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Rapid Induction of Multiple Terpenoid Groups by Ponderosa Pine in Response to Bark Beetle-Associated Fungi

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Abstract Ponderosa pine (*Pinus ponderosa*) is a major and widely distributed component of conifer biomes in western North America and provides substantial ecological and economic benefits. This tree is exposed to several tree-killing bark beetle-microbial complexes, including the mountain pine beetle (*Dendroctonus ponderosae*) and the phytopathogenic fungus *Grosmannia clavigera* that it vectors, which are among the most important. Induced responses play a crucial role in conifer defenses, yet these have not been reported in ponderosa pine. We compared concentrations of terpenes and a phenylpropanoid, two phytochemical classes with strong effects against bark beetles and their symbionts, in constitutive phloem tissue and in tissue following mechanical wounding or simulated *D. ponderosae* attack (mechanical wounding plus inoculation with *G. clavigera*). We also tested whether potential induced responses were localized or systemic. Ponderosa pines showed pronounced induced defenses to inoculation, increasing their total phloem concentrations of monoterpenes 22.3-fold, sesquiterpenes 56.7-fold, and diterpenes 34.8-fold within 17 days. In contrast, responses to mechanical wounding alone were only 5.2, 11.3, and 7.7-fold, respectively. Likewise, the phenylpropanoid estragole (4-allyanisole) rose to 19.1-fold constitutive levels after simulated attack but only 4.4-fold after mechanical wounding. Overall, we found no evidence of systemic induction after 17 days, which spans most of this herbivore's narrow peak attack period, as significant quantitative and compositional changes within and between terpenoid groups were localized to the wound site. Implications to the less frequent exploitation of ponderosa than lodgepole pine by *D. ponderosae*, and potential advantages of rapid localized over long-term systemic responses in this system, are discussed.

Keywords Inducible plant defenses · Conifer · *Pinus ponderosa* · Fungi · *Grosmannia* · *Dendroctonus* · Terpenoids · Estragole

Introduction

Conifers are confronted with multiple biotic agents that potentially can exploit their tissues, of which bark beetles and their associated microorganisms pose the greatest threat (Safranyik and Carroll 2006). Because these insects and their associated fungi colonize subcortical tissues, their reproduction and development typically impair trees' abilities to translocate water and nutrients, and hence cause host death. The mortality pressures exerted by these herbivore-fungal complexes have selected for sophisticated defensive adaptations (Franceschi et al. 2005; Hamberger et al. 2011; Huber et al. 2004).

Conifer defenses are multi-faceted, including physical defenses such as resin that can entomb or delay beetles (Popp et al. 1991). chemical defenses such as terpenoids and phenolics that can repel, inhibit, or kill beetles and their symbionts

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(Bohlmann 2012; Brignolas et al. 1998; Faccoli and Schlyter 2007; Klepzig et al. 1996). and histological reactions such as autonecrosis and hypersensitivity that can confine biotic agents and deprive them of essential nutrients (Paine et al. 1993). Chemical defenses include both constitutive and induced components (Zulak and Bohlmann 2010). and are supported by structures such as resin ducts and glands (Berryman 1972; Ferrenberg et al. 2014; Kane and Kolb 2010) that provide storage and transport and are likewise dynamic. The various processes of conifer defense are highly integrated. For example, resin provides an important physical barrier, but also contains compounds that can be toxic, repellant, or inhibit attraction to the aggregation pheromones (Erbilgin et al. 2006) that bark beetles need to overwhelm tree defenses (Blomquist et al. 2010). Likewise, strong resin flow can delay entering beetles, thus providing critical time for biosynthesis of insecticidal and antimicrobial compounds that might otherwise be negated by mass attack (Raffa et al. 2005).

Each of these various defense components has been associated with successful defense in some systems. For example, the density and size of resin ducts were higher in ponderosa and lodgepole pines that had survived attack than those observed in killed trees (Ferrenberg et al. 2014; Kane and Kolb 2010). Higher constitutive and induced resin flow in response to simulated attack (mechanical wound accompanied by challenge with the beetles' symbiotic fungus), reduced the likelihood of lodgepole pines subsequently being killed by mountain pine beetles (Boone et al. 2011). Lodgepole pines, grand firs, and Norway spruce that showed large accumulation of induced monoterpenes in response to similarly simulated attack were likewise less likely to be killed (Boone et al. 2011; Raffa and Berryman 1987; Zhao et al. 2011b). Length of necrotic lesions commonly are not related to the likelihood of subsequent mortality (Boone et al. 2011; Raffa and Berryman 1982, 1983a). although the rate of lesion formation can be a good predictor in some systems (Wallin and Raffa, 2001.

There is increased recognition that induced chemical defenses are widespread across plant taxa, and can play crucial roles in plant defense and herbivore population dynamics (Underwood and Rausher 2002). Induced plant defenses can be either localized or systemic, depending on the system, tissue, stimulus, and other factors (Karban et al. 1999; Thaler et al. 2012). Examples of systemic induced defenses, i.e., plant- or tissue-wide alterations, have become increasingly common in recent years (Pieterse et al. 2013; Vos et al. 2013).

The mountain pine beetle is one of the most important tree-killing insects in North America (Negron and Fettig 2014). This insect undergoes extended periods at low populations, during which it is limited to trees with poor defenses compromised by stresses such as disease, lightning, and old age (Safranyik and Carroll 2006). Certain conditions can favor a sudden population increase, such as severe drought, warm temperatures, and homogeneous mature stand structure (Aukema et al. 2008; Carroll et al. 2004; Hicke et al. 2006; Logan and Bentz 1999; Preisler et al. 2012). At high densities, the pheromone-mediated mass attack behavior of mountain pine beetle enables them to kill trees across a broader range of vigor, and landscape-scale irruptions occur (Bleiker et al. 2014; Boone et al. 2011; Smith et al. 2011).

Most research on mountain pine beetle has been conducted with lodgepole pine, its most common host (Meddens et al. 2012). However, this insect also attacks ponderosa pine. For example, the current outbreak is causing substantial mortality in ponderosa pine in sections of Montana, western South Dakota, and the front range of the Rocky Mountains, necessitating increased attention to this resource (Costello et al. 2013; Knapp et al. 2013; West et al. 2014). Gaylord et al. (2011) found that several components of induced defense appear minimal in ponderosa pine, including induced resinosis and necrotic lesion formation. We currently lack data on the chemical dimension of response, including whether induction occurs, and if so what compounds are involved and whether the response is localized or systemic.

