.6 mL min^{1} using the following solvent gradient (percentages referring to solvent A only): 0-42 minutes 100%-50%; 42-45 minutes 50%-15%; 45-53 minutes 15%-0%; 53-56 minutes hold at 0%; 56-59 minutes 0%-100%; 59-65 minutes hold at 100%. Each sample was analyzed both in a full scan mode, which gives total ion counts, and in TurboDDSTM mode (i.e. MS^n). Peaks detected at 280 nm by the HPLC-PDA were matched to masses detected in the full scan mode based off retention time. These matched full scan masses were then analyzed in Turbo DDS mode to find their unique fragmentation patterns. UV patterns, full scan and turbo DDS data were used to assign tentative IDs to the matched UPLC peaks, based off matches to external standards and relevant literature. The following standards were used: catechin, *trans*-4-coumaric acid and taxifolin (Apin Chemicals, UK); ferulic acid and vanillic acid (Sigma-Aldrich, St. Louis MO, USA).

The following MS parameters were used for full scan: electron spray ionization; negative mode scanning 60-800 m/z; -80 capillary volts; ± 5000 needle volts; ± 600 spray shield volts; 50 psi nebulizer gas; 30 psi drying gas; 400°C drying gas temperature. The same conditions were used for MS^n analysis, with MS^1 fragmentation triggered at 5000 ion counts, subsequent MS^2 fragmentation triggered at 500 ion counts, MS^3 fragmentation triggered at 50 ion counts and MS^4 fragmentation triggered at 10 ion counts. For both full scan and MS^n, other parameters were left at instrumentation defaults. Phenolics are reported based on dry weight tissue (ng mg-1 of tissue).

Monoterpene analyses

Description of individual monoterpene analyses was provided in our accompanying paper (Lusebrink et al. 2016). Briefly, we ground tissues using mortar and pestle in liquid nitrogen, and 100 mg of ground samples was transferred to microcentrifuge tubes (1.5 mL) and extracted twice with 0.5 mL dichloromethane and 0.01% tridecane as a surrogate standard. When the ground tissue was mixed with the solvent, samples in the tubes were vortexed for 30 sec, sonicated for 10 min, subsequently centrifuged at 13,200 rpm and 0 °C for 15 min, and placed in a freezer for at least 2 h to let the pellet freeze. Extracts were then transferred into GC vials and 3 µL of extracts were injected at a split ratio of 20:1 in a GC-MS (7890A-5062C, Agilent Tech., Santa Clara, CA, USA) with a HP-Chiral-20B column (I.D. 0.25 mm, length 30 m; Agilent Tech.), helium carrier gas flow at 1.1 mL/min, temperature 75°C for 15 min, increased to 230 °C by 5 °C/min. Using the following standards, we identified the peaks: Borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol (Sigma-Aldrich), camphor, 3-carene, α-humulene, terpinolene, α- and β-thujone, (-)-α- and β-pinene, (+)-α- and β-pinene, (S)-(-)- and (R)-(+)-limonene, sabinene hydrate, myrcene, camphene, p-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate, cis-ocimene, α-phellandrene (SAFC Supply Solutions, St. Louis, MO, USA), β-phellandrene (Glidco Inc.,

Jacksonville, FL, USA). Monoterpenes are reported based on fresh weight tissue (ng mg-1 of tissue).

Data analysis

Analyses used phenolic peak area (as integrated from chromatograms) and monoterpene concentrations (ng/mg fresh tissue). Constitutive monoterpenes and phenolics were those observed in control trees, without *G. clavigera* inoculation and growing in soil with ambient water conditions. Separate one-way ANOVAs that were blocked by individual tree were used to test differences in constitutive total and richness of phenolics and monoterpenes between phloem and foliar tissues from control lodgepole and jack pine trees. Separate two-way ANOVAs were used to test main effects of water deficit and fungal infection treatments as well as treatment interactions on the induced total and richness of phenolics and monoterpenes in phloem and foliage. Further, two-way ANOVA blocked by individual tree was used to test main effects of lesion occurrence (*G. clavigera*-induced lesions *vs*. non-inoculated phloem) and water deficit as well as their interaction on the induced total and richness of phenolics and monoterpenes. The potential effects of water and fungal inoculation treatments as well as their interaction were tested for statistical significance using two-way permutational MANOVA (PerMANOVA). All tests were performed separately for lodgepole and jack pine as species effects could not be separated from site effects because samples were collected from one site per species. Principal component analysis (PCA) was used to visualize PerMANOVA results as well as investigate the relationships between individual phenolics and tissues or treatment groups. Data were natural-log transformed to satisfy statistical assumptions of normality and heteroscedasticity, as necessary. Figures were generated using non-transformed data. All statistical analyses were performed using the R software environment version 3.2.1. (R Core Team 2015), and PerMANOVA and PCA

were performed using functions provided in R package "vegan" version 2.3-2 (Oksanen et al. 2015).

Results

Monoterpene analyses

Since individual monoterpene results were reported in our accompanying paper (Lusebrink et al. 2016), here we report test results for total monoterpenes and richness which were not reported in the previous paper.

Does constitutive monoterpene composition differ between phloem and foliage within a species?

The amount of total constitutive monoterpenes varied between phloem and foliage of both pine species (Table 1). For lodgepole pine, total monoterpenes were 6.5 times more concentrated in phloem than in foliage ($F_{1,\ 17}$=26.7, P<0.001). Conversely, foliage in jack pine contained 4.1 times more total monoterpenes than phloem ($F_{1,\ 17}$=7.2, P=0.016). Constitutive monoterpene richness differed between phloem and foliar tissues. Lodgepole pine phloem had 141% greater richness than foliage ($F_{1,\ 9}$=14.80, P=0.004), whereas jack pine phloem had 55% lower richness than foliage ($F_{1,\ 9}$=21.62, P=0.001) (Fig. 1A).

Are monoterpene differentially induced in tissue types in response to water-deficit and simulated fungal infection?

Total monoterpene concentrations in response to water and inoculation treatments differed between phloem and foliar tissues of both pines (Table 1). For lodgepole pine, although results are marginally significant ($F_{1,\ 36}$=2.53, P=0.12), total monoterpene concentrations were 71% larger in the phloem of *G. clavigera*-inoculated trees compared to non-inoculated trees. Foliar

monoterpenes did not vary with fungal inoculations. Further, the total monoterpene response of lodgepole pine did not change with water deficit, and there was no significant water deficit-inoculation interaction. Total monoterpenes in jack pine phloem ($F_{1, 36}=0.178, P=0.68$) or foliage did not respond to either treatment. Richness of induced monoterpenes in these tissues did not vary with either treatments for either species.

Do lesions contain higher levels of total monoterpene than non-infected phloem?

