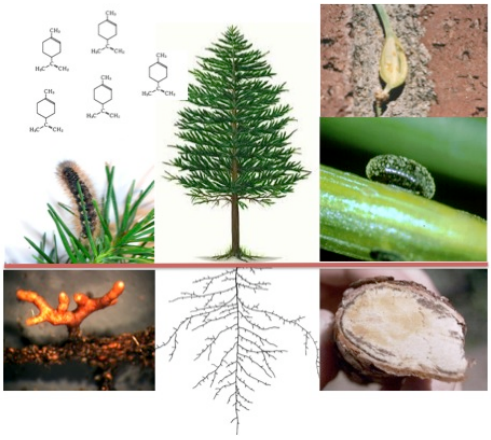
The Ups and Downs of Conifer Defense: Linking Aboveground Herbivory & Induced Needle and Root Defenses



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Abstract

Pinyon pines respond chemically to above- and belowground herbivory and pathogens by synthesizing and emitting secondary compounds known as monoterpenes (C_{10}). However, whether aboveground herbivory can alter belowground monoterpene concentrations and the ecological importance of this potential systemic response remain uncertain. The overarching goal of my thesis is to determine whether foliar damage elicits an induced response by altering monoterpene concentrations in needles and roots, thus potentially changing the susceptibility of pinyon pines to needle herbivores and root pathogens. I used mechanical damage (M), the application of methyl jasmonate (MeJA) and the combination of the two treatments (MeJA+M) on pinyon pine seedlings as proxies for foliar herbivore damage and assessed needle and root monoterpene concentrations periodically over 20 days following the treatments. Overall, my results show that simulated foliar herbivory significantly induced monoterpene production in both needles and roots. However, the specific compounds induced differed between needles and roots, and the direction and magnitude of these changes resulted in a significant damage effect on total needle monoterpenes, but not on total root monoterpenes. The significant increase observed for most of the needle and root individual compounds was due to the application of MeJA. Time and interaction of time and damage treatment did not exhibit a significant effect on most monoterpene compounds, which may be due to variation among individual seedlings at each time interval, resulting substantial variation in monoterpene concentrations. For future work, more detailed analyses are needed to be able to assess the time effect. Moreover, the effectiveness of the induced responses in both needles and roots in defense against future herbivory and root pathogens should be a focus of future investigations.

Introduction

For many years, plant secondary metabolites were thought to be metabolic waste products, with no specific benefit for plant fitness (Whittaker and Feeny, 1971). However, the importance of plant secondary compounds is now well known, as research over the past 50 years has described their roles in plant adaptation, fitness and survival (Feeny, 1976; Mithöfer et al., 2012; Paré and Tumlinson, 1999), including their function as plant defense responses to herbivores and pathogens (Bezemer and van Dam, 2005). The production of secondary metabolites as chemical defenses against herbivores can be constitutive (independent of herbivore/pathogen attacks) and/or inducible (after being attacked by herbivores/pathogens) (Bezemer and van Dam, 2005). Induced responses occur when plants alter concentrations of existing chemical compounds or synthesize metabolites *de novo* following damage or stress (Karban and Baldwin, 1997). Previous research has shown a wide range of herbivore-induced plant responses resulting from herbivore feeding, mechanical damage, and application of signaling compounds that induce defense chemical production through signal transduction, such as, methyl-jasmonate (MeJA), salicylic acid (SA) (Litvak and Monson, 1998; Martin et al., 2002; Kessler et al. 2004; Tholl, 2006). Induced responses are not only confined to leaf tissues, but can be found in different plant organs, including flowers, stems, and roots (Kaplan et al., 2008). In some species, this defense response is not only found in damaged tissues, but also systemically throughout the plant (Karban and Baldwin, 1997).

While folivory itself may not cause a significant risk of attack to plant roots (Coleman et al., 2004), subsequent changes in the resource allocation pattern of plants may influence root herbivores and/or infection by fungal pathogens (Kaplan et al., 2008).

