

ARTICLE

Chemical defense strategies, induction timing, growth, and trade-offs in *Pinus aristata* and *Pinus flexilis*David N. Soderberg^{1,2}  | Barbara J. Bentz³  | Justin B. Runyon⁴  | Sharon M. Hood⁵  | Karen E. Mock^{1,2} ¹Wildland Resources Department, Utah State University, Logan, Utah, USA²Ecology Center, Utah State University, Logan, Utah, USA³USDA Forest Service, Rocky Mountain Research Station, Logan, Utah, USA⁴USDA Forest Service, Rocky Mountain Research Station, Bozeman, Montana, USA⁵USDA Forest Service, Rocky Mountain Research Station, Missoula, Montana, USA

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Abstract

Trade-offs between plant defense investment and fitness traits, including growth, are often invoked to explain evolutionary strategies targeted at resisting herbivores. Many *Pinus* species have specialized herbivores, including the mountain pine beetle (MPB), *Dendroctonus ponderosae*, and have historically been a focus of defense investigations. We compared defense traits of two high-elevation *Pinus* species, *P. aristata* and *P. flexilis*, that are hosts to MPB and hypothesized to have different growth and defense traits and potential trade-offs. Interspecific differences were assessed by sampling trees within the same stands, and intraspecific differences were assessed by sampling stands at sites across latitudes where both species co-occurred. Constitutive defenses were measured at Day 0, and the timing, concentration, and composition of an induced resin defense response were assessed by sampling at 1, 4, and 30 days following either mechanical wounding only or a simulated MPB attack using its primary fungal symbiont *Grosmannia clavigera*. At Day 4, induced resin concentrations did not differ between mechanical wounding and simulated MPB attack in either species. By Day 30, resin defense concentrations in response to simulated MPB attack were greater than those in response to mechanical wounding and were >19-fold greater than constitutive levels. Results suggest that initial induced resin defense responses in the two species are likely generalized, with a delayed response that is targeted specifically at MPB and *G. clavigera*. At all sites, *P. aristata* had higher concentrations of constitutive and Day-30 induced resin defenses than *P. flexilis*, although *P. flexilis* induced proportionately more. Trade-offs in growth and defense between the species were only found at the two most climatically favorable sites where *P. aristata* grew slower than *P. flexilis*. No trade-offs were found between the two defense types at either biological scale. Overall, our findings highlight that the two pine species growing in the same stands (1) have a delayed response to a specialized native herbivore and fungal symbiont, (2) only exhibited interspecific defense-growth trade-offs at two climatically favorable sites, and showed

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no intraspecific defense–growth trade-offs, (3) showed no trade-offs between constitutive and induced defenses at either biological scale, and (4) have evolved different defense strategies.

KEYWORDS

induced defense, limber pine, mountain pine beetle, plant defense theory, plant–insect interaction, resource allocation, Rocky Mountain bristlecone pine, trade-offs

INTRODUCTION

Plants synthesize a diversity of secondary metabolites in resin to defend against herbivores and pathogens (Bennett & Wallsgrave, 2006). Resin defenses may be constitutive (i.e., always present) or induced (i.e., upregulated in response to a biotic challenge) (Karban & Baldwin, 1997; Rasmann & Agrawal, 2011) and are metabolically costly to produce (Agrawal et al., 1999; Cipollini & Heil, 2010; Strauss et al., 2002; Van Dam & Baldwin, 1998). Documenting and explaining patterns of variation in plant resin defenses has long been of interest to ecologists, often with a focus on potential trade-offs in resource allocation to defense versus growth (Coley et al., 1985; Herms & Mattson, 1992) or to constitutive versus induced defenses (Karban, 2011). Although multiple frameworks for describing plant defense strategies have been theorized (Stamp, 2003), a central tenet is that selection has optimized allocation of limited resources such that the benefits of defense outweigh the cost (Cipollini et al., 2014). Because variation in traits is typically greater between species than within species, hypotheses on the evolution of plant defenses were originally developed using interspecific comparisons (Coley et al., 1985; Herms & Mattson, 1992). More recently, evolutionary patterns of growth and defense investment for intraspecific comparisons have been shown to differ from interspecific comparisons (Hahn & Maron, 2016), resulting in the development of alternative hypotheses to explain observed patterns of resource allocation to growth and defense that are dependent on the biological scale of assessment (Agrawal, 2020; Hahn et al., 2021).

Plant growth and secondary metabolite production are two traits that compete for the same resources. Optimal levels of defense will therefore differ among species that have adapted to different environments with varying levels of resources and herbivory, highlighting evolved strategies that may differ among the species (Coley et al., 1985; Herms & Mattson, 1992). For example, inherently slow-growing and long-lived plant species, adapted to resource-limited environments with low levels of herbivory, are predicted to invest more heavily in constitutive defenses than growth (Coley et al., 1985; Fine

et al., 2004; Stamp, 2003). In high-resource and highly competitive environments, plant species may allocate more resources to growth, resulting in a lower relative level of investment in constitutive defenses (Coley et al., 1985). Clearly, adaptive specializations among species can result in trade-offs or negative associations between defense and growth (Endara & Coley, 2011; Sampedro et al., 2011; Züst et al., 2015). Trade-offs at the species level, however, may not manifest similarly when populations within a species are compared. Intraspecific populations have likely not evolved divergent resource investment strategies but instead have plastic responses across resource gradients that typically span less variation than comparisons across species (Hahn et al., 2019; Hahn & Maron, 2016). Moreover, because maximizing both constitutive and induced defenses is considered redundant, a trade-off between the two defense types has also been hypothesized (Karban & Baldwin, 1997; Rhoades, 1979) and observed among and within some species (Kempel et al., 2011; Koricheva et al., 2004; Moreira et al., 2014). Overall, while there is evidence that secondary metabolites can be metabolically costly, the effect of these costs on plant growth likely depends on multiple factors including the biotic and abiotic environment (Cipollini et al., 2014; de la Mata et al., 2017; Ferrenberg et al., 2017; Karban, 2020).

Pinus (pines) are among the longest-lived organisms (Piovesan & Biondi, 2021), and they deploy large amounts of carbon-based secondary metabolites, including terpenes, for both constitutive and induced defenses (Franceschi et al., 2005; Phillips & Croteau, 1999; Sampedro et al., 2011; Witzell & Martin, 2008). Considerable variability exists in constitutive resin defense strategies among and within pine species, with differences in concentrations (Bentz et al., 2017; Clark et al., 2012; Kichas et al., 2021; Latta et al., 2003; Raffa et al., 2013; Thoss et al., 2007), chemical combinations (i.e., “chemotypes”) (Davis & Hofstetter, 2012; Forrest, 1980; Latta et al., 2003; Taft et al., 2015; Thoss et al., 2007), and chemical defense types (Howe et al., 2020; Mason et al., 2019). Induced defenses, in terms of absolute resin secondary metabolite abundance and proportions of specific compounds, also vary among (Clark et al., 2014;

Erbilgin, Cale, Lusebrink, et al., 2017) and within (Villari et al., 2014; West, 2013) pine species. Studies that have examined relationships between growth and defense and between defense types among and within pine species have found mixed results, leading to scale-dependent trait associations (Howe et al., 2020; López-Goldar et al., 2020; Mason et al., 2019; Moreira et al., 2014).

Many pine species native to western North America have evolved relationships with a specialist herbivore, the mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins, Coleoptera: Curculionidae, Scolytinae), an ecologically and economically significant tree mortality agent that feeds upon and reproduces within the phloem, often killing the host tree in the process (Raffa et al., 2008). Following adult emergence from brood trees and attack of a new susceptible host tree, aggregation pheromones that rapidly attract conspecifics are released in an attempt to overwhelm tree defenses. A successful mass attack enables inoculation with mutualistic fungal associates (Safranyik & Carroll, 2006), host tree colonization, and subsequent brood production. Upon attack, which can peak within a few days (Bentz et al., 1996) and be sustained for a month or more (Bentz et al., 2014), most pine species produce a resinous induced response that contains secondary metabolites, including monoterpenes, sesquiterpenes, diterpenes and their derivatives, and phenolics. At low concentrations, host monoterpenes can function as aggregation pheromone precursors (Seybold et al., 2006) but can be toxic to adult beetles and their offspring when present at high concentrations (Chiu et al., 2017; Reid et al., 2017; Reid & Purcell, 2011). Some secondary metabolites are also toxic to beetle-associated fungi and bacteria (Adams et al., 2011; Paine & Hanlon, 1994; Raffa & Smalley, 1995) that can act in concert with MPB to neutralize host defenses (Six & Wingfield, 2010; Solheim, 1995). The role of monoterpenes in resistance to MPB is well studied, although relatively little is known about sesquiterpenes and their role in pine defense (Keefover-Ring et al., 2016). Research using wounding + inoculation with beetle-associated fungi (i.e., simulated MPB attack) has shown increased resin concentrations (Raffa & Smalley, 1995) and lesion lengths (Raffa & Berryman, 1983) in pines within a few days. Upregulation of genes controlling terpenoid production in *Picea* was also shown to occur within a few days after stimulus with methyl jasmonate, a general defense elicitor (Martin et al., 2002). Resin concentrations, relative to constitutive levels, were also elevated from 7 to 21 days following a simulated MPB attack in several pine species (Cale et al., 2017; Keefover-Ring et al., 2016; Raffa et al., 2017). Although they did not report constitutive levels, Burke et al. (2017) observed that an induced response 4 days

following treatments of either methyl jasmonate or simulated MPB attack was dependent on the host environment. Therefore, while it is clear that pines have both constitutive and induced responses, the early timing of an induced response that is specific to MPB and its fungal associates remains uncertain.

In addition to well-defined defenses, *Pinus* species can have widely varying growth rates (Burns & Honkala, 1990; Keeley, 2012) and include some of the world's oldest trees with the slowest growth rates (Piovesan & Biondi, 2021). Great Basin bristlecone pine (*P. longaeva* Bailey), a particularly long-lived and slow-growing species often found in low-resource environments, has higher levels of constitutive defenses than the relatively faster growing and co-occurring limber pine (*P. flexilis* James) (Bentz et al., 2017), consistent with interspecific predictions about trade-offs. Foxtail pine (*P. balfouriana*) and Rocky Mountain bristlecone pine (*P. aristata*) are close relatives of *P. longaeva* (Bailey, 1970), comprising the long-lived Balfourianae subsection of *Pinus* (Eckhart & Hall, 2006; Lanner, 2007). While *P. balfouriana* also has high levels of constitutive defenses relative to *P. flexilis* (Bentz et al., 2017), defense strategies and potential trade-offs within *P. aristata* are unknown.

