



# Drought-Mediated Changes in Tree Physiological Processes Weaken Tree Defenses to Bark Beetle Attack

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## Abstract

Interactions between water stress and induced defenses and their role in tree mortality due to bark beetles are poorly understood. We performed a factorial experiment on 48 mature ponderosa pines (*Pinus ponderosa*) in northern Arizona over three years that manipulated a) tree water stress by cutting roots and removing snow; b) bark beetle attacks by using pheromone lures; and c) phloem exposure to biota vectored by bark beetles by inoculating with dead beetles. Tree responses included resin flow from stem wounds, phloem composition of mono- and sesqui-terpenes, xylem water potential, leaf gas exchange, and survival. Phloem contained 21 mono- and sesqui-terpenes, which were dominated by (+)- $\alpha$ -pinene, (-)-limonene, and  $\delta$ -3-carene. Bark beetle attacks (mostly *Dendroctonus brevicomis*) and biota carried by beetles induced a general increase in concentration of phloem mono- and sesqui-terpenes, whereas water stress did not. Bark beetle attacks induced an increase in resin flow for unstressed trees but not water-stressed trees. Mortality was highest for beetle-attacked water-stressed trees. Death of beetle-attacked trees was preceded by low resin flow, symptoms of water stress (low xylem water potential, leaf gas exchange), and an ephemeral increase in concentrations of mono- and sesqui-terpenes compared to surviving trees. These results show a) that ponderosa pine can undergo induction of both resin flow and phloem terpenes in response to bark beetle attack, and that the former is more constrained by water stress; b) experimental evidence that water stress predisposes ponderosa pines to mortality from bark beetles.

**Keywords** *Dendroctonus* · Drought · *Pinus ponderosa* · Terpenes · Tree chemical defense · Induced defenses · Tree mortality

## Introduction

Lethal bark beetle attacks on coniferous trees often occur during drought. In western North America, for example, bark

beetle (Coleoptera: Curculionidae, Scolytinae) populations often transition from endemic to epidemic during drought, resulting in landscape-scale tree mortality (Raffa et al. 2008). Projected increases in drought frequency and intensity with climate warming in many forests suggest bark beetles will have increasing opportunities to attack water-stressed conifers in the future (Bentz et al. 2010; Fettig et al. 2013; Raffa et al. 2015). Yet, the connection between drought and bark beetle colonization success is based primarily on non-controlled field observations (Dobbertin et al. 2007; Kolb et al. 2016). Strong evidence from manipulative experiments for a role of drought in bark beetle attack success on mature trees is scarce, in part due to the challenges of experimentally controlling both water stress and bark beetle attacks on mature trees. Likewise, understanding of mechanisms by which drought alters bark beetle attack success, such as compromised tree defenses, remains largely hypothetical (Hermes and Mattson 1992; Mattson and Hack 1987) due to the lack of experiments on mature trees that are preferentially attacked by most tree-killing bark beetles under field conditions. Experiments on seedlings can provide insight on response mechanisms (e.g., Lusebrink et al. 2011; Turtola et al.

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2003), but may not necessarily reflect responses of mature trees given the substantial ontogenetic changes that occur in trees (Erbilgin and Colgan 2012; Fredericksen et al. 1996; Schoettle 1994; Yoder et al. 1994).

Conifer defenses against bark beetles are multifaceted and temporally dynamic (Franceschi et al. 2005; Lieutier 2004). Defenses include: a) quantitative physical defenses, such as the amount of resin flow from bark wounds (e.g., Strom et al. 2002); b) qualitative and quantitative chemical defenses, such as the composition and concentration of resin constituents, such as monoterpenes, sesquiterpenes, diterpenes, and phenolics (Raffa et al. 2017); and c) anatomical, such as the frequency and size of structures or tissues that produce and transport defensive chemicals, such as resin ducts (Hood and Sala 2015; Kane and Kolb 2010). These defense components can be a) constitutive, i.e., the amount of quantitative or qualitative defenses in tree tissues before bark beetle attack (Nebeker et al. 1992), or b) induced, i.e., changes in quantitative or qualitative defenses in response to bark beetle attack, including microorganisms vectored by beetles, such as fungi (Arango-Velez et al. 2018; Klepzig et al. 1995; Roth et al. 2018). Conifer anatomical defense against bark beetles can be uncoupled from chemical constitutive and induced terpenoid and phenolic defenses (Mason et al. 2018). Various combinations of these defense mechanisms have been linked with tree resistance to bark beetle attacks in several study systems (Erbilgin 2019; Franceschi et al. 2005; Krokene 2015; Lieutier 2004; Raffa et al. 2017).

Stress and physical damage to conifers often alters tree capacity for induced defense. Stresses or disturbances shown to alter induced quantitative and qualitative conifer defenses include drought or water stress (Arango-Velez et al. 2016; Erbilgin et al. 2017; Hodges and Lorio 1975; Lorio et al. 1995), prior root and lower-stem colonization by insects and fungi (Boone et al. 2011; Keefover-Ring et al. 2016; Klepzig et al. 1995), canopy and bole burning (Powell et al. 2012; Wallin et al. 2003), insect defoliation (Wallin and Raffa 1999; Wright et al. 1979), and low light (Klepzig et al. 1995). The effectiveness of both constitutive and induced, and both physical and chemical, defenses depends on bark beetle population dynamics, with diminishing protection as beetle populations increase to epidemic levels that overwhelm all defenses (Boone et al. 2011; Raffa and Berryman 1982).

Few studies have investigated effects of water stress on defenses of mature conifer trees against bark beetles using manipulative experiments. Most studies on trees larger than seedlings focused on loblolly pine (*Pinus taeda*) defense against southern pine beetle (*Dendroctonus frontalis*). Consistent with the growth-differentiation hypothesis (Herms and Mattson 1992; Mattson and Haack 1987), mature loblolly pine exhibited a non-linear response of quantitative resin defense to experimental drought; resin flow increased in response to moderate drought, and then decreased when

drought was severe (Dunn and Lorio 1993; Lombardero et al. 2000; Lorio et al. 1995; Reeve et al. 1995). Drought also altered the chemical composition of resin in pole-size and mature loblolly pine (Gilmore 1977; Hodges and Lorio 1975). More recent studies with mature trees of other pine species indicate a role of drought in weakening tree defenses. For example, experimental drought increased lethal attacks by *Ips confusus* on pinyon pine (*Pinus edulis*), which were associated with reductions in xylem resin duct size (Gaylord et al. 2013). Experimental drought on mature Norway spruce (*Picea abies*) increased attack success of *Ips typographus* via unresolved mechanisms (Matthews et al. 2018; Netherer et al. 2015). Additionally, experimental drought weakened the induced chemical defense of mature lodgepole pine (*Pinus contorta*) to the bark-beetle vectored fungus *Grosmannia clavigera* for several monoterpenes (e.g., myrcene,  $\delta$ -3-carene,  $\alpha$ -pinene), whereas there was little effect on jack pine (*Pinus banksiana*) (Arango-Velez et al. 2016; Erbilgin et al. 2017). These scattered and contrasting results underscore that understanding of drought impacts on tree defense against bark beetles is incomplete.

