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Research paper

Drought impairs herbivore-induced volatile terpene emissions by ponderosa pine but not through constraints on newly assimilated carbon

Shealyn C. Malone[1,2,5,†], Austin Simonpietri[2,3,†], Walter B. Knighton[4] and Amy M. Trowbridge[1,2]

[1]Department of Forest and Wildlife Ecology, University of Wisconsin, Madison, WI 53711, USA; [2]Department of Land Resources & Environmental Sciences, Montana State University, Bozeman, MT 59717, USA; [3]Center for Ecosystem Science and Society, Northern Arizona University, Flagstaff, AZ 86011, USA; [4]Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, USA; [5]Corresponding author scmalone2@wisc.edu

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Volatile terpenes serve multiple biological roles including tree resistance against herbivores. The increased frequency and severity of drought stress observed in forests across the globe may hinder trees from producing defense-related volatiles in response to biotic stress. To assess how drought-induced physiological stress alters volatile emissions alone and in combination with a biotic challenge, we monitored pre-dawn water potential, gas-exchange, needle terpene concentrations and terpene volatile emissions of ponderosa pine (*Pinus ponderosa*) saplings during three periods of drought and in response to simulated herbivory via methyl jasmonate application. Although 3-, 6- and 7-week drought treatments reduced net photosynthetic rates by 20, 89 and 105%, respectively, the magnitude of volatile fluxes remained generally resistant to drought. Herbivore-induced emissions, however, exhibited threshold-like behavior; saplings were unable to induce emissions above constitutive levels when pre-dawn water potentials were below the approximate zero-assimilation point. By comparing compositional shifts in emissions to needle terpene concentrations, we found evidence that drought effects on constitutive and herbivore-induced volatile flux and composition are primarily via constraints on the de novo fraction, suggesting that reduced photosynthesis during drought limits the carbon substrate available for de novo volatile synthesis. However, results from a subsequent $^{13}CO_2$ pulse-chase labeling experiment then confirmed that both constitutive ($<3\%$ labeled) and herbivore-induced ($<8\%$ labeled) de novo emissions from ponderosa pine are synthesized predominantly from older carbon sources with little contribution from new photosynthates. Taken together, we provide evidence that in ponderosa pine, drought does not constrain herbivore-induced de novo emissions through substrate limitation via reduced photosynthesis, but rather through more sophisticated molecular and/or biophysical mechanisms that manifest as saplings reach the zero-assimilation point. These results highlight the importance of considering drought severity when assessing impacts on the herbivore-induced response and suggest that drought-altered volatile metabolism constrains induced emissions once a physiological threshold is surpassed.

Keywords: $^{13}CO_2$ pulse-chase labeling, drought, herbivory, methyl jasmonate, terpenes, volatile organic compounds (VOC).

Introduction

Forest ecosystems are major sources of volatile terpenes that serve numerous physiological, atmospheric and ecological roles (Tholl 2015, Boncan et al. 2020). Volatile terpenes can help plants alleviate environmental stress, for example, by protecting membrane integrity and quenching reactive oxygen species (Vickers et al. 2009, Loreto and Schnitzler 2010). They can also directly induce biosynthetic pathways involved in plant chemical defense and resistance by serving as metabolic regulators of cellular signaling networks (Erb and Kliebenstein 2020, Monson et al. 2021*a*). Once emitted to the atmosphere, terpenes impact tropospheric chemistry and regional climate (Wu et al. 2020),

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[†]These authors contributed equally to this work.

whereas at the organismal level they play several ecological functions including attracting mutualists, deterring herbivores, pathogen resistance and recruiting natural enemies (Trowbridge and Stoy 2013). The co-occurrence of drought and herbivory has emerged as a major stressor to forest ecosystems (Bansal et al. 2013) and is altering volatile fluxes (Trowbridge et al. 2014) with cascading effects on atmospheric, physiological and ecological processes. Despite this, the mechanistic underpinnings of drought × herbivory-induced shifts in volatile synthesis and emission across conifer species remain elusive and the levels of physiological stress that must be surpassed to observe such changes have been largely unexplored.

In conifers like the Pinaceae family, needle terpene emissions originate from both stored terpene resins and de novo biosynthesis (Ghirardo et al. 2010). Stored terpenes experience very little metabolic turnover (Gershenzon et al. 1993, Rissanen et al. 2021) and volatilization is largely temperature-driven. Similar to stored pools, de novo synthesis and emission patterns are also dependent on prevailing temperature conditions, but in contrast are concurrently controlled by numerous genes and biosynthetic pathways and tend to be more tightly coupled to photosynthesis (Loreto and Schnitzler 2010). Stable isotope-labeling studies in conifers have shown that up to ~80% of the carbon substrate used to synthesize constitutive de novo terpene emissions can originate from recently fixed carbon (Ghirardo et al. 2010), with the remaining substrate originating from older xylem-transported and/or leaf-stored carbon sources (Kreuzwieser et al. 2002, Harley et al. 2014, Wu et al. 2017). Furthermore, the relative contribution of these de novo terpene pools to the overall constitutive emission profile is highly variable (ranging from ~10 to 60% among studied species) (Wu et al. 2017, Ghirardo et al. 2020) and remains relatively unknown among most conifer taxa. Studies have shown that the relative contribution of newly fixed versus older carbon toward constitutive de novo synthesis changes in response to environmental stressors, with light and temperature being the most important drivers (Lüpke et al. 2017, Wu et al. 2017, Kreuzwieser et al. 2021). In addition, carbon allocation dynamics associated with de novo synthesis can also be affected by specific stressors that induce emissions via oxidative (e.g., drought, excessive light/temperature) or mechanical stress (e.g., herbivory, wind) (Grote et al. 2019). Although stressors such as drought and herbivory have been shown to independently alter carbon allocation patterns towards de novo terpene emissions, the consequences in the face of both stressors concurrently remain relatively unknown.

The ways in which drought and herbivory modulate canopy-level emissions depend on how they interact to affect terpene synthesis and emissions from both stored and de novo pools. In terms of drought, recent studies show sophisticated molecular regulation of resource allocation toward volatile terpene production under carbon-limiting conditions (Huang et al. 2019, Kreuzwieser et al. 2021). For example, drought-stressed Norway spruce (*Picea abies*) can redirect carbon metabolism to increase the contribution of stored carbon toward maintaining monoterpene emissions in the absence of photosynthesis (Kreuzwieser et al. 2021). This may explain why terpene emission rates remain relatively stable under mild and moderate drought stress (but not always; Lüpke et al. 2017) and diminish only when drought is severe (Llusià and Peñuelas 1998, Lavoir et al. 2009, Kreuzwieser et al. 2021, Werner et al. 2021). This, however, assumes that de novo-produced terpenes represent a significant fraction of the overall emissions, and that much of these de novo terpenes are derived from new photoassimilates, which may not be the case for all conifer species. For example, in ponderosa pine (*Pinus ponderosa*), terpene emissions appear to be largely driven by volatilization from stored needle pools (Lerdau et al. 1994) with minimal contribution from de novo terpene synthesis. As such, drought-induced perturbations to photosynthesis and the availability of new photosynthates may have little to no effect on net emissions, but this has yet to be tested.

In contrast to drought, herbivory has been shown to consistently alter induced de novo synthesis of volatiles. Although terpene emissions increase immediately following herbivory via volatilization from damaged storage structures, herbivore-associated molecular patterns (HAMPs) also induce de novo synthesis of terpene volatiles in the hours, days and weeks that follow through jasmonic acid-activation of terpene-related enzymes (Martin et al. 2002, Miller et al. 2005). Studies using $^{13}CO_2$-labeling approaches have shown that induced de novo terpene emissions by multiple conifer species are synthesized predominantly from newly fixed carbon (Miller et al. 2005, Wu et al. 2017). This suggests that conditions that limit carbon fixation, such as drought, may lead to a constrained herbivore-induced response by reducing substrate availability via supply-side processes. Yet, recent conceptual frameworks have suggested that demand-side cellular coordination mechanisms (i.e., metabolic regulation) are also at play and may in fact dominate trait responses to stress (Monson et al. 2021*b*). We thus require a better understanding of the relative importance of substrate availability and metabolic regulation in explaining resource allocation dynamics under stress conditions.