Our goal was to investigate phloem resin defenses and the growth of *P. aristata* relative to co-occurring *P. flexilis* to gain insight into evolved defense strategies of these long-lived species that grow at high elevations. We assessed growth rates and the concentration, composition, and timing of constitutive resin defenses and induction following either a simulated MPB attack or mechanical wounding only. We tested for species differences by sampling similarly sized individuals within the same stands, thereby controlling for differences between the species in defense expression caused by varying environments. Differences among populations within each species were assessed by sampling stands at sites across latitudes where the two species co-occurred. Similar to its close relative *P. longaeva*, we expected *P. aristata* to have slower growth and higher constitutive defenses than co-occurring *P. flexilis*. Although induced defenses have not been investigated for *P. longaeva*, *P. balfouriana*, or *P. flexilis*, we expected to see a trade-off between defense types, with low induced response in *P. aristata* due to its putatively slower growth rate, and high induced response in *P. flexilis* due to its expected faster growth rate and low levels of constitutive compounds (Bentz et al., 2017). Among populations within each species, we expected an absence of trade-offs between growth and defense and constitutive and inducible resin defenses, in addition to intraspecific variability in chemotypes, due to variation among environments. The early timing of an induced response was assessed on 1 and 4 days following either a

simulated MPB attack or mechanical wounding only. In response to our treatments, we expected a rapid increase in resin defense concentrations as found previously (Burke et al., 2017) but hypothesized that a simulated MPB attack would result in a stronger and more rapid response than mechanical wounding only. An induced response targeted at sustained attacks was also hypothesized and tested at 30 days following treatment. We specifically hypothesized the following:

H1. Timing and composition of induction: *Induced responses to simulated MPB attack will be rapid (within 1–4 days) and stronger than mechanical wounding alone. Compounds that putatively confer toxicity to MPB and its fungal associates will increase in relative proportion within the resin chemistry profile.*

H2. Growth–defense trade-offs: *Slower-growing species will invest more in constitutive defenses (relative to induced defenses) than faster growing species. Within a species, neutral or positive relationships between growth rate and constitutive defenses are expected.*

H3. Constitutive-induced trade-offs: *Species with high concentrations of constitutive defenses will invest less in inducible defenses. Within a species, neutral or positive relationships between constitutive and induced defenses are expected.*

MATERIALS AND METHODS

Climate, site, and tree selection

Intraspecific comparisons were made by sampling *P. aristata* and *P. flexilis* in the same stands to control for differences in defense expression that may be caused by varying environments. Differences among populations within each species were assessed by sampling five stands across latitudes where the two species co-occurred, encompassing the range of *P. aristata* (Figure 1, Table 1). As a proxy for resource availability, climate at the five sites was described using modeled 30-year normals at 800-m resolution (1991–2020) (PRISM Climate Group, n.d.) (Table 1). The two most southern latitude sites had the highest mean annual precipitation and temperature and were considered the most climatically favorable of the five sites. The study site in Arizona (AZ) is within a zone of extensive *P. strobiformis* Engelm. and *P. flexilis* introgression (Menon et al., 2018), but for the purposes of this study, we report these trees as

P. flexilis. Trees were sampled at Days 0, 1, 4, and 30. Although the timing of sampling varied among the sites due to logistical constraints, all constitutive sampling was conducted in 2018 during the typical flight period of MPB, June through early August, and both species were sampled at each site and time period. At each site, we sampled 12 unattacked and live mature trees per species in the range of 23.4–45.7 cm dbh, for a total of 120 trees (Table 1). No recent MPB attacks were observed within or adjacent to the sampled stands.

Constitutive and induced defense sampling

To assess constitutive resin levels, a 22.2 × 6.4 mm phloem plug was removed with an oblong punch (Osborn Number 2; UPC 01646) at dbh from six equidistant locations circumnavigating the bole of each sampled tree (Appendix S1: Figure S1). Phloem thickness (in millimeters) was measured and averaged across all samples per tree, and the removed tissue was pooled and placed in a vial, sealed, immediately placed on dry ice for transport, and stored at −40°C to reduce volatile loss. To measure induced responses, the 12 trees of each species at a site were randomly chosen to receive one of two treatments following the constitutive mechanical sample: (1) simulated MPB attack via fungal inoculation + wounding ($n = 6$ per species per site) or (2) mechanical wounding only ($n = 6$ per species per site). MPB attack was simulated by immediately placing a fungal inoculation of *Grosmannia clavigera* (University of British Columbia; M002-06-03-05, UC21G26; Canmore, AB, Canada), a fungal symbiont of MPB, into all six wounds created by the constitutive sample. Inoculating trees with beetle-vectored fungi has shown successful activation of induced resin defense response that is similar to attacks by live beetles (Keefover-Ring et al., 2016; Raffa & Berryman, 1983; Villari et al., 2014). To mitigate the possibility that systemic defense induction (Blodgett et al., 2007; Bonello et al., 2003; Wallis et al., 2008) contributed to the observed effects of our treatments, induced samples were all localized within 1 cm of the constitutive sample (Appendix S1: Figure S1). *G. clavigera* was grown on 2% Malt Extract Agar for 14 days at room temperature prior to inoculation. The inoculum was cut to size with the oblong punch and placed “spore-side” toward the tree (Erbilgin, Cale, Lusebrink, et al., 2017) to produce full contact with the phloem and xylem. Mechanically wounded trees received the constitutive sampling wound but were not treated with fungal inoculum. Wounds on all trees, with and without the fungal inoculum, were sealed with parafilm (Bemis; PM999) to minimize desiccation and deter environmental contamination.

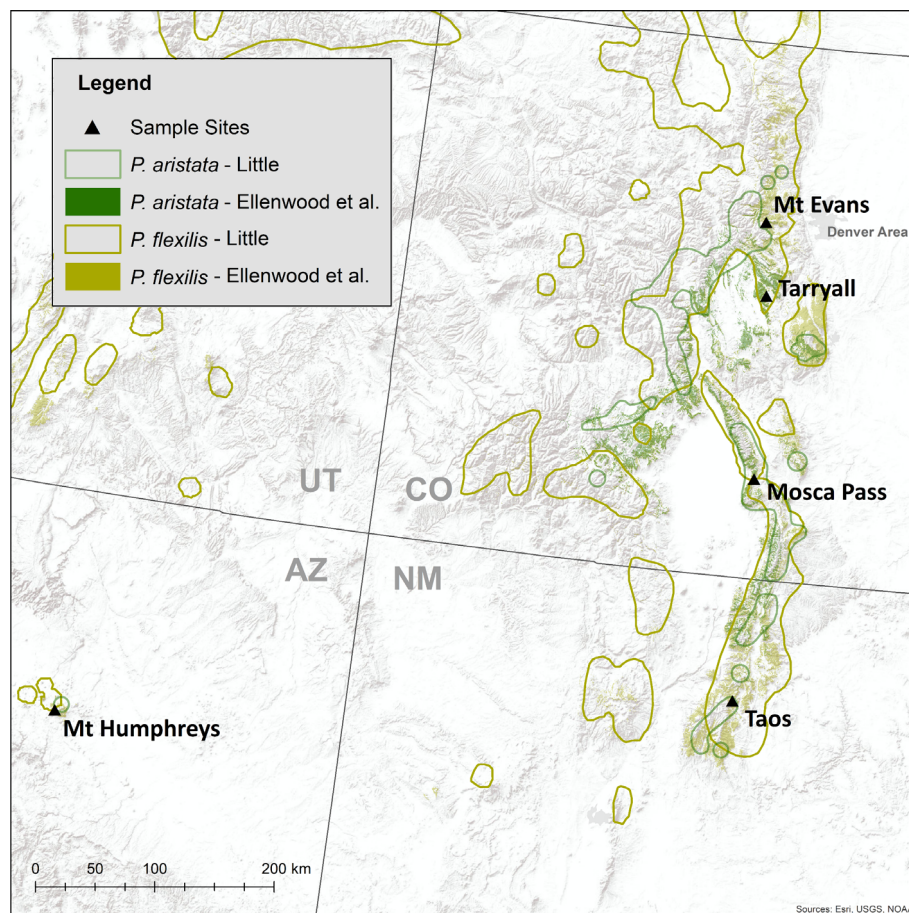


FIGURE 1 Study site locations. The distribution of Rocky Mountain bristlecone pine (*Pinus aristata*) is limited to Colorado (CO), New Mexico (NM), and an isolated population in Arizona (AZ). Limber pine (*P. flexilis*), which has a larger distribution across the western United States and Canada, co-occurs with *P. aristata*. Distributions based on Little (1971) and Ellenwood et al. (2015).

TABLE 1 Stand metrics of site location, elevation, mean annual temperature and precipitation, species sampled, and mean $\pm$ SE of dbh, age, basal area increment (BAI) (mean BAI of most recent 10 years), and phloem thickness of study trees.

Site	Latitude, longitude	Elevation (m)	Temperature ($^{\circ}$ C)	Precipitation (cm)	Species	dbh (cm)	Age (years)	BAI ($\text{mm}^2 \text{year}^{-1}$)	Phloem thickness (mm)
Mt Evans, CO	39.66243772, -105.5859309	3350	1.33	69.5	<i>Pinus aristata</i>	26.2 $\pm$ 0.7	223.2 $\pm$ 2.5	18.4 $\pm$ 3.7	3.64 $\pm$ 0.18
					<i>P. flexilis</i>	28.5 $\pm$ 1.0	203.0 $\pm$ 4.2	33.6 $\pm$ 4.8	3.10 $\pm$ 0.17
Tarryall, CO	39.11328439, -105.5114408	2820	4.11	41.6	<i>P. aristata</i>	31.3 $\pm$ 1.1	288.1 $\pm$ 26.2	21.0 $\pm$ 4.7	3.21 $\pm$ 0.34
					<i>P. flexilis</i>	31.7 $\pm$ 1.4	206.9 $\pm$ 26.3	29.3 $\pm$ 6.9	2.35 $\pm$ 0.25
Mosca Pass, CO	37.74113797, -105.4546224	3144	3.44	53.4	<i>P. aristata</i>	36.5 $\pm$ 1.1	267.8 $\pm$ 16.6	33.7 $\pm$ 6.0	4.06 $\pm$ 0.22
					<i>P. flexilis</i>	37.7 $\pm$ 1.3	195.7 $\pm$ 19.8	46.0 $\pm$ 8.7	3.77 $\pm$ 0.16
Taos, NM	36.07267572, -105.4496995	2930	5.44	70.1	<i>P. aristata</i>	33.1 $\pm$ 1.5	118.8 $\pm$ 7.0	31.9 $\pm$ 2.7	4.04 $\pm$ 0.26
					<i>P. flexilis</i>	33.7 $\pm$ 1.7	101.5 $\pm$ 6.1	88.6 $\pm$ 13.1	3.88 $\pm$ 0.29
Mt Humphreys, AZ	35.33692229, -111.717213	2740	6.56	82.8	<i>P. aristata</i>	31.8 $\pm$ 1.8	44.9 $\pm$ 3.2	217.4 $\pm$ 20.1	7.75 $\pm$ 0.32
					<i>P. flexilis</i>	36.6 $\pm$ 1.5	39.3 $\pm$ 2.0	329.3 $\pm$ 46.1	5.33 $\pm$ 0.36

Note: Sites are presented on a north-south climate gradient spanning 4.3 $^{\circ}$ of latitude with 12 trees/species sampled per site. BAI is the cross-sectional area of wood produced per year and accounts for decreasing ring width as the tree gets larger.