Terpenes have a broad range of activities that contribute to conifer defenses against bark beetles (Keeling and Bohlmann 2006). Most analyses have been done on monoterpenes, largely due to technical challenges associated with other groups. The available evidence suggests that diterpene acids can play important complementary roles (Hall et al. 2013). by having stronger anti-fungal but weaker anti-beetle properties than monoterpenes (Boone et al. 2013; Kopper et al. 2005). Sesquiterpenes likewise have been reported in conifers (Zhao et al. 2011b). but their defensive functions with regards to bark beetle-fungal complexes are largely unknown. Additionally, most work on conifer induction has focused on rapid localized induction, but recent reports have described systemic induction in Norway spruce (Krokene 2015). We tested whether ponderosa pine undergoes induced chemical changes in terpenes and a phenylpropanoid that may inhibit mountain pine beetle and its symbionts, the extent to which various groups of terpenes may undergo quantitative and compositional changes, and whether such changes may be localized or systemic.

Methods and Materials

Site Selection and Defense Induction The study site is situated on a south-facing slope north of Storm Castle Creek in Montana, USA at 45.44° N, 111.22° W, 1680 m above sea level. Ponderosa pine saplings were planted in the 1930s, and the site has not been actively managed since. Only mature trees that had no apparent symptoms of disease, defoliation, or wounding were selected for sampling. Diameter at breast height (DBH), 1.3 m above the ground, ranged from 21 to 61 cm with an average of 40.6 (± 9.0 SD) cm. Temperatures at the nearby Gallatin Gateway, MT weather station averaged

30.8 (± 2.8 SD) °C during the experiment (11–29 July 2013). At the beginning of the experiment, the year-to-date precipitation was 7.2 mm, and the area received only an additional 0.1 mm of rain by July 29th, resulting in a year-to-date accumulation about 2 mm below normal.

To assess constitutive levels of secondary metabolites, we collected phloem from 40 trees from 11 to 12 July 2013 (T_0). The cardinal direction from which this sample was collected was selected using a random number generator. We then randomly assigned half of the trees to one of two treatments: mechanical wounding or mechanical wounding + inoculation with mountain pine beetle's primary symbiont, *Grosmannia clavigera* (Univ. MT isolate 1747). The wounding treatment was applied to the side of the tree directly opposite the site where the constitutive sample was collected. Mechanical wounding was applied by removing a bark plug using a 6 mm tree borer and then reinserting the plug. Fungal inoculation was administered by wounding as above, but applying a 5 mm plug of actively growing mycelium to the phloem. Detailed descriptions are in Boone et al. (2011). All trees were resampled from 28 to 29 July 2013 (T_1), approximately 17 d following the wounding treatments. Lesions from both fungal-inoculated and mechanically damaged areas were removed with a scalpel, and these samples were termed "local." To test for systemic induction, we also collected phloem tissue from the opposite side of the trees, at the same time lesions were sampled. These "systemic" samples were taken above, slightly off-center, and about 30 cm away from where constitutive samples were obtained.

We placed all samples into vials immediately following sampling and stored them in a cooler over dry ice before being brought to Montana State University on the same day. They were stored at −80 °C. Samples were shipped on 1 August 2013 on dry ice to the University of Wisconsin-Madison for chemical analysis.

Terpenoid Extraction We extracted terpenoids separately from the three different phloem locations from each tree, including the constitutive samples taken at T_0, and systemic and local samples collected at T_1. We removed samples from the freezer as needed, cut phloem into small cubes (~2–3 mm), and divided them into two portions. We immediately submerged one portion in 1 ml of 95 % *n*-hexane with 0.2 μl ml^{-1} of toluene and nonyl acetate, as internal standards, in 2 ml GC vials with PTFE screw caps for mono- and sesquiterpene extraction. We placed the second portion of phloem into 1 ml of 200 proof ethanol in 2 ml microcentrifuge tubes with sealed screw caps, for diterpene extraction. We placed all vials and tubes in a sonication bath for 10 min, briefly vortex mixed them, and allowed them to shake overnight on an orbital mixer. After shaking, the solvent from the mono−/sesquiterpene samples was decanted into fresh GC vials. The diterpene sample tubes were centrifuged at 14, 000 rpm for 10 min, and clear ethanol solution was drawn off with a micropipette and into fresh tubes. Due to the much higher concentrations of terpenes in wounded samples, we diluted them 1 in 11 with their respective solvents prior to chemical analysis.

Chemical Analyses We analyzed the composition of the various mono- and sesquiterpenes across treatments by gas chromatography (GC) using an enantioselective column. The GC system consisted of a Hewlett Packard 5890 GC equipped with a flame ionization detector (FID) and a Cyclodex-B capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm; Agilent Technologies) with helium as the carrier gas at a flow rate of 1.0 ml min^{-1}. We injected 2 μl of each mono−/sesquiterpene sample directly, with a split flow ratio of 30:1, using an oven profile of 40 °C for 5 min, followed by a ramp of 3 °C min^{-1} to 200 °C, and then a second ramp at 25 °C min^{-1} to 220 °C. Injector and detector temperatures were set at 260 °C and 250 °C, respectively.

We used GC-FID for diterpene analysis, converting them to their methyl esters prior to analysis, using a method modified from Robert et al. (2010) and Keefover-Ring and Linhart (2010). We mixed 75 μl of each diterpene sample with 50 μl of a 2.0 M (trimethylsilyl) diazomethane (TMS-DAM) solution in diethyl ether (Sigma-Aldrich, St. Louis, MO, USA) and after brief vortex mixing allowed the solution to react for 20 min at ambient temperature. Samples then were vacuum centrifuge dried and resuspended with 75 μl of methanol with 0.8 μl ml^{-1} of carvacrol as an internal standard. We used the same GC and most of the conditions, as above, except for a DB-Wax capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm; Agilent Technologies) and an oven profile of an initial temperature of 160 °C followed by an immediate ramp of 2 °C min^{-1} to 250 °C, and then held at this final temperature for 20 min.

We conducted additional terpenoid identification analyses with a Shimadzu GC-2010 Plus gas chromatograph coupled with a QP-2010SE quadrupole mass spectrometer with an ion source of 70.0 eV at 230 °C, using helium as the carrier gas at 36 cm sec^{-1} (1.0 ml min^{-1}) with the injector temperature set at 250 °C. Oven conditions included an initial temperature of 40 °C followed by an immediate ramp of 3 °C min^{-1} to 200 °C. We injected 1 μl of selected mono−/sesquiterpene and diterpene samples, available standards, and a continuous series of *n*-alkanes (C_8–C_{20}; Sigma-Aldrich) in the splitless mode onto a ZB-5 capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm; Phenomenex, Torrance, CA, USA). We identified mono- and sesquiterpenes with retention time matches to pure standards, mass spectra, and/or linear retention indexes calculated with the alkane series (Adams 2007; El-Sayed 2013; NIST 2008). Diterpenes were identified by retention time matches to standards, mass spectra (Dethlefs et al. 1996; NIST 2008; Popova et al. 2010). and their relative retention times on both polar (Lewinsohn et al. 1993) and nonpolar columns (Dethlefs et al. 1996; Popova et al. 2010).