Trees are often exposed to multiple stressors, and it is likely that the severity and duration of prior stress events determine the metabolic shifts affecting trait responses to subsequent stress events (Holopainen and Gershenzon 2010). For example, mild drought has been shown to prime trees to increase herbivore-induced volatile emissions (Copolovici et al. 2014), but whether these herbivore-induced responses can persist as drought stress increases is unclear. Thus, knowledge about the level of drought stress and the physiological status of a tree are critical in predicting responses to future biotic attack. This is because herbivore-induced emissions demand a relatively high flux of carbon for synthesis, and therefore the resource and/or metabolic buffers that maintain herbivore-induced emissions during mild drought may not suffice when trees are experiencing more severe drought stress. Rather, trade-offs between drought- and herbivore-resistance may quickly emerge as pools of available carbon become increasingly limited under more severe drought stress (McDowell et al. 2011, Huang et al. 2020, Erbilgin et al. 2021). Prolonged drought stress may also trigger the downregulation of volatile terpene synthesis in favor of supporting other processes essential to survival during drought (i.e., osmoregulation, maintenance respiration, etc.) (Perreca et al. 2022).

Here we investigated the impact of varying levels of drought stress on the physiology of ponderosa pine saplings and the effects on carbon contributions to herbivore-induced mono- and sesqui-terpene emissions. Ponderosa pine is a dominant species of North American mid-elevation forests, but vegetation models predict range contraction in the face of hotter droughts and increased biotic disturbances (Rehfeldt et al. 2014). Sapling regeneration of this species faces similar threats and is projected to drastically decrease in the second half of the 21st century (Petrie et al. 2017), emphasizing the need to understand how saplings will respond to drought in combination with biotic stressors like herbivory. Regenerating ponderosa pine saplings are susceptible to numerous mining and defoliating insects, including pine tip moth (*Rhyacionia* spp.), ponderosa pine budworm (*Choristoneura lambertiana*), pine butterfly (*Neophasia menapia*) and pine needle-sheath miner (*Zelleria haimbachi*) that due to their chewing nature, are likely to upregulate terpene defense pathways via the phytohormone methyl jasmonate (MeJA) (Walling 2000, Thaler et al. 2012).

To understand the independent and interactive effects of drought and herbivory on terpene volatile emissions, ponderosa pine saplings were either well-watered or exposed to three levels of drought. We then elicited a herbivore-induced volatile response (herein referred to as either 'herbivore-induced' or 'MeJA-induced') from trees in each group by applying MeJA. MeJA induces emissions like the biotic agents themselves (Miller et al. 2005), and importantly, allowed us to assess de novo emissions independent of mechanical damage that would increase emissions through volatilization from stored resin. In tandem with sampling volatile emissions, we also measured needle terpene concentrations to determine the degree to which foliar concentrations explained emissions across treatments via volatilization from stored pools. Finally, to assess the contribution of new and stored carbon toward constitutive and herbivore-induced emissions independent of drought, we subsequently performed a $^{13}CO_2$-labeling experiment on well-watered trees treated with MeJA. Given the different pools and mechanisms controlling drought- and herbivore-induced terpene emissions, we hypothesized that if emissions by ponderosa pine are primarily governed by volatilization from stored pools, then terpene emissions will remain largely unaffected by reductions in photosynthesis during all levels of drought stress. We expected that constitutive and drought-induced emissions would be similar in composition to the needle terpene pools from which they originate, and that the constitutive emissions from well-watered trees would exhibit low incorporation of the ^{13}C-label. We then hypothesized that if herbivore-induced emissions are driven by the volatilization of newly synthesized (de novo) terpenes, reliance on new photoassimilates for their synthesis would make them more sensitive to drought. Specifically, herbivore-induced emissions would decrease shortly after the drought-caused cessation of photosynthesis due to a lack of substrate. If herbivore-induced emissions are indeed primarily of de novo origin and derived from newly fixed carbon, then we expected that the herbivore-induced emissions of well-watered trees would differ substantially from the needle compositional profile and exhibit high incorporation of the ^{13}C-label.

Materials and methods

Plant material and greenhouse conditions

In March of 2018, we obtained 2-year-old ponderosa pine saplings from the Colorado State Forest Service Seedling Tree Nursery (Fort Collins, CO, USA). Rooted saplings were planted in 2 l pots with Sunshine Mix #1 soil (Gro Horticulture Inc., Bellevue, WA, USA) and placed in the Montana State Plant Growth Center where they acclimated for 3 months prior to experimental treatments. Throughout the course of the study, supplemental light maintained a 16 h photoperiod (GE Multi-Vapor MVR1000/C/U, GE Lighting, Cleveland, OH, USA), and mean day and night temperatures were 22 and 20 °C, respectively (±1.5 °C). Saplings were fertilized once per week with 20-20-20 NPK GP Scott's Fertilizer (Marysville, OH, USA), watered to field capacity every other day and rotated biweekly to control for environmental variations along the greenhouse bench. During summer 2018, a subset of saplings ($n = 6$) was randomly selected for the $^{13}CO_2$-labeling experiment and the remaining saplings ($n = 71$) were used in the herbivory and drought experiment (Figure S1, available as Supplementary data at *Tree Physiology* Online). To manage greenhouse aphids, all saplings received a single application of M-Pede insecticidal soap (Gowan Company, Yuma, AZ, USA) just prior to the start of the drought treatment.

Drought and MeJA treatments

To explore the interaction of drought and simulated herbivory on terpene production and emissions, saplings were randomly assigned to one of four treatments: well-watered (W, $n = 18$), well-watered with MeJA (W + MJ, $n = 17$), drought-stressed (D, $n = 18$) and drought-stressed with MeJA (D + MJ, $n = 18$). On 26 July 2018, all saplings were watered to field capacity, after which water was withheld from saplings assigned to drought treatments (D and D + MJ). Watered treatments (W and

W + MJ) continued to receive water every other day. Variation in drought intensity was employed by withholding water for either 3, 6 or 7 weeks. At the conclusion of each drought period, we sampled saplings from all four treatments ($n = 6$, except at 6 weeks when $n_{\mathrm{W+MJ}} = 5$).

To determine the effects of simulated herbivory on terpene emissions of well-watered and drought-stressed trees, saplings were treated with a solution of either MeJA (W + MJ and D + MJ treatments) or a control solution (W and D treatments) 1 day prior to volatile collections. Saplings treated with MeJA were sprayed with a solution of 10 mM MeJA (Sigma-Aldrich, St Louis, MO, USA) in distilled water and 0.1% Tween 20 (Sigma-Aldrich), a surfactant that solubilizes MeJA to increase MeJA penetration of the needles and stem (Martin et al. 2002). Saplings that were not treated with MeJA were instead sprayed with a solution of 0.1% Tween 20 dissolved in distilled water. Prior to spraying, pots were covered with foil to avoid drainage of excess solution into the soil. To achieve full saturation of the leaf tissues and sufficient uptake of solution, saplings were placed in a fume hood, sprayed with 25 ml of either solution and left to dry for 1 h under illumination. To avoid contamination of MeJA and volatile signaling among trees, Tween and MeJA-treated saplings were sprayed and sampled on separate, consecutive days (Figure S1, available as Supplementary data at *Tree Physiology* Online). Volatile terpene emission measurements were conducted in separate but adjacent greenhouse rooms that maintained the same temperature, humidity and light:dark cycle as the room in which saplings were maintained throughout the study.

Pre-dawn water potential and gas-exchange measurements

To characterize the level of drought-stress experienced by individual saplings, we determined the stem pre-dawn water potential (Ψ_{pd}) using a Scholander pressure chamber (PMS Instruments, Albany, OR, USA). Because this method requires destructive harvest of the saplings, which would alter terpene emissions, water potential measurements were taken during the pre-dawn hours immediately following volatile collections.