Our study focuses on the southwestern variety of ponderosa pine (*Pinus ponderosa* var. *scopulorum*), which is one of several western North American pines that have experienced a recent pulse of tree mortality attributed to bark beetle attacks during severe drought (Hicke et al. 2016). The taxonomy of this variety is under debate (Willyard et al. 2017), but here we use the traditional scientific moniker *P. ponderosa* var. *scopulorum*. The southwestern US is characterized by a regular dry period in late spring and early summer, and high interannual variation in precipitation leading to severe drought in some years (Sheppard et al. 2002). The frequency of severe drought in the region is projected to increase in the future due to warming (Seager and Vecchi 2010).

Drought increases water stress and reduces carbon assimilation of ponderosa pine in the southwestern US (Gaylord et al. 2007; Skov et al. 2004), but effects on tree defense mechanisms against bark beetles are poorly understood. Investigations on effects of water stress on ponderosa pine defense in this region are limited to non-experimental observations of constitutive resin flow over wet and dry periods (Gaylord et al. 2007), and comparisons among forest stands differing in water stress due to experimental manipulation of tree density. These studies have produced mixed results, with evidence for both a negative effect (Feeney et al. 1998; Kolb et al. 1998; McDowell et al. 2007) and little effect (Gaylord et al. 2007; Zausen et al. 2005) of water stress on constitutive resin flow. Controls over induced defenses of ponderosa pine also are poorly understood. Ponderosa pine in Arizona had little induction of resin flow in response to physical bole wounding and exposure to bark-beetle vectored fungi (Gaylord et al. 2011). Ponderosa pine in Montana rapidly increased total phloem concentrations of monoterpenes,

sesquiterpenes, and diterpenes, as well as the relative proportions of certain specific terpenoids, in response to exposure to bark-beetle vectored fungi (Keefover-Ring et al. 2016). No previous studies, to our knowledge, have investigated direct impacts of water stress on any type of defense using manipulative experiments on mature ponderosa pines.

Here we describe an experiment that manipulated water stress, bark beetle attacks, and exposure to bark-beetle vectored biota (e.g., fungi) in mature southwestern ponderosa pines under field conditions. Our overall goal was to understand the role of drought in tree susceptibility to bark beetle attacks, and to elucidate drought impacts on mechanisms of tree defense against bark beetles. We test the following hypotheses: H<sub>1</sub>, beetle attacks and inoculation with beetle-vectored biota induce quantitative and qualitative/chemical resin defenses; H<sub>2</sub>, water stress constrains the induction of tree defenses.

## Methods and Materials

**Study Site** We conducted the study in a naturally regenerated ponderosa pine-dominated stand at the Northern Arizona University's Centennial Forest (35° 11' 9.65" N, 111° 45' 38.25" W; elevation 2250 m). Soils are primarily shallow lithic eutroboralfs, clayey-skeletal, on a bedrock of benmoreite (Miller et al. 1995).

Drought occurred during most of the study period (2013–2015). Based on the Palmer Drought Severity Index for the study region (Western Regional Climate Center; <https://wrcc.dri.edu>), drought in 2013 was severe to extreme in winter, spring, and summer, and moderate in fall. In 2014, drought was severe for late winter, and extreme in spring, summer, and fall. In 2015, drought was moderate to extreme in winter and spring, but normal to moderate in summer and fall. We measured additional climatic conditions at the study site with a weather station (Campbell Scientific CR-10X datalogger; Campbell Scientific HMP45C humidity sensor; Licor LI-190 PAR sensor). For daylight hours of May through September of 2013 to 2015, temperature averaged 22.9 °C, air humidity averaged 36%, photosynthetically active radiation averaged 1476  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and vapor pressure deficit averaged 1.9 kPa.

**Experimental Design.** We used a full factorial block design with two levels of tree stress, produced by cutting tree roots beneath the drip line (trenched, untrenched), crossed with three bark beetle treatments 1) induced bark beetle attacks ("attacked"), 2) inoculations with dead beetles ("inoculated"), and 3) a control, which lacked beetle attacks and inoculations. We applied the treatments to individual trees in a random block design with eight blocks ( $N=48$  trees;  $n=8$  trees per treatment combination). Each block had six trees and was approximately 1300 m<sup>2</sup> in area. We selected trees for the study

based on a moderate height (< 12 m) that allowed access to the canopy with ladders, diameter at breast height of at least 20 cm, and no signs of recent mechanical injury or insect attacks. Based on ring counts from increment cores, the trees used in the study were approximately 60 years old. Tree height averaged  $8.0 \pm 0.12$  m, ranged between 5.2 to 11.5 m, and was similar among treatments ( $P=0.464$ ). Diameter at breast height averaged  $24.2 \pm 0.46$  cm, ranged between 20.0 to 31.9 cm, and was similar among treatments ( $P=0.133$ ).

**Trenching Treatment** On May 20–21, 2013, we cut surface roots of 24 trees using methods modified from Devine and Harrington (2008). We cut trenches beneath the drip line of each tree to a depth of 0.33 m with a "Ditch Witch" trencher (The Charles Machine Works Inc. Perry, OK USA). We lined trenches with 6 mm flexible polyethylene sheeting (TRM Manufacturing Corona, CA USA) to restrict root regrowth, and then backfilled with soil. Trenching has been used to induce water stress in ponderosa pine in previous studies (McCullough and Wagner 1987a; McCullough and Wagner 1987b). Additionally, since winter precipitation is the dominant source of water for mature ponderosa pine in northern Arizona (Kerhoulas et al. 2013), we removed snow within drip lines of trenched trees by shoveling after every major snowfall during the winter of 2013.

**Bark Beetle Treatments** The first bark beetle treatment was initiated in June of 2014, over a year after the trenching treatments were initiated, and consisted of beetle attacks on trees induced by *Dendroctonus brevicomis* pheromone lures (western pine beetle lures adapted for Arizona: #3151, Synergy Semiochemicals Corp. Burnaby, BC CAN; (+)- $\alpha$ -pinene, *exo*-brevicommin, frontalin). Initiating beetle attacks with aggregation pheromones bypasses the process by which beetles select trees and allows direct investigation of the tree defense capability. Beginning June 2, 2014, we attached lures at a height of 1.5 m to initiate bark beetle attacks on one randomly selected tree per block in the untrenched treatment, and one randomly selected tree per block in the trenched treatment (16 total over all blocks). We monitored every tree for attacks at least twice weekly. Each pitch tube on the bark surface was classified as one attack. All attacks between ground level and a height of 3.5 m were counted, marked with pins, and mapped (height and aspect). Beetle attacks introduced the natural suite of biota carried by beetles (e.g., fungi, bacteria, mites). We removed the lure from each tree after 150 beetle attacks had occurred on that tree. Attacks continued after lure removal and averaged 230 (SE 13.2) per tree, or 90 (SE 9) m<sup>-2</sup> of bark, and did not differ between trenched and untrenched trees ( $P=0.161$ ). Our inspection of pitch tubes and of galleries beneath the bark of heavily attacked trees revealed that >90% of attacks were by *D. brevicomis*, with trace amounts by *D. frontalis* and *D. valens*.