The timing, concentration, and composition of induced responses on each mechanically wounded and simulated MPB attack tree were measured 1, 4, and 30 days after wounding and/or inoculation using a Trephor tool to collect microcores of 2 mm diameter and 12-mm length (Rossi et al., 2006). At each sampling time, a microcore was collected systematically from either 1 cm above or below each fungal inoculation or mechanically wounded site ($n = 6$ per tree; Appendix S1: Figure S1). Samples were pooled by tree and placed in a vial, sealed, immediately placed on dry ice for transport, and stored at -40°C to reduce volatile loss. To test for changes in constitutive resin defenses due to differences in “sampling period” across time, phloem of an additional six randomly chosen and untreated “control” trees of each species at each site on Day 30 was collected and stored identically to the mechanically wounded tree samples, using the same methodology described above for constitutive samples. Hereafter, samples that were taken after the Day 0 constitutive sample are referenced according to their treatment as “simulated MPB attack,” “mechanically wounded,” or “control.” All tools were washed with 95% ethanol between each sample.

Determination of age and growth rate

After chemical defense sampling was completed on Day 30, we collected tree cores from all trees to determine age and growth rate for the previous 10 years. Using a manual increment borer (Haglöf), a 5.15-mm-wide core was taken from dbh on each cross-slope side of the tree to minimize sampling compression wood. Cores were prepared using standard techniques (mounted and sanded until the cellular structure was visible through a binocular microscope) and scanned using an Epson platform scanner at 1200 dpi. To estimate tree age and calculate radial growth rates, ring widths were measured and assigned the correct calendar year to each tree ring (i.e., cross-dated), species-specific chronologies were developed for each sampling area, and the number of rings and the distance to the pith were approximated using CooRecorder and CDendro v. 8.8.1 (Cybis Elektronik & Data AB, Saltsjöbaden, Sweden). Cross-dating was additionally checked with COFECHA (Holmes, 1983). Cores that could not be confidently cross-dated were removed from further analyses (<5% of cores). To assess recent growth rates, we calculated basal area increment (BAI). BAI is the cross-sectional area of wood produced per year and accounts for decreasing ring width as the tree gets larger. Annual BAI values for the most recent 10 years (2008–2017) were calculated using the dplR package v. 1.6.0 in R v. 3.0.1 (Bunn, 2008).

Resin compound extraction and identification

We extracted and analyzed the monoterpenoids, sesquiterpenoids, and low molecular weight benzenoids and hydrocarbons in phloem resin using gas chromatography following methods in Powell and Raffa (2011). These compounds, especially monoterpenes, make up a substantial fraction of constitutive and inducible secondary compounds in *Pinus* phloem (Raffa et al., 2017). Frozen phloem was cut into small pieces (ca. $2\text{--}3\text{ mm}^2$), and for each sampling date and treatment, all phloem samples from each tree were pooled (mean $\pm$ SE phloem dry weight [d.w.]; 27.27 ± 0.76 mg). Phloem was submerged in 1 ml of 95% n-hexane (Sigma-Aldrich, St Louis, MO, USA; ACS reagent grade) in a 2-ml glass gas chromatograph (GC) vial with a polytetrafluoroethylene (PTFE) screw cap (Agilent Technologies, Wilmington, DE, USA) and agitated on a shaker plate for 24 h (20°C , 250 rpm). After shaking, the solvent was transferred into new GC vials using a pipette with low-retention, extra-fine point tips (Denville Scientific Inc., Metuchen, NJ, USA). The phloem vial was rinsed twice with 0.25 ml of hexane and added to the second vial for a final volume of 1.5 ml; 100 μg of 2-nonanone was added as the internal standard. Phloem was dried in an oven at 30°C for 1 week and then weighed.

Samples were analyzed using an Agilent 7890A GC coupled with a 5975C mass spectrometer and separated on a chiral Cyclodex B column (Agilent; $30\text{ m} \times 0.25\text{ mm}$ inner diameter, $0.25\text{-}\mu\text{m}$ film thickness) with helium as the carrier gas at a constant flow rate of 1.0 ml min^{-1} . One microliter of each sample was injected using splitless mode (injector 225°C) with the GC oven maintained at 60°C for 10 min, followed by a ramp of $2.5^{\circ}\text{C min}^{-1}$ to 160°C and then a second ramp at $30^{\circ}\text{C min}^{-1}$ to 225°C . Quantifications were made relative to the internal standard using the ChemStation software (Agilent Technologies, Wilmington, DE, USA). Compounds were identified by comparison between chromatographic retention times and mass spectra with those of commercially available standards. Standards were purchased from Sigma-Aldrich (St Louis, MO, USA) except for (E)- β -farnesene, which was obtained from Bedoukian Research, Inc. (Danbury, CT, USA). For compounds without available standards, mass spectra and NIST 08 Mass Spectral Search Program (National Institute of Standards and Technology, Gaithersburg, MD, USA) were used to classify unidentified compounds by isoprenoid-based (i.e., monoterpenoids and sesquiterpenoids) and non-isoprenoid-based (i.e., benzenoids and hydrocarbons) classes. The occurrence of unknown compounds across species was verified by comparing retention times and mass spectra, and using the

NIST 08 Mass Spectral Search Program. Compound concentrations were standardized by dry phloem weight (in grams) to calculate the concentration (in milligrams per gram of phloem). In many samples, β -pinene enantiomers were often present but not able to be uniquely quantified. Subsequently, we pooled β -pinene enantiomer quantifications into a single compound ([+/-]- β -pinene). Of the two benzenoids observed, we only analyzed results for estragole (i.e., 4-allylanisole), a compound that has been previously shown to influence bark beetle behavior (Emerick et al., 2008; Werner, 1995).

Data analysis

We used absolute values of constitutive and induced concentrations for H1, where our objective was to compare the timing and concentration of defenses produced by simulated MPB attack and mechanically wounded trees. For analyses aimed to evaluate trade-offs between growth and defenses (H2) and constitutive versus induced defenses (H3), we calculated inducible defenses as the difference between absolute induced concentrations due to a simulated MPB attack and constitutive concentrations (induced – constitutive, Δ_{I-C}). Calculations were made on a per tree basis. For H2 and H3, secondary metabolite concentrations were z-score-standardized ($[\text{observed} - \text{mean}]/\text{SD}$) by species. Thus, data were centered on zero and intercepts were excluded from the models. For each analysis, resin defense concentrations were estimated by pooling concentrations of all measured secondary metabolites (i.e., total compounds) and subdividing by monoterpenoids, sesquiterpenoids, and the benzenoid estragole. Changes in absolute concentrations of individual compounds between constitutive and induced samples were also assessed.

We tested for trait differences by species and population by assessing differences among sites, treatments (simulated MPB attack, mechanically wounded, and control), and sampling period (Days 0, 1, 4, and 30). Depending on the hypothesis, individual tree traits tested included absolute constitutive and induced concentrations, inducible concentrations, individual constitutive and inducible compound proportions, age, dbh, phloem thickness, and growth rate. The effect of species, treatment, and sampling period on tree defense traits (H1) was assessed with ANOVA, and linear regression analysis was used to assess the relationships between growth rate and defense (H2) and constitutive and inducible defenses (H3) among species and their respective populations.

All analyses were conducted in R version 4.0.0 (R Core Team, 2020). Bayesian hierarchical models, accounting for variation among sites, were conducted via

Markov chain Monte Carlo (MCMC) sampling. The package “brms” (Bürkner, 2017, 2018) was used to compute four MCMC for 4000 iterations, discarding the first 2000 iterations as burn-in and sampling each iteration thereafter. All models were checked graphically for convergence and $\hat{r}$ values (i.e., the Gelman-Rubin convergence diagnostic [Gelman & Rubin, 1992], a ratio of variation within and between MCMC) were equal to 1, indicating thorough MCMC sampling and convergence of the posterior distributions.

Using Bayesian MCMC estimates, a median estimate and quantified uncertainty were derived for each model parameter. The median effect size (ES) and 95% Bayesian credible intervals (CI_{Bayes}) were then calculated as the median difference in model parameter estimates between comparison samples, bounded by the range of values indicating the equal-tail 95% CI of the true parameter estimate, given the data. The marginal probability (MP) that a parameter estimate is statistically different (greater or less than, given the direction of the ES) than the test comparison (ANOVA in H1) or slope of zero (linear regression analysis in H2 and H3) was estimated by calculating the proportion of parameter MCMC estimates greater (or less) than the comparison. We specify “credible” differences between species when MP > 90% (Buonanuci et al., 2020). For more information on model equations and prior distributions, see Appendix S1: Statistics.

H1: Timing and composition of induction

To evaluate induced response timing for each species, we compared concentrations of monoterpenoids, sesquiterpenoids, estragole, and total compounds (in milligrams per gram of d.w.) in simulated MPB attack trees by sampling date (i.e., Day 0 vs. Day 1; Day 0 vs. Day 4; and Day 0 vs. Day 30) using ANOVA. To evaluate whether the induced response on Day 1, Day 4, and Day 30 differed between simulated MPB attack and mechanically wounded trees, concentrations of each secondary metabolite group were compared for each species and treatment using ANOVA. Increased mobilization and/or upregulation of specific compounds in response to simulated MPB attack, relative to mechanically wounded trees, was assessed by calculating the relative proportion of individual compounds (% of total quantity) within the same tree between sample dates using ANOVA.

The potential effect of the sampling period for each species was tested by comparing concentrations of constitutive samples taken from all trees on Day 0 with those of samples taken from control trees (i.e., trees that had not been previously sampled) on Day 30. To assess the

diversity of secondary metabolite compounds within each species and treatment, we calculated the diversity of the mono- and sesquiterpenoid constitutive and inducible response fractions for each tree using the Shannon-Wiener index: $H' = -\sum_{i=1}^n p_i \ln p_i$, where p_i is the relative proportion of a compound within the resin chemistry profile. Intraspecific differences in the diversity of mono- and sesquiterpenoid fractions were assessed with ANOVA.

H2: Growth–defense trade-offs

We examined growth–defense trade-offs by first comparing growth rates (most recent 10-year average) among species at each site using ANOVA. A similar analysis was then used to test for differences in concentrations between the species in monoterpenoids, sesquiterpenoids, estragole, and total compounds at Day 0 (constitutive), and inducible responses at Days 1, 4, and 30. To test for the strength of the relationship between growth rate and constitutive and inducible defenses within each species, a Bayesian linear regression analysis (R package “brms”; Bürkner, 2018) was used to assess the relationship between growth rate and constitutive and inducible concentrations of individual trees. Bayesian linear regression slope coefficients were calculated as the response variable with monoterpenoids, sesquiterpenoids, estragole, and total compound concentrations assessed separately for each species. The same analysis was used to evaluate the relationships between growth rate and concentrations of constitutive and inducible monoterpenoids, sesquiterpenoids, estragole, and total compounds.