Differences between non-infected phloem and lesion tissues were detected in the total monoterpene concentration to fungal inoculation for both pine species (Table 1). Total monoterpenes were 13 times more concentrated in lesions compared to non-infected phloem for lodgepole pine ($F_{1, 36}=198.4$, $P<0.001$) and 140 times higher in lesions than non-infected phloem for jack pine ($F_{1, 36}=475.2$, $P<0.001$). Total monoterpene concentration did not respond to water deficit (main effect) and tissue type-water deficit interaction for either pine species. Induced monoterpene richness did differ between phloem and *G. clavigera*-induced lesions for lodgepole and jack pines. In the former species, richness was 25% greater in phloem compared to lesions ($F_{1, 36}=14.94$, $P<0.001$), phloem richness was 88% lower than that in lesions for the latter pine species ($F_{1, 36}=40.73$, $P<0.001$) (Fig. 1B). However, these differences were not significantly affected by water-deficit.

Phenolic analyses

Overall, we identified 13 phenolic compounds from different tissues of pines, including catechin dimer, coumaric acid hexocide, ferulic acid glucoside, ferulic acid hexoside, ferulic acid hexoside-like compound, hydroxypropiovanillone hexoside, lignan deoxyhexoside, lignan

derivative, lignan xyloside, phenolic hexoside, taxifolin hexoside, unknown 1, and vanillic acid hexoside (Tables S1, S2). None of the tissue types contained all 13 compounds.

Does constitutive phenolic composition differ between phloem and foliage within a species?

We detected differences in levels of constitutive phenolics between phloem and foliage of both pine species. Total phenolics were 2.9 times greater in lodgepole pine phloem (33061156 ± 3591420 mAU) than foliage (8404527 ± 338442 mAU) ($F_{1, 17}$=128.6, P<0.001) and 5.7 times greater in jack pine phloem (36734720 ± 3184596 mAU) than foliage (5448853 ± 515151 mAU) ($F_{1, 17}$=196.8, P<0.001). Phenolic profiles also varied between these two tissue types in both lodgepole ($F_{1, 18}$=59.6, P<0.001; Fig. 2A) and jack ($F_{1, 18}$=114.2, P<0.001; Fig. 2B) pines. Interestingly, in both species, foliage was characterized by the abundance of catechin dimer, while the remaining phenolic compounds were associated with the phloem. Similarly, phenolic richness differed between phloem and foliage within each species. For lodgepole pine, phenolic richness in phloem (8.7 ± 0.3 compounds) was 38% greater ($F_{1, 17}$=48.4, P<0.001) than that in foliage (6.3 ±0.2 compounds) and phenolic richness was 29% greater in jack pine phloem (7.5 ± 0.6 compounds) compared to foliage (5.8 ± 0.3 compounds) ($F_{1, 18}$=5.6, P=0.030).

Are phenolics differentially induced in tissue types in response to water-deficit and simulated fungal infection?

We detected several differences in the total phenolics of phloem and foliage of both species in response to the water deficit and *G. clavigera* inoculation treatments. For lodgepole pine, both water ($F_{1, 36}$=39.1, P<0.001; Fig. 3A) and inoculation ($F_{1, 36}$=4.5, P=0.041; Fig. 3A) treatments reduced total phloem phenolics, which were 50% and 20% lower in water deficit and inoculated treatments, respectively, than those in control trees (those exposed to ambient water levels and

did not receive inoculations). Conversely, lodgepole pine foliage had 13% more total phenolics under water-deficit ($F_{1, 36}$=4.4, P=0.044; Fig. 3B), but did not respond to fungal inoculations. For jack pine, total phenolics in phloem were 34% lower in response to water-deficit ($F_{1, 36}$=20.6, P<0.001; Fig. 3C), but did not differ with fungal inoculation. There was no difference in total phenolics in jack pine foliage in response to either treatment.

Phenolic richness in response to water-deficit and inoculation treatments varied between foliage and phloem. In lodgepole pine phloem, phenolic richness was 9% lower in the water-deficit (7.9 ± 0.4 compounds) compared to the ambient water (8.7 ± 0.2 compounds) treatment ($F_{1, 36}$=4.6, P=0.039), but did not vary with fungal inoculation. Phenolic richness of lodgepole pine foliage and jack pine phloem did not respond to either treatment. However, richness in jack pine foliage was lower by 9% in the inoculation (5.5 ± 0.1 compounds) treatment compared to the non-inoculated (6.0 ± 0.2 compounds) treatment ($F_{1, 36}$=4.8, P=0.035).

Do lesions contain higher levels of total phenolics than non-infected phloem?

Phloem obtained from lesions and non-infected phloem in the same trees differed in total phenolic concentrations for both pine species in response to the water-deficit treatment. For lodgepole pine, total phenolics were 7.1 times greater in non-infected phloem compared to lesions ($F_{1, 33}$=164.9, P<0.001), and 43% lower in water-deficit trees ($F_{1, 33}$=4.5, P=0.042) (Fig. 4A). Total phenolics in jack pine showed a significant interaction between tissue types (phloem and lesion) and water treatment ($F_{1, 36}$=11.7, P=0.002) (Fig 4B), with lesions from ambient or water-deficit trees having marginally (P=0.082) different levels of total phenolics. However, total phenolics were higher in non-infected phloem of trees with ambient moisture compared to that of water-deficit trees (Fig. 4B).

Phenolic profiles in pine tissues varied in their quantitative responses to water-deficit and *G. clavigera* inoculation. For both pines, we detected significant profile responses of phenolics in non-infected phloem to water treatment (lodgepole pine: $F_{1, 36}$=22.0, P<0.001, Fig. 5A; jack pine: $F_{1, 36}$=11.2, P<0.001, Fig. 5B), but not fungal inoculation. However, foliar profiles did not vary with inoculations in either pine species. When comparing phloem and lesions within each species, we detected a significant interaction (lodgepole pine: $F_{1, 33}$=4.5, P=0.022, Fig. 6A; jack pine: $F_{1, 36}$=3.4, P=0.048, Fig. 6B) between tissue types and water treatments. For lodgepole pine, profiles in non-infected phloem differed between ambient and water-deficit treatments, but did not differ between these treatments in lesion tissue (Fig. 6A). Similar differences were also observed in jack pine (Fig. 6B).

For trees receiving fungal inoculations, we detected differences in phenolic richness between non-infected phloem and lesions as well as in response to water-deficit for the pine species. For lodgepole pine, phenolic richness was 64% lower in lesions compared to non-infected phloem ($F_{1, 33}$=146.4, P<0.001) (Fig. 7A), but did not significantly respond to water deficit. For jack pine however, we detected a significant interaction between these tissues and moisture deficit that affected phenolic richness ($F_{1, 36}$=5.6, P=0.023) (Fig. 7B). This interaction showed phenolic richness was higher in lesions of water-deficit trees than trees experiencing ambient moisture conditions (Fig. 7B). However, the opposite pattern was observed in non-infected phloem in which ambient-moisture trees had higher phenolic richness than water-deficit trees.