The second beetle treatment consisted of the inoculation of trees with biota carried by bark beetles, but without beetle feeding and gallery construction. Bark beetles carry a suite of fungal, bacterial, mite, and nematode symbionts (Adams et al. 2008; Adams et al. 2013; Boone et al. 2013; Davis 2015; Delalibera et al. 2005; Hofstetter et al. 2015). We inoculated trees with beetle-carried biota by crushing dead, recently captured beetles into the phloem-xylem interface (Klepzig et al. 2005). We collected beetles near the study site the previous summer (2013) from Lindgren funnel traps (same pheromone lures as the tree attack treatment) and stored them at  $-10^{\circ}\text{C}$  until use. Viable fungi have been extracted from frozen bark beetles in earlier investigations (Hofstetter 2004; Waalberg 2015). We used a 3:1 ratio of *D. brevicomis* to *D. frontalis* in the inoculation, based on the ratio of beetles captured the previous year. We made inoculations of *D. brevicomis* and *D. frontalis* at a bole height between 1 and 3.5 m. In addition, we included inoculations with six *D. valens* at a bole height of 0.5 m because that beetle typically attacks the lower bole in low numbers. We began inoculations on June 4 and ended them August 5, 2014. We inoculated two randomly selected trees per block (16 total), one in each of the untrenched and trenched treatments. Inoculation locations on the tree bole were based on the location and number of attacks on a paired attack tree within the same stress treatment and block; e.g. when a beetle attacked a trenched/attack tree on the north side at a height of 1 m, an inoculation was placed at the same location on the paired inoculation tree within the same block. The inoculation consisted of removing a plug of bark with a 4-mm diameter metal punch, inserting one beetle into each resulting cavity, and crushing the beetle into the phloem/xylem interface by replacing the bark plug. We conducted all inoculations on the same day that attacks on paired attack trees within the same block were recorded (e.g., twice weekly).

We prevented bark beetle attacks on all trees in non-attacked treatments (control, inoculated) by spraying the entire tree bole with 2.0% carbaryl (Sevin SL) on May 17, 2014. Appropriately applied carbaryl prevents attacks by *Dendroctonus* and *Ips* spp. (DeGomez et al. 2006; Shea and McGregor 1987). We did not spray branches because the *Dendroctonus* species in our study primarily attack the mid and lower bole of host trees (Six and Bracewell 2015; Wood 1982). The carbaryl spray was effective; we observed only six attacks in total on two sprayed trees and those attacks were unsuccessful based on a lack of boring dust and frass.

**Tree Stress** We measured stress on trees at two-week intervals during the warm months of the study in three ways. First, we assessed tree survival status (dead or alive). Second, we measured leaf xylem water potential at predawn and midday for a direct assessment of tree water condition. Third, we measured leaf gas exchange because stomatal conductance and net photosynthetic rate are well known to be sensitive to water stress in ponderosa pine (Gaylord et al. 2007; Kolb and Stone 2000).

We defined tree death as browning or reddening of  $>90\%$  of the tree canopy. Four observers independently scored the percentage of canopy browning/reddening in 10% increments on digital photographs of each tree acquired every two weeks (May through September) during the study. We averaged these scores for each tree and date.

We measured leaf xylem water potential at predawn ( $\Psi_{\text{pre}}$ , 0400–0600 h, depending on day length) and midday ( $\Psi_{\text{mid}}$ ; 1200–1300 h). We measured  $\Psi_{\text{pre}}$  and  $\Psi_{\text{mid}}$  on all 48 trees starting one week prior to the onset of the trenched treatment in May 2013, and from May until September in 2013, 2014, and 2015, or until tree death. We excised leaves from branches at mid-canopy with razor blades, and sealed them in plastic bags. Bags were held in a cool dark environment until water potentials were measured using a pressure chamber (Model 1000, PMS Instruments, Corvallis, OR, USA). We made all measurements within a half-hour of needle excision. This procedure yields  $\Psi$  values similar to those taken immediately after excision (Kaufmann and Thor 1982), and has been successfully used on ponderosa pine in prior studies (Kolb and Stone 2000; Koepke and Kolb 2013). Treatment effects and trends were similar for  $\Psi_{\text{pre}}$  and  $\Psi_{\text{mid}}$  ( $r^2 > 0.56$ ,  $P < 0.0001$  each year), consequently we present results for  $\Psi_{\text{pre}}$  for brevity, and because we could not measure  $\Psi_{\text{mid}}$  on several dates due to dangerous lightning storms.

We measured net photosynthetic rate and stomatal conductance on needles of all trees at mid-morning (0945–1145 h) using a Li-Cor 6400 IRGA (Li-Cor Inc., Lincoln, NE, USA) on the same dates when  $\Psi$  was measured. We maintained the cuvette at a photosynthetically active radiation of  $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $\text{CO}_2$  concentration of  $400 \mu\text{mol mol}^{-1}$  air, flow rate of  $500 \mu\text{mol s}^{-1}$ , and relative humidity of 40%. We placed two fascicles of one-year-old needles from mid-canopy exposed to full sun in the cuvette for approximately one minute, allowing gas exchange to stabilize before recording values. We removed the portion of each leaf inside the cuvette, returned it to the laboratory, and measured projected leaf area using WinFOLIA (Regent Instruments Inc. Nepean, ON, CAN). We entered these leaf areas into the Li-Cor 6400 computer for final calculation of gas exchange.

**Tree Defenses** We measured tree defense against bark beetles throughout the experiment two ways. First, we measured the amount of resin that flowed from wounds to the bark and phloem to assess quantitative resin defense (“resin flow” hereafter). Resin flow has been positively associated with ponderosa pine defense against bark beetle attacks in earlier investigations (Wallin et al. 2008). Second, we measured the concentrations of mono- and sesqui-terpenes in phloem to assessment treatment effects on the chemical composition of terpenes encountered by beetles when feeding. Ponderosa pine has many terpene components and the composition can change in response to biotic attacks (Keefover-Ring et al.

2016). For example, in *Montana ponderosa* pine the phloem tissue concentrations of terpenes are 61% diterpenes, 37% monoterpenes and 2% sesquiterpenes (Keefover-Ring et al. 2016). For brevity, we refer to the sum of all measured mono- and sesqui-terpenes as “total terpenes” in this study.

We measured resin flow monthly in 2014 (May to October) and 2015 (May to September) following methods developed earlier for pines (Lorio 1993; Gaylord et al. 2007). At each month near midday, we wounded each tree at two locations with a 1.25 cm diameter metal punch (#16 punch, C.S. Osborne, Harrison, NJ USA), 1.4 m above the ground at a random azimuth. Resin was collected for 24 h into vials, weighed (g) on an analytical balance, and averaged for the two vials sampled from each tree.

For the terpene analyses, we sampled phloem once a month between May–October 2014, and May–September 2015. In each month, we sampled all trees on the same day; each tree was sampled once by taking two phloem punches from opposite sides of the tree. Samples were immediately sealed in plastic bags and kept frozen ( $-20\text{ }^{\circ}\text{C}$ ) until analysis.

