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Research article

Volatile and Within-Needle Terpene Changes to Douglas-fir Trees Associated With Douglas-fir Beetle (Coleoptera: Curculionidae) Attack

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Abstract

Mass attack by tree-killing bark beetles (Curculionidae: Scolytinae) brings about large chemical changes in host trees that can have important ecological consequences. For example, mountain pine beetle (*Dendroctonus ponderosae* Hopkins) attack increases emission of terpenes by lodgepole pine (*Pinus contorta* Dougl. ex Loud.), affecting foliage flammability with consequences for wildfires. In this study, we measured chemical changes to Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Mirb.) Franco) foliage in response to attack by Douglas-fir beetles (*Dendroctonus pseudotsugae* Hopkins) as trees die and crowns transitioned from green/healthy, to green-infested (year of attack), to yellow (year after attack), and red (2 yr after attack). We found large differences in volatile and within-needle terpene concentrations among crown classes and variation across a growing season. In general, emissions and concentrations of total and individual terpenes were greater for yellow and red needles than green needles. Douglas-fir beetle attack increased emissions and concentrations of terpene compounds linked to increased tree flammability in other conifer species and compounds known to attract beetles (e.g., α-pinene, camphene, and D-limonene). There was little relationship between air temperature or within-needle concentrations of terpenes and emission of terpenes, suggesting that passive emission of terpenes (e.g., from dead foliage) does not fully explain changes in volatile emissions. The potential physiological causes and ecological consequences of these bark beetle-associated chemical changes are discussed.

Key words: bark beetle, terpene, wildfire, foliage, host attraction

Interactions between bark beetles (Coleoptera: Curculionidae, Scolytinae) and their conifer hosts are regulated by a diverse array of terpene compounds (Raffa and Berryman 1987, Erbilgin and Raffa 2000, Martin et al. 2003, Gershenzon and Dudareva 2007). Terpenes play important roles in tree defenses against bark beetles (Smith 1965, Reid and Gates 1970, Keeling and Bohlmann 2006), and serve as host location cues for beetles (Brattli et al. 1998, Seybold et al. 2000, Gray et al. 2015). Bark beetle mass attack induces large changes to host tree chemistry. This occurs, in part, through beetle feeding triggering tree defenses, severing vascular tissue, and introducing blue-stain fungi that penetrate xylem tissue, effectively blocking water transport (Paine et al. 1997, Lewinsohn et al. 1994). Crowns of beetle-killed trees usually transition through four phases: non-infested (green, G), current year's infestation (green-infested, GI), previous year's infestation (yellow, Y), and 2 yr post infestation (red, R; Amman 1982, Wulder et al. 2006, Jenkins et al. 2008). Over the course of this period, tree chemistry changes and foliar moisture declines as photosynthesis ceases and needles desiccate (Parmeter et al. 1989). For example, large changes in moisture content, and emissions and concentrations of foliar terpenes occur in *Dendroctonus*-attacked lodgepole pine (*Pinus contorta* Douglas) and Engelmann spruce (*Picea engelmannii* Parry ex Engelm.), as needles fade over the course of an outbreak (Page et al. 2012, 2014).

Bark beetle-induced alterations to tree terpenes are particularly important because terpenes released from and within foliage can alter foliage flammability based on their high heat values, low flash points, and low flammability limits (Nuñez-Regueira et al. 2005, Alessio et al. 2008, Jenkins et al. 2014, Pausas et al. 2016). For example, concentrations of the monoterpenes bornyl acetate and limonene in Ashe juniper (*Juniperus ashei* Buchholz) had a significant positive effect on the amount of plant material that burned (Owens et al. 1998). Among six Mediterranean plant species, those with higher terpene concentrations in their foliage had higher flammability, sustainability, ignitability, and combustibility rates (Ormeño et al. 2009). Several terpenes in beetle-attacked lodgepole pine needles, including β-pinene, camphene, tricyclene, D-limonene, and *p*-cymene, were correlated with shortened time to ignition and

lowered temperature at ignition (Page et al. 2012). Bark beetle alteration of foliage terpene quantity and quality may elevate crown fire hazard during the period needles remain on the tree.

In the central Rocky Mountains, interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Mirb.) Franco) is a dominant tree species and the exclusive host for the Douglas-fir beetle (*Dendroctonus pseudotsugae* Hopkins) (Rudinsky 1966, McGregor et al. 1984, Schmitz and Gibson 1996, Negrón et al. 2001). The Douglas-fir forest type occurs predominantly in the middle montane zone between 900 and 1,500 m (Bailey 1980). Bark beetle-associated changes to Douglas-fir tree chemistry and forest fuels have been studied less than upper montane lodgepole pine or spruce/fir forests (Page et al. 2012, 2014; Amin et al. 2013; Berg et al. 2013; Donato et al. 2013). Identifying the compounds produced and how their rates change as needles progress through each crown stage following a Douglas-fir beetle attack will fill these gaps in understanding terpene response of bark beetle-infested trees.

We examined changes in foliage chemistry of Douglas-fir beetle-attacked Douglas-fir trees as needles change from G to GI, Y, and to R focusing on volatile emission rates and within-needle terpene concentrations. We also evaluated the influence of ambient air temperature and within-needle terpene concentrations on volatile terpene emission rates. We hypothesized that GI crowns will contain higher within-needle and volatile terpene concentrations compared with G crowns due to the production of terpene-based, anti-herbivore defenses. We further hypothesized that Y and R crowns will have greater terpene content and emissions than G crowns, as terpene storage vesicles break down and passively release terpenes as foliage dies.