Discussion

This is the first study to report changes in the phenolic composition of mature lodgepole and jack pine trees due to water-deficit and simulated infection by a MPB-vectored pathogenic fungus, *G. clavigera*. We found that the changes in phloem chemistry varied within and between pine

species depending upon the class of defense chemicals and stressor types. Our results have important ecological and evolutionary implications for understanding MPB-host pine interactions and the factors underlying jack pine suitability to MPB infestation.

Water-deficit inhibited the accumulation of phenolics but had no effect on monoterpene production in both lodgepole and jack pine phloem

In both pines, water-deficit consistently impeded the accumulation of phenolics but had no effect on monoterpene synthesis. Reduced soil water availability can affect terpene production in conifers (Turtola et al. 2003, Lusebrink et al. 2011, Arango-Velez et al. 2014, 2016), but this is the first demonstration of the differential effects of water-deficit on the simultaneous production of phenolics and monoterpenes in any species of conifers, supporting similar results in non-conifer woody plants (Thomas and Schafellner 1999, McKiernan et al. 2014). Reductions in soil water availability likely lowered stomatal conductance and photosynthetic rates in both study pines (Arango-Velez et al. 2016). Lodgepole and jack pines have different stomatal response mechanisms to cope with reduced water—lodgepole pine has isohydric mechanism which provide a lower stomatal conductance threshold than the near-isohydric stomatal response of jack pine (Arango-Velez et al. 2016). Thus, reduction in phenolics is likely a direct result of reduced stomatal/vascular conductance and/or an indirect result of proportionally greater allocation of available resources to monoterpene production, as the synthesis of both phenolics and monoterpenes is carbohydrate limited (Goodsman et al. 2013). Indeed, the differential effects of resource availability on phenolic and terpenoid production have been reported in other woody plants (Koricheva et al. 1998, Blodgett et al. 2005, Roitto et al. 2009, Wallis et al. 2011).

Constant monoterpene production in lodgepole and jack pines is likely an adaptation to their enemies as plant-herbivore/pathogen interactions often favor a certain class of defense chemical

over others (Ledig 1998, Haukioja and Koricheva 2000, Franceschi et al. 2005). Both pines have likely evolved with common enemies such as dwarf mistletoe (*Arceuthobium* spp.), western gall rust (*Endocronartium harknessii*), Armillaria root disease and several species of bark beetles and defoliating insects against which monoterpenes seem to be favored defense chemicals (Franceschi et al. 2005, Colgan and Erbilgin 2011, Erbilgin and Colgan 2012). Additional studies are needed to elucidate the inhibitory effects of these chemicals on particular pest species as well as understanding the activities of phenolics and monoterpenes in pine resistance to drought.

Simulated fungal infection differentially altered phenolic and monoterpene production in both lodgepole and jack pine phloem

Fungal infection reduced phenolic production and increased monoterpene synthesis in lodgepole pine by 71% but, in jack pine, phenolic or monoterpene production did not change with fungal infection between infected and non-infected trees. Similarly Goodsman et al. (2003) found significant increase in total monoterpenes in lodgepole pine trees inoculated with *G. clavigera*. Likewise, a recent study by Keefover-Ring et al. (2016) reported a several fold increase in monoterpene concentration in another historical host, ponderosa pine (*P. ponderosa*), of MPB in response to *G. clavigera* inoculations. We suspect that increased monoterpene production in the beetle's historical host, but not in the novel host, might be a result of a co-evolutionary relationship between lodgepole pine and MPB (Sequeira et al. 2000, Raffa et al. 2005), and defenses in lodgepole pine have developed to match the selective pressures exerted by MPB (Raffa and Berryman 1987; Sequeira et al. 2000, Huber et al. 2004, Franceschi et al. 2005). In the evolutionary arms race between plants and their herbivores, co-evolved hosts possess more effective defenses against a given enemy than hosts without such an evolutionary history (Berenbaum 1995, Becerra 1997, Futuyma 2008). In contrast, jack pine is considered a novel host

(Cullingham et al. 2011, Erbilgin et al. 2014), without such an evolutionary history with MPB, and thus may lack effective defense mechanisms against the beetle.

Trees accumulated more monoterpenes but less phenolics in fungal lesions

Lesions in both lodgepole and jack pines had quantitatively and qualitatively fewer phenolics than non-lesion phloem, irrespective of water availability. In contrast, there were more total monoterpenes in lesions relative to the non-lesion phloem in both species. Monoterpene accumulation in lesions suggests they play a critical role in pine defense against *G. clavigera*, either by killing the fungus or inhibiting its growth (Krokene et al. 2008, Erbilgin and Colgan 2012, Keefover-Ring et al. 2016, Klutsch et al. 2016). Similar discrepancies have been observed in other pine-fungal pathosystems, where terpenoids can have more pronounced negative effects on lesion development than phenolics (Wallis et al. 2011). However, phenolics are a major component of induced resistance to several biotic agents, including a variety of bark beetles and their symbiotic fungi (Brignolas et al. 1995, Evensen et al. 2000, Faccoli and Schlyter 2007, Schiebe et al. 2012, Sherwood and Bonello 2013). Perhaps other types of phenolics (e.g., condensed tannins) or cell wall-bound compounds are involved in jack and lodgepole pine defenses (Strack et al. 1988, Maie et al. 2003). Alternatively, jack and lodgepole pine phenolics might show differential responses to the other fungal associates of MPB (e.g., *Ophiostoma montium* and *Leptographium longiclavatum*), as some bark beetle-associated fungi seem to be more sensitive to phenolics than others (Evensen et al. 2000). Additional investigations are needed to further elucidate the role of phenolics in pine defenses against MPB and its fungi.