We extracted terpenoids from each phloem sample. We removed samples from the freezer as needed, cut the phloem into small cubes ( $\sim 2\text{--}3\text{ mm}$ ), and immediately submerged the cubes in 1 ml of 95% *n*-hexane with  $0.2\text{ }\mu\text{l ml}^{-1}$  of toluene and nonyl acetate, as internal standards, in 2 ml GC vials with polytetrafluoroethylene lined screw caps to insure no loss due to evaporation. We placed all samples in a sonication bath for 10 min, briefly vortex mixed them, and allowed them to shake overnight on an orbital mixer. After shaking, we pored the solvent into fresh gas chromatography vials. Many of the resulting solutions were analyzed as is, but due to the much higher concentrations of terpenes in some samples from attacked and inoculated trees, we diluted them to fall within the concentration range of the standard curves.

We analyzed composition of mono- and sesqui-terpenes in phloem samples 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 ( $30\text{ m} \times 0.25\text{ mm}$  I.D., film thickness  $0.25\text{ }\mu\text{m}$ ; Agilent Technologies, Santa Clara, CA, USA) with helium as the carrier gas at a flowrate of  $1.0\text{ ml min}^{-1}$ . We injected  $2\text{ }\mu\text{l}$  of each sample directly, with a split flow ratio of 30:1, using an oven profile of  $40\text{ }^{\circ}\text{C}$  for 5 min, followed by a ramp of  $3\text{ }^{\circ}\text{C min}^{-1}$  to  $200\text{ }^{\circ}\text{C}$ , and then a second ramp at  $25\text{ }^{\circ}\text{C min}^{-1}$  to  $220\text{ }^{\circ}\text{C}$ . Injector and detector temperatures were set at  $260\text{ }^{\circ}\text{C}$  and  $250\text{ }^{\circ}\text{C}$ , respectively.

We conducted additional terpenoid identification analyses with a Thermo Trace 1310 GC coupled to a Thermo ISQ mass spectrometer (MS) with electron ionization (EI) at  $70.0\text{ eV}$  at  $250\text{ }^{\circ}\text{C}$ , using helium as the carrier gas at  $1.0\text{ ml min}^{-1}$  with the injector temperature set at  $250\text{ }^{\circ}\text{C}$ . Oven conditions included an initial temperature of  $40\text{ }^{\circ}\text{C}$  followed by an immediate

ramp of  $3\text{ }^{\circ}\text{C min}^{-1}$  to  $200\text{ }^{\circ}\text{C}$ . We injected one  $\mu\text{l}$  of representative samples, available standards, and a continuous series of *n*-alkanes ( $\text{C}_8\text{--C}_{20}$ ; Sigma-Aldrich St. Louis, MO, USA), in the split mode onto a TR-5MS capillary column ( $30\text{ m} \times 0.25\text{ mm}$  I.D., film thickness  $0.25\text{ }\mu\text{m}$ ; Thermo Fisher Scientific). We identified mono- and sesqui-terpenes 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).

We dried phloem samples to a constant weight at  $60\text{ }^{\circ}\text{C}$ , and used dry weight (DW) values to calculate compound concentrations ( $\text{mg compound g}^{-1}\text{ DW}$ ) 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. Purified  $\beta$ -phellandrene came from Glidco Organics (Jacksonville, FL, USA).

**Statistical Analysis** For resin flow data we used a  $\log_{10}(x + 1)$  transformation to stabilize variances and obtain a normal distribution. We then used a repeated measures MANOVA on data in each year to investigate whether treatment effects varied over sample months. Factors in the MANOVA were block, month, trenching treatment, beetle treatment, and all two- and three-way interactions. For brevity, we report MANOVA results for the univariate adjusted G-G test; results for this test were similar to other MANOVA tests (Wilks' Lambda, Pillai's Trace, Hotelling-Lawley, Roy's Max Root). For the 2014 data all these tests showed that treatment differences varied among months (e.g., interactions between date and treatments were significant,  $P \leq 0.05$ ). Consequently, we then analyzed 2014 data separately by month with ANOVA using block, trenching treatment, beetle treatment, and the trenching treatment  $\times$  beetle treatment interaction as factors. We used Tukey's HSD comparisons to separate means. We removed trees from the analyses that died ( $>90\%$  canopy browning/reddening) during the month of measurement. This resulted in the removal of one tree from analysis of the August data, two trees from analysis of the September data, and three trees from analysis of the October data. Finally, we removed all trees from the attacked/trenched treatment combination from analysis of 2015 resin flow data because six of the eight trees in this treatment were dead by May 2015.

For the 2014 data we also compared resin flow between trees that eventually died during the two years of the experiment (“doomed trees”) and trees that survived (“survived trees”) using a similar approach of first using MANOVA. If differences between doomed and survived trees varied among months, we then conducted ANOVAs by month with tree survival status and block as factors. We deleted trees from each monthly analyses that died in that month or an earlier month. For example, tree 40 died by August 15, 2014 and was not included in analyses of August, September and October data. We report results for resin flow in original units of  $\text{grams } 24\text{ h}^{-1}$ .

The analysis approach for the  $\Psi$  and leaf gas exchange data was identical to that for the resin flow data, except that those data were normally distributed on most dates and were not transformed. For brevity, we focus on treatment effects on  $\Psi_{\text{pre}}$  and net photosynthetic rate. Comparisons of  $\Psi_{\text{pre}}$  and leaf gas exchange between doomed and survived trees are presented in the supplementary materials and in Burr (2016).

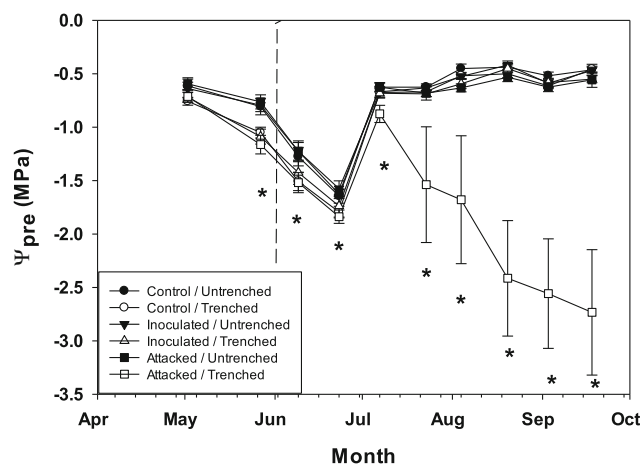
For the terpene data we used SAS (ver. 9.4; SAS Institute 2013) to examine variable distributions and apply square root transformations when distributions were non-normal and for subsequent statistical analyses. We used a three-factor repeated measures ANOVA with the factors of treatment (control, attack, or inoculation) and trenching (untrenched or trenched) with date as the repeated measure. Consistent with other analyses, we deleted trees from the analyses when they died.

## Results

**Tree Mortality** Eight of the 48 total trees died during the two-year experiment (“doomed trees” hereafter). Six (75%) doomed trees were in the attacked/trenched treatment, and the other two (25%) were in the attacked/untrenched treatment. No trees died in the inoculation or control treatments, with or without trenching. Three doomed trees died between August and October 2014 (all in the attacked/trenched treatment), three died between October 2014 and May 2015 (all in the attacked/trenched treatment), one died in September 2015 (attacked/untrenched treatment), and one died in November 2015 (attacked/untrenched treatment). Tree height and diameter were similar for doomed and survived trees ( $P > 0.42$ ).