We dried all phloem samples to a constant weight at 60 °C, and we used dry weight (d.w.) values to calculate compound concentrations (μg or mg compound g^{-1} d.w.) with standard curves of authentic standards, when available, injected on the GC-FID. We purchased standards for all but two identified monoterpenes, longifolene, and estragole from Sigma-Aldrich (St. Louis, MO, USA). Purified β-phellandrene came from Glidco Organics (Jacksonville, FL, USA) and α-thujene had no available standard. Abietic acid was from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). The USDA Forest Service Forest Products Laboratory in Madison, WI, supplied the remaining diterpene standards, except for sandaracopimaric and isocupressic acids. We calculated the concentrations of all unknowns and identified compounds that had no standards with the nearest eluting standard compound.

Terpenoid Diversity We calculated chemical diversity separately for mono-, sesqui-, and diterpenes for each individual phloem sample. We used Shannon's index $H = -\Sigma p_i \ln(p_i)$, where p_i is the proportion, i.e., abundance of the ith terpenoid of an individual sample, and converted these values to "true diversities" by raising the constant e to the power of H [exp(H)] for each sample (Jost 2006). We then used these computed values to test for differences among the six treatments.

Resin Viscosity Conifer oleoresin consists of non-volatile diterpene resin acids (C_{20}) dissolved in lower molecular weight, volatile mono- (C_{10}) and sesquiterpenes (C_{15}), with the relative composition of these two main groups determining viscosity. Thus, for each sample we calculated the ratio of total mono- plus sesquiterpenes to total diterpenes {[(M + S)/D] = mg mono- plus sesquiterpenes g^{-1} d.w. divided by mg diterpenes g^{-1} d.w.)}. Lower ratios of mono- plus sesquiterpenes to diterpenes would create more viscous resin.

Statistical Analyses We used SAS software version 9.4 (SAS Institute 2013) to examine potential differences among the terpene profiles of the six different treatment combinations (constitutive, systemic, and local in either wounded only or wounded plus inoculation with *G. clavigera* trees). We performed separate single-factor multivariate analysis of variance (MANOVA; PROC GLM function with the MANOVA statement) tests for all mono-, sesqui-, and diterpenes using terpene concentration data (mg terpenes g^{-1} d.w.), with treatment combination as the factor. For all terpenoid classes, significant MANOVA tests were followed with separate single-factor ANOVAs for individual compounds and total mono-, sesqui-, and diterpenes. Separate single-factor ANOVAs were used to test for treatment differences in the amounts of estragole (also called 4-allylanisole), chemical diversity, and terpenoid viscosity. We followed all ANOVAs with a Tukey *post hoc* test to determine differences among the six treatment combinations.

We also conducted non-metric multidimensional scaling analyses (MDS) on the three terpenoid groups to assess the influence of the different treatments on ponderosa pine phloem chemistry. Mono-, sesqui-, and diterpenes were analyzed individually in the same manner. Samples were log(y + 1) transformed and standardized by the total in the sample. Euclidean pairwise dissimilarity matrices were computed between samples and used to compute an MDS plot for each group of compounds in Primer-E v. 7.0.

Results

Because our study includes multiple groups of compounds, comparisons, and approaches to data analysis, we provide here a general roadmap for the results. We describe the three classes of terpenes in order of increasing molecular weight. Within each class, we first describe total concentrations, then relative composition of individual compounds. Within each class, we compare constitutive, mechanically wounded, and simulated *D. ponderosae* attacked phloem samples, and also local changes at the treatment site vs. systemic changes on the opposite side of the tree.

Monoterpenes Monoterpenes were the second most abundant group of terpenes in ponderosa pine phloem, after diterpenes (Fig. 1a). The total quantities of monoterpenes varied among the six treatments (F = 80.0, $P < 0.001$). In trees that were mechanically wounded only, concentrations at the wound site (local samples) rose to 5.2 times that of constitutive levels. In trees that were subjected to simulated *D. ponderosae* attack, i.e., combined mechanical wounding with inoculation of the beetle's fungal symbiont, concentrations at the wound site increased 22.3 times that of constitutive levels. We did not find evidence of systemic induction of total monoterpenes in response to either the mechanical wounding or fungal inoculation, as there was no within-tree change in phloem tissue distant from the treatment point (systemic samples).

We quantified the concentrations of 24 monoterpenes, including the (−) and (+) enantiomers of α-pinene, camphene, β-pinene, limonene, and linalool, in addition to α-thujene, tricyclene, myrcene, α-terpinene, δ-3-carene, *p*-cymene, β-phellandrene, γ-terpinene, terpinolene, bornyl acetate, terpinyl acetate, geranyl acetate, and two unknowns (Table 1, Suppl. Table 1). The most abundant monoterpenes were δ-3-carene (1.1 mg g^{-1} d.w. ± 0.1 SE) and (−)-limonene (1.7 mg g^{-1} d.w. ± 0.3 SE), which each accounted for approximately one quarter of total monoterpenes in constitutive phloem.

The MANOVA showed compositional changes in the monoterpene profiles among the six treatments (Wilks' $\lambda = 0.01$, $F_{120,\ 437} = 5.1$, $P < 0.001$). ANOVA results for individual compounds showed that all monoterpenes, except geranyl acetate differed with treatment (Table 1). In general,