Materials and Methods

Study Site and Stand Characteristics

Study site selection was made using Forest Health Monitoring Aerial Detection Survey maps (ADS), showing recent (<2-yr-old) Douglas-fir beetle outbreaks greater than 10 ha in size on U.S. National Forest System lands. The site chosen for this study is located in the northern portion of the Bear River Range in southeastern Idaho on the Caribou-Targhee National Forest (42° 42′9″ N, 111° 10′36″ W). The 10-ha study site was on a southwest aspect at an elevation of 2,250 ± 25 m and a mean slope of 30%. Overstory vegetation consisted of an open, mixed-aged stand of interior Douglas-fir (100% overstory composition), surrounded by Engelmann spruce and subalpine fir (*Abies lasiocarpa* Hook. Nutt.) intermixed with lodgepole pine on north and east aspects. Understory vegetation was predominantly grasses and chokecherry (*Prunus virginiana* Nutt. Torr.). The climate for this area is characterized by a 30-yr mean maximum July temperature of 25°C and January mean low temperature of −12°C, with total annual precipitation normal of 864 mm (PRISM 2015). Stand characteristics include mean basal area of 41.3 m^2 ha^{-1}, density of 399 stems ha^{-1}, a diameter at breast height (dbh) range of 18.8 to 59.4 cm, and mean tree height of 17.9 m. The Douglas-fir beetle outbreak had been ongoing for 4 yr (2011–2014) prior to the sampling season.

Experimental Design

Four crown condition phases associated with Douglas-fir beetle outbreaks were included in this study as assessed at the start of sampling on 12 June 2014 (Fig. 1). G trees were healthy, non-infested by Douglas-fir beetle, and had live, green foliage. GI trees were those with current year's Douglas-fir beetle attack based on the presence of entrance holes, frass accumulation around the base of the tree, and observations of adult Douglas-fir beetle actively infesting hosts. The majority of needles on GI trees are green, but begin to turn yellow over the season. Y trees were classified as those that were successfully infested the previous year based on presence of Douglas-fir beetle galleries, emergence holes, and foliage that had transitioned to a yellow coloration. R trees were those killed by Douglas-fir beetle evidenced by characteristic Douglas-fir beetle galleries, frass accumulation, exit holes, and red crown foliage.

Six trees of similar heights and dbh and showing no signs of secondary disturbance (e.g., herbivory, root disease, drought stress, frost damage, or fire damage) were selected per crown class. To obtain infested (GI) trees, six healthy trees were each baited using one Douglas-fir beetle w/Seudenol lure from Pherotech (now Contech, Victoria, BC) composed of 0.3 g frontalin, 121 g ethanol, and 0.2 g seudenol, following Ross and Daterman (1997). The Douglas-fir beetle lure was attached 1.8 m above the surface of the forest floor on the north aspect of each prospect GI tree bole for one week during the Douglas-fir beetle flight period (week 1). The mean dbh (±SE) values of trees were: G 46.0 ± 4 cm, GI 39.6 ± 3 cm, Y 49.3 ± 2 cm, and R 47.8 ± 4 cm, and mean heights (± SE) were: G 18.1 ± 1.2 m, GI 15.4 ± 1.8 m, Y 17.3 ± 0.3 m, and R 21.8 ± 1.1 m. Volatile terpene emissions and within-needle terpene concentrations were measured every 2 wk between early June and early October (nine sampling periods total) coinciding with a typical fire season in southern Idaho. All sampling took place between 1000 and 1800 local Mountain Standard Time (MST). Prior to the start of each sampling period, air temperature was measured using a sling psychrometer.

Field Sampling

Volatile emissions were collected from one randomly selected branch (approximately 50 cm in length) less than 1.5 m above the forest floor following methods of Page et al. (2012) and Gray et al. (2015). The same branch on each sample tree was used for all sampling periods to minimize any confounding factors associated with branch condition, aspect, and location. Branches were enclosed in a clear Teflon bag (50 by 75 cm; American Durafilm Co., Holliston, Massachusetts) for 30 min to sample volatiles, and then removed between sample dates. Volatiles were collected by using a portable vacuum pump (SKC AirLite Sampler Model 110–100) pulling air out of the bag through a volatile trap (Volatile Assay Systems, Rensselaer, New York) containing 30 mg of the adsorbent material HayeSep-Q (Restek, Belefonte, Pennsylvania). Air was sampled at a rate of 0.5 l min^{-1}. After the last sampling period (October 4), branches were clipped and needles removed and weighed. To measure within-needle terpene concentrations, 15–20 g of foliage from the lower third of each tree crown were clipped on each sampling date, labeled, placed in plastic bags, and stored in a freezer at −80°C until processed. Samples from G and GI trees included both previous and current year's needles.

Laboratory Analyses

Terpene compounds and emission rates were identified and quantified based on procedures following Runyon et al. (2008) and Page et al. (2012). Volatiles were eluted from traps using 200 µl of dichloromethane, and 1,000 ng of *n*-nonyl-acetate was added as an internal standard. An Agilent 7890A gas chromatograph (GC) coupled with a 5975C mass spectrometer was used to analyze samples, with helium as the carrier gas. Internal temperature of the GC oven was set at 35°C for 3 min, before being increased by 5°C min^{-1} up to 125°C, followed by an increase of 25°C min^{-1} up to 250°C. Quantification of volatile compounds was determined through comparison with the internal standard using ChemStation software