Taken both phloem and lesion chemistry together, our results suggest that water-deficit and fungal infection treatments differentially affect defense-related signaling pathways—phenolics are produced via the shikimic acid pathway whereas the mevalonic acid pathway produces

monoterpenes (Franceschi et al. 2005). While both treatments inhibited the shikimic acid pathway in both pines, fungal infection promoted the mevalonic acid pathway only in lodgepole pine. These results have two important implications for forest health under future disturbance conditions: (1) Lodgepole and jack pines subjected to drought will likely have reduced phenolic defenses—compared to those growing under conditions of normal water availability—and thus might be more susceptible to attack by organisms otherwise defended against by these compounds. This mechanism might explain why some drought stressed trees are preferentially targeted by herbivores or pathogens (Niinemets 2010, Sherwood et al. 2015, McKiernan et al. 2016), but the degree of water stress is apparently crucial. (2) Lodgepole pine trees previously experiencing pathogen attack will likely have a greater induced monoterpene response—than those without such experience—and thus might be better defended against future attacks by organisms deterred by monoterpenes. For jack pine, prior pathogen attack apparently does not affect monoterpene synthesis and thus may not influence tree susceptibility to subsequent attacks. Stress-specific regulation of defense pathways, particularly the roles of different enzymes in production of defense chemicals, and their changing roles due to prior stress in tree susceptibility to subsequent attacks should be investigated in more detail (Bonello et al. 2006, Erbilgin and Colgan 2012, Sherwood and Bonello 2016)

Constitutive and induced phenolics of phloem differed from lodgepole and jack pine foliage

Phloem contained qualitatively and quantitatively more constitutive phenolics than foliage in both pines. In contrast, each species showed a different pattern of constitutive monoterpenes between phloem and foliage. While lodgepole pine phloem contained qualitatively and quantitatively more monoterpenes than foliage, jack pine foliage had more monoterpenes than phloem, suggesting inter-specific variation in the production of monoterpenes and phenolics

among different organs (Wallis et al. 2010, Villari et al. 2014). Constitutive phenolics are not known to contribute to defense against MPB nor other bark beetles, but this may due largely to a lack of investigation and these compounds thus may have unknown defensive activities.. However, high constitutive phenolic diversity can be a predictor of conifer resistance against some insects and pathogens (Witzell and Martin 2008, Delvas et al. 2011). In addition, constitutive phenolics in conifers can have other physiological functions including UV photoprotection and cold-hardiness (Witzell and Martin 20083). Some phenolics are also precursors for other defensive compounds, such as tannins and lignin (Boerjan et al. 2003).

In the current study, the phenolic composition of foliage and phloem was differentially affected by the same induction treatment. For example, fungal infection inhibited phenolic production in lodgepole pine phloem but had no effect on foliar phenolics. Conversely, infection increased the production of total monoterpenes in lodgepole pine phloem, but did not affect foliar monoterpenes. In jack pine, fungal infection had no effect on phloem or foliar monoterpenes. These findings suggest resources are differentially allocated toward biosynthesis of phenolics and monoterpenes as well as between different plant organs (Zangerl and Bazzaz 1992). Such differences may be stress-specific (attacks on stem *vs*. foliage) (Wallis et al. 2010, Erbilgin and Colgan 2012), defense compound-specific (Wallis et al. 2010), or simply driven by differences in the enzymatic activities of each tissue in support of defense or repair (Cheynier et al. 2013).

In conclusion, we provide three lines of evidence for monoterpenes being more critical components of pine defenses against MPB and its associated fungus than phenolics, and that tree physiology is an important determinant of host suitability and thus invasion success by forest insects. First, water limitation consistently inhibited phenolic production in both pines but had no effect on monoterpenes in either species. Second, simulated fungal infection reduced phenolic

production but marginally increased monoterpene production in phloem of lodgepole pine, but had no effect on jack pine phloem phenolics or monoterpenes. Third, lesions contained less phenolics but more monoterpenes than non-lesion phloem in both species. Based on our results, we hypothesize that MPB may have capitalized on the 'evolutionary naiveté' of jack pine and is thus successfully exploiting this novel host (Mooney and Cleland 2001, Walther et al. 2009, Erbilgin et al. 2014). More specifically, a lack of increased monoterpene response to *G. clavigera* infection is an important evolutionary factor defining jack pine susceptibility to the MPB invasion in western Canada.

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Figure Captions

Figure 1. Mean (±SE) richness of constitutive monoterpenes in phloem and foliar tissues of lodgepole (*Pinus contorta*) and jack pines (*P. banksiana*) (A) and induced monoterpene richness in phloem and *Grosmannia clavigera*-induced lesions for the same trees (B). Statistical significance of P=0.01 – 0.001 and P<0.001 indicated by "**" and "***", respectively.

Figure 2. Differences in constitutive phenolic profiles in lodgepole (*Pinus contorta*; A) and jack pine (*P. banksiana*; B) phloem (black) and foliage (grey). Confidence ellipses (95%) indicate differences between principle component clusters for each tissue. Phenolics displayed are as follows: catechin dimer (Cat), coumaric acid hexocide (CAHx), ferulic acid glucoside (FAGl), ferulic acid hexoside (FAHx), ferulic acid hexoside-like compound (FAHx2), hydroxypropiovanillone hexoside (HHx), lignan deoxyhexoside (LDeox), lignan derivative (LDer), lignan xyloside (LXy), phenolic hexoside (PHx), taxifolin hexoside (THx), and vanillic acid hexoside (VAH), as well as an unknown 1 (Unk).

Figure 3. Mean (±SE) total peak area (mAU) of induced phenolics in lodgepole pine (*Pinus contorta*) phloem (A) and foliage (B) as well as jack pine (*P. banksiana*) phloem (C) in response to soil water treatments (deficit or ambient) and *Grosmannia clavigera* treatments (inoculated or non-inoculated). Statistical significance of less than P=0.001 and P=0.05 – 0.01 indicated by "***" and "*", respectively, whereas "NS" indicates a non-significant difference between neighboring bars.

Figure 4. Mean (±SE) total peak area of induced phenolics in lodgepole pine (*Pinus contorta*; A) and jack pine (*P. banksiana*; B) in tissues (*Grosmannia clavigera*-infected lesions or healthy

phloem) and soil water treatments (deficit [triangles] or ambient [circles]). Statistical significance of main effects for lodgepole pine (A) of less than P=0.001 and P=0.05 – 0.01 indicated by "***" and "*", respectively.

Figure 5. Differences in induced phenolic profiles in lodgepole (*Pinus contorta*; A) and jack pine (*P. banksiana*; B) phloem for trees growing in soil water-deficit (grey) or ambient (black) conditions. Confidence ellipses indicate differences between principle component clusters for each treatment. Phenolics displayed are as follows: coumaric acid hexocide (CAHx), ferulic acid glucoside (FAGl), ferulic acid hexoside (FAHx), ferulic acid hexoside-like compound (FAHx2), hydroxypropiovanillone hexoside (HHx), lignan deoxyhexoside (LDeox), lignan derivative (LDer), lignan xyloside (LXy), phenolic hexoside (PHx), taxifolin hexoside (THx), and vanillic acid hexoside (VAH), as well as an unknown 1 (Unk).