Both the production of herbivore-induced defense compounds and the application of plant hormones known to elicit changes in defensive chemistry and the resulting induced defenses have been shown to result in higher plant fitness, by increasing abundance of natural enemies (indirect defense) and decreasing herbivore attacks (direct defense) (Baldwin, 1998). According to optimal defense theory, defenses against herbivores are costly to plants and hence should be allocated to parts that are most valuable to plants and at higher risk of herbivore attacks (Zangerl and Bazzaz, 1992).

Induced plant defense responses caused by foliage-feeding insects can also play important roles in affecting belowground organisms associated with the plant. Root herbivory by insects, nematodes, root pathogens and colonization by mycorrhizal fungi can also induce aboveground plant defense responses by increasing foliar concentrations of secondary metabolites (Bezemer and van Dam, 2005). Previous studies have demonstrated that root herbivory by insects may increase aboveground plant defensive compounds in a similar manner as foliar-feeding insects (Bezemer et al., 2004; van Dam et al., 2005). For example, root-feeding nematodes can either increase or decrease the concentrations of plant defensive compounds in aboveground tissues, depending on the susceptibility of the plant to nematodes and the type of feeding behavior (van Dam et al., 2005). In addition, bacterial or fungal root pathogens can increase plant defense responses against aboveground pathogens (Pieterse et al., 2002) and mycorrhizal infection influences plant aboveground defenses differently (decrease, increase, or no effect on plant defenses) depending on types and species of mycorrhizae (Strack et al., 2003). Unfortunately, the role of secondary metabolites as resistance factors has mainly been studied for aboveground plant parts and their associated herbivores, despite the fact

that roots contain just as rich a variety of secondary compounds as shoots and belowground herbivores can do as much damage to plants as aboveground feeding (Gerber et al. 2007).

Although effects of herbivory on defense responses of aboveground tissues have been extensively studied, studies focused on how foliage feeding could influence the secondary chemistry of belowground tissues are lacking. Some studies have found that foliage feeding by caterpillars induce *lower* alkaloid concentrations in ragwort roots (*Senecio jacobaea*), which in turn, promote higher risk of pathogenic root fungi infection, or "induced susceptibility" (Bezemer et al., 2005; Hol et al., 2004). However, other studies demonstrate no significant change in root concentrations of defensive compounds after damage to aboveground tissues (van Dam et al., 2004). These differences in aboveground-belowground effects might be caused by the degree to which the source-sink relationships are altered within a plant species, where some plants allocate higher amounts of carbon to roots after shoot herbivory to contribute to root growth rather than root defense, while others may be more constrained by other resources present (e.g., N, P, etc.) (Bezemer et al., 2005).

One of the most diverse groups of secondary metabolites that have been shown to be systemically linked between above and belowground parts of some groups of plants is the terpenoids (Bezemer and van Dam, 2005; Gershenzon and Dudareva, 2007). Within the terpenoid group, monoterpenes (C_{10}), are the primary constituents of conifer resin, and can easily volatilize into the atmosphere (Litvak and Monson, 1997). These compounds can serve as both constitutive and inducible defensive compounds against herbivores and pathogens (Paine and Hanlon, 1994; Hummelbrunner and Isman, 2001),

have been shown to be nematicidal (toxic to nematodes) (Kong et al., 2007), toxic to mammalian herbivores (Iason, 2005), and mycotoxic (Ludley et al., 2008). Monoterpenes as volatiles may potentially act as host location cues for the natural enemies of herbivores, but have also been shown to serve as attractant signals for feeding or oviposition by herbivores or aggregation promoters, which may serve as a disadvantage to the plant (Carisey and Bauce, 1997).