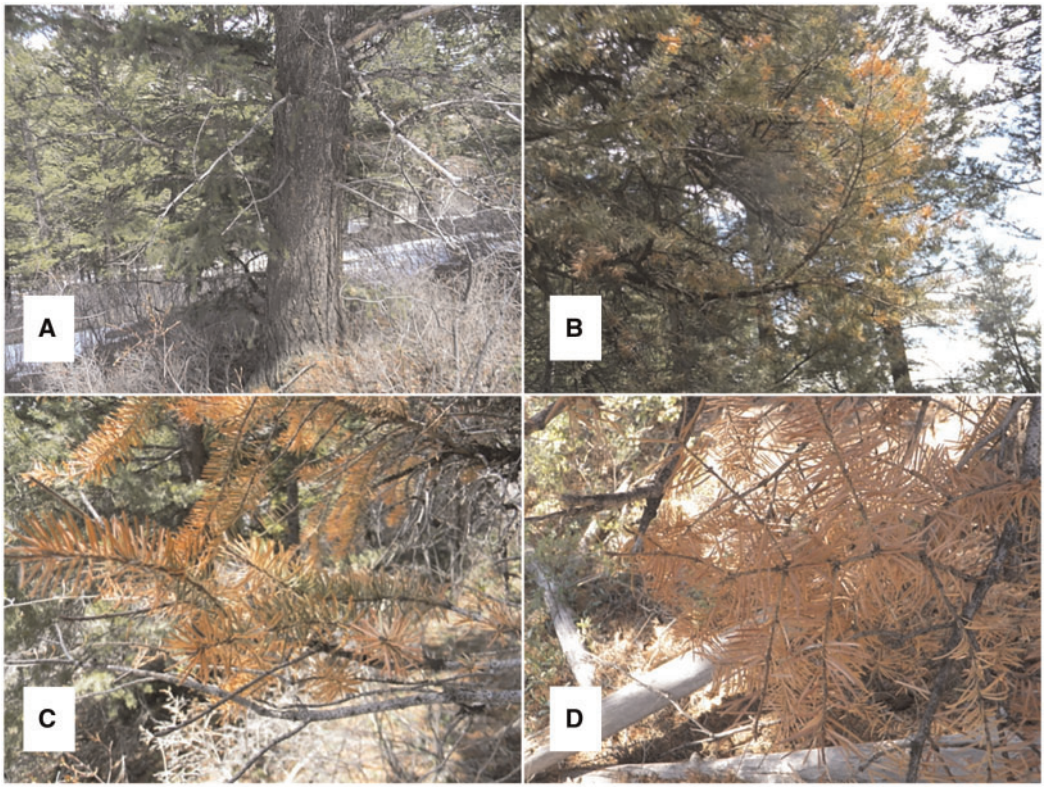

Fig. 1. Four crown condition classes, (A) green (G), (B) green-infested (GI), (C) yellow (Y), and (D) red (R), associated with Douglas-fir beetle (*Dendroctonus pseudotsugae* Hopkins) infestations in interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Mirb.) Franco) forests.

(Agilent Technologies). Volatile emission rates are reported on a fresh needle weight basis (ng h^{-1} g^{-1} fresh weight). Compounds were identified using the NIST 08 Mass Spectral Search Program (National Institute of Standards and Technology, Gaithersburg, MD) and confirmed by comparing retention times and mass spectra with commercial standards, when available. If commercial standards were not available, compounds were named if they matched with a probability greater than 0.5 using the NIST 08 Mass Spectral Search Program and supported by compounds previously reported in the literature to occur in Douglas-fir (Sakai et al. 1967, von Rudolph 1971, Gambliel and Cates 1995). The remaining unidentified compounds were labeled as unidentified monoterpenoids (MT 1, MT 2, etc.) or unidentified sesquiterpenoids (ST 1, ST 2, etc.).

Terpenes were extracted from foliage following Ormeño et al. (2009) and Page et al. (2014). Five grams of needles were randomly pulled from the 15–20 g collected and ground into fine powder in liquid nitrogen using a mortar and pestle. Approximately 0.1 g of powdered needles was transferred into 2-ml FastPrep tubes (MP Biomedicals, Solon, OH), and 1.5 ml of cyclohexane was added and sonicated at room temperature for 20 min. Vials were then centrifuged at 13,000 g for 1 min and 200 µl of cyclohexane (top layer) was transferred to a GC vial for analysis. Compounds were identified based on peak retention times, and within-needle terpene concentrations were quantified following procedures described previously for volatile analyses. Within-needle terpene concentrations are reported on a µg g^{-1} fresh needle weight basis.

Statistical Analyses

Eight compounds were chosen for statistical analyses based on rank abundance and established role in affecting foliage flammability and Douglas-fir beetle host attraction (Furniss and Schmitz 1971; Page et al. 2012, 2014). The terpene compounds included for analyses were α-pinene, β-pinene, β-myrcene, camphene, *E*-β-ocimene, *p*-cymene, D-limonene, and tricyclene. Data were analyzed with a repeated-measures analysis of variance (ANOVA) using the Proc Glimmix procedure in SAS (version 9.4, SAS Institute, Inc. 2014) to identify significant differences in terpene emission rates and within-needle terpene concentrations between crown condition classes and across time. Crown condition class and sampling date were set as fixed effects. Measures from six sample trees per crown class were averaged together to produce one sample mean for each crown class per sampling period. To account for the appropriate covariance structure, the models that minimized Akaike's information criterion (AIC) were selected, which included autoregressive with random effect (AR1 + RE) structure and compound symmetry (heterogeneous) structure. Log and square root transformations were used to meet assumptions of normality and homogeneity of variance. Tests of significant differences between crown condition classes were carried out using a post hoc means comparison approach with the experimental-wise error rate controlled using the Tukey–Kramer method, and performed at $\alpha = 0.05$ level.

Pearson's correlation coefficients (r) were used to identify linear relationships between ambient air temperature and volatile emission rates, as well as any linear relationships between within-needle concentrations and volatile emission rates. A simple linear regression model, $Y_i = \beta_0 + \beta_1 X_1 + \varepsilon_i$, where Y = response variable (volatile emission rate), $_i$ = observation number (tree sampled), β_0 = intercept, β_1 = slope of X_1, X_1 = air temperature, and ε_i = error term for subject $_i$, was performed to test for significant relationships between temperature (°C) and volatile emission rates per crown class for individual terpene compounds. The model $Y_i = \beta_0 + \beta_2 X_2 + \varepsilon_i$, where Y = response

Table 1. Volatile compounds emitted (ng h^{-1} g^{-1} fresh weight) by Douglas-fir trees (*Pseudotsuga menziesii* var. *glauca* (Mirb.) Franco) that are uninfested (G) or infested by Douglas-fir beetle (*Dendroctonus pseudotsugae* Hopkins) in the current year (GI), the previous year (Y), and 2 yr prior (R)