To assess the effect of water availability on needle physiology, we measured net photosynthesis (A) and stomatal conductance (g_s) from a random subset of watered and drought-stressed saplings (n = 4–8 trees) prior to the application of MeJA or control spray. We used a portable photosynthesis system with a transparent conifer chamber attachment (LI-6400 infrared gas-exchange analyzer system, Li-Cor, Lincoln, NE, USA) and all measurements were taken at 400 p.p.m. CO_2 with a flow rate of 500 μmol s^{-1}. Saplings were measured mid-morning under controlled greenhouse conditions (as described above) so as not to introduce diurnal biases in temperature and light over the course of the experiment. Measurements were corrected for needle area as described by Chen et al. (1997).

Volatile terpene emission collection

We measured volatile terpene emission rates from sapling canopies enclosed in chambers constructed from polyamide nylon bags (482 × 596 mm, Reynolds Consumer Products, Lake Forest, IL, USA) that were secured with garden twist ties below the lowest branch (Figure S2, available as Supplementary data at *Tree Physiology* Online). A custom-built push-pull pump system was used to create a dynamic headspace from which volatiles were collected. Diaphragm pumps (Reitschle Thomas, Sheboygan, WI, USA), powered by 12 V batteries were set to push air through Teflon tubing and pull air through tygon tubing. Flow rates were controlled with in-line rotameters (Brooks Instrument, Hatfield, PA, USA) and verified with a mass flow controller (Alicat Scientific, Tucson, AZ, USA) before and after every volatile collection.

To flush volatile compounds from the chamber prior to sampling, ambient air was filtered using two solid-phase adsorbent traps containing 30 mg of Super-Q (Alltech Associates, Inc., Deerfield, IL, USA) and delivered into the chamber at 500 ml min^{-1} for 10 min prior to sampling. Sample air was then collected by pulling headspace air over single solid-phase adsorbent traps at a flow rate of 450 ml min^{-1} for 2 h. Sample traps were then wrapped in aluminum foil, stored at 4 °C and eluted within 24 h for chemical analysis. We measured each chamber's temperature throughout the volatile collection period using Thermochron iButtons (iButtonLink Technology, Whitewater, WI, USA). To report volatile terpene emission rates on a per gram of fresh weight (gFW) basis, canopy needles were collected and weighed immediately after water potential measurements.

We used gas chromatography–mass spectrometry (GC–MS) to determine the identities and relative concentrations of hydrocarbon monoterpenes, oxygenated monoterpenes, monoterpene esters, hydrocarbon sesquiterpenes, oxygenated sesquiterpenes and sesquiterpene esters present in sapling emissions (Text S1, available as Supplementary data at *Tree Physiology* Online). Emission rates are reported as basal emission rates standardized to 25 °C (Guenther 1997) and are expressed as the amount of volatile compound emitted per gram of fresh tissue per hour (μg gFW^{-1} h^{-1}).

Needle tissue collection and terpene extraction

Immediately after volatile collection, six to eight fascicles of 1-year-old needles were harvested, flash frozen in liquid nitrogen and stored at −80 °C until extraction. To avoid volatilization of terpene metabolites, needles were ground in liquid nitrogen using a chilled mortar and pestle. Frozen needle powder was then weighed into a 2-dram glass vial (exact weights between 0.4 and 0.5 g) and terpenes were extracted in 4 ml of *n*-hexane (Fischer Scientific, Fair Lawn, NJ, USA) containing 0.1 μl ml^{-1} (+)-fenchone (Sigma-Aldrich) as an internal standard. Tissue samples were sealed with Teflon lined caps and allowed to soak for 1 week at −20 °C. Next, 100 μl of supernatant was transferred into a 200 μl glass insert placed in a 2 ml glass vial for chemical analysis. Needle extractions were analyzed according to Trowbridge et al. (2014) and are expressed as mg gFW^{-1}.

$^{13}CO_2$-labeling of individual saplings

To determine the relative contribution of newly assimilated carbon allocated toward constitutive and herbivore-induced terpene emissions from well-watered saplings, we employed a $^{13}CO_2$-pulse chase labeling approach (see Figure S3, available as Supplementary data at *Tree Physiology* Online for a detailed schematic). The experimental set-up consisted of a custom 7000 ml glass chamber (Sigma Scientific LLC, Micanopy, FL, USA) sealed at the top with a Teflon lid comprised of one inlet and one outlet port. One sapling would be placed in the chamber and a closed system was created by securing one end of an open Teflon bag (ClearBags, El Dorado Hills, CA, USA) around the base of the stem with a garden twist tie and the other end around the base of the glass chamber with a rubber band. The inlet air consisted of 300 standard cubic centimeters per minute (s.c.c.m.) of synthetic volatile-free air (21.0% v/v O_2, 79.0% v/v N_2, American Welding and Gas, Bozeman, MT, USA) that was humidified through a glass bubbler before mixing with an additional 1700 s.c.c.m. of synthetic air, creating ~15% relative humidity in the chamber. Flow rates were set using two mass flow controllers (Alicat Scientific, Tucson, AZ, USA) and ~ 0.85 s.c.c.m. of CO_2 was added to the inlet flow to supply ambient levels at 425 ± 15 p.p.m. that were confirmed with the empty chamber and an IRGA (infrared gas analyzer, LI-850, LI-COR Inc., Lincoln, NE, USA) immediately prior to placing the tree in the chamber. From the chamber outlet air, 600 s.c.c.m. was continuously directed into the IRGA via a mass flow controller and a sealed diaphragm pump to monitor CO_2 levels. Approximately 200 s.c.c.m. was pulled constantly by a proton transfer reaction-mass spectrometer (PTR-MS) for volatile emissions monitoring.

All labeling experiments took place at the Montana State Plant Growth Center in a temperature- and light-controlled growth room held at 25 °C and a 16:8-h light:dark cycle, respectively. Each sapling was moved from the greenhouse to the growth room housing the experimental set-up and allowed to acclimate for 48–72 h prior to the start of the labeling experiment. First, the empty chamber was purged with the synthetic air mixture until the chamber CO_2 concentration reached ~425 p.p.m., as confirmed by the IRGA. One tree was then placed in the chamber and allowed to equilibrate for 2 h prior to initiating volatile collection. The 2-h equilibration time not only ensured that the CO_2 concentration had stabilized, but also that any volatiles emitted because of handling the tree in the chamber had dissipated (as confirmed with the PTR-MS). Within 30 min of being placed in the chamber, photosynthesis caused a CO_2 drawdown to ~310 (±25) p.p.m. and an increase in relative humidity to ~50%. A bypass leg was plumbed into the IRGA sample line so that the flow could be either routed directly to the IRGA, or alternatively through an adsorption trap and then to the IRGA.

To determine the labeling dynamics of constitutive terpene emissions by ponderosa pine ($n = 3$), emissions were measured first under ambient $^{12}CO_2$ conditions and then after applying $^{13}CO_2$ in the light for 3 h. To measure volatile emissions under ambient $^{12}CO_2$ conditions, air flow was redirected through the bypass leg where 600 s.c.c.m. of air was pulled for 1 h through an adsorption trap containing 25 mg of Hayesep Q (Volatile Assay Systems, Rensslaer, NY, USA). Following sampling, the bypass flow path was valved off and the normal flow path was restored. The adsorption tube was then removed and wrapped in aluminum foil and analyzed using GC–MS (Text S1, available as Supplementary data at *Tree Physiology* Online). Following adsorbent collection of the volatiles under $^{12}CO_2$ conditions, the inlet CO_2 was switched to the $^{13}CO_2$ source (>99% $^{13}CO_2$, Cambridge Isotope Laboratories, Tewksbury, MA, USA) at the same flow rate and the tree was allowed to equilibrate to the new conditions over a 3-h period before again collecting volatile emissions for 1 h on another sample tube as described above. Because a single mass flow controller was used to introduce both the $^{12}CO_2$ and $^{13}CO_2$, it took ~1 h to purge the mass flow controller and chamber to a stable $^{13}CO_2$ environment. As such, the 3-h interval allowed the establishment of a stable $^{13}CO_2$ environment (~1 h) in addition to a 2-h equilibration period for the tree under the stable environment (as confirmed by PTR-MS).