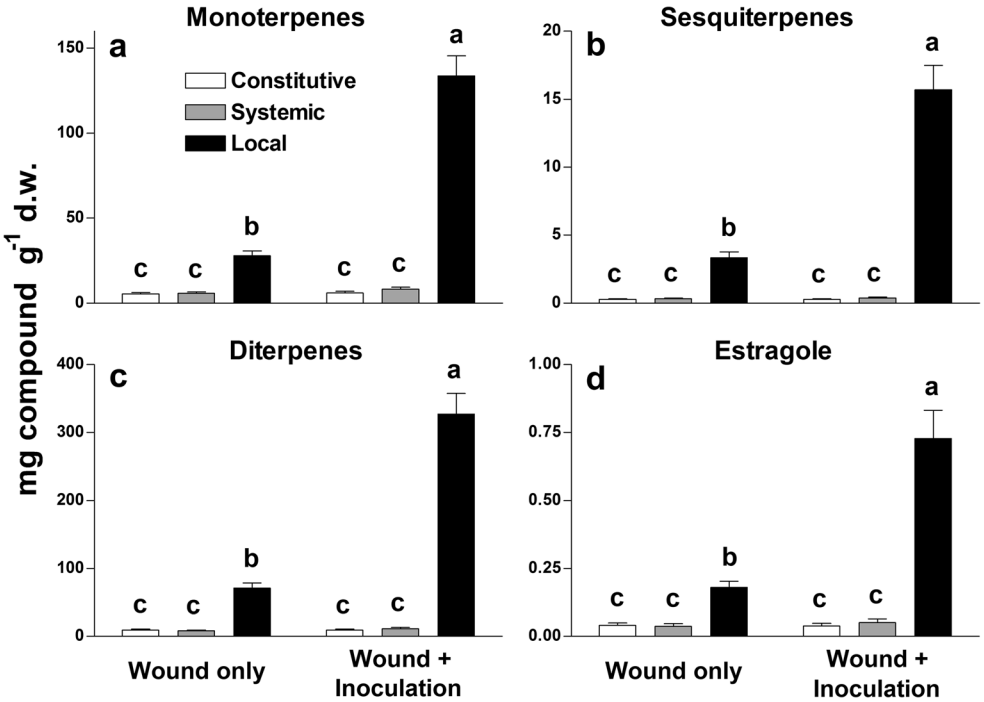

Fig. 1 Total concentrations (mg compound g^{-1} d.w.; Mean ± SE) of three terpene classes and the phenylpropanoid estragole (4-allylanisole) in *Pinus ponderosa* phloem. Note different scales among classes. Terpenes were measured in constitutive phloem (Constitutive; initial time T_0), and in phloem that had either been subjected to a mechanical wound (Wound only) or to a simulated *Dendroctonus ponderosae* attack that entailed a mechanical wound plus inoculation with *D. ponderosae's* predominant fungal symbiont *Grosmannia clavigera* (Wound + inoculation). Treated samples were collected either within the hypersensitive lesion formed in response to the wounding treatment (Local) or on the opposite side of the tree at the same height (Systemic). The local and systemic samples were collected 17 d (T_1) after constitutive samples from the same trees. The mechanical wounding only and wounding plus fungal inoculation treatments were applied to separate trees

the *post hoc* analyses pattern for individual monoterpenes that varied matched that of the total monoterpenes. While many individual monoterpenes changed due to wounding, the most notable percentage changes involved 23 and 121-fold increases in (−)-β-pinene and almost 10 and 60-fold increases in δ-3-carene in wounded only and wounded plus inoculation samples, respectively (Table 1). Increases in the levels of these two monoterpenes appeared to be mostly at the expense of (−)-limonene and geranyl acetate. The wound-only trees showed percentage decreases of more than half for (−)-limonene and greater than 7-fold for geranyl acetate compared to initial and systemic damage (Suppl. Table 1). The percentages of these two monoterpenes decreased about 5.5-fold and greater than 28-fold, respectively, for the same comparison in damaged plus inoculated trees. Similar relative declines also were seen for both the (+) and (−) isomers of camphene, (+)-α-pinene, (+)-limonene, and β-phellandrene.

Sesquiterpenes Constitutive phloem tissue contained much lower concentrations of total sesquiterpenes than the other terpene classes (Fig. 1b). However, this group responded to wounding plus inoculation by undergoing greater increases relative to the other two terpenoid classes. Total quantities of sesquiterpenes also varied among the six treatments (F = 139.8, $P < 0.001$). In trees that were mechanically wounded only, concentrations at the local wound site were 11.3 times that of constitutive levels. In trees that were subjected to both mechanical wounding and inoculation with the mountain pine beetle fungal symbiont, concentrations at the local wound site were 56.7 times that of constitutive levels. We did not find evidence of systemic induction of total sesquiterpenes in response to either the mechanical wounding or fungal inoculation.

Longifolene dominated the sesquiterpene component, accounting for more than 50 % of this fraction in the constitutive and systemic samples (Table 1, Suppl. Table 1). The levels of longifolene in wounded phloem represented more than 70 % of total sesquiterpenes. We also found λ-murolene, α-murolene, and four unknown sesquiterpenes. The MANOVA showed the sesquiterpene composition changed among the six treatments (Wilks' $\lambda = 0.11$, $F_{35,\ 444} = 9.0$, $P < 0.001$). ANOVA results for individual compounds showed that four of the seven sesquiterpenes differed with treatment, not including α-murolene and unknowns 3 and 4 (Table 1). The *post hoc* analyses for single sesquiterpenes also found relative differences among the treatments similar to the pattern for total sesquiterpenes.

Diterpenes Diterpene resin acids were the most abundant group of terpenes in ponderosa pine phloem (Fig. 1c).

Table 1 Compound concentrations [μg compound g^{-1} d.w.; Mean (± SE)] of mono-, sesqui-, and diterpenes in ponderosa pine phloem from six treatment combinations, including mechanically wounded (Wound only) and mechanically wounded plus inoculation with *Grosmannia clavigera* (Wound + inoculation), both with samples collected just prior to treatment (T_0, Constitutive), at the wound site after 17 days (T_1, Local), and on the opposite side of the tree at 17 d (T_1, Systemic). *F*-ratios and *P*-values from ANOVA tests comparing amounts of each compound among the six treatments. Letters after the concentration values show the Tukey *post hoc* results for each significant compound. Treatments with the same letter did not differ. Boldface *P*-values indicate significance at $\alpha = 0.05$. RT = retention times for mono- and sesquiterpenes on a Cyclodex-B column and diterpenes on a DB-Wax column. See Supplementary Table 1 for compound percentages