H3: Constitutive-induced trade-offs

Using simulated MPB attack trees only, we tested for a trade-off between constitutive and inducible defenses among and within species using a Bayesian linear regression analysis (R package “brms”; Bürkner, 2018) to assess the relationship between constitutive and inducible concentrations. Bayesian linear regression coefficients were calculated for concentrations of monoterpenoids, sesquiterpenoids, estragole, and total compounds separately. Inter- and intraspecific compositional differences in constitutive and inducible defenses were assessed using hierarchical clustering. Clustering was performed on normalized compound concentrations similar to methods from Taft et al. (2015), with each compound converted to a proportion of the total compounds for each sample. Cluster analysis was initially conducted

separately on monoterpenoids, sesquiterpenoids, and total compounds. Because monoterpenoids comprise a majority fraction of the total in both species, clustering based on total compounds and monoterpenoids only did not differ, and further analyses were focused on monoterpenoids and sesquiterpenoids separately. We applied nonmetric multidimensional scaling (NMDS) and bootstrap resampling analysis of compound profiles using R packages “vegan” (Oksanen et al., 2007) and “pvclust” (Suzuki & Shimodaira, 2015), respectively, to visualize and statistically test for dissimilarities in resin defense composition among and within species. In NMDS visualizations, all detected compounds within their secondary metabolite classes were included, with the Bray–Curtis dissimilarity index used as the multidimensional distance measure. We performed hierarchical clustering of individual trees into compositional classes with the “average” distance clustering method on a Euclidean distance matrix and 10,000 bootstrap resamples that allows for p value assignments to clusters (Efron et al., 1996; Shimodaira, 2002, 2004). Individual clusters were assigned chemotype labels based on the predominant secondary metabolite compound within a cluster.

RESULTS

H1: Timing and composition of induction

A total of 47 compounds were identified from the phloem of 60 *P. aristata* and 60 *P. flexilis* including 30 monoterpenoids, 13 sesquiterpenoids, 2 benzenoids, and 2 non-isoprenoid hydrocarbons (Appendix S1: Table S1). Monoterpenoids represented 91% of the constitutive composition in *P. aristata* and 79% in *P. flexilis* (Appendix S1: Table S1). Chemical differences between the species were largely quantitative in that phloem for both species each contained 45 of the 47 identified compounds (and all 30 monoterpenoids). The hydrocarbon tridecane and an unidentified sesquiterpenoid (only found in *P. flexilis*) were the only compounds quantified that were not shared among both species tested.

Resin concentrations and subsequent proportional changes following treatment (i.e., simulated MPB attack or mechanical wounding alone) varied by species, component compounds, and time since treatment. *P. aristata* resin concentrations on Day 1 following a simulated MPB attack were not different from constitutive concentrations, although Day 1 monoterpenoid concentrations in mechanically wounded *P. aristata* were greater than constitutive concentrations. At Day 1 following both treatments, *P. flexilis* mono- and sesquiterpenoid

TABLE 2 Bayesian model results testing for the effect of (A) simulated mountain pine beetle (MPB) attack (i.e., fungal inoculation + wounding) and (B) mechanical wounding treatments on induced (Day 1, Day 4, and Day 30) relative to constitutive (Day 0) secondary metabolite (SM) concentrations: Day 1 versus Day 0; Day 4 versus Day 0; and Day 30 versus Day 0.

Temporal duration	<i>Pinus aristata</i>			<i>Pinus flexilis</i>		
	ES	MP (%)	Proportion change	ES	MP (%)	Proportion change
(A) Simulated MPB attack						
Total compounds						
Day 1 vs. Day 0	1.37 (−4.26, 6.19)	71.0	1.05 (0.87, 1.23)	5.29 (−0.14, 9.56)	97.2	3.48 (0.97, 49.1)
Day 4 vs. Day 0	23.8 (8.06, 39.6)	99.9	1.81 (1.27, 2.41)	18.6 (2.65, 34.5)	99.2	9.60 (1.85, 141.5)
Day 30 vs. Day 0	547.0 (361.9, 722.4)	100	19.7 (13.1, 26.4)	274.2 (89.2, 446.6)	100	126.7 (28.1, 1703.7)
Monoterpenoids						
Day 1 vs. Day 0	1.52 (−3.75, 5.99)	73.0	1.05 (0.88, 1.23)	4.27 (−0.78, 8.26)	95.8	3.25 (0.84, 47.1)
Day 4 vs. Day 0	23.6 (9.78, 39.9)	99.9	1.85 (1.33, 2.50)	16.3 (2.83, 32.4)	99.2	9.46 (1.95, 151.1)
Day 30 vs. Day 0	547.1 (380.0, 720.7)	100	20.7 (14.4, 27.5)	248.2 (85.8, 421.8)	99.9	127.4 (28.6, 1826.2)
Sesquiterpenoids						
Day 1 vs. Day 0	0.02 (−0.56, 0.76)	53.6	1.02 (0.58, 1.63)	1.09 (0.52, 1.82)	99.9	7.90 (2.57, 74.2)
Day 4 vs. Day 0	0.47 (−0.83, 2.14)	75.5	1.37 (0.36, 2.76)	2.76 (1.35, 4.38)	100	18.3 (5.60, 174.8)
Day 30 vs. Day 0	4.45 (−0.80, 15.1)	91.9	4.49 (0.37, 13.2)	29.5 (20.4, 40.5)	100	185.1 (66.7, 1808.0)
Estragole						
Day 1 vs. Day 0	0.95 (0.56, 1.60)	42.7	0.95 (0.56, 1.60)	3.03 (−22.3, 26.6)	60.2	1.32 (0.08, 31.0)
Day 4 vs. Day 0	1.31 (0.80, 2.17)	86.6	1.31 (0.80, 2.17)	3.30 (−22.8, 31.0)	60.6	1.35 (0.08, 31.4)
Day 30 vs. Day 0	21.6 (12.8, 36.6)	100	21.6 (12.8, 36.6)	144.2 (−5.56, 539.7)	95.8	15.7 (0.62, 370.9)
(B) Mechanical wounding						
Total compounds						
Day 1 vs. Day 0	9.39 (0.27, 19.5)	97.7	1.32 (1.01, 1.70)	6.51 (−1.95, 16.4)	92.7	4.10 (0.47, 60.6)
Day 4 vs. Day 0	25.9 (16.6, 34.9)	100	1.89 (1.53, 2.27)	10.9 (1.91, 20.0)	98.9	6.09 (1.41, 84.3)
Day 30 vs. Day 0	47.7 (20.4, 77.6)	100	2.63 (1.67, 3.71)	35.6 (7.86, 66.0)	99.2	17.7 (3.22, 250.0)
Monoterpenoids						
Day 1 vs. Day 0	9.05 (1.26, 18.1)	98.7	1.32 (1.04, 1.68)	4.93 (−2.07, 14.0)	91.3	3.60 (0.39, 60.2)
Day 4 vs. Day 0	25.6 (16.6, 34.9)	100	1.92 (1.55, 2.32)	8.83 (0.74, 17.8)	98.3	5.63 (1.19, 80.3)
Day 30 vs. Day 0	47.4 (26.0, 72.2)	100	2.70 (1.90, 3.67)	28.5 (7.83, 53.0)	99.8	15.6 (1.19, 80.3)
Sesquiterpenoids						
Day 1 vs. Day 0	0.47 (−0.55,)	83.3	1.37 (0.58, 2.39)	1.56 (0.53, 2.79)	99.9	1.56 (0.53, 2.79)
Day 4 vs. Day 0	0.63 (−0.55, 2.30)	85.2	1.50 (0.57, 2.85)	2.34 (1.15, 3.96)	100	2.34 (1.15, 3.96)
Day 30 vs. Day 0	1.07 (−1.15, 6.09)	73.4	1.85 (0.10, 5.89)	8.20 (4.34, 13.4)	100	8.20 (4.34, 13.4)
Estragole						
Day 1 vs. Day 0	−1.01 (−56.2, 57.2)	48.6	0.98 (0.19, 2.09)	17.6 (−17.8, 67.7)	81.3	2.84 (0.15, 62.3)
Day 4 vs. Day 0	43.1 (−3.85, 90.4)	96.4	1.67 (0.95, 2.84)	7.96 (−20.7, 48.6)	69.3	1.85 (0.07, 42.4)
Day 30 vs. Day 0	232.1 (75.9, 394.0)	99.8	4.63 (2.10, 8.53)	54.2 (−11.3, 190.4)	91.5	6.47 (0.27, 137.0)

Note: Responses were analyzed by secondary metabolite group (monoterpenoids, sesquiterpenoids, and the benzenoid estragole) and total quantified SM (i.e., total compounds) concentrations (in milligrams per gram) in *P. aristata* and *P. flexilis*. The median effect size (ES) is the median difference in model parameter estimates between comparison samples. Values in parentheses are the 95% Bayesian credible intervals. The marginal probability (MP) is the probability that pairwise comparisons are statistically different, given the direction of the ES. Values in boldface are estimates that are credibly different (MP > 90%). Proportional change is the proportional difference of the Day 1, 4, and 30 induced concentrations, relative to the constitutive concentrations.

TABLE 3 Bayesian model results testing for differences between simulated mountain pine beetle (MPB) attack and mechanical wounding trees in monoterpenoid, sesquiterpenoid, estragole, and total quantified secondary metabolite (SM) (i.e., total compounds) concentrations (in milligrams per gram) at Day 1, Day 4, and Day 30 in *Pinus aristata* and *P. flexilis*.