To determine labeling of herbivore-induced emissions ($n = 3$), saplings were measured on two consecutive days. On the first day, pre-MeJA treatment emissions were measured under ambient $^{12}CO_2$ conditions as described above. At the conclusion of the sampling period on Day 1, the tree was then sprayed with MeJA solution as described above. The following day, ~16 h post-MeJA application, the tree was sampled again as described above, first in the presence of $^{12}CO_2$ and subsequently in the presence of $^{13}CO_2$ to measure ^{13}C incorporation into MeJA-induced emissions.

Calculation of ^{13}C-incorporation into volatile emissions

Using mean emission rates from the drought × herbivory experiment, we found that in well-watered trees, over 95% of the constitutive (W) and MeJA-induced terpene emissions (W + MJ) were due to the contribution of 17 and 22 terpene compounds, respectively (Tables 1 and 2). Therefore, to estimate the contribution of newly assimilated carbon toward constitutive and MeJA-induced terpene emissions, we calculated the mean percent ^{13}C-incorporation for these compounds, corrected for each compound's relative emission abundance, and then summed the values to determine the total percent ^{13}C-incorporation for constitutive ($n = 3$) and MeJA-induced volatiles ($n = 3$; Tables 1 and 2, respectively). We determined the percent ^{13}C-incorporation for each tree following

Table 1. Percent incorporation of newly assimilated $^{13}CO_2$ [mean (SD)] for the 17 most abundant terpenes constitutively emitted by well-watered ponderosa pine that together comprise > 95% of the total constitutive terpenes emitted.

Terpene	Percent relative abundance in constitutive emissions	*n*	*m*/*z*	% incorporation into individual compound	% contribution of incorporation into total emissions
δ-3-carene	27.6	3	na	0	0
(−)-β-pinene	21.8	3	na	0	0
(+)-α-pinene	13.0	3	na	0	0
(−)-α-pinene	6.5	3	na	0	0
SQT18 (α-farnesene)	6.3	3	161	29.03 (5.72)	1.83 (0.36)
SQT11 (*trans*-β-farnesene)	4.4	3	161	22.79 (4.29)	1.00 (0.19)
β-phellandrene	3.7	3	na	0	0
β-myrcene	2.0	3	na	0	0
terpinolene	1.8	3	na	0	0
S-(−)-limonene	1.7	3	na	0	0
bornyl acetate	1.5	3	na	0	0
trans-β-ocimene	1.0	3	na	0	0
caryophyllene	1.0	3	na	0	0
(−)-camphene	1.0	3	na	0	0
SQT16 (copaene)	0.8	2	na	0	0
SQT5 (longifolene)	0.6	3	na	0	0
γ-terpinene	0.6	3	na	0	0
Total ^{13}C-incorporation into constitutive emissions					2.83 (0.55)

n is the number of trees represented in the mean percent incorporation; not all trees emitted each compound and when emissions were present but low, percent incorporation was unquantifiable and thus omitted. ^{13}C-labeling of individual terpene compounds was determined by visual inspection of the mass spectra and when labeling was evident, percent incorporation was calculated using the fragment noted (*m*/*z*). ^{13}C-incorporation is expressed for individual compounds (% incorporation into individual compound) and in terms of total emissions (% contribution of incorporation into total emissions) where % incorporation into individual compounds is weighted by the percent relative abundance in constitutive emissions. Total ^{13}C-incorporation is expressed as a weighted average to represent the percent incorporation of ^{13}C into the volatile terpene profile. When synthetic standards were unavailable, the NIST mass spectral search program (NIST) was used for comparison of mass spectra to determine putative compound identities. The putative compound identity (in parentheses) is used only when the NIST match quality was greater than 80%. SQT = unidentified sesquiterpene, na = not applicable.

Ghirardo et al. (2020) and the methods are outlined in Text S2, available as Supplementary data at *Tree Physiology* Online

Statistical analyses

To assess the physiological effects of drought on saplings, we fit linear models with drought treatment (well-watered or drought-stressed), drought duration (3, 6 or 7 weeks) and their interaction as predictors, and pre-dawn water potential, net photosynthesis and stomatal conductance as the response variables. To assess the effect of drought-stress and MeJA on volatile emissions of individual compounds, classes of compounds (i.e., hydrocarbon monoterpenes, oxygenated monoterpenes and sesquiterpenes) and the sum of all measured compounds, hereafter called 'total', we fit linear models with treatment (W, W + MJ, D, D + MJ), drought duration and their interaction as the predictors. Model emission rates were square root-transformed to meet assumptions of normality. When predictor variables accounted for variation in the model, as determined by type II ANOVA, post hoc differences were assessed among treatments within a single drought duration using pairwise comparisons using the 'emmeans' function (Lenth 2022).

To assess compositional shifts in volatile emissions by ponderosa pine, we evaluated differences among treatments by calculating Bray–Curtis dissimilarities among individual trees (Oksanen et al. 2020), followed by permutational multivariate ANOVA (PERMANOVA; Anderson 2014) where treatment (W, W + MJ, D, D + MJ), drought duration and their interaction were the predictor variables. To visualize differences among treatment at each drought duration, we used non-metric multidimensional scaling (NMDS) and plotted the first two axes, and stress values were calculated to determine the NMDS ordination goodness-of-fit. To assess potential relationships between the relative abundance of needle terpenes with the relative abundance of constitutive and herbivore-induced emissions, we used correlation analysis (Harrell 2021). All analyses were performed in R version 1.2.5001 (R Core Team 2020).

Results

Drought decreases pre-dawn water potential, photosynthesis and stomatal conductance

Drought-stressed saplings experienced more negative Ψ_{pd} ($P < 0.0001$) and reduced A ($P < 0.0001$) and g_s ($P < 0.0001$) compared with well-watered saplings (Figure 1; Table S1, available as Supplementary data at *Tree Physiology* Online). At 3 weeks, Ψ_{pd} of drought-stressed trees was

Table 2. Percent incorporation of newly assimilated $^{13}CO_2$ [mean (SD)] for the 20 most abundant terpenes emitted by MeJA-induced, well-watered ponderosa pine that together comprise > 95% of the total induced terpenes emitted.

Terpene	Percent relative abundance in MeJA-induced emissions	*n*	*m/z*	% incorporation into individual compound	% contribution of incorporation into total emissions
SQT11 (*trans*-β-farnesene)	21.8	3	161	10.94 (3.75)	2.39 (0.82)
(−)-β-pinene	15.9	3	na	0	0
δ-3-carene	15.9	3	na	0	0
linalool	9.0	3	121	29.75 (7.45)	2.68 (0.67)
(+)-α-pinene	7.9	3	na	0	0
SQT8 (Bergamotene)	6.6	2	121	27.21 (11.44)	1.80 (0.76)
(−)-α-pinene	4.1	3	na	0	0
SQT18 (α-farnesene)	2.1	2	161	8.86 (2.31)	0.19 (0.05)
β-phellandrene	1.7	3	na	0	0
terpinolene	1.4	1	na	0	0
β-myrcene	1.4	3	93	4.76 (4.27)	0.07 (0.06)
SQT19 (bisabolene)	1.4	1	93	11.25 (na)	0.16 (na)
bornyl acetate	1.0	3	na	0	0
linalool oxide	1.0	3	111	21.96 (15.80)	0.22 (0.16)
S-(−)-limonene	0.9	3	na	0	0
(−)-camphene	0.7	3	na	0	0
trans-β-ocimene	0.7	3	93	0.62 (1.08)	0.004 (0.01)
caryophyllene	0.7	2	na	0	0
SQT16 (copaene)	0.5	3	na	0	0
SQT4	0.5	2	na	0	0
Total ^{13}C-incorporation into MeJA-induced emissions					7.49 (2.52)

n is the number of trees represented in the mean percent incorporation; not all trees emitted each compound and when emissions were present but low, percent incorporation was unquantifiable and thus omitted. ^{13}C-labeling of individual terpene compounds was determined by visual inspection of the mass spectra and when labeling was evident, percent incorporation was calculated using the fragment noted (*m/z*). ^{13}C-incorporation is expressed for individual compounds (% incorporation into individual compound) and in terms of total emissions (% contribution of incorporation into total emissions) where % incorporation into individual compounds is weighted by the percent relative abundance in MeJA-induced emissions. Total ^{13}C-incorporation is expressed as a weighted average to represent the percent incorporation of ^{13}C into the volatile terpene profile. When synthetic standards were unavailable, the NIST mass spectral search program (NIST) was used for comparison of mass spectra to determine putative compound identities. The putative compound identity (in parentheses) is used only when the NIST match quality was greater than 80%. SQT = unidentified sesquiterpene, na = not applicable.