Figure 6. Differences in induced phenolic profiles in *Grosmannia clavigera*-infected (lesion; grey) and healthy phloem (black) of lodgepole (*Pinus contorta*; A) and jack pine (*P. banksiana*; B) phloem for trees growing in ambient (circles) or soil water-deficit (triangles) conditions. Confidence ellipses (95%) indicate differences between principle component clusters for each treatment. Phenolics displayed are as follows: coumaric acid hexocide (CAHx), ferulic acid glucoside (FAGl), ferulic acid hexoside (FAHx), ferulic acid hexoside-like compound (FAHx2), hydroxypropiovanillone hexoside (HHx), lignan deoxyhexoside (LDeox), lignan derivative (LDer), lignan xyloside (LXy), taxifolin hexoside (THx), and vanillic acid hexoside (VAH), as well as an unknown 1 (Unk).

Figure 7. Mean (±SE) total peak area of induced phenolics in lodgepole pine (*Pinus contorta*; A) and jack pine (*P. banksiana*; B) in tissues (*Grosmannia clavigera*-infected lesions or healthy phloem) and soil water treatments (deficit [triangles] or ambient [circles]). Statistical

significance of main effects for lodgepole pine (A) of less than P=0.001 and P=0.05 – 0.01 indicated by "***" and "*", respectively.

Table 1. Mean total monoterpene concentrations (ng/mg) for lodgepole (*Pinus contorta*) and jack pine (*P. banksiana*) phloem (from between lesions), foliage, and *Grosmannia clavigera*-induced lesions under different treatments: constutitive (ambient moisture and without *G. clavigera* inoculation), ambient soil moisture, restricted soil moisture, non-inoculated, and inoculated with *G. clavigera*.

Treatments	Species	Mean total monoterpene concentration per tissue (±s.e.)		
		Phloem	Foliage	Lesion
Constitutive	Lodgepole pine	8881.7 (1348.3)	1360.9 (686.42).	-
	Jack pine	457.4 (136.0)	1854.3 (656.5)	-
Ambient water	Lodgepole pine	12338.0 (2209.0)	1458.4 (435.8)	-
	Jack pine	457.7 (94.7)	1917.8 (411.9)	-
Water deficit	Lodgepole pine	8090.1 (1063.2)	2245.2 (466.5)	-
	Jack pine	972.3 (295.2)	2203.5 (336.0)	-
Non-inoculated	Lodgepole pine	7532.9 (826.8)	2067.7 (530.3)	-
	Jack pine	648.1 (181.4)	2168.4 (426.1)	-
Inoculated	Lodgepole pine	12895.2 (2246.0)	1635.9 (371.1)	167255.6 (9108.2)
	Jack pine	781.8 (264.0)	1952.8 (319.3)	109728.4 (5993.0)

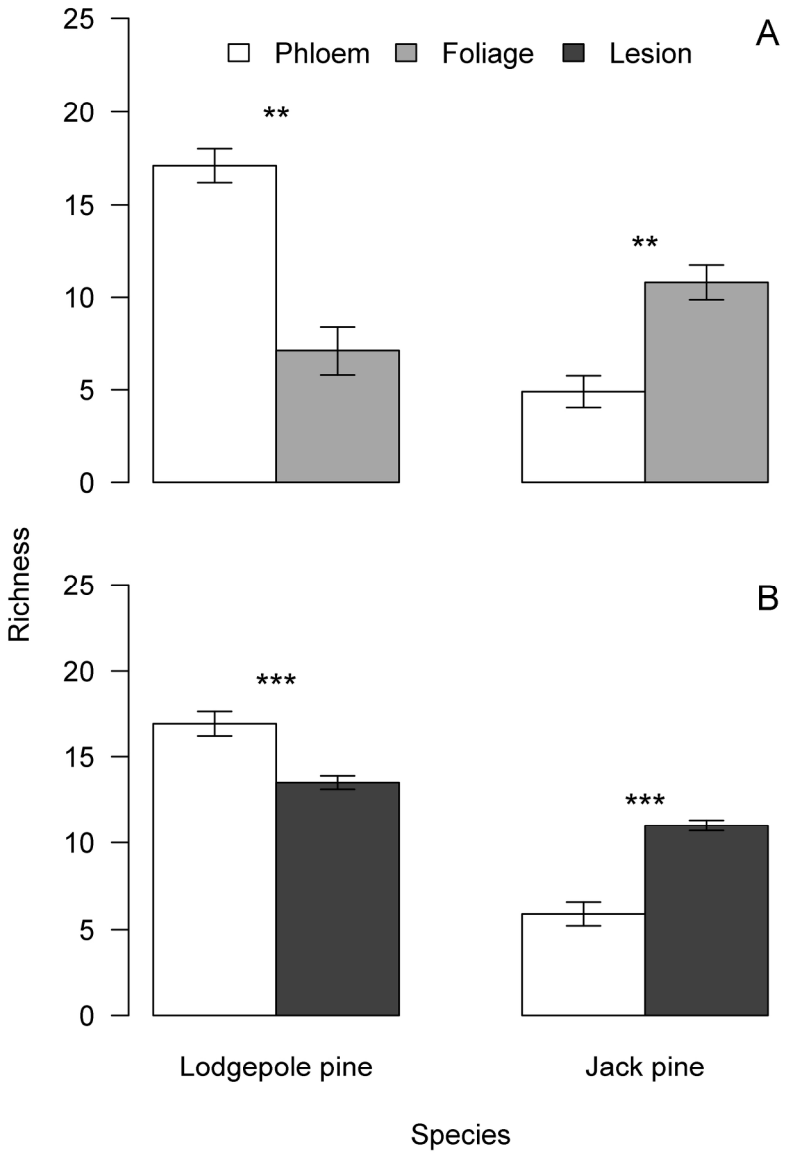

Figure 1. Mean (±SE) richness of constitutive monoterpenes in phloem and foliar tissues of lodgepole (Pinus contorta) and jack pines (P. banksiana) (A) and induced monoterpene richness in phloem and Grosmannia clavigera-induced lesions for the same trees (B). Statistical significance of P=0.01 – 0.001 and P<0.001 indicated by "**" and "***", respectively.