Time period	<i>Pinus aristata</i>		<i>Pinus flexilis</i>	
	ES	MP (%)	ES	MP (%)
Total compounds				
Day 1	−7.75 (−16.4, 0.99)	96.2	−1.03 (−5.92, 3.81)	69.0
Day 4	−1.93 (−21.9, 18.0)	58.6	7.89 (−9.79, 25.1)	84.7
Day 30	506.1 (365.8, 641.3)	100	246.3 (176.3, 313.6)	99.9
Monoterpenoids				
Day 1	−7.32 (−15.6, 0.55)	96.4	−0.54 (−4.15, 3.16)	64.0
Day 4	−1.70 (−22.4, 18.7)	57.2	7.44 (−10.4, 25.0)	83.5
Day 30	505.0 (373.8, 629.6)	100	231.4 (172.9, 289.7)	100
Sesquiterpenoids				
Day 1	−0.48 (−1.84, 0.90)	80.9	−0.51 (−2.04, 1.01)	78.1
Day 4	−0.10 (−1.46, 1.26)	57.8	0.53 (−1.75, 2.82)	68.9
Day 30	2.84 (−0.95, 6.99)	94.0	22.0 (9.76, 34.5)	99.9
Estragole				
Day 1	−0.00 (−0.08, 0.08)	55.5	−0.01 (−0.09, 0.07)	61.9
Day 4	−0.02 (−0.11, 0.07)	70.6	−0.00 (−0.08, 0.09)	50.3
Day 30	1.13 (0.18, 2.04)	98.6	0.01 (−0.92, 0.93)	51.0

Note: The median effect size (ES) is the median difference in model parameter estimates between comparison samples. Values in parentheses are the 95% Bayesian credible intervals. The marginal probability (MP) is the probability that pairwise comparisons are statistically different, given the direction of the ES. A negative ES suggests that the SM group concentrations were greater in mechanically wounded than in the simulated attack treatment. Values in boldface are estimates that are credibly different (MP > 90%).

concentrations were greater than constitutive concentrations. Simulated MPB attack and mechanically wounded trees of both species exhibited greater monoterpenoid and total concentrations at Day 4 than at Day 0 constitutive samples (Table 2). Simulated MPB attack and mechanically wounded *P. flexilis* also showed an increase in sesquiterpenoids within the first 4 days relative to constitutive samples (Table 2). In both species, however, the treatments did not differ at Day 4 for concentrations of any secondary metabolite class, and in *P. flexilis*, concentrations did not differ between the two treatments at Day 1 or Day 4 (Table 3). Therefore, while by Day 4 both species had greater monoterpenoid concentrations and *P. flexilis* also had greater sesquiterpenoid concentrations, relative to constitutive levels, both concentrations did not differ between the two treatments in either species. Estragole concentrations did not differ between Day 1 or Day 4 and constitutive nor between the two treatments on those days in either species.

By Day 30, monoterpenoids, sesquiterpenoids, estragole, and total compounds in simulated MPB attack trees of both species were >19-fold greater than those in constitutive levels by Day 0 (Figure 2, Table 2). All

concentrations, except for *P. aristata* sesquiterpenoids, were also greater in mechanically wounded trees by Day 30, relative to constitutive levels. Concentrations of all compounds were greater in simulated MPB attack trees of both species by Day 30 than in mechanically wounded trees (Table 3). In addition, following the treatment-induced changes in absolute concentrations, *P. flexilis* exhibited greater proportional changes in concentrations across all time periods, relative to *P. aristata* (MPs > 99.0%) (Table 2). Most notably, in response to simulated MPB attack at Day 30 relative to constitutive levels, *P. flexilis* exhibited >126-fold proportional increase in concentrations of total compounds, while *P. aristata* exhibited >19-fold proportional increase. In response to mechanical wounding alone, *P. flexilis* exhibited >17-fold proportional increase in concentrations of total compounds, while *P. aristata* exhibited >twofold proportional increase.

Individual compound proportions within the resin chemistry profile of either species did not exhibit differences between simulated MPB attack and mechanical wounding treatments in comparison between Day 0 to Day 1 and Day 4 samples (MPs < 90.0%). By Day 30,

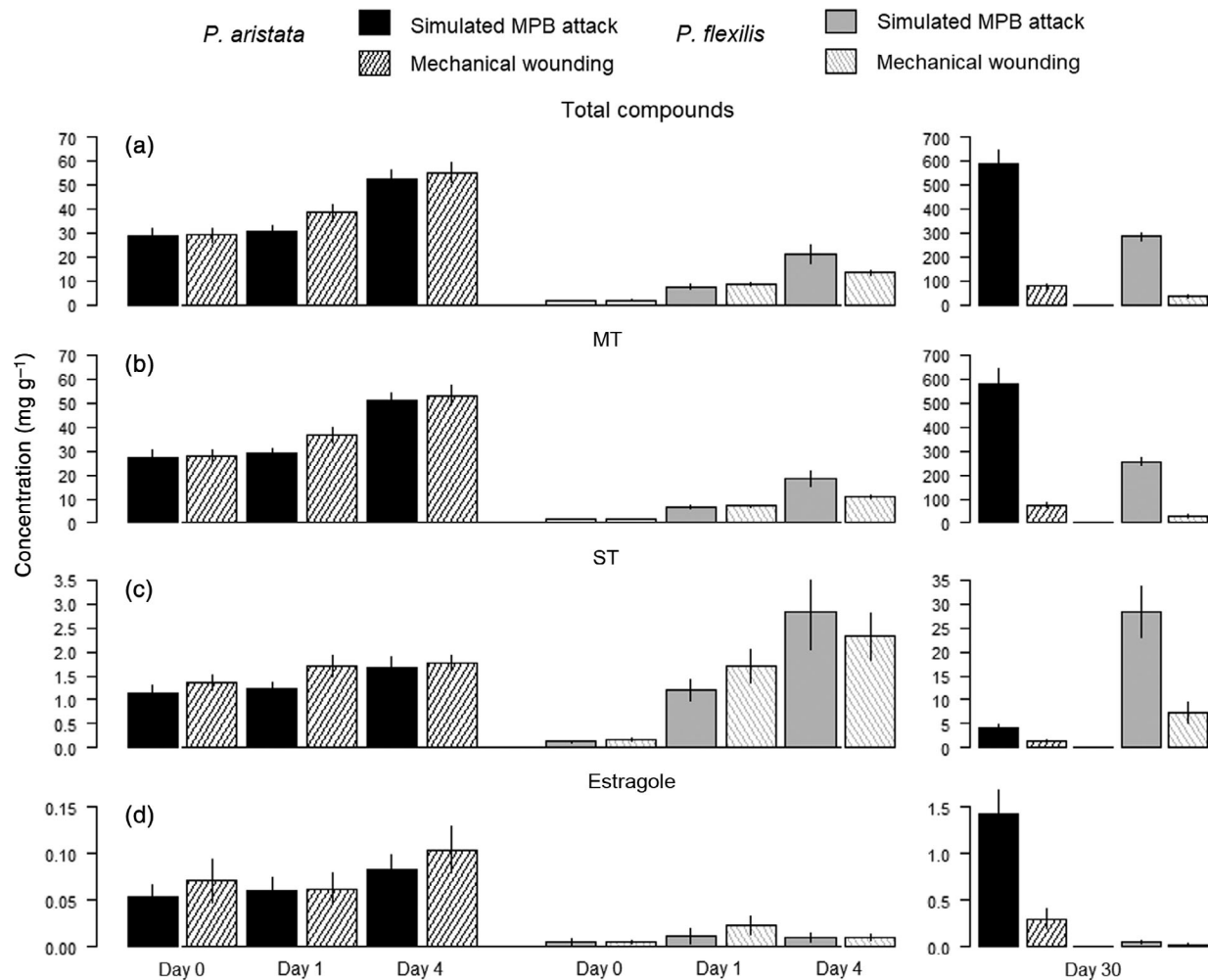


FIGURE 2 Measured secondary metabolite phloem concentrations (in milligrams of compound per gram of dry weight, mean $\pm$ SE) of (a) total compounds measured, (b) monoterpenoids (MT), (c) sesquiterpenoids (ST), and (d) the benzenoid estragole among *Pinus aristata* and *P. flexilis* in constitutive (Day 0) and induced (Day 1, Day 4, and Day 30) treatments by simulated mountain pine beetle (MPB) attack and mechanical wounding only.

however, simulated MPB attack trees of both species had greater proportions of certain compounds (relative to constitutive levels) than mechanically wounded trees, although only five monoterpenoids were statistically credible between the treatments (Figure 3). Individual compounds that exhibited greater induction by Day 30 in simulated MPB attack trees than in mechanically wounded trees include the following: δ -3-carene (ES = 25.9; MP = 98.8%) and terpinolene (ES = 3.2; MP = 99.7%) in *P. aristata*; and sabinene (ES = 3.0; MP = 90.0%), (–)- α -pinene (ES = 3.6; MP = 96.5%), and (+)- α -pinene (ES = 3.5; MP = 91.1%) in *P. flexilis* (Figure 3). In addition, monoterpenoid diversity decreased in response to simulated MPB attack relative to the constitutive profile in both species: *P. aristata* (ES = 0.48; MP = 99.3%) and *P. flexilis* (ES = 0.11; MP = 89.2%). These results indicate higher relative proportions of specific compounds within the inducible fractions in

response to simulated MPB attack. All further comparisons therefore are between constitutive (Day 0) and Day 30 simulated MPB attack concentrations.

We found no differences in monoterpenoid concentrations in *P. aristata* or *P. flexilis* between constitutive samples (i.e., Day 0) and Day 30 control trees (i.e., trees that had not been previously sampled), suggesting the timing of sampling did not influence monoterpenoid concentrations in either species (Appendix S1: Table S2). Similarly, no differences were observed in *P. flexilis* sesquiterpenoids or estragole between the treated and control trees. Although constitutive concentrations at several *P. aristata* sites did differ between temporal sampling of these compounds, differences between Day 30 simulated MPB attack and constitutive concentrations were much greater than those with control trees (sesquiterpenoid—2.6-fold; estragole—30-fold). These findings suggest that while the timing of sampling may

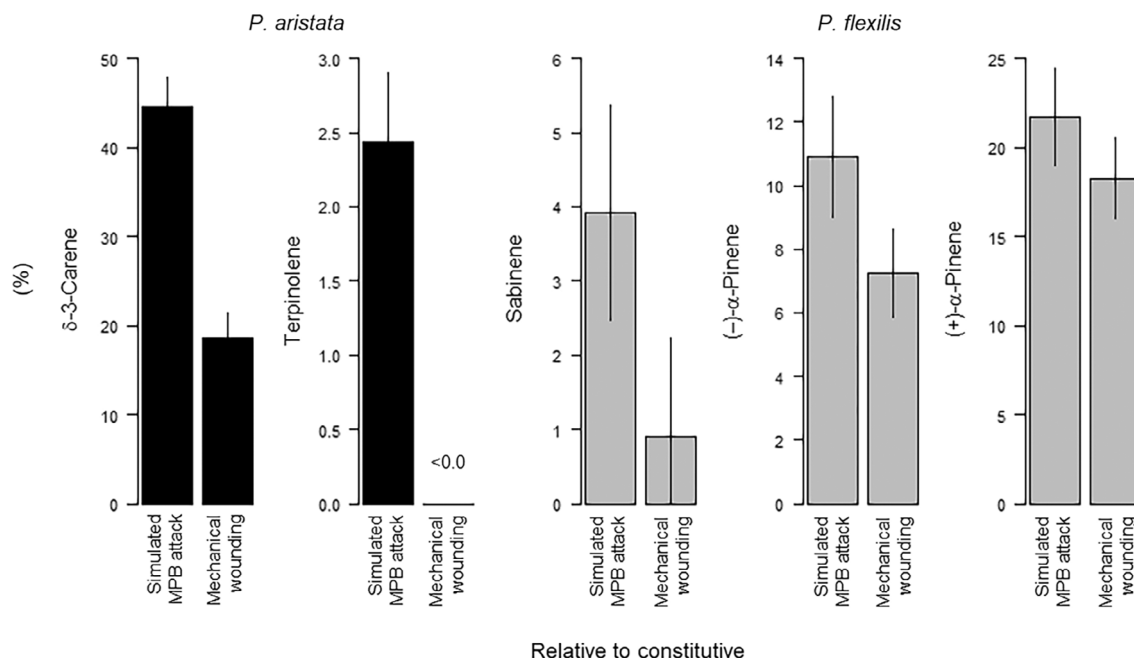


FIGURE 3 Induction patterns of individual compound proportions (mean $\pm$ SE) that showed statistically credible and greater proportional differences in simulated mountain pine beetle (MPB) attack (fungal inoculation + mechanical wounding) than in mechanical wounding only (Day 30) treatments relative to constitutive levels in *Pinus aristata* and *P. flexilis*.

influence sesquiterpenoid and estragole concentrations, the effects were minute compared with our simulated MPB attack treatments and likely confer a minimal effect on our experimental assessments.