Compound	Green (G)		Infested (GI)		Yellow (Y)		Red (R)		*P*-value
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
α-pinene	**322.13^{a}**	**165.84**	**813.9^{a}**	**482.29**	**1485.4^{b}**	**702.27**	**1097.70^{b}**	**580.34**	**0.0300**
3-carene	**64.01^{a}**	**38.27**	**371.93^{ab}**	**255.09**	**1652.18^{b}**	**818.51**	**834.03^{b}**	**541.82**	**0.0017**
D-limonene	**113.02^{a}**	**56.66**	**280.42^{ab}**	**201.62**	**696.03^{b}**	**364.87**	**599.60^{b}**	**334.57**	**0.0300**
camphene	**147.98^{a}**	**67.64**	**161.98^{a}**	**84.90**	**127.76^{a}**	**46.34**	**304.94^{b}**	**109.74**	**0.0037**
β-pinene	75.28	39.34	139.68	85.86	210.11	96.71	189.31	102.65	0.0978
sabinene	**113.02^{a}**	**56.66**	**280.42^{ab}**	**201.62**	**696.03^{b}**	**364.87**	**599.60^{b}**	**334.57**	**0.0217**
β-myrcene	36.58	18.76	109.55	75.62	110.02	40.82	109.23	52.29	0.0940
α-terpinene	**0.62^{a}**	**0.11**	**0.98^{a}**	**0.33**	**44.23^{a}**	**39.61**	**182.40^{b}**	**180.29**	**<0.0001**
terpinolene	**4.18^{a}**	**2.14**	**24.17^{a}**	**19.21**	**124.16^{b}**	**72.22**	**55.12^{a}**	**39.11**	**0.0144**
bornyl acetate	**36.50^{ab}**	**19.70**	**41.90^{a}**	**25.09**	**27.75^{a}**	**13.72**	**73.47^{b}**	**32.88**	**0.0246**
β-phellandrene	24.35	12.04	44.09	23.44	41.31	17.97	34.40	11.83	0.2752
linalool	12.32	10.08	64.80	46.19	0.80	0.35	0.43	0.27	0.0682
tricyclene	**30.05^{a}**	**14.07**	**32.97^{a}**	**17.29**	**34.40^{a}**	**13.63**	**62.57^{b}**	**23.95**	**0.0078**
verbenone	0.34	0.16	1.30	0.82	1.27	0.80	1.00	0.55	0.4207
E-β-ocimene	1.03	0.72	2.72	2.38	3.71	2.06	15.59	12.19	0.1031
santene	2.22	1.02	9.31	6.15	2.25	0.69	3.37	1.45	0.8048
p-cymene	**2.80^{a}**	**1.11**	**7.43^{b}**	**4.33**	**21.63^{b}**	**10.72**	**21.27^{b}**	**11.46**	**0.0023**
MT1	**0.88^{a}**	**0.28**	**2.71^{ab}**	**1.67**	**13.00^{c}**	**7.01**	**7.59^{b}**	**4.45**	**0.0074**
benzenoid 1	**0.51^{a}**	**0.27**	**1.85^{ab}**	**1.11**	**14.62^{bc}**	**9.38**	**6.3^{c}**	**4.11**	**0.0032**
α-phellandrene	**0.45^{a}**	**0.15**	**0.95^{b}**	**0.50**	**1.45^{b}**	**0.66**	**1.39^{b}**	**0.67**	**0.0096**
β-phellandrene	24.34	12.04	44.09	23.44	41.31	17.97	34.40	11.83	0.2752
Z-β-ocimene	**0.79^{a}**	**0.22**	**1.94^{bc}**	**0.94**	**20.17^{b}**	**12.16**	**11.09^{c}**	**7.30**	**0.0067**
γ-terpinene	**0.91^{a}**	**0.42**	**4.49^{ab}**	**3.35**	**27.65^{bc}**	**17.50**	**12.16^{c}**	**9.11**	**0.0119**
MT3	0.24	0.10	1.16	0.77	2.76	1.93	1.36	0.77	0.1611
p,α-dimethylstyrene	1.10	0.47	4.80	3.22	4.07	2.18	3.55	1.68	0.2337
MT4	**0.25^{a}**	**0.08**	**0.71^{ab}**	**0.43**	**2.35^{b}**	**1.35**	**1.18^{b}**	**0.62**	**0.0389**
MT5	**1.17^{a}**	**0.31**	**2.63^{ab}**	**1.32**	**3.91^{c}**	**1.56**	**3.94^{bc}**	**1.55**	**0.0092**
camphor	3.01	1.10	6.76	3.66	7.90	3.54	6.39	2.89	0.2730
MT6	**0.32^{a}**	**0.17**	**1.33^{ab}**	**0.92**	**3.54^{b}**	**2.28**	**2.35^{b}**	**1.50**	**0.0072**
MT7	**0.56^{a}**	0.26	**1.60^{ab}**	**1.22**	**7.55^{ab}**	**4.62**	**5.04^{b}**	**3.39**	**0.0446**
MT8	**0.50^{a}**	**0.19**	**1.34^{ab}**	**0.80**	**1.65^{b}**	**0.74**	**1.80^{b}**	**0.82**	**0.0288**
borneol	1.79	1.09	6.23	4.32	1.02	0.43	1.18	0.63	0.3399
p-cymen-8-ol	**0.90^{a}**	**0.50**	**3.00^{ab}**	**2.08**	**6.69^{ab}**	**3.84**	**5.83^{b}**	**3.65**	**0.0307**
cis-carveol	**0.17^{a}**	**0.10**	**1.75^{ab}**	**1.42**	**2.86^{b}**	**1.65**	**2.35^{b}**	**1.57**	**0.0522**
meSA	0.45	0.19	1.42	0.90	1.00	0.48	1.19	0.60	0.2481
α-terpineol	0.26	0.39	1.54	2.73	0.05	0.07	0.20	0.24	0.4388
thymol-methyl ether	**1.36^{a}**	**0.54**	**3.33^{ab}**	**1.68**	**6.38^{ab}**	**3.39**	**5.56^{b}**	**2.87**	**0.0524**
MT9	0.44	0.46	9.38	19.67	0.71	0.89	1.79	3.49	0.6531
MT10	**0.28^{a}**	**0.13**	**1.81^{ab}**	**1.16**	**4.21^{b}**	**2.15**	**2.90^{b}**	**1.82**	**0.0103**
ST1	0.24	0.09	0.70	0.40	0.92	0.49	0.74	0.37	0.0999
ST2	0.02	0.01	0.01	0.01	0.03	0.02	0.08	0.06	0.1053
ST3	0.37	0.22	0.12	0.06	0.18	0.10	0.12	0.05	0.5901
ST4	0.34	0.17	0.83	0.50	1.57	1.00	1.52	0.80	0.0961
ST5	**0.52^{a}**	**0.27**	**0.99^{ab}**	**0.52**	**1.71^{b}**	**0.80**	**2.08^{bc}**	**0.90**	**0.0033**
α-farnesene	2.92	1.91	5.47	4.55	5.03	4.65	0.57	0.21	0.1877
MT2	**0.49^{a}**	**0.08**	**0.95^{a}**	**0.38**	**6.99^{b}**	**3.12**	**4.30^{b}**	**2.15**	**<0.0001**
Total volatiles	**910.7^{a}**	**430.21**	**2223.3^{ab}**	**1329.50**	**4997.9^{b}**	**2415.41**	**3819.4^{b}**	**2104.50**	**0.0509**

Amounts are averaged across all sample periods (June-October). MT = unidentified monoterpene, ST = unidentified sesquiterpene. Significant differences are in bold.