Pinyon pines (*Pinus edulis,* Pinaceae) are rich in monoterpenes and dominate 20 million hectares of land in the western U.S., comprising the third largest vegetation type in the country (Mueller et al., 2005). In the last decade, pinyons have undergone severe drought stress and insect outbreaks, which have led to large-scale mortality events, thus changing the structure and function of the pinyon-juniper ecosystem (McDowell et al., 2008). To conserve these woodlands, recent research has focused on understanding how pinyon pines respond chemically to both above- and belowground herbivory and pathogens. Pinyon pines are attacked by a wide range of herbivores including tiger moth larvae (*Lophocampa ingens*: Arctiidae), pinyon "pitch mass" borer larvae (*Dioryctria ponderosae,* Pyralidae), ips beetle larvae (*Ips* spp., Scolytidae), pinyon needle scale (*Matsucoccus acalyptus,* Matsucoccidae) and pinyon spindlegall midge larvae (*Pinyonia edulicola,* Cecidomyidae) (Jacobi and Cranshaw, 2009). Black stain root fungi (*Leptographium wagneri*) and *Armillaria* fungi (*Armillaria* spp.) are two fungal strains that are known to infect the roots of pinyon pines and may have severe consequences for both pine physiology and overall fitness (Jacobi and Cranshaw, 2009). Furthermore, black stain root fungi can exacerbate pinyon pine mortality events, but the defensive role of monoterpenes in preventing infection is unknown. In addition, whether aboveground

herbivory can alter monoterpene concentrations belowground is uncertain. *The aim of my thesis is to investigate how aboveground herbivory alters the monoterpene concentrations in both needles and roots, thus potentially changing the susceptibility of pinyon pine to foliar and root herbivores and pathogens*. Knowledge of aboveground plant-herbivore interactions and their influence on belowground processes is essential to be able to effectively manage the pinyon-juniper ecosystem under predicted climate change (Trowbridge, 2012).

Using mechanical damage and application of methyl jasmonate (MeJA) as proxies for herbivore damage, this study aimed to determine if aboveground damage to needles can alter needle and root monoterpene concentrations. I addressed the following questions: 1) Does simulated aboveground herbivory alter the concentrations of needle or root monoterpenes and 2) Does mechanical damage alone, MeJA application alone, and the combination of the two treatments result in qualitative and quantitative changes in needle and root monoterpenes. To my knowledge, this is the first report of how aboveground simulated herbivory alters root chemical defenses, particularly monoterpene concentrations, in pinyon pine. These results have important implications for assessing monoterpene mediated multi-trophic interactions in this system and susceptibility of pinyon pines to future above and belowground attack following moderate herbivory.

Materials and Methods

Plant material and growth conditions

In April 2011, I obtained 120 3-year old pinyon pine seedlings that had been grown at the tree nursery at the Colorado State Forest Service (Fort Collins, CO). The trees were re-potted in two-liter pots using potting soil containing 70% Canadian sphagnum peat, perlite, and vermiculite (Fafard Growing Mix2 Professional Formula, Agawam, MA). The plants were then grown under ambient light and controlled temperature conditions in the University of Colorado greenhouse. Seedlings were watered every other day or as needed and received fertilizer once a week from 13 October 2011 to 30 January 2012.

Before the experiment began, 60 pinyon seedlings at a time were randomly selected from the greenhouse and placed in a growth chamber (Conviron, Winnipeg, Manitoba) where they equilibrated for one week at 25:21 °C day:night temperatures with 16:8 day:night photoperiods at a PPFD of 800 µmol photons m^{-2} s^{-1} at canopy level and 60% relative humidity (RH). The seedlings were watered every other day, or as needed, and fertilized once a week. After the treatments were applied (see section below), seedlings remained under these growth conditions until harvested. Due to the limited space in the growth chamber, the experiment was carried out in two different phases and 60 seedlings were in the growth chamber at a time, for a total of 120 seedlings.