179x259mm (300 x 300 DPI)

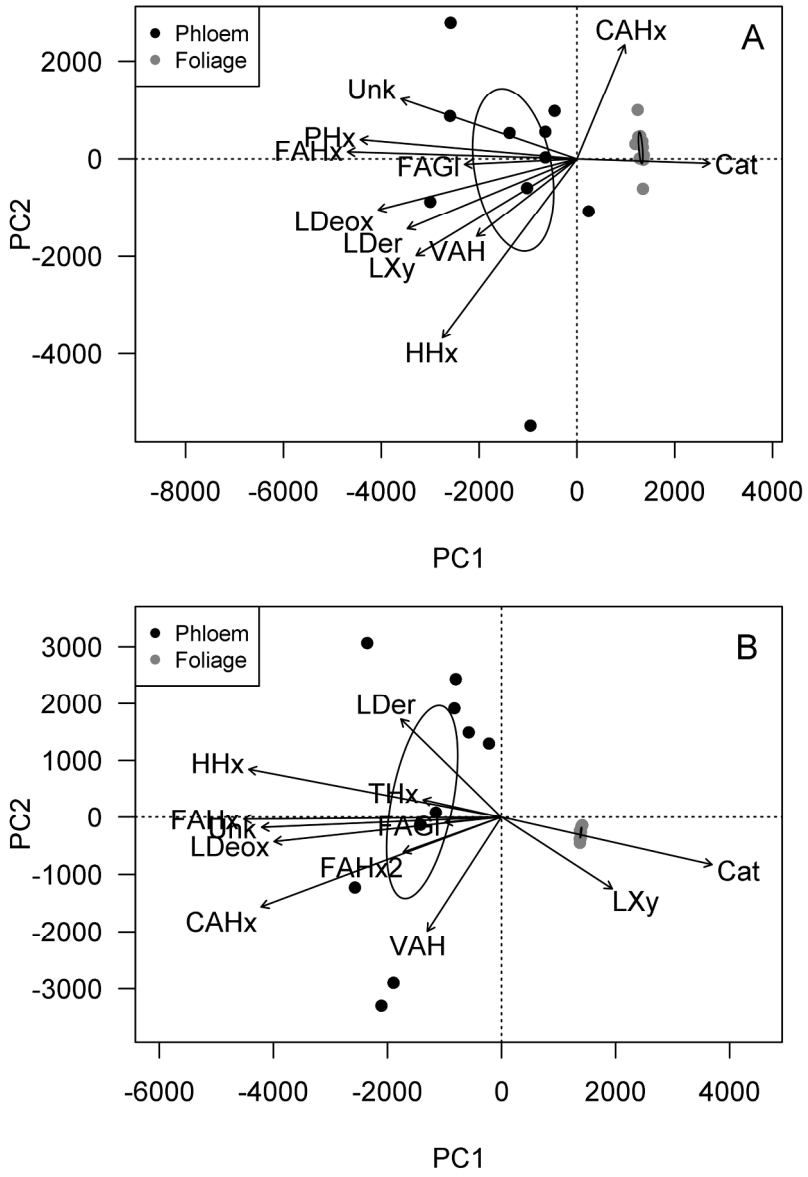

Figure 2. Differences in constitutive phenolic profiles in lodgepole (Pinus contorta; A) and jack pine (P. banksiana; B) phloem (black) and foliage (grey). Confidence ellipses (95%) indicate differences between principle component clusters for each tissue. Phenolics displayed are as follows: catechin dimer (Cat), coumaric acid hexocide (CAHx), ferulic acid glucoside (FAGl), ferulic acid hexoside (FAHx), ferulic acid hexoside-like compound (FAHx2), hydroxypropiovanillone hexoside (HHx), lignan deoxyhexoside (LDeox), lignan derivative (LDer), lignan xyloside (LXy), phenolic hexoside (PHx), taxifolin hexoside (THx), and vanillic acid hexoside (VAH), as well as an unknown 1 (Unk).

184x273mm (300 x 300 DPI)

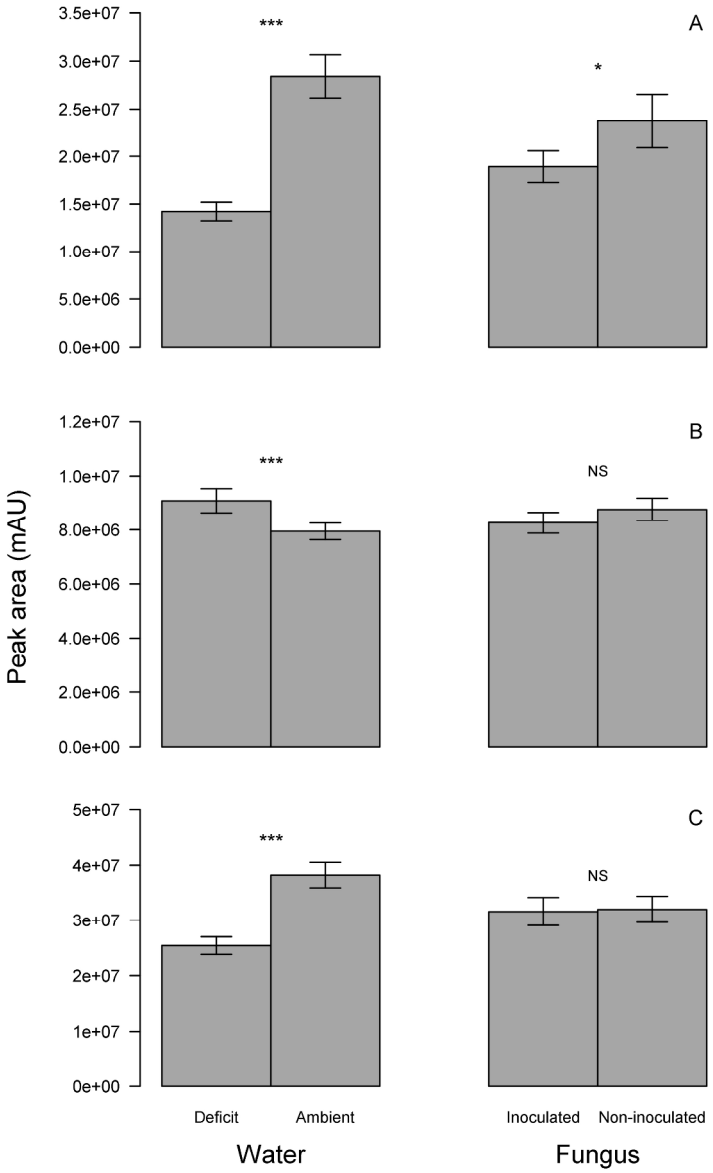

Figure 3. Mean (±SE) total peak area (mAU) of induced phenolics in lodgepole pine (Pinus contorta) phloem (A) and foliage (B) as well as jack pine (P. banksiana) phloem (C) in response to soil water treatments (deficit or ambient) and Grosmannia clavigera treatments (inoculated or non-inoculated). Statistical significance of less than P=0.001 and P=0.05 – 0.01 indicated by "***" and "*", respectively, whereas "NS" indicates a non-significant difference between neighboring bars.