Superscript letters indicate test of significance based on Tukey–Kramer pairwise comparisons; different letters indicate significant differences at the $\alpha = 0.05$ level.

variable (volatile emission rate), $_i$ = observation number (tree sampled), β_0 = intercept, β_2 = slope of X_2, X_2 = within-needle terpene concentration, and ε_i = error term for subject $_i$, was performed to test for significant relationships between within-needle terpene concentrations and volatile emission rates. The R statistical software package was used for regression analyses (R Development Core Team 2014).

Results

Volatile Emissions

We identified 46 volatile compounds emitted by the Douglas-fir trees sampled, the majority of which were monoterpenoids (38 compounds; Table 1). These 46 compounds were produced by all trees throughout the study period (June–October) regardless of crown

Table 2. Mean total volatile terpene emission rates (ng h^{-1} g^{-1} fresh weight) and within-needle terpene concentrations (μg g^{-1} fresh weight) with standard errors for each sample period for each crown condition class ($n = 6$).

Date	Green (G)		Infested (GI)		Yellow (Y)		Red (R)	
	Volatiles (ng h^{-1} g^{-1})	Within-needle (μg g^{-1})	Volatiles (ng h^{-1} g^{-1})	Within-needle (μg g^{-1})	Volatiles (ng h^{-1} g^{-1})	Within-needle (μg g^{-1})	Volatiles (ng h^{-1} g^{-1})	Within-needle (μg g^{-1})
6/12[a]	870 ± 417[a]	3781 ± 382[a]	4472 ± 2759[a]	6452 ± 650[bc]	1998 ± 1008[a]	9514 ± 966[b]	2615 ± 1739[a]	17197 ± 1906[c]
6/28	1515 ± 737[a]	5565 ± 858[a]	1472 ± 834[a]	4635 ± 524[a]	4458 ± 2343[a]	6984 ± 1423[a]	2048 ± 724[a]	14191 ± 1717[b]
7/14	1695 ± 797[a]	6287 ± 689[a]	5881 ± 3685[a]	4390 ± 503[a]	28112 ± 14285[a]	12613 ± 595[b]	17835 ± 7415[a]	18699 ± 933[c]
7/30	571 ± 226[a]	8052 ± 637[a]	2480 ± 1094[ab]	7486 ± 783[a]	4466 ± 2402[ab]	12698 ± 2079[b]	1257 ± 440[b]	19005 ± 1745[c]
8/7	284 ± 59[a]	2000 ± 329[a]	462 ± 189[a]	2831 ± 497[a]	536 ± 221[a]	8738 ± 974[b]	725 ± 214[a]	15690 ± 1919[c]
8/31	557 ± 251[ab]	7250 ± 832[a]	229 ± 68[a]	2885 ± 481[b]	1590 ± 432[b]	12785 ± 1009[c]	736 ± 214[ab]	15337 ± 742[c]
9/16	842 ± 268[a]	7695 ± 1184[a]	724 ± 243[a]	8309 ± 521[a]	611 ± 281[a]	14911 ± 1045[b]	1066 ± 547[a]	17283 ± 1988[b]
10/2	948 ± 684[a]	8948 ± 697[a]	2124 ± 1782[a]	9778 ± 1112[a]	1086 ± 163[a]	13313 ± 887[b]	882 ± 157[a]	18798 ± 1087[c]

Different lower case letters (a, b, c) indicate significant differences within a sampling date between crown classes ($\alpha = 0.05$).

[a] Douglas-fir beetle flight period.

phase (Table 1). Volatile emissions varied greatly among crown phases and among sampling periods for total amounts and individual compounds (Table 2, Fig. 2). Differences in total volatiles emitted among crown phases were marginally significant at $P = 0.051$ when averaged across all sampling periods (Table 1). Trees in the Y crown phase emitted the most total volatiles, 40% more on average than G trees. Emissions differed among crown classes for 24 of the 46 volatile compounds when averaged across all sample periods (Table 1). The most abundant compounds tended to be emitted in greater quantities by Y and/or R foliage than G and/or GI foliage (Table 1, Fig. 3). For example, D-limonene was emitted in greater amounts by R than G trees ($t = -2.95$; df = 3, 20; $P = 0.04$). Emissions of camphene were higher in R than G ($t = -2.98$; df = 3, 20; $P = 0.03$), GI ($t = -1.24$, df = 3, 20; $P = 0.003$), and Y trees ($t = 0.94$; df = 3, 20; $P = 0.03$), while emission of *p*-cymene was higher in Y compared with G trees ($t = -1.03$; df = 3, 20; $P = 0.04$). In addition, 3-carene had higher emissions in R than G trees ($t = -3.17$; df = 3, 20; $P = 0.02$) and Y than G trees ($t = -4.52$; df = 3, 20; $P = 0.001$). Tricyclene emission peaked in GI foliage (100.10 ng h^{-1} g^{-1} fresh weight) following the Douglas-fir beetle flight period (June 12). From early July to early October, Y foliage produced significantly ($F = 3.08$; df = 3, 20; $P = <0.0001$) greater emissions of tricyclene compared with G, GI, and R crowns. When averaged across all sample periods, tricyclene showed a pattern similar to camphene, with emissions higher from R than G ($t = -2.96$; df = 3, 20; $P = 0.04$) or GI ($t = -1.15$; df = 3, 20; $P = 0.006$) trees. For total and individual compounds, α-pinene, β-myrcene, camphene, tricyclene, *p*-cymene, β-pinene, *E*-β-ocimene, and D-limonene, significant emission increases were detected in Y and R trees on the July 14 sampling date (Table 2; Fig. 2).