**Tree Water Potential and Gas Exchange** The trenching treatment increased tree water stress. In early May 2013, three weeks before trenching,  $\Psi_{\text{pre}}$  was similar for trees assigned to the trenched and untrenched treatments ( $P = 0.537$ ). MANOVA on data pooled over post-treatment dates in 2013 showed that the trenching treatment significantly ( $P < 0.001$ ) reduced  $\Psi_{\text{pre}}$  and differences between the trenched and untrenched treatments were consistent over measurement dates (date  $\times$  treatment interaction  $P > 0.61$ ). Mean  $\Psi_{\text{pre}}$  averaged over all post-treatment dates in 2013 was  $-0.825$  (SE 0.170) MPa in the untrenched treatment and  $-0.971$  (SE 0.180) MPa in the trenched treatment. Photosynthetic rate measured in fall 2013 was similar ( $P = 0.322$ ) for untrenched trees (mean 6.9, SE 0.35  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and trenched trees (mean 6.5, SE 0.28  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ).

In 2014, the second year after trenching, MANOVA showed that differences in  $\Psi_{\text{pre}}$  among treatments varied over measurement dates (date  $\times$  trenching treatment interaction  $P = 0.001$ ). In May 2014,  $\Psi_{\text{pre}}$  was similar for all treatments (Fig. 1). In contrast,  $\Psi_{\text{pre}}$  was lower in the trenched treatment than the untrenched treatment between May 27 and July 7.



**Fig. 1** Mean (1 SE) leaf xylem predawn water potential ( $\Psi_{\text{pre}}$ ) in 2014 of ponderosa pines in six experimental treatments that combined different levels of root trenching, bark beetle attacks, and inoculation with beetle-vectored biota. Months with significant ( $P \leq 0.05$ ) treatment effects in ANOVA are marked \*. The vertical dashed line shows the start of the attack and inoculation treatments in June

Between July 23 and September 18,  $\Psi_{\text{pre}}$  was lower in the attacked/trenched treatment than other treatments. The dramatic decrease in  $\Psi_{\text{pre}}$  in the attacked/trenched treatment started in July approximately one month before the onset of tree death in mid-August based on canopy reddening and browning.

Trenching reduced photosynthetic rate in 2014 before onset of the bark beetle treatments in May, and after the treatments on most dates between June and September (Supplementary Table 2). Differences in photosynthetic rate between untrenched trees and trenched trees ranged from 14% in early May (mean of 6.1 and 5.3  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively) to 90% in late June (mean of 0.4 and 0.04  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , respectively). Similar differences occurred for stomatal conductance (data not shown).

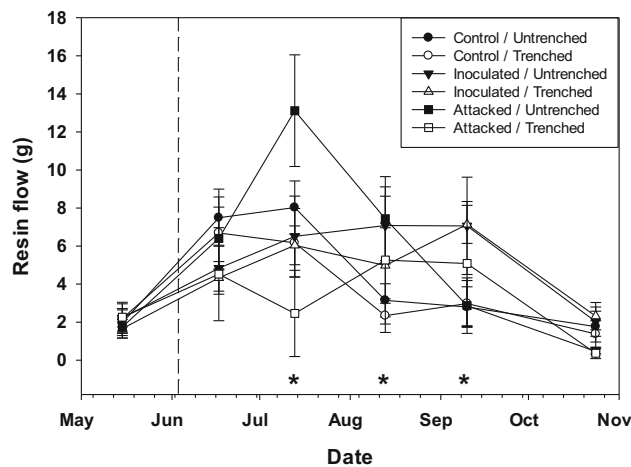
Effects of trenching on  $\Psi_{\text{pre}}$  were smaller in 2015 than 2014, in part due to trees in the attacked/trenched treatment not being included in the analysis because most (75%) were dead by May, 2015. MANOVA on 2015 data for the other treatments showed differences in  $\Psi_{\text{pre}}$  among treatments that depended on sample date (date  $\times$  treatment interaction  $P = 0.047$ ). Significant differences in  $\Psi_{\text{pre}}$  occurred only on June 23, 2015, when  $\Psi_{\text{pre}}$  was significantly lower in the control/trenched ( $-0.99$  MPa) and inoculated/trenched ( $-0.98$  MPa) treatments compared with the control/untrenched ( $-0.80$  MPa) and inoculated/untrenched ( $-0.75$  MPa) treatments.

The doomed trees showed symptoms of severe water stress after inoculations and attacks. The doomed trees had significantly more negative  $\Psi_{\text{pre}}$  and  $\Psi_{\text{mid}}$  than survived trees on most dates starting in May or June 2014 (Fig. S1), and lower net photosynthetic rate and stomatal conductance starting in July 2014 (Fig. S2).

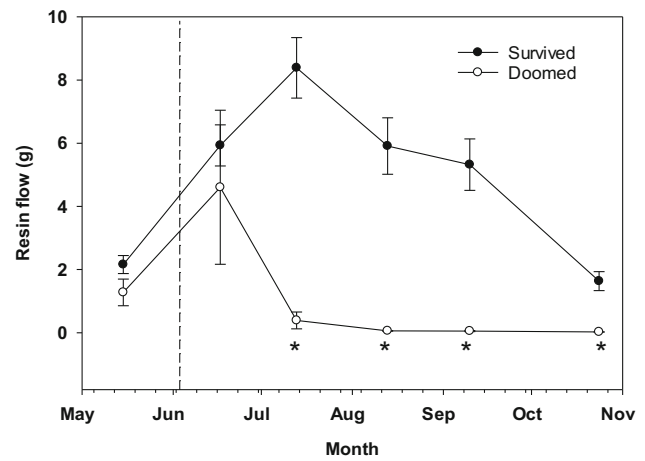
**Resin Flow** In 2014, resin flow was initially similar for all treatments, but subsequent differences arose during the summer (Fig. 2). These differences had significant date x treatment interactions ( $P \leq 0.05$ ), with beetle attack, trenching, or their combination always resulting in the lowest resin flow. In July 2014, one month after the onset of treatments, the beetle x trenching treatment interaction was significant ( $P = 0.007$ ), with resin flow lowest in the attacked/trenched treatment, highest in the attacked/untrenched treatment, and intermediate in other treatments. In August, only trenching was significant ( $P = 0.036$ ), with lower resin flow in trenched than untrenched trees. In September, only the beetle treatment was significant ( $P = 0.003$ ); resin flow was higher in the inoculated treatment than the attacked treatment, whereas flow in the control treatment was intermediate. Resin flow in late October was low and similar for all treatments.

Resin flow in 2014 was initially similar for doomed trees and survived trees, but differences arose after the onset of beetle attack treatments in June (date x survival status interaction  $P < 0.001$ ). Resin flow after June was consistently lower for doomed trees than survived trees in all months (Fig. 3). In 2015, resin flow was similar for all treatments ( $P > 0.28$  for treatment and date x treatment interaction), but did vary among sample dates ( $P = 0.002$ ), with greater flow in August than other months.