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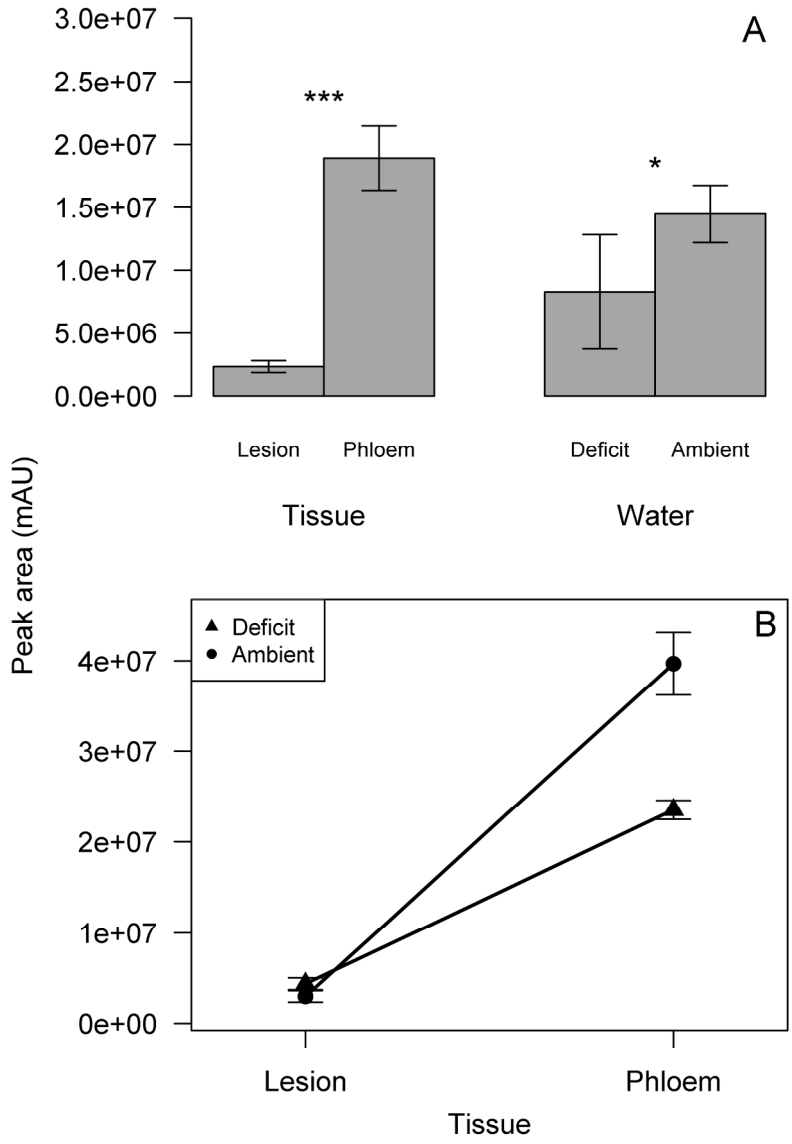

Figure 4. Mean (±SE) total peak area of induced phenolics in lodgepole pine (Pinus contorta; A) and jack pine (P. banksiana; B) in tissues (Grosmannia clavigera-infected lesions or healthy phloem) and soil water treatments (deficit [triangles] or ambient [circles]). Statistical significance of main effects for lodgepole pine (A) of less than P=0.001 and P=0.05 – 0.01 indicated by "***" and "*", respectively.

179x259mm (300 x 300 DPI)

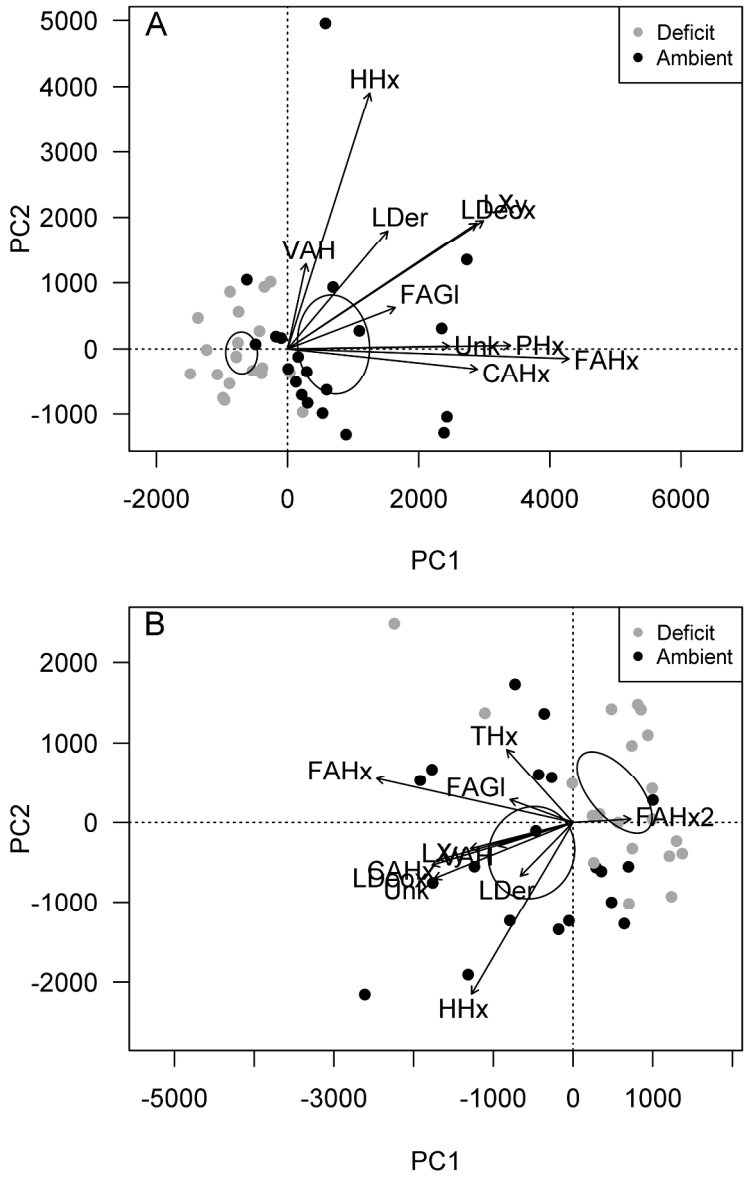

Figure 5. Differences in induced phenolic profiles in lodgepole (Pinus contorta; A) and jack pine (P. banksiana; B) phloem for trees growing in soil water-deficit (grey) or ambient (black) conditions. Confidence ellipses indicate differences between principle component clusters for each treatment. Phenolics displayed are as follows: coumaric acid hexocide (CAHx), ferulic acid glucoside (FAGl), ferulic acid hexoside (FAHx), ferulic acid hexoside-like compound (FAHx2), hydroxypropiovanillone hexoside (HHx), lignan deoxyhexoside (LDeox), lignan derivative (LDer), lignan xyloside (LXy), phenolic hexoside (PHx), taxifolin hexoside (THx), and vanillic acid hexoside (VAH), as well as an unknown 1 (Unk).