H2: Growth–defense trade-offs

Overall, *P. aristata* grew more slowly than *P. flexilis* but the differences were greatest at the two most climatically favorable sites where trees were also the youngest (Taos, NM, USA; Mt Humphreys, AZ, USA) (MPs > 99.9%) (Figure 4a, Table 1; Appendix S1: Table S3). Across and within all sites, there were no age differences between *P. aristata* and *P. flexilis* (Figure 4b; Appendix S1: Table S4). Although dbh of both species at the Mt Humphreys, AZ site, was similar to all other sites (Figure 4c; Appendix S1: Table S5), trees at this site were much younger (~ 2 – $6\times$) and both species had $\sim 10\times$ the growth rate of trees at other sites (Table 1). While all study trees were randomly selected within the range of 23.4–45.7 cm dbh, *P. aristata* were smaller than co-occurring *P. flexilis*, particularly at the Mt Humphreys, AZ site (Figure 4c; Appendix S1: Table S5). Phloem thickness in *P. aristata* was greater than in *P. flexilis* at three of the five sites (Figure 4d; Appendix S1: Table S6). Age, dbh, and phloem thickness were not credible covariates in describing the relationship between growth rate and constitutive or inducible

responses or between constitutive and inducible resin concentrations.

Across all sites, concentrations of constitutive monoterpenoids, sesquiterpenoids, estragole, and total compounds were greater in *P. aristata* than in *P. flexilis* (Figure 2, Table 4; Appendix S1: Tables S7 and S8). *P. aristata* also invested more in inducible monoterpenoids and estragole than *P. flexilis* at Day 30, although inducible sesquiterpenoids were greater in *P. flexilis* (Table 4; Appendix S1: Tables S7 and S8). Across all sites, both constitutive and inducible total concentrations were greater in *P. aristata* than in *P. flexilis*, including at the two climatically favorable sites where the growth of *P. aristata* was slower than that of *P. flexilis* (MPs = 100%). However, across all time periods the proportional increase in both mono- and sesquiterpenoids was greater in *P. flexilis* than in *P. aristata* (Table 4). Among populations of *P. aristata*, regression slope coefficients between growth rate and constitutive concentrations of monoterpenoids, estragole, and total compounds were positive and credible, with mixed results and no credible relationships in *P. flexilis* (Figure 5; Appendix S1: Table S9). Growth rate and inducible concentrations of monoterpenoids, sesquiterpenoids, and estragole were also positive and credible for *P. aristata*, and for sesquiterpenoids in *P. flexilis* (Figure 5; Appendix S1: Table S9). These results do not support evidence for trade-offs in either species between growth and constitutive or inducible resin defenses.

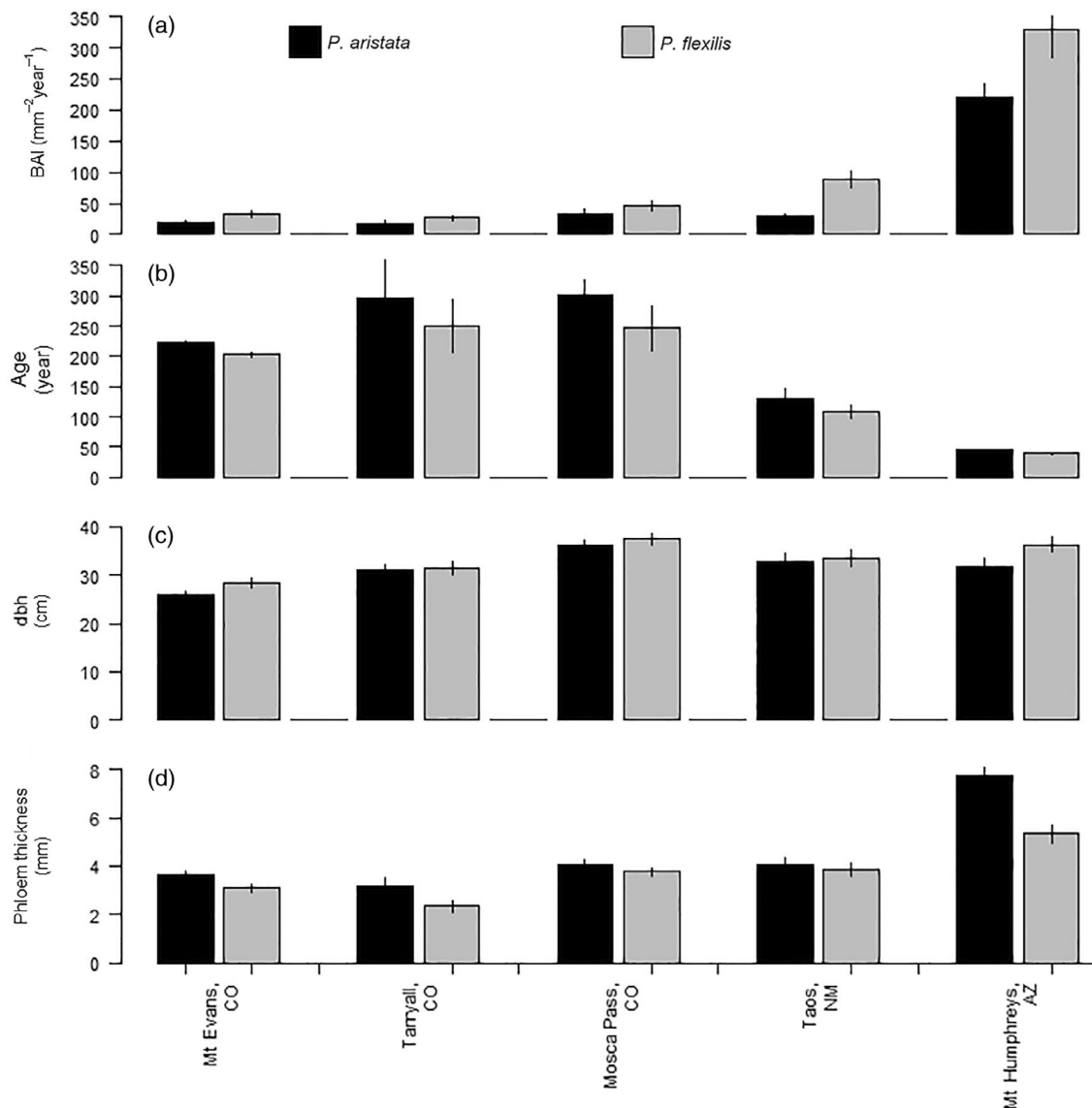


FIGURE 4 Mean $\pm$ SE of (a) growth rate (recent 10-year basal area increment, BAI), (b) age, (c) dbh, and (d) phloem thickness of *Pinus aristata* and *P. flexilis* at each study site. Sites are arranged on a north-to-south gradient on the x-axis.

TABLE 4 Bayesian model results testing for species-level differences between *Pinus aristata* and *P. flexilis* in constitutive (Day 0) and Day-30 inducible (Δ_{I-C}) monoterpene, sesquiterpene, the benzenoid estragole, and pool secondary metabolite (SM) concentrations (in milligrams per gram).

Compounds	<i>P. aristata</i> versus <i>P. flexilis</i>			
	Constitutive		Inducible (Δ_{I-C})	
	ES	MP (%)	ES	MP (%)
Total compounds	27.3 (23.3, 31.3)	100	274.1 (163.2, 387.1)	100
Monoterpenoids	26.1 (22.1, 30.0)	100	297.7 (1.87.8, 408.6)	100
Sesquiterpenoids	1.10 (0.87, 1.32)	100	−25.3 (−34.6, −16.2)	100
Estragole	0.06 (0.03, 0.08)	99.9	1.33 (0.78, 1.88)	100

Note: The median effect size (ES) and 95% Bayesian credible intervals (in parentheses) are shown. The marginal probability (MP) is the probability that coefficient estimates are greater or less than zero, given the direction of the ES. A positive ES indicates *P. aristata* had a greater response, and negative ES indicates *P. flexilis* had a greater response. Values in boldface are estimates that are credibly different (MP > 90%).