Simulated herbivory treatments

After equilibrating for a week in the growth chambers, 30 seedlings were randomly placed in one of four simulated herbivory treatments: control (C), mechanical damage (M), methyl jasmonate application (MeJA), or mechanical damage + MeJA application (MeJA+M) (total N = 120 individual plants). I simulated mechanical damage by removing ~25-30% of the total needle area with single cuts using scissors (see Litvak et al. 1998). For the MeJA application, 150 ml of a 10 mM MeJA solution (0.1% Tween-20 detergent and distilled water) was applied to each tree under a fume hood using a spray bottle (>95% MeJA FG grade, Sigma-Aldrich, St. Louis, MO) (see Heijari et al. 2008). The trees were coated as evenly as possible and allowed to dry for ~30 minutes before returning them to the chamber. The seedlings in the MeJA+M treatment had ~25-30% of their needle area removed just prior to the MeJA application, as described above. Control plants were left alone.

As previously mentioned, due to the limited space in the growth chamber, the experiment was carried out in two different phases. I first investigated trees in the C and M treatments, which were transferred to the growth chamber on 13 October, 2011. To examine how monoterpenes may be induced over time, following the treatments, three seedlings from each treatment were destructively harvested for needles and roots at intervals over a three-week period. I began harvesting C and M trees on 21 October, a week after the plants were placed in the growth chamber (designated Day 0) and continued sampling for 20 days at days 1, 2, 3, 5, 7, 11, 14, 17 and 20. For the second phase, I began harvesting MeJA and MeJA+M trees one week after placing them in the chamber (Day 0), on 2 February 2012, for 20 days at intervals of 1, 2, 3, 5, 11, 17 and 20.

Day 7 and 14 of these treatments were not harvested because 6 of the MeJA trees and 6 of MeJA+M trees died. Needles were harvested first, placed in aluminum foil bags and immediately immersed in liquid nitrogen. Roots were harvested immediately afterward and were also flash-frozen in liquid nitrogen. All samples were then stored in a -80°C freezer until chemical analysis.

Chemical analyses

Needles were removed from the freezer and immediately weighed to obtain the total mass. I removed a sub-sample of all the needles collected, and ground them in liquid nitrogen using a chilled mortar and pestle to minimize monoterpene loss. Samples of between 0.4 and 0.6 g of powder were weighed into 2 dram glass vials to the nearest 0.01 g, exact weights were recorded, and 2 mL of GC-grade n-hexane (Fisher Scientific) containing 0.1 μL mL^{-1} (+)-fenchone (Sigma-Aldrich) as an internal standard were added to the vials. Vials were immediately closed with teflon lined caps, mixed with a vortexer, and allowed to soak for one week at ambient temperature. After the seven-day soaking period, 100 μL aliquots of each sample were transferred into micro-inserts in small mouth clear vials capped with PTFE liners (Alltech Associates, Deerfield, IL) for chemical analysis.

Roots were removed from the freezer and first washed in cool tap water and then rinsed in room temperature distilled water to remove all soils. After blotting roots with paper towels to remove excess water, the total fresh weight of each root sample was determined using a digital scale. Clean roots were cut with scissors to obtain a sub-

sample of the taproot and fine roots for grinding. For monoterpene extraction, root samples were ground in liquid nitrogen using a chilled mortar and pestle. The frozen powder was then weighed to the nearest 0.01 g into 2 dram glass vials, exact weights (between 0.6 and 1.5 g) were recorded, and the same internal standard as used in needles was added to the vials. After this, the same process used for needles was repeated.

One µL of each sample was then injected on an Agilent Technologies 6890 gas chromatograph-5975 mass spectrometer (GC-MS) fitted with a Cyclodex-B chiral column (30 m × 250 µm × 0.25 µm; J&W Scientific). Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} with a split flow ratio of 25:1. Injector temperature was set at 230 ºC. The oven profile consisted of an initial temperature of 60 ºC followed by a ramp of 5 ºC min^{-1} to 200 ºC, then a second ramp of 25 ºC min^{-1} to 230 ºC. Monoterpene enantiomers were identified by comparing retention times of known standards and mass spectra using the NIST library and MSD Chemstation software. Concentrations for each compound were calculated using 4-point calibration curves with injections of known amounts of pure standards and the internal standard, fenchone. All standards were purchased from Sigma Aldrich (Saint Louis, MO) with the exception of β-phellandrene which was obtained from Glidco Organics (Jacksonville, FL).