**Terpene Composition** Phloem of trees in our study contained 19 monoterpenes (Table 1) and two sesquiterpenes (longifolene and  $\beta$ -caryophyllene). Total terpenes (monoterpenes plus sesquiterpenes) were mostly comprised of monoterpenes over all trees and dates (96.5%, SE = 0.159); sesquiterpenes were only 3.5% (SE 0.159) of the total. (+)- $\alpha$ -Pinene was the most common and least variable monoterpene over



**Fig. 2** Mean (1 SE) 24-h resin flow in 2014 from phloem wounds of ponderosa pines in six experimental treatments that combined different levels of root trenching, bark beetle attacks, and inoculation with beetle-vectored biota. Months with significant ( $p < 0.05$ ) treatment effects in ANOVA are marked \*. The vertical dashed line shows the start of the attack and inoculation treatments in June



**Fig. 3** Mean (1 SE) 24-h resin flow of ponderosa pine trees that died during the experiment (doomed) and trees that survived by month in 2014. Months with significant differences between survived and doomed trees are marked \*. The vertical dashed line shows the start of the attack and inoculation treatments in June

trees and dates. The three most common monoterpenes ((+)- $\alpha$ -pinene, (-) limonene,  $\delta$ -3-carene) constituted 76.5% of the total. (-)- $\alpha$ -Pinene, myrcene, (-)- $\beta$ -pinene,  $\beta$ -phellandrene, bornyl acetate, terpinolene, and (+)-limonene each constituted greater than 1% of total monoterpenes. The remaining nine monoterpenes each constituted less than 0.53% of the total. Variation over trees and dates was greatest

**Table 1** Mean (SE) percent composition and coefficient of variation (CV) of monoterpenes detected in ponderosa pine phloem pooled over treatments and dates. Specific monoterpenes are listed from highest to lowest in percent composition

Monoterpene	Mean % (SE) total monoterpenes	CV
(+)- $\alpha$ -Pinene	38.21 (0.579)	33.1
(-)-Limonene	19.42 (0.556)	62.7
$\delta$ -3-Carene	18.96 (0.640)	73.9
(-)- $\alpha$ -Pinene	5.25 (0.159)	66.5
Myrcene	4.28 (0.095)	48.6
(-)- $\beta$ -Pinene	4.10 (0.360)	192.5
$\beta$ -Phellandrene	3.14 (0.187)	129.9
Bornyl acetate	1.52 (0.060)	86.5
Terpinolene	1.32 (0.042)	70.6
(+)-Limonene	1.17 (0.039)	72.1
Tricyclene	0.53 (0.014)	58.8
Unknown monoterpene	0.51 (0.014)	61.6
(+)-Camphene	0.40 (0.009)	48.4
(-)-Camphene	0.30 (0.007)	55.4
(+)- $\beta$ -Pinene	0.24 (0.006)	51.2
(-)-Linalool	0.24 (0.006)	140.1
<i>p</i> -Cymene	0.18 (0.009)	112.9
$\gamma$ -Terpinene	0.12 (0.012)	215.6
(+)-Linalool	0.11 (0.011)	219.4

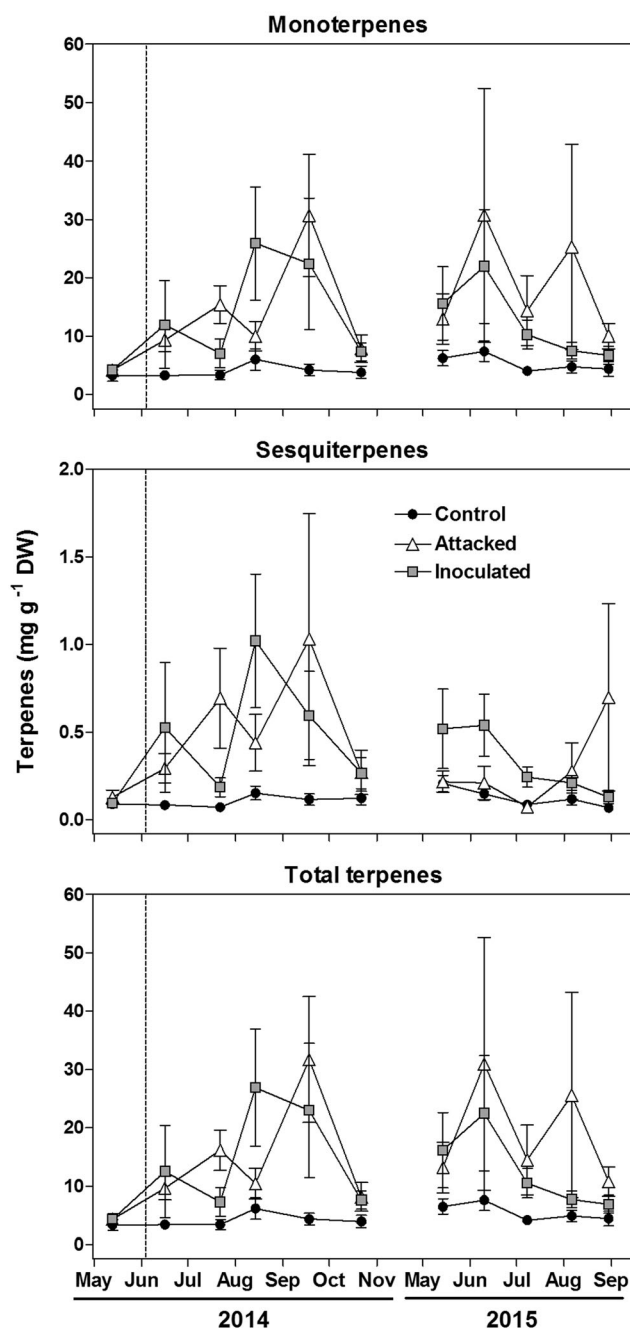
for (–)- $\beta$ -pinene,  $\beta$ -phellandrene, (–)-linalool, *p*-cymene,  $\gamma$ -terpinene, and (+)-linalool, all of which have a coefficient of variation greater than 100%.

The ANOVA showed that bark beetle treatment and date were significant ( $P \leq 0.05$ ) sources of variation for total monoterpenes, total sesquiterpenes, and their combined total, whereas the trenching main effect was not significant ( $P > 0.11$ ) (Fig. 4, Supplementary Table 1). Concentrations of monoterpenes, sesquiterpenes, and their combined total were similar for the control, attacked, and inoculated treatments in May 2014, before the onset of beetle treatments. Attacks and inoculations increased concentrations of monoterpenes, sesquiterpenes, and their combined total starting in June 2014 compared with the control. Trees in the attacked and inoculated treatments continued to have higher concentrations of both terpene groups than the control for most dates in 2014 and 2015. Trees in the control had little temporal variation in terpene concentrations. Also, the treatment  $\times$  trenching interaction was a significant influence ( $P < 0.001$ ) on combined monoterpenes and sesquiterpenes due to a smaller increase in concentrations of attacked trees in the trenched treatment than the untrenched treatment (Fig. 5).