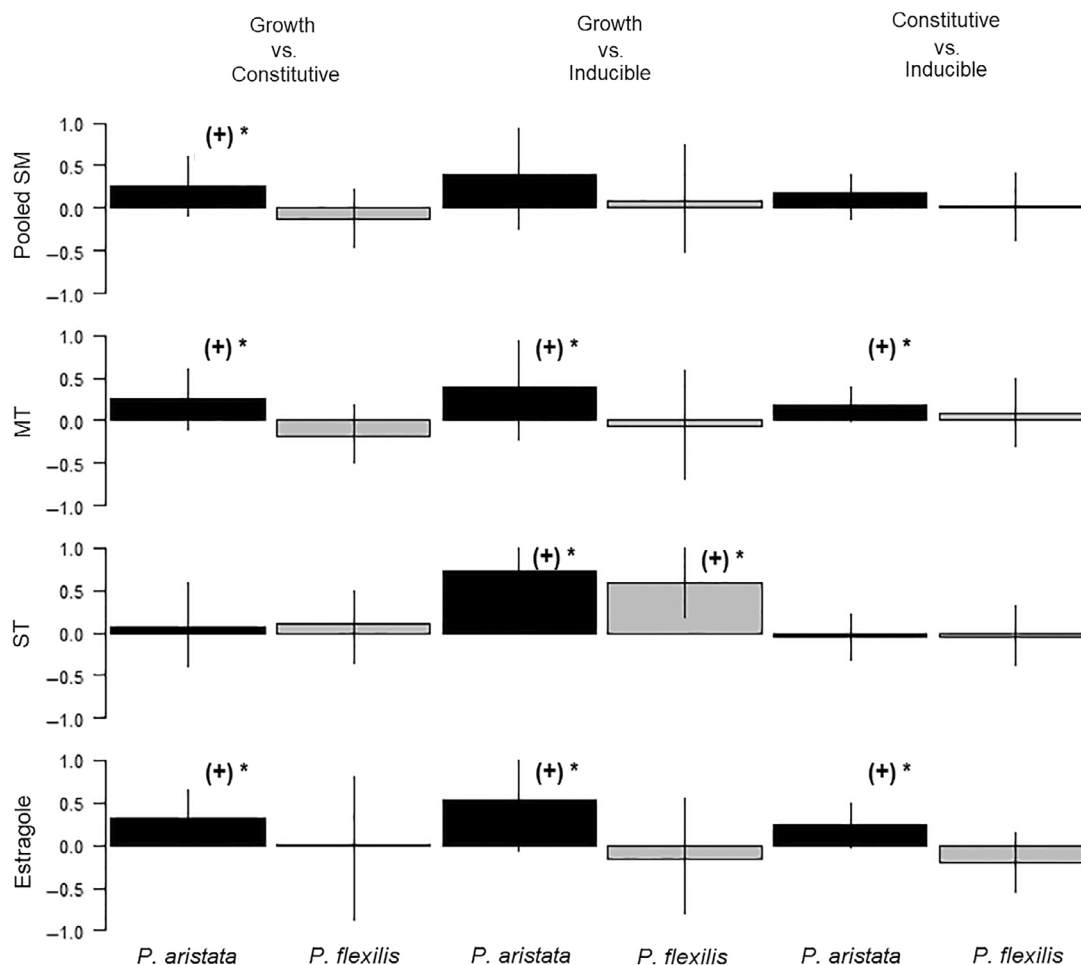


FIGURE 5 Relationships between growth rate (basal area increment) and constitutive and inducible (Δ_{I-C}) (Day 30 simulated mountain pine beetle attack – Day 0 constitutive) concentrations (H2) and between constitutive and inducible concentrations (H3) among populations of *Pinus aristata* and *P. flexilis*. Shown are concentrations of total compounds measured, monoterpenoids (MT), sesquiterpenoids (ST), and estragole (in milligrams per gram). Bayesian linear regression slope median estimates and 95% Bayesian credible intervals are shown. An asterisk (*) signifies a slope estimate that was credibly (marginal probability > 90%) different from zero, with corresponding directionality (e.g., “(+)”—positive) of the relationship. See Appendix S1: Table S9 for slope median estimates (effect size) and Bayesian 95% credible intervals (CI_{Bayes}) for all shown relationships. SM, secondary metabolite.

H3: Constitutive-induced trade-offs

P. aristata had greater concentrations of both constitutive and inducible monoterpenoids, estragole, and total compounds (MPs > 99.9%) than *P. flexilis*, although *P. flexilis* induced greater concentrations of sesquiterpenoids (Table 4). At the intraspecific level, the relationship between constitutive and inducible defenses in *P. aristata* was positive and credible for monoterpenoids and estragole, with no credible relationships between defense types in *P. flexilis* (Figure 5; Appendix S1: Table S9). For individual compounds, relationships between *P. aristata* and *P. flexilis* were neutral to positive (Appendix S1: Table S10). Therefore, there were no credible negative correlations for either species, suggesting no trade-offs between constitutive and inducible resin defenses.

In *P. aristata*, we found that the two most climatically favorable sites had greater constitutive and inducible concentrations of monoterpenoids and total compounds than the three other more northern sites (MPs > 99.9%; Appendix S1: Table S8). Although site-level differences in constitutive concentrations were not found in *P. flexilis*, across all sites concentrations of inducible sesquiterpenoids and total compounds were greatest at the two climatically favorable sites (MPs > 85.0%) where tree growth was also the greatest (Figure 4). Hierarchical cluster analysis using constitutive and Day-30 inducible proportions identified single chemotypes based on predominant monoterpenoid compounds in *P. aristata* (δ -3-carene), and two chemotypes in *P. flexilis* (δ -3-carene: bootstrap p value = 0.03 or (+)- α -pinene: bootstrap p value = 0.03).

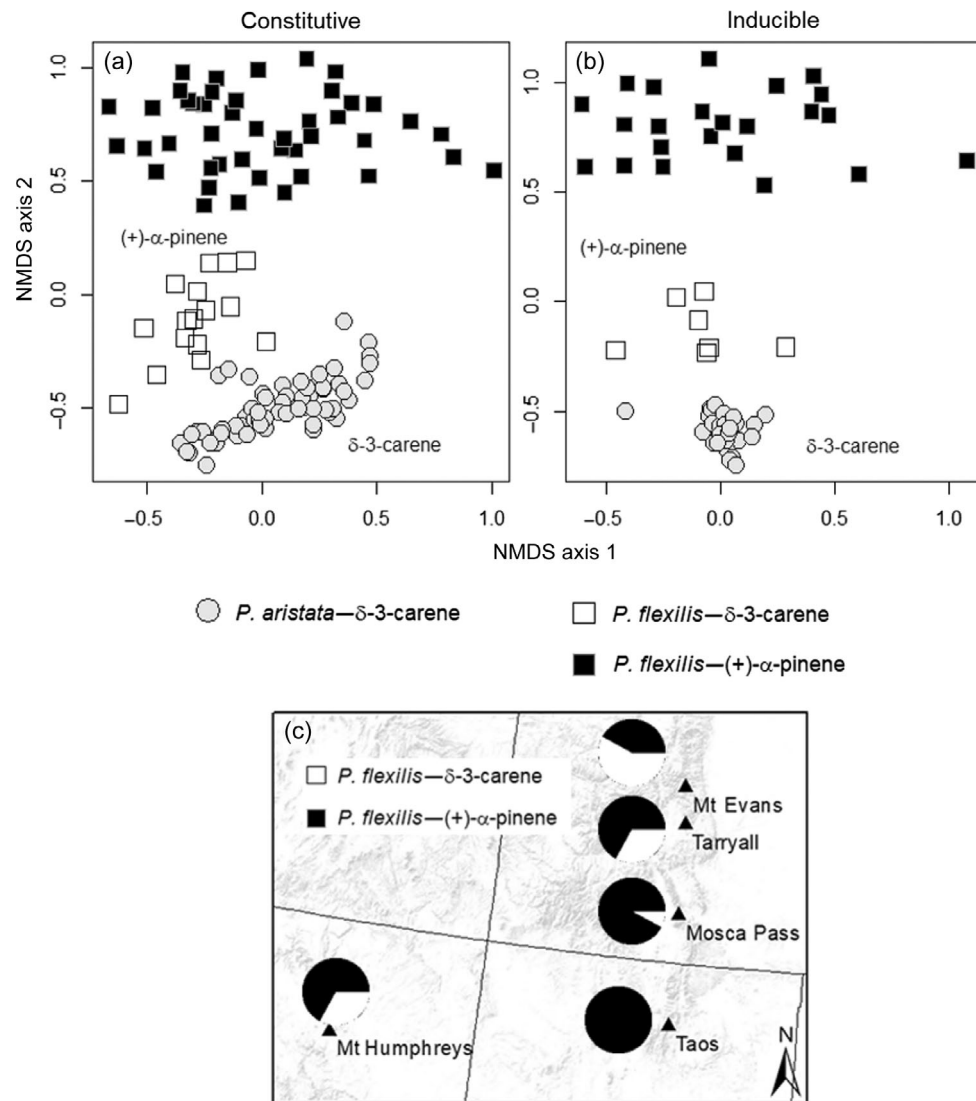


FIGURE 6 Nonmetric multidimensional scaling (NMDS) ordination of constitutive (Day 0) and inducible (Δ_{I-C}) (Day 30 simulated MPB attack – Day 0 constitutive) concentrations of monoterpenoids in *Pinus aristata* and *P. flexilis* (a, b). Two-dimensional stress values for the ordinations were 0.1173 for constitutive concentrations and 0.0921 for inducible (Δ_{I-C}) concentrations. Point character and/or shading differences separate species and respective chemotype (predominant compound within the resin composition). *P. flexilis* chemotype proportions across study sites (c) show both site variability and geographic structuring with respect to the dominant compound within its resin chemistry profile.

(Figure 6a,b). Within *P. flexilis*, the δ -3-carene chemotype was predominant at Mt Evans, CO; Tarryall, CO; and Mt Humphreys, AZ (Figure 6c). In contrast, (+)- α -pinene was the predominate chemotype at Mosca Pass, CO; and Taos, NM. At Mosca Pass, CO, only one tree contained δ -3-carene, which was completely absent in all trees at Taos, NM. These results suggest both intrasite variation and geographic population structure by chemotype within our sampled range (Figure 6c). Importantly, all individuals of *P. aristata* and *P. flexilis* presented the same chemotypes in both constitutive and inducible resin chemistry profiles (Figure 6a,b).

DISCUSSION

Warming temperatures associated with climate change are threatening high-elevation pines by increasing MPB population survival and persistence (Bentz et al., 2016; Buotte et al., 2016). Multiple high-elevation pine species have experienced extensive MPB-caused tree mortality in recent decades (Buotte et al., 2016; Millar et al., 2012), including *P. aristata* and *P. flexilis* (Bentz et al., 2021; Cleaver et al., 2015). To gain insight into defensive strategies of these two long-lived pine species, we investigated chemical defenses, including constitutive concentrations and induction timing (H1), and potential trade-offs

between growth and defense (H2) and between defense types (H3) between *P. aristata* and *P. flexilis* growing in the same stands, and among populations of each species in the same stands at five sites across a latitudinal gradient.

H1: Timing and composition of induction

Contrary to our expectations, we did not find an induced resin chemical response to a simulated MPB attack in either species within 4 days that was greater than the response to mechanical wounding alone. The induced response in both species at Day 30, however, was greater than the response to mechanical wounding alone and also greater than the response to constitutive concentrations. Proportions of specific resin compounds also did not change within 4 days of either treatment. These results highlight that a response specific to MPB likely does not occur within the first few days of attack. Similar to our results, Raffa and Smalley (1995) showed an induced resin response to simulated bark beetle attack within 3 days, with a greater change in concentration than proportionate compositions, although they did not compare with wounding only at that time period. In contrast to our results, Burke et al. (2017) demonstrated an induced response to MPB-simulated attack within 4 days that was greater than the response to methyl jasmonate but only in trees located at a single site. While it is clear that pines have a rapid initial response to simulated bark beetle attacks, our results demonstrate this response likely does not differ from mechanical wounding alone. A rapid (i.e., within 4 days) induced response in *P. aristata* and *P. flexilis* may therefore be considered adaptive to generalist herbivores or injury (Karban, 2020), rather than specific to MPB and its fungal associates. We cannot, however, rule out a role for these compounds in impeding MPB pheromone communication (Erbilgin et al., 2003, 2006; Schiebe et al., 2012).