Compound	RT (min)	*F*	*P*	Wound only			Wound + inoculation		
				Constitutive	Systemic	Local	Constitutive	Systemic	Local
Monoterpenes									
α-Thujene	17.22	137.1	**<0.001**	5.9 (0.8) c	6.3 (0.9) c	48.0 (4.4) b	6.5 (1.2) c	8.0 (1.5) c	294 (32) a
(−)-α-Pinene	19.09	66.8	**<0.001**	166 (29) c	187 (27) c	951 (119) b	197 (44) c	275 (50) c	4363 (516) a
(+)-α-Pinene	19.41	14.2	**<0.001**	724 (210) c	875 (250) c	3563 (1128) b	552 (145) c	784 (186) c	4145 (701) a
Tricyclene	19.86	9.4	**<0.001**	6.2 (0.9) bc	7.9 (1.1) bc	23.5 (5.2) ab	6.4 (1.8) c	8.8 (2.2) bc	38.9 (5.1) a
(−)-Camphene	20.20	18.7	**<0.001**	10.2 (1.8) c	13.7 (2.4) c	36.9 (5.7) ab	14.9 (4.5) c	20.9 (5.1) c	90.8 (14.5) a
(+)-Camphene	20.40	6.4	**<0.001**	26.3 (20.4) bc	7.9 (2.3) bc	25.2 (8.2) b	6.8 (2.4) c	9.3 (2.5) bc	36.3 (5.6) a
Myrcene	20.83	75.6	**<0.001**	342 (61) c	368 (71) c	2052 (279) ab	379 (71) c	500 (80) c	11,734 (1527) a
α-Terpinene	21.32	114.3	**<0.001**	37.4 (5.6) c	42.4 (7.5) c	265 (30) b	46.5 (10.7) c	50.1 (8.1) c	1672 (172) a
(+)-β-Pinene	22.02	92.0	**<0.001**	5.6 (0.9) c	6.4 (1.3) c	59.9 (10.6) b	5.3 (0.9) c	6.1 (1.1) c	256 (33) a
(−)-β-Pinene	22.13	105.5	**<0.001**	198 (31) c	224 (53) c	4558 (750) b	220 (27) c	221 (44) c	26,574 (3710) a
δ-3-Carene	22.21	145.5	**<0.001**	1140 (174) c	1265 (244) c	11,302 (1134) b	1132 (182) c	1434 (230) c	67,496 (6908) a
(−)-Limonene	23.49	5.4	**<0.001**	1556 (385) b	1516 (284) b	2398 (443) ab	1893 (384) b	2591 (437) ab	7010 (1646) a
(+)-Limonene	23.71	13.4	**<0.001**	91.2 (25.5) b	113 (25) b	183 (37) b	127 (29) b	183 (35) b	517 (55) a
p-Cymene	23.95	80.1	**<0.001**	10.7 (4.1) c	4.8 (0.8) c	42.8 (3.8) b	7.0 (1.2) c	6.1 (1.4) c	93.6 (8.8) a
β-Phellandrene	24.29	12.9	**<0.001**	319 (92) b	386 (106) b	605 (95) b	454 (183) b	708 (198) b	1899 (173) a
γ-Terpinene	25.14	49.7	**<0.001**	7.1 (1.2) c	8.9 (1.7) c	92.1 (12.8) b	7.4 (1.4) c	10.3 (1.6) c	639 (77) a
Terpinolene	26.39	70.5	**<0.001**	147 (31) c	150 (26) c	824 (102) b	146 (29) c	193 (32) c	5022 (614) a
(+)-Linalool	32.17	15.4	**<0.001**	20.0 (8.1) c	25.8 (10.5) c	87.0 (20.5) b	21.6 (7.1) c	23.6 (7.7) c	367 (68) a
(−)-Linalool	32.38	3.7	**<0.001**	6.6 (2.5) b	5.5 (1.4) b	19.9 (7.8) b	6.4 (2.1) b	8.8 (2.6) b	87.3 (20.0) a
Unknown 1	33.82	6.3	**<0.001**	22.4 (6.3) a	24.6 (4.8) a	13.7 (3.4) abc	31.0 (9.2) a	78.5 (42.2) a	13.2 (4.9) c
Bornyl acetate	38.46	5.4	**<0.001**	62.6 (10.8) bc	72.3 (12.4) bc	152 (29) ab	70.8 (23.7) c	98.6 (26.8) bc	179 (29) a
Unknown 2	40.04	62.0	**<0.001**	9.4 (1.0) c	14.2 (2.5) c	119 (16) b	26.1 (10.1) c	27.1 (11.0) c	492 (53) a
Terpinyl acetate	40.51	29.9	**<0.001**	25.9 (4.4) c	27.0 (5.0) c	163 (31) b	45.1 (10.1) c	83.6 (27.1) c	511 (73) a
Geranyl acetate	42.75	1.5	0.191	395 (92)	503 (105)	271 (64)	612 (165)	826 (215)	301 (107)
Sesquiterpenes									
Unknown 1	40.75	50.8	**<0.001**	13.8 (3.8) c	12.1 (3.5) c	91.7 (18.5) b	16.4 (3.7) c	17.8 (4.3) c	302 (36) a
Unknown 2	41.25	33.6	**<0.001**	31.6 (6.4) c	35.6 (7.4) c	171 (25) b	34.8 (7.5) c	38.8 (6.3) c	769 (108) a
Longifolene	41.68	127.8	**<0.001**	143 (19) c	179 (42) c	2556 (342) b	134 (31) c	167 (44) c	11,153 (1262) a
Unknown 3	42.96	1.8	0.121	21.4 (5.4)	26.3 (5.0)	32.8 (9.8)	34.5 (10.0)	40.5 (8.8)	67.2 (12.3)
λ-Murolene	45.75	10.4	**<0.001**	15.3 (4.0) b	18.6 (4.9) b	361 (101) b	19.7 (3.1) b	28.9 (7.0) b	3232 (73) a
α-Murolene	47.05	0.7	0.662	24.7 (13.0)	28.9 (14.3)	66.0 (37.7)	2.7 (1.1)	17.7 (13.6)	62.0 (32.5)
Unknown 4	49.62	1.7	0.149	43.4 (23.0)	34.4 (14.3)	39.6 (26.3)	34.7 (13.4)	67.3 (22.2)	101 (64)
Diterpenes (resin acids)									
Sandaracopimaric	33.95	177.7	**<0.001**	251 (40) c	226 (41) c	4500 (687) a	272 (42) c	271 (65) c	21,780 (2309) b
Pimaric	35.22	177.4	**<0.001**	97.7 (17.2) c	81.3 (10.9) c	956 (115) a	88.8 (10.7) c	101 (16) c	4479 (435) b
Palustric/Levopimaric	37.88	414.7	**<0.001**	626 (74) c	628 (74) c	22,665 (2843) a	709 (82) c	774 (119) c	141,707 (14,632) b
Isopimaric	38.43	177.8	**<0.001**	760 (94) c	711 (104) c	7352 (1087) a	777 (96) c	885 (130) c	37,728 (3580) b
	43.34	123.4	**<0.001**	1447 (254) c	1260 (266) c		1490 (270) c	1576 (461) c	60,690 (5495) b

Table 1 (continued)

Compound	RT (min)	*F*	*P*	Wound only			Wound + inoculation		
				Constitutive	Systemic	Local	Constitutive	Systemic	Local
Dehydroabietic/ Abietic						16,870 (1758) a			
Neoabietic	45.73	313.8	**<0.001**	350 (40) c	375 (58) c	9764 (1167) a	360 (42) c	399 (62) c	53,385 (5499) b
Isocupressic	49.07	2.3	0.050	310 (87)	398 (94)	482 (108)	352 (90)	661 (171)	886 (165)
Acetylisocupressic	57.19	1.4	0.247	3832 (770)	4595 (768)	4945 (899)	5164 (1214)	6670 (1130)	6430 (861)

The total quantities of diterpenes varied among the six treatments (F = 142.4, P < 0.001). In trees that were mechanically wounded only, concentrations at the local wound site rose to 8.8 times that of constitutive levels. In trees that were subjected to combined mechanical wounding and inoculation with the beetle's fungal symbiont, concentrations at the wound site were 35.5 times greater than constitutive levels. As with the other two terpene classes, we did not find evidence of systemic induction of total diterpenes in response to either mechanical wounding or fungal inoculation.