The ANOVA showed that differences in combined monoterpene and sesquiterpene concentration between doomed and survived trees depended on sample date (survival  $\times$  date interaction  $P = 0.038$ ; Fig. 6). Combined monoterpene and sesquiterpene concentrations were initially similar for doomed and survived trees, but differences emerged after the onset of attack and inoculation treatments. In May before the onset of treatments, doomed and survived trees had similar concentrations of combined monoterpenes and sesquiterpenes ( $P = 0.806$ ) and each of the 21 individual terpenes ( $P > 0.262$ ). Combined monoterpene and sesquiterpene concentrations were again similar ( $P = 0.598$ ) for doomed and survived trees in June 2014 when treatments started. After the onset of treatments, combined monoterpene and sesquiterpene concentrations were greater in doomed trees than survived trees in late July ( $P = 0.045$ ), but again became similar for all subsequent time periods ( $P = 0.385$ , 0.137, and 0.568 for August, September, and October 2014, respectively).

## Discussion

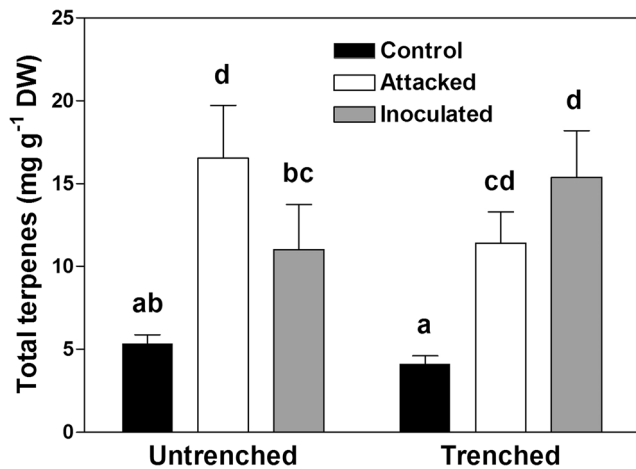
We describe the first experimental manipulation of water stress and bark beetle attacks on mature ponderosa pine in the southwestern US. Our first hypothesis was that bark beetle attacks and inoculation with beetle-vectored biota would induce quantitative chemical and resin defenses. Consistent with the hypothesis, both pheromone-induced beetle attack and controlled inoculation treatments induced quantitative chemical defenses via an increase in phloem concentrations of both classes of terpenoids tested, monoterpenes and sesquiterpenes



**Fig. 4** Mean (1 SE) of phloem total monoterpenes, total sesquiterpenes, and both combined (total terpenes) of ponderosa pines in the control, attacked, or inoculated treatments. See Supplementary Table 1 for statistical results. The vertical dashed line shows the start of the attack and inoculation treatments in early June 2014

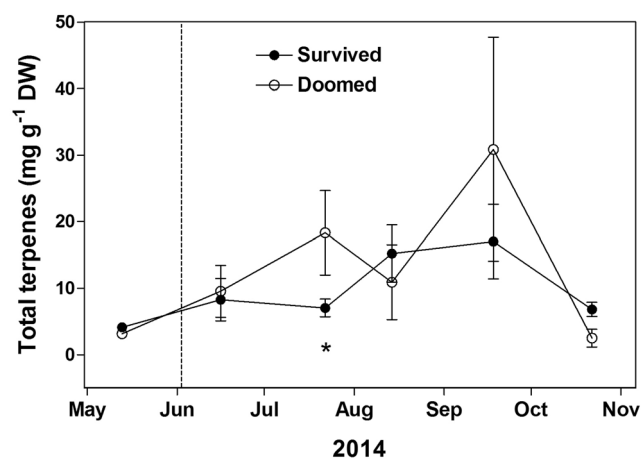
(Fig. 4). The quantitative defense of resin flow from bole wounds, however, was induced only by direct beetle attacks, not inoculations with beetle-vectored biota (Fig. 2). We found variable support for our second hypothesis, that water stress would constrain the induction of tree defenses. Consistent with our hypothesis, water stress constrained induction of the quantitative defense of resin flow from bole wounds. In particular, resin flow was higher in beetle-attacked than





**Fig. 5** Mean (1 SE) of phloem combined monoterpenes and sesquiterpenes (total terpenes) of ponderosa pines in the control, attacked, and inoculated treatments pooled over 2014 and 2015 that were either untrenched or trenched. Means followed by the same letter do not differ significantly ( $P \leq 0.05$ ) in Tukey HSD comparisons

unattacked trees when they were not water-stressed, but the opposite was true when they were stressed (Fig. 2). Also consistent with our hypothesis, water stress dampened the induced chemical defense of increasing phloem terpene concentrations in response to beetle attacks, but had less effect on the response to inoculations (Fig. 5). Our comparisons of terpene concentrations between trees that ultimately died and those that survived were not consistent with our hypothesis. There was one sampling period shortly after the onset of treatments when these concentrations were higher in attacked and stressed trees that ultimately died compared with trees that survived (Fig. 6). Our finding that water stress can have variable impacts on specific tree-induced defenses highlights the complexity of tree responses to water stress and biotic attacks.



**Fig. 6** Mean (1 SE) phloem total terpene concentration (mono- plus sesqui-terpenes) of ponderosa pine trees that died during the experiment (doomed) and trees that survived (survived) by month in 2014. Months with significant ( $P \leq 0.05$ ) differences between doomed and survived trees are marked \*. The vertical dashed line shows the start of the attack and inoculation treatments in early June

Overall, our finding that experimental water stress predisposed trees to bark-beetle induced mortality provides strong evidence for an important role of drought in host-bark beetle dynamics.

Our investigation revealed a diverse composition of phloem terpenoids in southwestern ponderosa pine. Phloem in our study contained 21 terpenoids (not including diterpenes), which were dominated by the monoterpenes (+)- $\alpha$ -pinene, (-)-limonene, and  $\delta$ -3-carene. Only two sesquiterpenes (longifolene and  $\beta$ -caryophyllene) were detected and together comprised only about 3% of the total measured terpene fraction. Our finding of a much lower concentration of sesquiterpenes relative to monoterpenes, and a dominant role of longifolene in sesquiterpenes, is similar to reports for ponderosa pine in Montana (Keefover-Ring et al. 2016). However, we detected fewer phloem sesquiterpenes in Arizona ponderosa pine (two) than reported for Montana ponderosa pine (seven, including four unknowns). Similar to monoterpenes (Keefover-Ring and Linhart 2010; Smith 2000), sesquiterpenes appear to be highly variable among regional populations of ponderosa pine with unknown potential effects on tree defense. Fifteen monoterpenes occurred at low (1–5%) or trace (<1%) concentrations (Table 1). Several studies have demonstrated that the terpene resin chemistry of southwestern ponderosa pine can be clustered into distinctive, geographically interspersed chemotypes (Davis and Hofstetter 2012; Latta and Linhart 1997; Latta et al. 2003). The terpene composition of phloem in our study matches several of the chemotypes described in Davis and Hofstetter (2012) for resin of ponderosa pines in Arizona. Ponderosa pines in Montana had phloem concentrations of (-)-limonene and  $\delta$ -3-carene similar to our study, but 33% lower concentrations of (+)- $\alpha$ -pinene (Keefover-Ring et al. 2016). These results are consistent with previously documented regional variation in monoterpene composition of ponderosa pine (Smith 2000). An important caveat about our results is that we did not measure diterpenes. The potential role of phloem diterpenes in defense of southwestern ponderosa pine against bark beetles requires further investigation.