H2: Growth–defense trade-offs

As expected, *P. aristata* had higher concentrations of all measured constitutive secondary metabolite classes and compounds than *P. flexilis* at all sites. *Pinus aristata* growth, however, was only slower than *P. flexilis* at the two sites with the most favorable climate. As predicted, the faster growing *P. flexilis* invested less in constitutive defenses at these two sites. A trade-off between growth and constitutive defenses between the species was therefore found but only at the two most climatically favorable sites. Several traits in both species that were positively

related to the climatic favorability at these two sites were as follows: (1) young age, despite being of similar size to other sites; (2) high growth rate; (3) thick phloem; and (4) high levels of inducible concentrations, suggesting an influence of resource quality and phloem thickness on resin defenses as has been found in other conifers (Hood & Sala, 2015; Huang et al., 2019; López-Goldar et al., 2020). The low concentrations of defensive compounds in *P. flexilis* across all sites, compared with other pines, have been found previously (Bentz et al., 2017; Ferrenberg et al., 2017) and may be related to the broad distribution of this species (Burns & Honkala, 1990). We also found no negative correlations between resin defense compound concentrations and growth among populations within either species, supporting our hypothesis of no trade-off between these fitness traits in intra-specific comparisons. However, intraspecific variation in defenses can be influenced by plastic responses to environmentally determined cues, potentially reducing or eliminating signatures of trade-offs between growth and defenses or between the two defense types (Hahn et al., 2019; López-Goldar et al., 2019).

H3: Constitutive-induced trade-offs

In contrast to our expectations, the species with the greatest constitutive concentrations, *P. aristata*, also had greater inducible monoterpenoids and estragole (after 30 days) than *P. flexilis*. Similarly, positive or neutral relationships were found between the two defense types among populations within the two species. These results add to the growing body of evidence, suggesting a lack of trade-off between constitutive and induced resin defenses in plants and that investment in both is likely not redundant (Brody & Karban, 1992; Howe et al., 2020; Koricheva et al., 2004). Because the two species were growing in the same environment, our results also suggest different inherent defense strategies between the species. Overall, *P. aristata* had greater concentrations of constitutive and induced monoterpenoids, sesquiterpenoids, and estragole, although the proportional increase in mono- and sesquiterpenoids at all time periods was greater in *P. flexilis*. While absolute resin concentration increases are considered to confer greater resistance than proportional increases (Morris et al., 2016), a proportional increase in key compounds with biological activity could be advantageous (Raffa et al., 2005). The specific compounds that were induced also varied between the species. Increases in monoterpenoids were greater in *P. aristata*, and sesquiterpenoids were more inducible in *P. flexilis*. *P. albicaulis*, another high-elevation five-needle pine, was

also found to contain greater constitutive and induced sesquiterpenes than *P. contorta* (Kichas et al., 2021; Raffa et al., 2017). Our results support the hypothesis that defense strategies among pine species may differ not only in total concentrations but also in the secondary metabolite class and its proportional level of investment (López-Goldar et al., 2019; Mason et al., 2019).

The terpene composition of pine phloem can have both genetic and environmental influences, often resulting in distinct chemotypes (individuals that vary in their chemistry and defined by the dominant compound produced) among and within populations (Baradat & Yazdani, 1988; Davis & Hofstetter, 2012; Forrest, 1980; Hanover, 1966; Smith, 2000; Thoss et al., 2007). For example, three constitutive chemotypes were found within stands and across the extensive range of jack pine (*P. banksiana* Lamb.) (Taft et al., 2015) and the proportion of two chemotypes in *P. ponderosa* also differed between populations in Arizona and Colorado (Latta et al., 2003). Across the range of *P. aristata* in Colorado, New Mexico, and Arizona, we found no evidence of multiple chemotypes, with δ -3-carene dominant in all individuals in both the constitutive and inducible resin chemistry profiles. Zavarin et al. (1976) also found that δ -3-carene was predominant across the range of *P. aristata*. Although we only sampled a portion of the *P. flexilis* distribution, we observed two chemotypes, (-)- α -pinene and δ -3-carene, with the ratios of the two varying across the sites. The δ -3-carene chemotype was predominant at the northern Mt Evans, CO; Tarryall, CO; and Mt Humphreys, AZ sites, but absent in Taos, NM, and present in a single tree at Mosca Pass, CO. Similarly, Zavarin et al. (1993) and Bentz et al. (2017) found that (+)- α -pinene or δ -3-carene were predominant compounds in *P. flexilis*, and both studies found variability among populations, with the δ -3-carene chemotype absent in some populations. Across all individuals, the predominant compound remained consistent in constitutive and inducible samples, suggesting that constitutive and inducible defenses share the same metabolic pathways for these compounds (Keeling & Bohlmann, 2006). δ -3-carene and (+)- α -pinene chemotypes are common in multiple conifer species and often vary among populations (Davis & Hofstetter, 2012; Emerick et al., 2008; Erbilgin, 2019; Kannaste et al., 2013; Latta et al., 2003), highlighting the roles of both genetic and environmental influences on resin chemistry composition. As variation in the chemical makeup of *Pinus* resin may facilitate ecosystem processes through a variety of mechanisms (e.g., Adler & Karban, 1994), including interactions within the MPB–fungi–bacteria complex (Adams et al., 2009, 2011), an increased understanding of the composition of these heritable traits may have

important implications for future management and forest dynamic forecasting.

Significance of a delayed induced response

Although we did not observe a response specific to simulated MPB attack at 4 days post-inoculation, Keefover-Ring et al. (2016) reported a greater induced response 17 days following a simulated MPB attack than to trees with mechanical wounding alone. Our results suggest that this delayed response extends to at least 30 days. In both species, we found that on Day 30, an induced response to simulated MPB attack was >19-fold greater than to constitutive concentrations and also greater than the response in mechanically wounded trees. In previous studies, elevated monoterpenoid levels in MPB-attacked *P. contorta* were found 6–8 weeks post-attack (Clark et al., 2012; Roth et al., 2018) and 6 (Goodsman et al., 2013) and 8 (Erbilgin & Colgan, 2012) weeks post-fungal inoculation, suggesting that the temporal extent of elevated resin defense levels extends beyond our 30-day sample. In addition to increases in overall resin concentrations, by Day 30, monoterpenoid diversity decreased in both species in response to simulated MPB attack relative to the constitutive resin chemistry profile, and relative proportions of specific compounds differed between simulated MPB attack and mechanically wounded trees. Others have found similar changes in simulated MPB attack treatments with a disproportionate increase in compounds with fungicidal or insecticidal properties (Keefover-Ring et al., 2016; Raffa et al., 2017; Tomlin et al., 2000). δ -3-carene, which exhibited the largest proportional increase in response to simulated MPB attack in *P. aristata*, can be toxic to MPB (Chiu et al., 2017; Reid et al., 2017), can disrupt MPB pheromone communication (Borden et al., 2008), can inhibit growth of MPB-associated bacteria (Adams et al., 2011; Raffa et al., 2005), and was found in higher concentrations in MPB-resistant *P. contorta* (Erbilgin, Cale, Hussain, et al., 2017). *P. aristata* also induced greater concentrations of estragole, a compound that may inhibit attraction to bark beetle pheromones (Hayes & Strom, 1994). (+)- α -pinene and terpinolene, compounds that are increased in representative proportion within both species, are known MPB pheromone synergists or attractants, and were also found to be less concentrated in resistant trees (Erbilgin, Cale, Hussain, et al., 2017). Both *P. aristata* and *P. flexilis* therefore responded to a simulated MPB attack with compounds that are known to have both positive and negative effects on MPB host colonization.

A delay in specific compounds that were induced in response to a simulated MPB attack could be due to

physiological limitations on the synthesis and mobilization of defenses (reviewed in Keeling & Bohlmann, 2006). In addition, other secondary metabolites that we did not measure, including phenolics and diterpenes, both of which have shown fungicidal effects within conifers (Evensen et al., 2000; Hammerbacher et al., 2013; Hart, 1981; Kopper et al., 2005), could have been rapidly induced. Alternatively, the delayed but sustained response we observed could be adaptive, targeting fungal symbiont growth (i.e., in an effort to compartmentalize spread), and/or MPB eggs and neonates that may be more vulnerable than adult beetles to resin toxins (Raffa et al., 2005; Raffa & Berryman, 1983). Although investment in prolonged elevated defenses is costly (Gershenson, 1994), a delayed induced response is common among plants (Karban, 2011). Whereas investment in prolonged induced defenses in short-lived plants may prove maladaptive (Wilkinson et al., 2019), our observations suggest that prolonged induced defenses in long-lived conifers may be adaptive against MPB attack and developing brood (Clark et al., 2012).

CONCLUSION

P. aristata and *P. flexilis* are five-needle white pines that are long-lived, slow-growing, and typically found at high elevations. *P. flexilis* often co-occurs across the range of *P. aristata*, although *P. flexilis* has a much larger distribution, and the two species appear to be attacked by MPB at similar frequencies when growing in the same stand (Bentz et al., 2021). Both species had a delayed (i.e., >4 days) but strong induced response to simulated MPB attack. From an evolutionary perspective, a delayed response could be advantageous for the tree in terms of reducing fungal symbiont spread and increasing the toxic environment of the developing bark beetle brood. Despite their similar growing conditions, susceptibility to MPB, and strong induced response, we found that the two species have evolved different strategies in terms of concentration and type of resin terpenoid defenses. *P. aristata* had higher concentrations of both constitutive and inducible resin defenses, predominately monoterpenoids, and *P. flexilis* induced greater concentrations of sesquiterpenoids. Our findings are consistent with other studies showing that co-occurring *Pinus* species can have varying defense strategies despite similar MPB attack levels (Bentz et al., 2015; West et al., 2014, 2016), highlighting the diversity of defensive tactics and resource allocation strategies among and within *Pinus* species. The observed neutral or positive correlations between constitutive and induced terpenoid resin defenses suggest that these defense strategies may not be

redundant in *Pinus*. Finally, similar to other conifers (Huang et al., 2019; Kichas et al., 2021), resource quality likely influences *P. aristata* and *P. flexilis* growth and defense strategies. Interspecific trade-offs between growth and resin defenses were only found at the two most climatically favorable sites where absolute concentrations of constitutive and inducible terpenoids were greatest, and trees of both species were the youngest and the fastest growing.

AUTHOR CONTRIBUTIONS

The experiment was designed by Barbara J. Bentz, David N. Soderberg, Justin B. Runyon, and Sharon M. Hood. David N. Soderberg and Barbara J. Bentz collected the data. David N. Soderberg and Justin B. Runyon conducted resin chemistry analyses. David N. Soderberg conducted the statistical analysis and led the writing of the manuscript with assistance from Barbara J. Bentz, Justin B. Runyon, Sharon M. Hood, and Karen E. Mock.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Soderberg, 2022) are available from Dryad: <https://doi.org/10.5061/dryad.fxpvnv0r1>.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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