The relationship between temperature and volatile emission rates and the effect of within-needle terpene concentrations on volatile emission rates were generally found to be weak among crown classes for total and individual compounds. Emissions of only one compound in G, three in GI, one in Y, and 12 in R trees were positively correlated with temperature (out of 46 total volatile compounds). Only two of these compounds have been linked in another study to increased flammability: camphene ($t = 2.29$; df = 2, 46; $P = 0.02$) and *p*-cymene ($t = 2.00$; df = 2, 46; $P = 0.05$) in R trees (Page et al. 2012). Increased emissions of only a few compounds were positively correlated with within-needle concentrations: five in G, one in GI, one in Y, and four in R trees. Of these, only camphene ($t = 2.83$; df = 2, 46; $P = 0.007$) in R trees has been implicated in enhancing tree flammability in other studies (Page et al. 2012, Pausas et al. 2016). Emission of no compounds was negatively correlated with temperature or within-needle concentration.

Within-Needle Terpene Concentrations

Results for within-needle terpene concentrations generally mirrored those found with volatile emissions. In total, 85 compounds, including the 46 identified in volatile emissions, were found within needles, the majority of which were monoterpenoids (45 compounds) and sesquiterpenoids (36 compounds). Mean total within-needle terpene concentrations averaged across all sample periods were significantly higher in R compared with G foliage ($t = -9.87$; df = 3, 20; $P = <0.0001$), GI foliage ($t = -10.39$; df = 3, 20; $P = <0.0001$), and Y foliage ($t = 3.85$; df = 3, 20; $P = <0.005$); Y foliage also contained more terpenes than G foliage ($t = -6.01$; df = 3, 20; $P = <0.0001$) and GI foliage ($t = -6.54$; df = 3, 20; $P = <0.0001$; Fig. 4). Over the course of the season, total within-needle terpene concentrations varied considerably both within crown classes and between crown classes (Table 2). The concentration of mean total within-needle terpenes in Y foliage increased over the season (Table 2).

Measures of within-needle concentrations of individual terpene compounds also varied greatly between crown classes (Fig 5). Concentrations of tricyclene were significantly higher in R compared with G foliage ($t = -12.21$; df = 3, 20; $P = <0.0001$), GI foliage ($t = -12.13$; df = 3, 20; $P = <0.0001$), and Y foliage ($t = 4.01$; df = 3, 20; $P = 0.0007$). Similar results were found for α-pinene, whose concentrations were significantly higher in R than G foliage ($t = -10.97$; df = 3, 20; $P = <0.0001$), GI foliage ($t = 3.51$; df = 3, 20; $P = 0.001$), and Y foliage ($t = 3.51$; df = 3, 20; $P = 0.01$). Y foliage also contained more terpenoids than G foliage ($t = -7.05$; df = 3, 20; $P = <0.0001$). There was a 65% increase in the mean amount of D-limonene in Y compared with G foliage when averaged across all sample periods (Fig. 5). Y foliage had the highest concentrations of camphene, D-limonene, tricyclene, and *p*-cymene compared with other crown classes.

Discussion

We found that Douglas-fir trees attacked and killed by Douglas-fir beetle undergo large chemical changes as needles transition from green (G) to green-infested (GI) to yellow (Y), and finally to red (R). Total and individual volatile emission rates changed greatly both within and among crown classes over the course of the sampling period (from June–October). Mean total volatile emission rates tended