194x304mm (300 x 300 DPI)

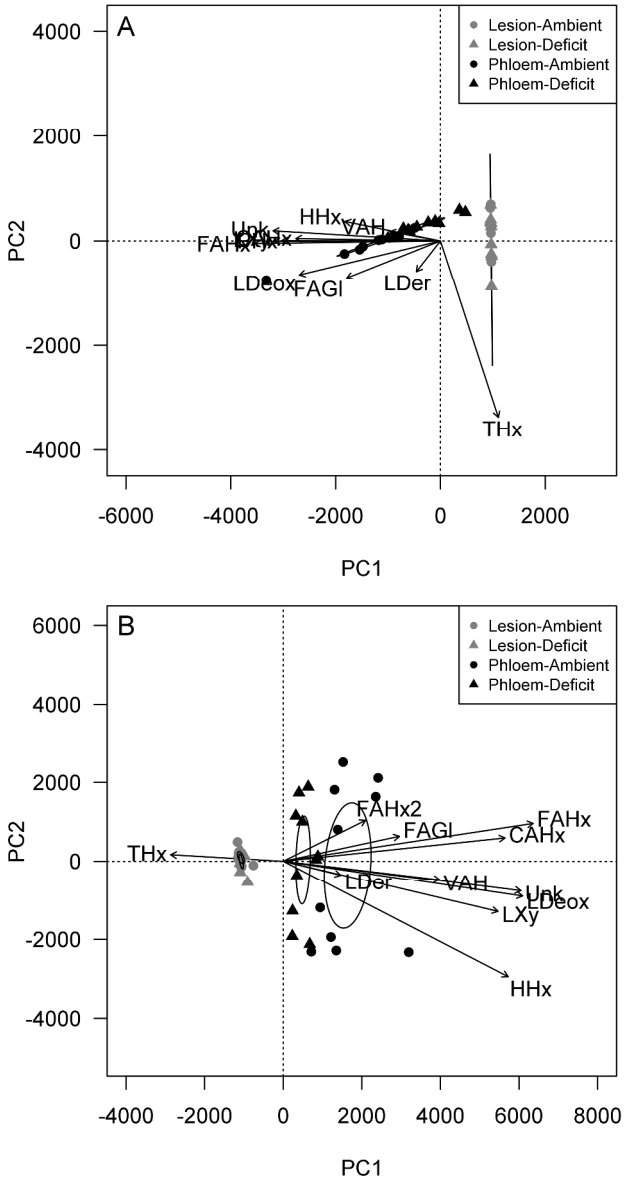

Figure 6. Differences in induced phenolic profiles in Grosmannia clavigera-infected (lesion; grey) and healthy phloem (black) of lodgepole (Pinus contorta; A) and jack pine (P. banksiana; B) phloem for trees growing in ambient (circles) or soil water-deficit (triangles) conditions. Confidence ellipses (95%) indicate differences between principle component clusters for each treatment. Phenolics displayed are as follows: coumaric acid hexocide (CAHx), ferulic acid glucoside (FAGl), ferulic acid hexoside (FAHx), ferulic acid hexoside-like compound (FAHx2), hydroxypropiovanillone hexoside (HHx), lignan deoxyhexoside (LDeox), lignan derivative (LDer), lignan xyloside (LXy), taxifolin hexoside (THx), and vanillic acid hexoside (VAH), as well as an unknown 1 (Unk).

234x441mm (300 x 300 DPI)

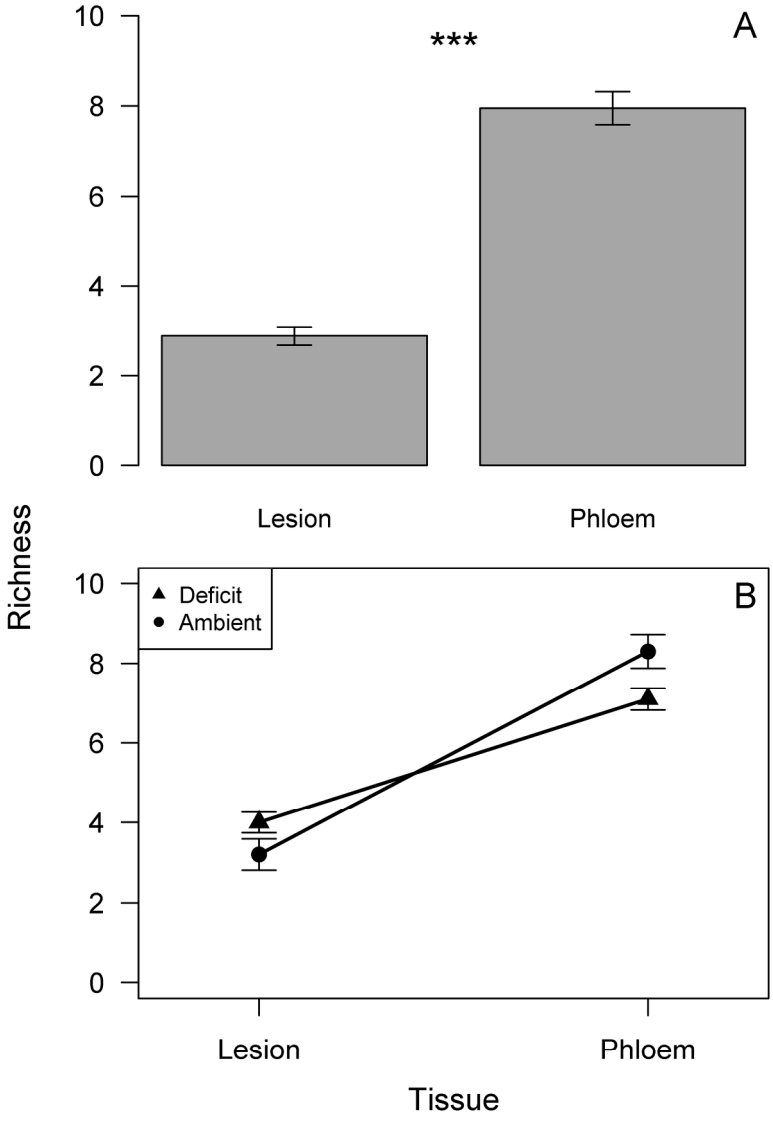

Figure 7. Mean (±SE) total peak area of induced phenolics in lodgepole pine (Pinus contorta; A) and jack pine (P. banksiana; B) in tissues (Grosmannia clavigera-infected lesions or healthy phloem) and soil water treatments (deficit [triangles] or ambient [circles]). Statistical significance of main effects for lodgepole pine (A) of less than P=0.001 and P=0.05 – 0.01 indicated by "***" and "*", respectively.

179x259mm (300 x 300 DPI)