The root trenching-snow removal treatment was effective in causing water stress as shown by more negative  $\Psi_{pre}$  for trenched trees over the first summer after treatment implementation in 2013 and in the dry part of the second summer in 2014. Similar impacts of root trenching on ponderosa pine  $\Psi_{pre}$  were reported in Arizona by McCullough and Wagner (1987a, 1987b). Root trenching also reduced photosynthesis in the dry part of 2014 before and after beetle treatments. Root trenching may have had other effects on our study trees, but there is little evidence for strong or lasting impacts based on our measurements one and two years after treatment. For example, trenching without bark beetle attacks and inoculation did not induce resin flow or change phloem terpene concentrations. These results are similar to reports of no stem

induction of monoterpene defenses by root infestations by weevils in lodgepole pine (Boone et al. 2011). The lowering of  $\Psi_{pre}$  by 0.2 to 0.4 MPa during in the driest periods of the study, late spring and early summer of 2014 (Fig. 1), simulated impacts of moderate drought on  $\Psi_{pre}$  of ponderosa pine. For example, severe droughts in 2000 and 2002 reduced ponderosa pine  $\Psi_{pre}$  by up to 0.6 MPa compared with wetter periods (Gaylord et al. 2007; Skov et al. 2004), which is more than the effect of trenching in our study. Additionally, the most negative  $\Psi_{pre}$  in the root trenching treatment before implication of the beetle treatments ( $-1$  MPa) is less stressful than  $\Psi_{pre}$  of  $-2$  MPa measured on ponderosa pines in the same region during earlier severe droughts (Gaylord et al. 2007). Thus, our results for the trenching/snow removal treatment should be interpreted in the context of moderate drought.

Our results show that ponderosa pines in northern Arizona rapidly induce defenses in response to both direct bark beetle attacks and inoculation of phloem with biota carried by bark beetles. The first type of induced defense in our study was a rapid increase in terpene concentration in phloem. This result is similar to reports for ponderosa pine in Montana in response to bark beetle-vectored fungi (Keefover-Ring et al. 2016), and to studies of other pines (Reid et al. 1967; Arango-Velez et al. 2018). Our study cannot distinguish which of the many organisms carried by bark beetles in northern Arizona (Hofstetter et al. 2015) were responsible for tree defense induction, but fungi are likely candidates based on investigations of ponderosa pine in other regions (Keefover-Ring et al. 2016). We observed fungal-induced lesions in phloem of all beetle attacked or inoculated trees, and blue-stain fungi in xylem sapwood of all trees that died (Burr 2016). However, bark beetle-associated microbes, such as mycangial fungi and bacteria, are also responsive to variation in the monoterpene composition of host trees, and metabolism of phloem and xylem tissues by these microbes can alter the concentration of monoterpenes (Boone et al. 2013; Davis and Hofstetter 2011; Hofstetter et al. 2005; Hofstetter et al. 2007).

The second type of induced defense in our study was an increase in resin flow from phloem wounds. This induced defense can prevent beetle penetration into bark by producing a terpene-laden river of resin that lethally encapsulates or delays beetles (Boone et al. 2011; Raffa and Berryman 1983; Wallin et al. 2008), or by inhibiting pheromone communication (Erbilgin et al. 2003). Induced resin flow only occurred in trees attacked by bark beetles without trenching, and induction only occurred for about one month after bark beetle attacks (Fig. 2). There was no evidence of induced resin flow in the more water-stressed trenched trees at this time; in fact, they had the lowest resin flow of all treatments (Fig. 2). These results show that induced resin flow occurred only when water stress was low, and was impeded by water stress typical of moderate drought. Our findings help explain the results of earlier studies of ponderosa pine in the southwestern US that

reported little induction of resin flow in response to severe mechanical wounding of the phloem to simulate bark beetle attacks or inoculation with fungi carried by bark beetles (Gaylord et al. 2011; Wallin et al. 2003). Unlike our study, these earlier studies did not include stress treatments, which likely prevented detection of stress-dependent responses.

The lack of induced resin flow by water-stressed trees during bark beetle attack likely resulted from a combination of lower turgor pressure in epithelial cells of resin ducts (Vite 1961; Vite and Wood 1961), depletion of phloem carbohydrates due to induced synthesis of terpenes (Roth et al. 2018), and constraints on overall supply of carbohydrates for resin production caused by low canopy photosynthesis (Wallin et al. 2003). Our finding that the trenching treatment reduced both tree water potential and photosynthesis is consistent with constraints on resin flow by low turgor pressure and reduced carbohydrates. The role of induced chemical defense in depleting carbohydrate supply for resin synthesis in stressed trees, however, will require more investigation for ponderosa pine in the southwestern USA. Interestingly, the first type of induced defense, increased terpene concentration in phloem, was somewhat decoupled from the second type of defense, increased resin flow. Trees showed the first type of induction in response to bark beetle attacks and biotic inoculation with and without stress, whereas the second type only occurred for non-water-stressed trees attacked by bark beetles. This agrees with the lack of correlation between terpene concentrations and resin duct metrics reported by Mason et al. (2017). Also, Davis and Hofstetter (2013) found that ponderosa resin chemotype was not related to resin flow or phenotypic traits such as tree size and phloem thickness, suggesting that these traits are independent of chemotypic variation, which is thought to be under strong genetic control (Smith 1970; Squillace 1971). Overall, our results show that water stress can have variable effects on ponderosa pine induced defenses, with the largest impacts on turgor-dependent induced resin flow.

Our results provide insight into the interactive roles of water stress and bark beetle attacks in ponderosa pine mortality. The lethality of bark beetle attacks depended on tree stress. Seventy-five percent of trees that died in the study had been subjected to water-stress treatments. Mortality of water-stressed trees that were mass-attacked following pheromone deployment occurred despite a pulse of increased concentrations of monoterpenes and sesquiterpenes, which did not provide enough protection against beetles. The lower leaf water potential and stomatal conductance prior to canopy browning of doomed trees than survived trees are consistent with impairment of sapwood water flow by bark-beetle-vectored fungi (Hubbard et al. 2013). Water potentials of doomed trees in the first summer after bark beetle attacks were at levels that initiate xylem cavitation of ponderosa pine in northern Arizona (Koepke and Kolb 2013), which likely accelerated tree desiccation and death.

In summary, our study produced three major findings about ponderosa pine defense against bark beetles in northern Arizona. First, bark beetle attacks and biota carried by beetles induce a general increase in concentration of terpenes in phloem regardless of tree stress. Second, water stress constrains the induction of resin flow during attacks more than the induction of terpene concentrations. Third, water stress and low resin flow override the effects of induced phloem monoterpenes and sesquiterpenes following bark beetle attacks, resulting in tree mortality. The negative influence of drought on tree defense and mortality during bark beetle attacks portends more frequent episodes of bark-beetle-induced tree mortality if ponderosa pine forests become more arid.

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