reduced compared with well-watered trees (−0.90 ± 0.11 and −0.67 ± 0.11 MPa, respectively, $P = 0.01$), and *A* was also reduced in these trees by 20% (1.53 ± 0.12 and 1.93 ± 0.10 μmol m^{-2} s^{-1}, respectively, $P = 0.02$). After 6 and 7 weeks of drought, Ψ_{pd} of drought-stressed trees fell to −3.0 ± 0.11 MPa and −3.3 ± 0.11 MPa, respectively. The mean *A* of drought-stressed trees neared the zero-assimilation point at 6 weeks (0.19 ± 0.11 μmol m^{-2} s^{-1}) and dropped below the zero-assimilation point by 7 weeks (−0.11 ± 0.09 μmol m^{-2} s^{-1}), resulting in an 89 and 105% reduction in *A* compared with well-watered trees, respectively. Stomatal conductance was also reduced in drought-stressed trees by 95 and 97% at 6 and 7 weeks, respectively.

Methyl jasmonate treatment reduces photosynthesis but does not affect pre-dawn water potential

Gas-exchange measurements were conducted immediately prior to MeJA treatment, precluding us from determining the effect of MeJA on net photosynthesis and stomatal conductance during the drought × herbivory experiment. However, during $^{13}CO_2$-labeling experiments, we observed reduced rates of photosynthesis for MeJA-treated saplings, as evidenced by weaker CO_2 drawdown ($\Delta CO_2 = 24 \pm 13$ p.p.m.) compared with control trees ($\Delta CO_2 = 122 \pm 24$ p.p.m.). Despite reductions in carbon assimilation, well-watered saplings still showed a strong induction of terpene emissions in response to MeJA (see below). Methyl jasmonate did not influence pre-dawn water potential ($P = 0.5$).

Drought and MeJA alone have contrasting effects on volatile terpene emission rates

Across all treatments, 52 compounds were emitted and quantified from constitutive and induced terpene emissions by ponderosa pine (Table S2, available as Supplementary data at *Tree Physiology* Online). Compounds included 19 hydrocarbon monoterpenes, 5 oxygenated monoterpenes, 2 monoterpene esters, 1 C_{10} phenylpropene, 22 sesquiterpenes, 2 oxygenated sesquiterpenes and 1 sesquiterpene ester. Across all

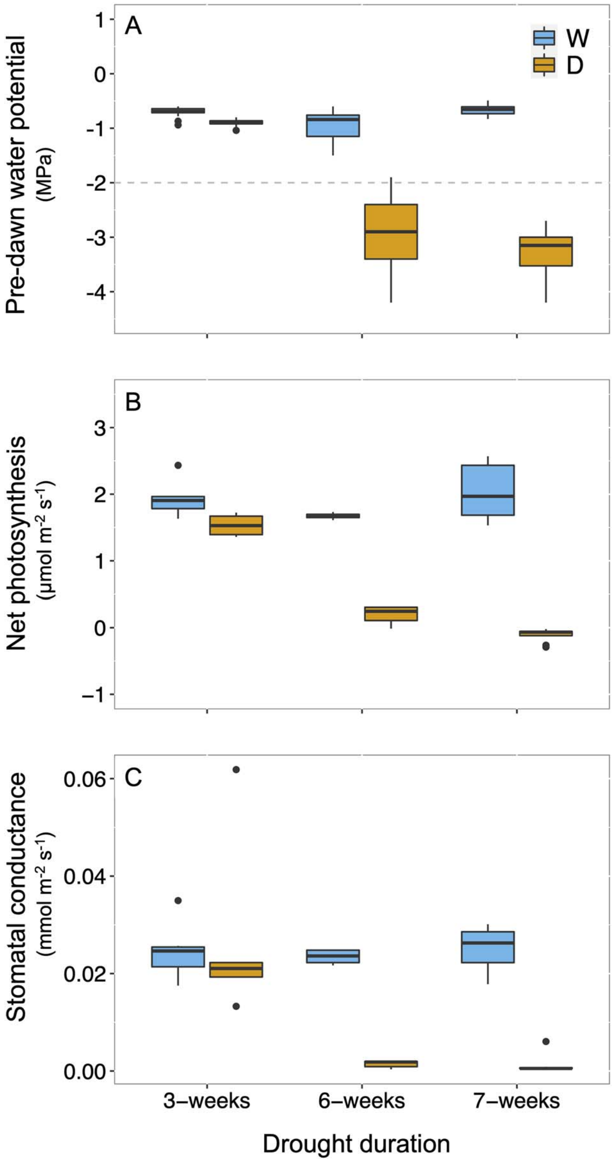

Figure 1. Pre-dawn water potential (panel A; MPa), net photosynthesis (panel B; μmol m^{-2} s^{-1}) and stomatal conductance (panel C; mmol m^{-2} s^{-1}) of well-watered (W; light blue) and drought-stressed (D; light orange) trees across three drought durations. The dashed line in panel (A) represents the pre-dawn water potential corresponding to the approximate zero-assimilation point for ponderosa pine (∼2 MPa).

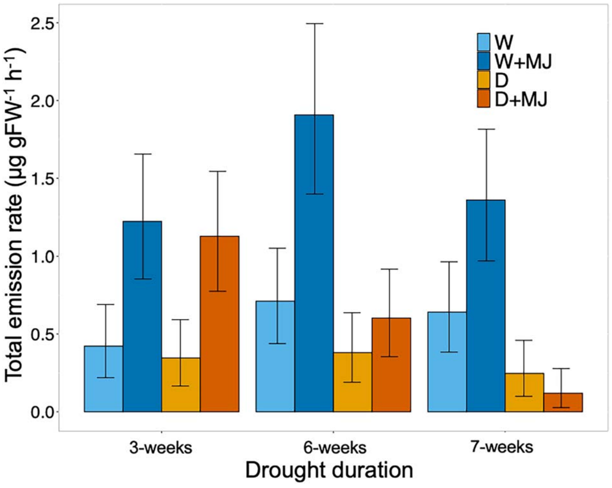

Figure 2. Total terpene emission rates (μg gFW^{-1} h^{-1}) from ponderosa pine. Saplings were either well-watered (W; light blue bars), well-watered and treated with MeJA (W + MJ; dark blue bars), drought-stressed (D; light orange bars) or drought-stressed and treated with MeJA (D + MJ; dark orange bars). Model means ± 95% confidence intervals are shown.