We found ten different diterpenes in the phloem of ponderosa pine, including sandaracopimaric, pimaric, palustric, levopimaric, isopimaric, dehydroabietic, abietic, neoabietic, isocupressic, and acetylisocupressic acids (Table 1, Suppl. Table 1). The MANOVA test revealed compositional differences in diterpenes among the treatments (Wilks' λ = 0.03, $F_{40,\ 456}$ = 13.8, P < 0.001). ANOVA results for individual compounds showed that all diterpenes, except isocupressic and acetylisocupressic acids, differed among the six treatments and had the same *post hoc* pattern as total diterpenes (Table 1). The most abundant diterpenes in constitutive phloem were acetylisocupressic and abietic/dehydroabietic acids, which together accounted for two thirds of the total diterpene fraction. In the induced local samples, the levels of palustric/levopimaric acid increased more than 36-fold for mechanically wounded only and almost 200-fold for mechanically wounded plus inoculation. Wounding also caused increases in amounts of sandaracopimaric and neoabietic acids. These compositional changes that occurred at the point of the wounding treatment did not occur systemically.

Estragole The amounts of the phenylpropanoid estragole varied among the six treatments (F = 48.3, P < 0.001; Fig. 1d). In trees that were mechanically wounded only, concentrations at the treatment site rose to 4.4 times that of constitutive levels. In trees with combined mechanical wounding with fungal inoculation, concentrations at the treatment site were 19.1 times higher than constitutive levels. We did not find evidence of systemic induction of estragole in response to either of the wounding treatments.

MDS Plots on Terpenoid Profiles Monoterpenes and diterpene acids had clear shifts in profile composition with distinct separations between local samples and both constitutive and systemic (Fig. 2a and c), compared to very little separation for these sample types observed for sesquiterpenes (Fig. 2b). Composition of constitutive and systemic tissues exhibited a greater amount of variability among the trees. Compositional variability within a tree was greater with diterpenes than with the monoterpenes. Sesquiterpene wound responses, however, grouped tighter than the constitutive and systemic samples, indicating more similar composition.

Terpenoid Diversity In general monoterpenes showed the highest diversity of the three terpenoid classes (Table 2). Within this group, chemical diversity declined slightly more than 20 % in the local wounded only and almost 33 % in the local wounded plus inoculation samples compared to respective constitutive samples. This, combined with lack of differences in monoterpene diversity between constitutive and systemic samples in both wound types, resulted in a pattern inverse of total monoterpene amounts (Fig. 1a). Overall, sesquiterpene diversity was the lowest of the three classes, and was also reduced in both of the local wounded samples, but they did not differ (Table 2). Sesquiterpene diversity in the local wounded only samples was about a third lower than that of related systemic samples and the constitutive samples were intermediate between these two. Local wounded plus inoculation samples had a sesquiterpene diversity of about 40 % less than constitutive phloem samples in inoculated trees, which did not differ from respective systemic samples. Diterpene diversity was intermediate between the other two groups and showed less pronounced differences among the treatment combinations (Table 2). In both wounding treatments the trend was slightly lower diterpene diversity

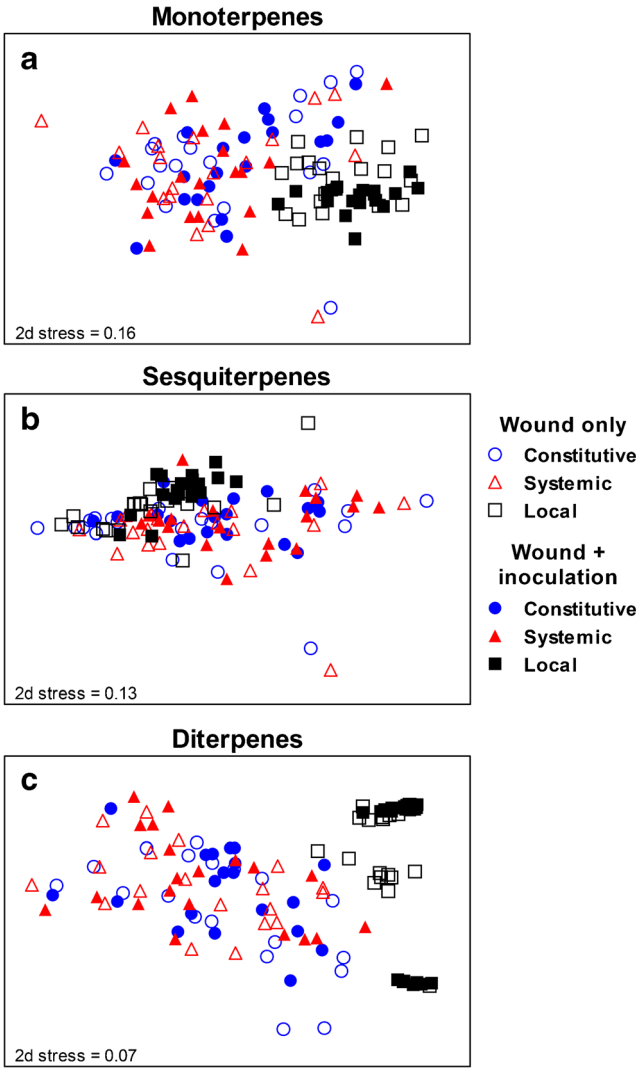

Fig. 2 Nonmetric multidimensional scaling (MDS) plots of relative proportions of monoterpenes (**a**), sesquiterpenes (**b**), and diterpenes (**c**) present in ponderosa pine phloem. Stress values indicate ease of fit of the data in two dimensions, where lower indicates a better fit. MDS plots were computed using Euclidean distances from standardized log(y + 1) transformed data. Samples closer in space are indicative of having a more similar composition

in the systemic samples compared to either constitutive or local samples.