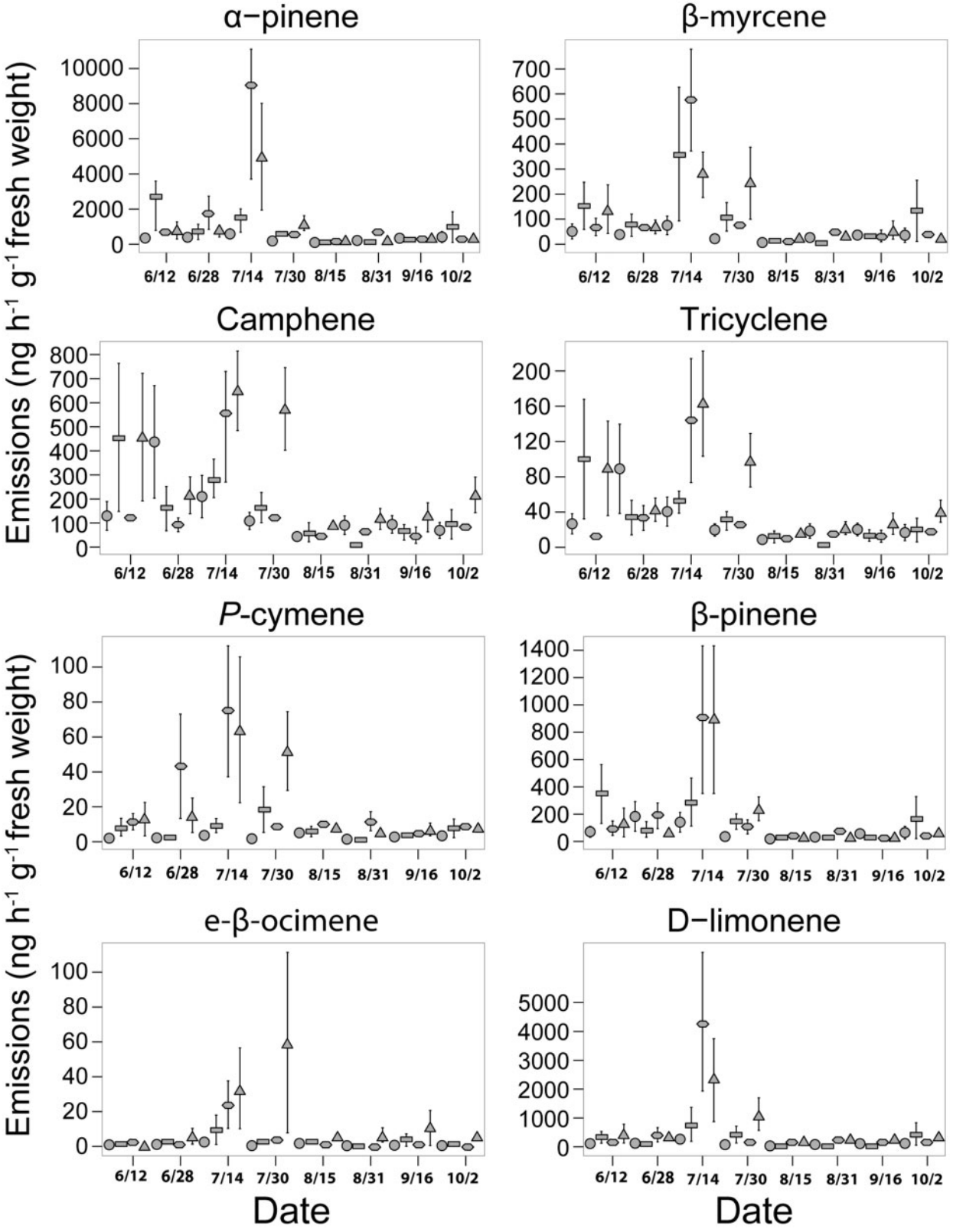

Fig. 2. Mean volatile emission rates (ng h^{-1} g^{-1} fresh weight) with associated standard error bars for α-pinene, β-myrcene, camphene, tricyclene, *p*-cymene, *E*-β-ocimene, β-pinene, and D-limonene per sampling period for each crown class. Symbols denote crown class as follows: G = circle, GI = square, Y = diamond, R = triangle, ($n = 6$). Note: Different Y-axis scales.

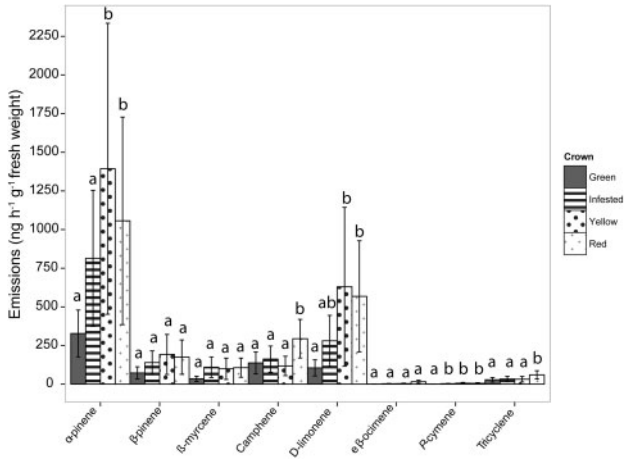

Fig. 3. Mean volatile emission rates (ng h^{-1} g^{-1} fresh weight) of select individual volatile compounds with associated standard error bars per crown class averaged over all sampling periods. Note: Different lowercase letters indicate significant differences between crown classes ($\alpha = 0.05$).

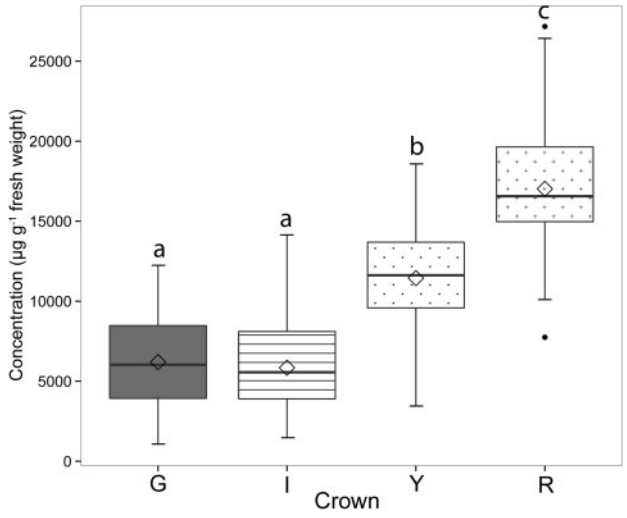

Fig. 4. Box and whisker plots of mean total within-needle terpene concentrations (μg g^{-1} fresh weight) measured over all sampling periods. The median, 25th percentile, and 75th percentiles are denoted by the center line and box edges, and the 10th and 90th percentiles by the whiskers. The mean is denoted by the diamond symbol and filled circles represent outliers. Note: Different lowercase letters indicate significant differences between crown classes ($\alpha = 0.05$).

to be higher in Y and R crowns than G crowns when averaged across all sample periods. Within-needle terpene content followed a similar pattern with the concentrations of many compounds (e.g., α-pinene, β-myrcene, camphene, D-limonene, *p*-cymene, and tricyclene) higher within Y and R foliage than G or GI foliage.

What causes these chemical changes to Douglas-fir crowns when attacked by Douglas-fir beetle? We hypothesized that terpene production induced by beetle attack would lead to an increase in terpene content of needles. However, we found little evidence of increased emission or content of terpenes in needles of attacked trees. Bark beetle attack is known to induce extensive terpene and resin defenses in conifer boles (Franceschi et al. 2005). Our findings indicate that these defenses may not be systemic and are not expressed in needles, but rather targeted to the site of beetle attack

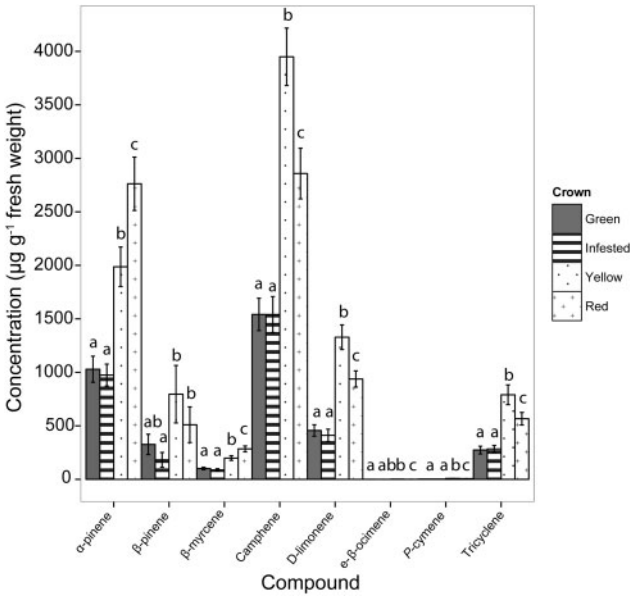

Fig. 5. Mean individual within-needle terpene concentrations (μg g^{-1} fresh weight) with associated standard error bars per crown class measured over all sampling periods. Note: Different lowercase letters indicate significant differences between crown classes ($\alpha = 0.05$).