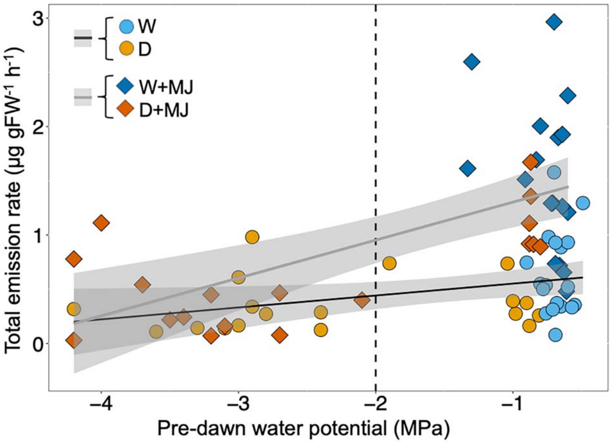

Figure 3. Terpene emission rates (μg gFW^{-1} h^{-1}) from ponderosa pine in response to pre-dawn water potential (MPa). Saplings were either well-watered (W; light blue), well-watered and treated with MeJA (W + MJ; dark blue), drought-stressed (D; light orange) or drought-stressed and treated with MeJA (D + MJ; dark orange). Circles and diamonds represent emissions in the absence and presence of simulated herbivory (MeJA), respectively. Solid lines represent the best-fit regression lines for emissions without MeJA (black; $P = 0.02$, $R^2 = 0.12$) and emissions with MeJA (gray; $P < 0.0001$, $R^2 = 0.43$). The vertical dashed line represents the approximate zero-assimilation point for ponderosa pine (∼2 MPa).

treatments, volatile profiles were dominated by four monoterpenes: δ-3-carene, (−)-β-pinene, (−)-α-pinene and (+)-α-pinene, which comprised ∼ 60% of the total emissions. Drought stress alone did not influence the magnitude of volatile flux by ponderosa pine at 3, 6 or 7 weeks (Figure 2, $P = 1.0$, $P = 0.3$ and $P = 0.09$, respectively); however, compositional shifts were observed after 7 weeks of drought when sesquiterpenes emissions were reduced by ∼85% (Table S2, available as Supplementary data at *Tree Physiology* Online; $P = 0.05$). Drought-induced emission rates were slightly reduced as sapling pre-dawn water potential became more negative (Figure 3, $P = 0.02$, $R^2 = 0.12$). Specifically, for every 1 MPa decrease in water potential, terpene emission rate decreased by 0.08 μg gFW^{-1} h^{-1}. In contrast, MeJA alone dramatically increased the mean emission rate of well-watered saplings; MeJA-induced emissions were three times greater than the constitutive emissions of well-watered controls across all time points (Figure 2, $P = 0.0008$).

Drought severity differentially influences the induced emissions of MeJA-treated trees

For trees experiencing both drought-stress and simulated herbivory, volatile emission rates varied depending on the duration of drought (Figure 2; Table S3, available as Supplementary data at *Tree Physiology* Online; $P < 0.0001$). After 3 weeks when photosynthesis of drought-stressed trees was reduced by ~20%, the magnitude of herbivore-induced terpene emissions did not differ among drought-stressed and well-watered trees. However, after 6 and 7 weeks of drought, herbivore-induced emission rates were reduced by 68 and 91% relative to the well-watered group that was also treated with MeJA (Figure 2, $P = 0.0002$ and $P > 0.0001$, respectively). Overall, the relative magnitude of herbivore-induced emission rates decreased as water potential became more negative ($P < 0.0001$, $R^2 = 0.43$). Volatile emissions were strongly induced in response to MeJA when the water potential was above −2 MPa, the approximate zero-assimilation point for ponderosa pine, but beyond this physiological threshold, emissions from MeJA-treated trees were no greater than the constitutive emissions of untreated trees, and never above ~1 μg gFW^{-1} h^{-1} (Figure 3).

The composition of MeJA-induced volatile emissions is altered by drought

Saplings emitted distinct volatile blends depending on drought duration and herbivory treatment (Figures 4 and 5; Table S4, available as Supplementary data at *Tree Physiology* Online). Although 70% of constitutive terpene emissions from well-watered trees consisted of δ-3-carene, (−)-β-pinene, (+)-α-pinene and (−)-α-pinene (Table 1), well-watered MeJA-treated trees had volatile profiles dominated by *trans*-β-farnesene, (−)-β-pinene, δ-3-carene and linalool, and these compounds accounted for ~60% of the total induced volatiles emitted (Table 2). In well-watered trees, application of MeJA increased the relative abundance of oxygenated monoterpenes and sesquiterpenes (Figure 4) in the volatile profile. Specifically, following MeJA application, oxygenated monoterpenes and sesquiterpenes comprised 10.25 ± 3.03% and 33.1 ± 11.1% of total emissions by well-watered trees, respectively, whereas they represented only 2.5 ± 0.7% and 13.1 ± 5.7% of total constitutive emissions, respectively.

When drought duration was relatively short (3 weeks), MeJA application resulted in volatile blends similar in composition to well-watered trees also treated with MeJA (Figures 4A and 5A). As the duration of drought lengthened (6 and 7 weeks), the MeJA treatment resulted in volatile blends increasingly similar to trees experiencing drought stress alone, and less similar to well-watered trees treated with MeJA (Figures 4B and 4C, 5B and 5C). At 6 and 7 weeks of drought, trees treated with MeJA were unable to induce emissions of oxygenated monoterpenes and sesquiterpenes (Figure 4), and thus their blends were most similar to trees experiencing drought-stress alone (Figure 5).

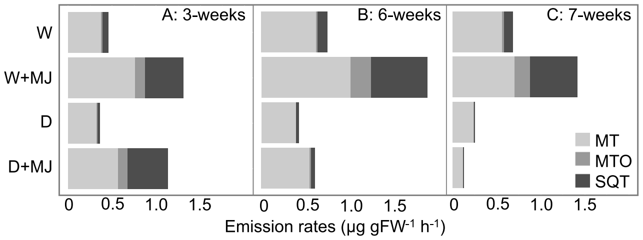

Figure 4. Mean emission rates (μg gFW^{-1} h^{-1}) of hydrocarbon monoterpenes (MT), oxygenated monoterpenes (MTO) and sesquiterpenes (SQT). Trees were either well-watered (W), well-watered and treated with MeJA (W + MJ), drought-stressed (D) or drought-stressed and treated with MeJA (D + MJ).

Constitutive emissions are driven by volatilization from stored terpene pools

Across all treatments, a total of 29 terpene compounds were quantified from the needle tissue (Table S5, available as Supplementary data at *Tree Physiology* Online). Compounds included 20 hydrocarbon monoterpenes, one oxygenated monoterpene, two monoterpene esters and seven sesquiterpenes. Needle terpene concentrations were dominated by (−)-β-pinene, δ-3-carene, (+)-α-pinene and (−)-α-pinene, which together accounted for nearly 80% of total needle concentrations and notably, hydrocarbon monoterpenes contributed >90% to the total needle terpene composition (Table S5, available as Supplementary data at *Tree Physiology* Online). Treatment (W, W + MJ, D and D + MJ) had no effect on total needle terpene concentration ($P = 0.09$; Figure S4, available as Supplementary data at *Tree Physiology* Online; Table S3, available as Supplementary data at *Tree Physiology* Online) or composition ($P = 0.30$; Table S4, available as Supplementary data at *Tree Physiology* Online). Although MeJA has been shown to induce terpene resin biosynthesis in other conifer species, those studies observed tissue induction 5–15 days post-MeJA application (Martin et al. 2002, 2003, Miller et al. 2005), whereas our saplings were sampled only 1 day post-MeJA application.

The relative abundance of constitutive terpene emissions was highly correlated with the relative abundance of terpenes observed in needle tissue (Figure 6; Table S6, available as Supplementary data at *Tree Physiology* Online). For example, the four most abundant terpenes present in constitutive emissions were also the most abundant needle terpenes: δ-3-carene, (−)-β-pinene, (+)-α-pinene and (−)-α-pinene contributed to 28, 22, 13 and 7% of constitutive emissions (Table 1) and to 25, 30, 14 and 9% of needle terpene concentrations (Table S5, available as Supplementary data at *Tree Physiology* Online), respectively. In contrast, *trans*-β-farnesene, (−)-β-pinene, δ-3-carene and linalool were the four most