Resin Viscosity Wounded trees, both mechanical only and mechanical plus fungal inoculation, had [(M + S)/D] ratios of about a third less than their respective constitutive and systemic samples (Fig. 3; F = 3.6, P = 0.005). This pattern did not differ with wound type, indicating higher resin viscosity in wounded samples, regardless of inoculation.

Discussion

Ponderosa pines undergo substantial induction of chemical defenses in response to simulated bark beetle attack. This includes large increases in total concentrations of three groups of terpenes, monoterpenes, sesquiterpenes, and diterpenes, as well as pronounced changes in the relative abundances of some individual compounds. Induced reactions were much stronger in response to the combination of mechanical wounding and fungi vectored by bark beetles than to simple mechanical wounding alone. In this regard, induced chemical responses of ponderosa pine resemble those of many other conifer species, which likewise show stronger responses to biotic than physical elicitation (Franceschi et al. 2005; Raffa et al. 2005). Higher concentrations of monoterpenes and diterpenes, such as those observed here, can result in reduced beetle entry, higher toxicity to adults and brood, reduced likelihood of generating attraction by conspecifics, less substantial development among surviving brood, lower fungal spore germination, and lower fungal growth, all of which improve a tree's likelihood of surviving attack and reducing beetle fitness (Erbilgin et al. 2006; Manning and Reid 2013; Raffa et al. 2005; Zhao et al. 2011a). The bioactivity of sesquiterpenes to bark beetle-microbial complexes is not well understood, and merits evaluation.

We did not observe evidence of systemic induction, i.e., increased defensive compounds throughout the tissues

Table 2 Chemical diversity [Mean (± SE)] of mono-, sesqui-, and diterpenes in ponderosa pine phloem among the six treatment combinations. Treatments included, mechanically wounded (Wound only) and mechanically wounded plus inoculation with *Grosmannia clavigera* (Wound + inoculation), both with samples collected just prior to treatment (T_0, Constitutive), at the site treatment after 17 d (T_1, Local), and on the opposite side of the tree at 17 d (T_1, Systemic). F-ratios and P-values from ANOVA tests comparing compound diverstiy of each group among the six treatments. Letters after the diversity values show the Tukey *post hoc* results for each terpene class among the six treatments. Treatments with the same letter did not differ. Boldface P-values indicate significance at α = 0.05

Terpene class	F	P	Wound only			Wound + inoculation		
			Constitutive	Systemic	Local	Constitutive	Systemic	Local
Monoterpenes	21.8	**<0.001**	7.6 (0.2) a	7.5 (0.2) a	6.1 (0.2) b	7.5 (0.2) a	7.3 (0.3) a	5.0 (0.2) c
Sesquiterpenes	10.3	**<0.001**	3.0 (0.3) ab	3.2 (0.2) a	2.3 (0.2) b	3.7 (0.2) a	3.8 (0.2) a	2.3 (0.1) b
Diterpenes	3.8	**0.003**	4.4 (0.3) ab	4.3 (0.3) b	5.3 (0.1) a	4.5 (0.3) ab	4.0 (0.3) b	4.8 (0.1) ab

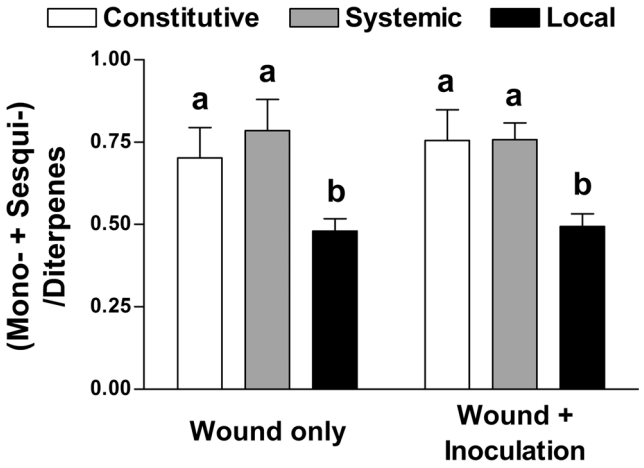

Fig. 3 Mono-plus-sesquiterpene to diterpene ratio [(M + S)/D] in the phloem of ponderosa pine that had either been subjected to a mechanical wound (Wound only) or to a mechanical wound plus inoculation with *Grosmannia clavigera* (Wound + inoculation), both with samples collected just prior to treatment (T_0, Constitutive), at the wound site after 17 d (T_1, Local), and on the opposite side of the tree at 17 d (T_1, Systemic). Lower [(M + S)/D] values indicate higher resin viscosity

colonized by this herbivore. The time course over which we assayed for systemic induction, 17 days, encompasses most of the within-season flight period during which *D. ponderosae* would attack trees. For example, Reid (1962) found that 96 % of beetles fly within a 3 week period, and Logan et al. (1998) reported peak flight occurs within 1–2 weeks. In contrast, there is strong evidence for plant- or tissue-wide induction of defense compounds that directly or indirectly inhibit the attacking agent in a large number of other plant-herbivore systems (Ankala et al. 2013; Ferrieri et al. 2015; Furstenberg-Hagg et al. 2013). In conifers, systemic induction has been demonstrated against the necrogenic canker pathogen *Diplodia pinea* in Austrian pine (*P. nigra*) and the invasive pitch canker *Fusarium circinatum* in Monterrey pine (*P. radiata*) (Eyles et al. 2010). The spatial scale at which we assayed for systemic induction likewise relates to the behavior by which *D. ponderosae* colonizes the entire perimeter of trees. The rapid, pheromone-mediated group attacks by *D. ponderosae* may select for mobilizing all available resources immediately to the point of initial invasion. That is, if the first bark beetles to enter succeed in eliciting mass attacks, trees have little chance of survival. Our results do not eliminate the possibility of diffusion of bioactive compounds or short-distance signaling, which is needed for lesions to proliferate in advance of and confine beetle-fungal complexes (Raffa and Berryman 1983b). They also are consistent with results with other bark beetle-fungal complexes, in which induced formation of traumatic resin ducts and swelling and proliferation of polyphenolic parenchyma cells in spruce occurred within or very near treated areas, and persistent elevated terpene levels induced by methyl jasmonate were localized within treated stem sections (Krokene et al. 2003, 2008).