(the bole). It is unknown whether the post-mortem chemical changes we observed are specific to Douglas-fir beetle-killed trees, or whether similar changes occur in all trees as they die.

Greater emissions from Y and R needles could be due to passive emission of terpenes as foliage dies and plant cell walls break down. If passive emission is occurring, then higher temperatures should increase emissions because ambient air temperature has been shown to have strong positive influence over plant emission rates, with some estimates indicating a two- to threefold increase in monoterpene emissions for every 10°C temperature increase (Schuh et al. 1997, Constable et al. 2001, Gouinguené and Turlings 2002, Niinemets et al. 2004). Temperatures varied throughout the study period from a maximum high temperature of 23°C on 14 July to a minimum temperature of 7°C on 2 October. Notably, peak volatile emissions in all crown types occurred on the warmest sampling day (14 July), suggesting a temperature effect. However, linear regression analysis showed little relationship between temperature and individual terpene emission rates (emission of only 9% of compounds was affected by temperature). Increased temperature was positively correlated most often in R (dead) crowns (26%), suggesting that passive emission plays a role for these compounds in this crown class. The mechanisms underlying the effect of temperature on volatile terpene emission rates are not fully understood (Kesselmeier and Staudt 1999, Penuelas and Llusia 2001) and other abiotic factors could influence volatile emissions from dead foliage, including the amount of irradiance (Yokouchi and Ambe 1984, Langenheim 1994, Bertin et al. 1997) and water availability (Penuelas and Llusia 2001).

Within-needle terpene concentration is another factor that could affect terpene emissions by Douglas-fir trees. A positive relationship between terpene concentration in plant tissues and emissions has been shown for several plant species (Lerdau 1991, Tingey et al. 1991, Lerdau et al. 1995). However, we found very low correlation between concentration and emission, with less than 6% of compounds seemingly affected. Plants have great control over the identity and amount of volatiles released into the surrounding

atmosphere (Widhalm et al. 2015), and these results indicate that at least some barriers to passive emission (e.g., cell walls, cuticle) remain intact in beetle-killed trees and prevent simple concentration-dependent diffusion. Needle age may have also affected the concentration of terpenes stored in needles (Kupcinskiene et al. 2008); however, we did not distinguish between previous and current year's needles in samples from G and GI trees.

One factor certainly affecting concentrations of terpenes in Douglas-fir beetle-killed Douglas-fir crowns is moisture content. Attack by *Dendroctonus* species in other conifer species is known to lead to large reductions in foliar moisture, especially in the Y and R stages (Jolly et al. 2012, Page et al. 2012, 2014, Jenkins et al. 2014). The overall mass of needles declines due to water loss between the G and R crown phase, translating into an increase in the proportion of terpenes concentrated in the needle mass. Although terpenes generally have low water solubility (Weidenhamer et al. 1993), terpene emission may increase if transported to the needle surface in water evaporating from Y and R needles.

The changes to Douglas-fir terpene content found in Douglas-fir beetle-attacked trees could potentially have important consequences for wildfire behavior. Several affected compounds identified in this study have been associated with increased flammability of foliage in other conifer species (Owens et al. 1998, Page et al. 2012). In mountain pine beetle-attacked lodgepole pine, for example, the monoterpene tricyclene shortened time to ignition (Page et al. 2012). This compound increased in Douglas-fir trees attacked by Douglas-fir beetle. Page et al. (2012) also found that total volatile terpene emissions and emission of nine individual monoterpenes were positively correlated with a key measure of burning rate (maximum rate of mass loss). We found that emission of four of these compounds, α-pinene and *p*-cymene in Y and R trees and camphene and tricyclene in R trees, increased in Douglas-fir beetle-attacked trees (Table 1; Fig. 3). Douglas-fir beetle attack also altered within-needle concentrations of flammable terpenes, including camphene, tricyclene, and β-myrcene in Y and/or R trees. These results suggest that Douglas-fir beetle-infested stands with many Y and R crowns emitting flammable terpenes may have a higher potential of surface fire transition to canopy fuels compared with stands comprised predominantly of G healthy trees. After the needles fall to the ground (typically about 4 yr after attack), crown fire hazard is expected to decrease due to the loss of canopy biomass (Hicke et al. 2012, Black et al. 2013). It should be noted that foliar moisture content, canopy bulk density, canopy base height, and presence or absence of ladder fuels are also critical factors that determine crown fire initiation and spread (Van Wagner 1977, Call and Albini 1997, Scott and Reinhardt 2001, Alexander 2010). However, increased relative content of flammable terpenes in a Douglas-fir beetle-affected stand could influence crown fire hazard when fire weather is conducive to crown fire initiation and spread.

Changes to Douglas-fir terpene emissions associated with Douglas-fir beetle attack could affect bark beetles attack behavior, as beetles have been shown to utilize volatiles from tree crowns to locate and choose suitable trees to attack (Gray et al. 2015). For example, α-pinene is a kairomone important in bark beetle host attraction (Heikkenen et al. 1965, Schroeder 1988, Miller and Rabaglia 2009) and is a known synergist for the Douglas-fir beetle attraction pheromone, frontalin (Furniss and Schmitz 1971). Emissions of α-pinene were greater in Y and R crowns and could prolong an outbreak by supplementing chemical signals in host-seeking beetles (Negrón et al. 2001, Furniss 2014). Camphene and D-limonene were also emitted in greater amounts after attack and are known to attract bark beetles (Rudinsky 1966).

Through this study, we identified the complex of terpene compounds that are present in interior Douglas-fir foliage following a Douglas-fir beetle infestation. We were able to demonstrate the rate of terpene emission and the amount of within-needle terpenes increase as crown foliage fades from the G to R phase during and after Douglas-fir beetle outbreaks. These chemical changes could have broad ecological consequences by affecting wildfires and bark beetle behavior. Understanding how bark beetles affect tree chemistry, especially compounds enhancing flammability, is important, given that global change is leading to increased beetle outbreaks (Bentz et at. 2010) and more frequent and larger wildfires (Jolly et al. 2015).

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