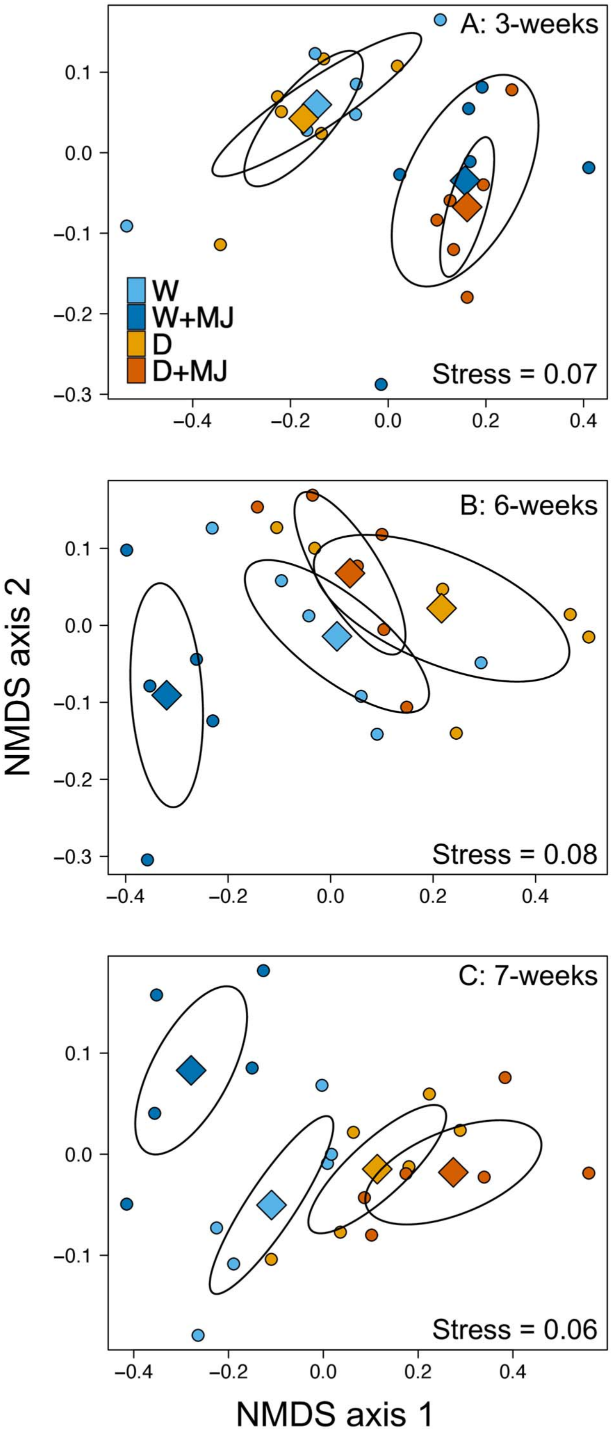

Figure 5. Nonmetric multidimensional scaling (NMDS) of treatment effect on the composition of terpene volatile emissions by ponderosa pine over three drought durations: 3, 6 and 7 weeks (panels A, B and C, respectively). Trees were either well-watered (W; light blue), well-watered and treated with MeJA (W + MJ; dark blue), drought-stressed (D; light orange) or drought-stressed and treated with MeJA (D + MJ; dark orange). Ellipses encircle the centroids (diamonds) and the relative composition of each individual tree (circles) from the same treatment.

abundant compounds of herbivore-induced emissions, making up 22, 16, 16 and 9% of total emissions, respectively (Table 2).

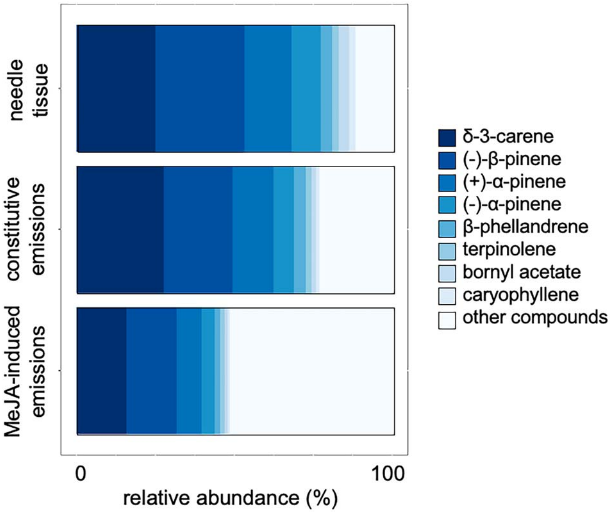

Figure 6. Relative abundance of terpene compounds present in the needle tissue, constitutive emissions and MeJA-induced emissions for well-watered trees. Terpenes listed are the eight most abundant terpenes present in the needle tissue and they are listed in decreasing order of abundance; any remaining terpene compounds present in the needle tissue and emissions are represented as 'other compounds'.

Constitutive and herbivore-induced volatile emissions of well-watered saplings exhibit low levels of ^{13}C incorporation

The constitutive volatile terpene emissions of well-watered saplings were almost entirely unlabeled (<3%), indicating a low contribution of newly assimilated carbon sources to their synthesis (Table 1). Similarly, the overall assimilation of ^{13}C into herbivore-induced emissions from well-watered trees was also minimal (<8%) despite emission rates being three times greater than trees untreated with MeJA (Table 2). Under simulated herbivory, compounds such as linalool, bergamotene, linalool oxide, *trans*-β-farnesene and α-farnesene showed relatively significant incorporation of ^{13}C (30, 27, 22, 11 and 9%, respectively), suggesting that at least a fraction of their synthesis occurred de novo and relied on recently fixed carbon for synthesis. Notably, for *trans*-β-farnesene and α-farnesene, the only compounds also labeled in the constitutive emissions, the percent ^{13}C-incorporation decreased following the MeJA treatment where photosynthesis was reduced, suggesting that their synthesis and carbon fixation are in part coupled.

Discussion

Volatile terpene emissions play important roles in forest ecosystems, but how they respond to concurrent abiotic and biotic stressors is unclear due to a lack of empirical studies and an incomplete understanding of the pools and controls driving volatile fluxes. This is particularly true for conifers where canopy-level emissions represent a mixture of terpenes originating from stored and de novo sources, making it difficult to disentangle contributions of each. In support of our hypotheses, we found that volatile emissions were generally resistant to drought, but when trees experienced concurrent herbivory and moderate to severe drought, herbivore-induced emissions were constrained. By comparing needle terpene composition to emissions, we confirmed that constitutive emissions from ponderosa pine saplings are predominantly of stored origin, and likely explain our observation that the magnitude of emissions were generally resistant to drought (though composition became altered during severe drought stress when the emissions of de novo sesquiterpenes were reduced). In contrast, herbivore-induced emissions from well-watered saplings varied significantly from needle terpene composition, highlighting a significant contribution of de novo terpenes to the induced volatile blend. Although we found drought to constrain herbivore-induced emissions when saplings exhibited assimilation rates of zero, in contrast to our hypothesis, ^{13}C-labeling revealed that these constraints were not due to reduced availability of newly fixed carbon. Together, these results provide key insights into the relative contributions of terpene pools and older carbon sources that control constitutive and stress-induced volatile emissions by ponderosa pine. Most notably, this work identifies a likely drought-induced physiological threshold (the zero-assimilation point) after which the magnitude of herbivore-induced volatile emissions become constrained and the composition is significantly altered.

Pools matter—mechanisms controlling constitutive and stress-induced emissions

Because terpene emissions from ponderosa pine result from two separate pools, the net effect of drought, herbivory and the combination of the two stresses on emissions will depend on (i) the relative contribution of each pool to canopy-level emissions, and (ii) the degree to which each pool is impacted (Lüpke et al. 2017). We find evidence that individually and in combination, drought and herbivory alter terpene emission dynamics in ponderosa pine by affecting de novo synthesis. Our results support prior work that constitutive emissions from ponderosa pine are driven primarily by volatilization from stored resin, with relatively smaller contributions from pools synthesized de novo (Lerdau et al. 1994). We found the composition of constitutive terpene emissions from well-watered trees to generally mirror the composition of needle terpene concentrations where both profiles were dominated by monoterpene hydrocarbons (>90%). In contrast, oxygenated monoterpenes and sesquiterpenes were of de novo origin (Harley et al. 2014, Kreuzwieser et al. 2021) as evidenced by their notable presence in volatile emissions but near-absence in needle tissue. With this knowledge, we were able to explain and partition the effects of drought, herbivory and their interaction based on how they impacted volatiles characteristic of stored (hydrocarbon) and de novo (oxygenated monoterpenes and sesquiterpenes) pools.