We found major compositional changes both among and within terpene groups as a result induction. In constitutive tissue, the ratio of the lower molecular weight mono- and sesquiterpenes to the heavier diterpenes averaged about 0.72. During induced responses, however, this ratio declined to about 0.48, indicative of a more viscous resin. This change, among terpene groups with substantially different physical properties, adds further complexity to relationships between resin flow and resin quantity in response to attack. A more viscous resin may delay beetle progress and reduce emission of volatile aggregation pheromones. Any delays in beetle progress, especially during the critical early stages of attack allow induced biochemical reactions more time to reach effective defense levels. Compositional changes within terpene classes yielded differences in overall chemical diversity, especially in the mono- and sesquiterpenes. Within these groups, diversity declined in locally induced samples, compared to constitutive and systemic samples. Large increases in the abundance of a few compounds in induced samples, e.g., the monoterpenes (−)-β-pinene and δ-3-carene and the sesquiterpenes longifolene and λ-murolene, drove these chemical diversity decreases. Others studies have reported variable changes in resin chemistry after biotic challenge. Tomlin et al. (2000) found compositional changes, especially among monoterpenes, in the resin of white spruce subjected to white pine weevil damage. Changes in monoterpene composition also occurred in Norway spruce after treatment with methyl jasmonate (Martin et al. 2002). In contrast, Erbilgin et al. (2006) found increased concentrations, but no differences in the chemical profile of Norway spruce, likewise treated with methyl jasmonate. In another study with ponderosa pine, only very small compositional differences were seen in the terpenes of resin collected over 24 h. between trees that had been mass-attacked vs. not attacked by roundheaded pine beetle (Fischer et al. 2010). Likewise *P. contorta* showed less pronounced compositional changes than *Abies grandis* (Raffa et al. 2005).

Ponderosa pine's induced response has similarities to those of other conifers subjected to biotic stress. For example, lodgepole pine increased production of both mono- and diterpenes after both wounding and inoculation with *G. clavigera* (Croteau et al. 1987). Lodgepole pine reacted to *G. clavigera* inoculation with similar percentage increases in mono- and diterpenes. In addition, white spruce (Tomlin et al. 2000) and Sitka spruce (Miller et al. 2005) displayed resinosis following simulated or actual white pine weevil damage. While all of these species showed induction of terpenes due to physical and/or biotic treatment, the among-terpene-class patterns differed. For instance, like ponderosa pine in our study, Sitka spruce both started with higher amounts of diterpenes in its constitutive resin, and also induced greater proportions of this terpene class (Miller et al. 2005). This likely yielded a more viscous resin. In contrast, although white spruce constitutive resin contained about twice the amount of diterpenes

compared to monoterpenes, simulated weevil damage led to it synthesizing proportionally more of the lower molecular weight monoterpenes (Tomlin et al. 2000).

Several features of ponderosa pine terpene chemistry may contribute to the lower overall exploitation of this host than lodgepole pine by *D. ponderosae*. First, the monoterpene fraction in these ponderosa pines consists of over 24 % limonene, which often is the most toxic, repellent, and fungicidal monoterpene in bioassays (Raffa et al. 2005; Smith 1963). By comparison, limonene typically accounts for less than 1 % of the monoterpenes in lodgepole pine (Boone et al. 2011; Raffa et al. 2012). Second, ponderosa pine is much higher in δ-3-carene, which can inhibit pheromone communication (Borden et al. 2008). is the most inhibitory terpene to beetle-associated bacteria (Adams et al. 2011). and has been associated with reduced incidence of mortality in lodgepole pine by *D. ponderosae* (Ott 2009). Third, ponderosa pine is much lower in β-phellandrene, which *D. ponderosae* exploit as a host-detection kairomone (Jost et al. 2008; Miller and Borden 2000). Fourth, in addition to differences in monoterpenes, ponderosa pine undergoes much higher induction of diterpenes, reaching concentrations of approximately 350 mg g^{-1} 17 days after inoculation with *G. clavigera*, compared to approximately 14 mg g^{-1} 12 days after inoculation with *G. clavigera* in lodgepole pine saplings (Croteau et al. 1987). Diterpenes have not been quantified in the phloem tissues of many North American conifer species, but by comparison, *Pinus resinosa* has only trace quantities in its constitutive phloem, and *Picea alba* contained only 5 mg g^{-1}, in Wisconsin (Mason et al. 2015). In some other regards, however, ponderosa and lodgepole pines are relatively similar, such as in the percent compositions of α-pinene and myrcene, which *D. ponderosae* exploits as kairomones, specifically by enhancing attraction to its aggregation pheromones (Miller and Borden 2000). The thicker bark of ponderosa pine also could pose a more formidable barrier than the relatively thinner bark of lodgepole pine. Further, it is difficult to make direct comparisons between ponderosa and lodgepole pine susceptibility, because they tend to occupy different sites, which might overlap differently with other features of *D. ponderosae* physiology and ecology. In a study of neighboring (~2 km) ponderosa and lodgepole sites, Lerch (2013) found higher attack rates on ponderosa than lodgepole pine over four years, even though background *D. ponderosae* populations were equivalent or higher in the ponderosa pine stands.

Ponderosa pine is a broadly distributed tree, and varies geographically in its terpenoid chemistry, possibly leading to regional differences in defense (Sturgeon and Mitton 1986). The area in this study belongs to the ponderosa pine monoterpene ecotype area designated Region III Cascade Northern by Smith (2000). As in our results, Smith (2000) found that δ-3-carene dominated the monoterpene profile of ponderosa pines in this area. Our trees, however, also had relatively high proportions of limonene [mostly the (−) enatiomer] which he found mostly in the two monoterpene ecotype regions that span the Northwest to southern California. In addition, we detected relatively high amounts of geranly acetate and some terpinyl acetate, two monoterpene acetates seen in ponderosa foliage from Washington state (Adams and Edmunds 1989). With regard to diterpenes, Zinkel and Magee (1991) also reported high levels of isocupressic and acetylisocupressic acids in the needles of ponderosa pine from Montana. We, however, also found relatively high levels of abietic-dehydroabietic acids, leading to diterpene profiles similar to those in ponderosa pine seed cones in Colorado (Keefover-Ring and Linhart 2010).

Future work is needed to further characterize the induced subcortical responses of ponderosa pine. First, whether changes in phenolics also accompany attack is unknown. Second, ponderosa pine shows strong geographic variation in constitutive phloem terpenes (Sturgeon and Mitton 1986). so there likely are genetic and environmentally based differences in inducibility. Third, controlled bioassays on potential effects of sesquiterpenes on bark beetles and their symbionts are needed. Although the responses in sesquiterpenes are dramatic, and some sesquiterpenes are known to have high activity in other, unrelated systems (Prasifka et al. 2015; Zhang et al. 2015). evidence of activities against bark beetles or their symbionts is needed to assign a defensive role in this system. Finally, future studies should evaluate whether the induced chemical changes described here, particularly high increases in diterpenes, may contribute to the much lower mortality of ponderosa than lodgepole pine to *D. ponderosae* (Meddens et al. 2012).

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