Drought-induced effects on emissions were observed via reduced synthesis of terpenes from the relatively small de novo pool. Although results show a decrease in the sesquiterpene emissions from the de novo pool under increasing drought stress, total terpene emission rates were largely buffered by the large flux of hydrocarbon monoterpenes from stored pools (Lüpke et al. 2017). Interestingly, drought-stressed trees trended toward greater needle terpene concentrations (Figure S4, available as Supplementary data at *Tree Physiology* Online), even though their constitutive emissions rates were reduced (Figure 3). These observations suggest that although needle terpene composition controls the composition of volatile fluxes, the concentration of needle terpenes is uncoupled from and exerts little control over the magnitude of terpene emissions (Trowbridge et al. 2014, 2019, Eller et al. 2016). In other words, if resin remains present in specialized storage structures, emissions from these pools will continue to occur in a temperature-dependent manner (Niinemets et al. 2004).

Like drought, simulated herbivory also affected emissions through changes to the de novo pool, but by increasing terpene synthesis. Unlike constitutive emissions, herbivore-induced emissions from well-watered saplings were dominated by de novo terpenes, as evidenced by significant increases in the proportion of oxygenated monoterpenes and sesquiterpenes in response to MeJA application alone. Given that drought also acts on emissions through the de novo pool, can this herbivore-induced increase in de novo emissions be sustained under drought? We observed that as drought progressed, the magnitude and composition of herbivore-induced emissions became more similar to constitutive emissions. By 6 weeks of drought, for example, the herbivore-induced emissions of oxygenated monoterpenes and sesquiterpenes were largely absent from the profile, suggesting that de novo synthesis had ceased, and that canopy-level emissions were now largely driven by the volatilization of hydrocarbon terpenes stored in resin ducts. This cessation of de novo terpene synthesis required to support herbivore-induced emissions under prolonged drought stress suggests that drought overrides the sink demand from biotic agents, but only under more severe conditions.

De novo volatile emissions are synthesized predominantly from older carbon

We find evidence that both constitutive and herbivore-induced de novo volatile terpene emissions from well-watered ponderosa pine are synthesized predominantly from older carbon sources. Based on work using Norway spruce (*P. abies*) and Scots pine (*Pinus sylvestris*), we hypothesized that de novo terpene synthesis would rely largely on recently assimilated carbon (Ghirardo et al. 2010, Wu et al. 2017). Thus, we expected that the mechanism by which drought might inhibit herbivore-induced volatiles was by reducing the availability of newly fixed substrates. Although we did indeed observe reduced herbivore-induced emissions from MeJA-treated trees near their zero-assimilation point, this was not due to a constraint on the availability of newly formed photosynthates. When well-watered MeJA-treated trees were fumigated with $^{13}CO_2$, <8% of the induced emissions became labeled—only ∼5% more than what was labeled constitutively. Here, it is important to note that unlike insect herbivory, needle resin ducts remain intact when subjected to MeJA-simulated herbivory, ensuring that MeJA-induced changes to terpene emissions originated entirely from de novo synthesis and not from volatilization of unlabeled stored pools exposed to the atmosphere through mechanical damage. Our observations are in stark contrast to labeling studies with species from several genera including *Pinus*, *Picea*, *Populus* and *Gossypium* where recent photosynthates were the primary substrate for herbivore-induced de novo terpene emissions (Paré and Tumlinson 1997, Miller et al. 2005, Brilli et al. 2009, Wu et al. 2017). Non-photosynthetic carbon sources, including xylem-transported glucose and leaf carbon pools (i.e., starch and other soluble sugars), are important substrates for the synthesis of constitutive de novo emissions (Kreuzwieser et al. 2002, 2021, Schnitzler et al. 2004, Brilli et al. 2007, Ghirardo et al. 2011, Wu et al. 2017), and our data suggest that they likely contribute to the synthesis of de novo herbivore-induced emissions in ponderosa pine.

Drought severity affects emissions alone and in combination with herbivory at different points of physiological stress

Our results emphasize that quantitative knowledge of drought severity is critical in determining the magnitude and composition of drought- and drought × herbivore-induced emissions from ponderosa pine. Consistent with other studies, we show that the magnitude of terpene emissions from ponderosa pine saplings are largely resistant to drought (Llusià and Peñuelas 1998, Lavoir et al. 2009, Trowbridge et al. 2019, Perreca et al. 2020), even under significant hydraulic stress (−3.3 ± 0.11 MPa) and complete cessation of photosynthesis (−0.11 ± 0.09 μmol m^{-2} s^{-1}). In contrast, we show that increasing drought severity constrains herbivore-induced emissions, and this aligns with prior research with piñon pine (*Pinus edulis*) and white spruce (*Picea glauca*) that also found drought to serve as the overriding factor governing the effect of current- and past-herbivory on volatile emissions (Trowbridge et al. 2014, Perreca et al. 2022). Herbivore-induced volatile emissions were largely constrained when sapling water potentials surpassed −2 MPa, the approximate zero-assimilation point for ponderosa pine (Gaylord et al. 2007, Kolb et al. 2019). The relative lack of induction beyond this hydraulic threshold is likely controlled by several mutually inclusive mechanisms influencing volatile synthesis and emission, and carbon metabolism more broadly. First, terpene pathways typically upregulated during biotic challenge have been shown to be attenuated by drought (Fox et al. 2018, Perreca et al. 2022) and carbon investment in de novo volatile synthesis may be downregulated during extended drought in favor of maintaining other critical processes including foliar defense accumulation (as observed in the present study; Figure S4, available as Supplementary data at *Tree Physiology* Online), osmoregulation and maintenance respiration. Second, recent research has highlighted the important roles of membrane proteins (Adebesin et al. 2017) and cuticle thickness (Liao et al. 2021) in controlling flower volatile emissions. Needle-produced volatiles must similarly transverse cellular membranes and the cuticle to exit the leaf, making it is likely that these biophysical factors also mediate needle volatile emissions (Widhalm et al. 2015), both of which are likely to be altered under drought. Clearly, emission mechanisms are complex, and under drought, environmental stress factors that previously exerted little control over emissions may become increasingly important.

Conclusions

This work demonstrates that the effects of abiotic and biotic stressors on conifer terpene emissions from de novo and stored pools vary, and that understanding the complex biophysical controls over their synthesis and emissions will be critical for predicting how volatile emissions will respond to future stresses. We find that herbivore-induced volatile terpene emissions from ponderosa pine are synthesized predominantly from older carbon, suggesting that drought-induced constraints on these emissions manifest through more sophisticated mechanisms beyond substrate limitations derived from photosynthesis. Importantly, our data highlight a key physiological tipping point of drought-stressed ponderosa pine saplings, beyond which they are unable to induce terpene volatile emissions in response to herbivory; we suspect that similar thresholds exist for other conifer species. Although this work focuses on saplings, an ontonogenetic stage of trees critical to recruitment and regeneration, it is unclear how de novo terpene synthesis or the contribution of de novo and stored terpenes to volatile emissions may vary with tree age. Therefore, determining whether mature tree responses to drought × herbivory exhibit similar patterns will be essential for predicting the impact of these stressors on forests in situ. This is critical because drought-stressed trees unable to induce volatile emissions may exhibit reduced resistance against forests pests (Ghirardo et al. 2012, Schiebe et al. 2019), increasing their likelihood of damage, disease and/or mortality. Though herbivore-induced volatile emissions represent only one dimension of a tree's stress response, their failed induction during drought may accompany or portend the failure of other metabolic and chemical traits critical to tree resistance in the face of multiple stressors. Therefore, it is essential to understand how failed induced emissions relate to other aspects of tree function along the drought continuum.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest statement

None declared.

Data and materials availability

The datasets for this manuscripts are available on Dryad.

Authors' contributions

A.M.T., W.B.K. and A.S. conceived and designed the project; A.S. and W.B.K. conducted the experiments with assistance from A.M.T. and S.C.M.; S.C.M. and A.S. analyzed the data; S.C.M., A.S. and A.M.T. drafted the initial manuscript; and all co-authors revised, read and approved the final manuscript.

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