Statistical analyses

All statistical analyses were performed using R (2.10.1, Vienna, Austria). Data for needles and roots were analyzed separately (see below). To meet the assumption of normality, individual and total monoterpene concentration data were square-root

transformed. First, I tested for correlations between total and individual monoterpenes in needles and roots. Then, I performed a two-way ANOVA to assess the effect of damage type, time, and their interaction on total and individual monoterpene concentrations in needles and roots separately. When a significant damage effect was observed, a Bonferroni-corrected post-hoc analysis of the pairwise comparisons was performed using a Tukey's honestly significant difference (HSD) test to test for differences among the damage treatments. Pinyon pine needle and root monoterpene composition was analyzed by examining individual monoterpenes as a proportion of total concentration.

Results

Monoterpene Distribution in Needles and Roots: General Patterns

There was no correlation between total monoterpenes in needles and roots. For individual compounds, only β–pinene showed a significant positive correlation between needles and roots ($P<0.005$; Table 1). As a result, the foliar and root monoterpene concentration data were analyzed separately and will be discussed in that way. Furthermore, the relative abundances of the individual monoterpenes in needles versus roots varied substantially (Table 2). Specifically, (+)-α–pinene, (-)-α–pinene and β–pinene comprised over 80% of the total monoterpene pool in pinyon pine needles, while (+)-α-pinene, carene and β–phellandrene comprised about the same percentage of total monoterpenes present in the roots (Table 2).

Effects of damage and time on foliar monoterpene concentrations

For clarity, data are presented in two ways. The first presents monoterpene concentrations as a function of time in control versus the three treatments (Fig. 1-9); however, the complexity of those graphs makes it difficult to see damage treatment effects. Therefore, data are also shown as box plots (Figs. 10–18), allowing clearer presentation of the effects of damage treatment on each monoterpene compound.

The total monoterpene needle concentration within pinyon pine seedlings was affected by the damage treatment, but not by time nor the interaction of the two variables (Fig. 10a; Table 3). This result is likely driven by the fact that the concentration of (+)-α-

pinene, the primary constituent of needle resin (45.9%; Table 2), was affected by the damage treatment (Fig. 12a). Needle concentrations of (+)-α–pinene, β–myrcene and β–phellandrene were also significantly affected by the damage treatment (Table 3; Fig. 12a, Fig. 17a and Fig. 18a). A similar treatment effect was observed across these three compounds; specifically, *post hoc* tests showed that the MeJA+M treatment resulted in a significant decrease in (+)-α–pinene, β–myrcene and β–phellandrene relative to the application of MeJA alone, yet plants in MeJA+M treatment were not significantly lower than those in the mechanical damage treatment or control trees (Fig. 12a, Fig. 17a and Fig. 18a). Damage did not significantly alter the concentrations of (-)-α–pinene, β–pinene, camphene, carene nor S-limonene[1] (Table 3; Fig. 11a, Fig. 13a, Fig. 14a, Fig. 15a and Fig. 16a). Similarly to the total monoterpene concentration, neither time nor the interaction between time and treatment significantly affected any of the individual monoterpene compounds present within the needles (Table 3), probably because the concentration of each individual compound fluctuated over the 20 day sampling period in all damage treatments (Fig. 2a- Fig. 9a).

Effects of damage and time on root monoterpene concentrations

In roots, total monoterpene concentration was not affected by damage treatment, time nor their interaction (Table 4; Fig. 10b). Similar to the needles, (+)-α–pinene was also the primary constituent of root resin (45.9%; Table 1); however, amounts of this compound were not affected by the damage treatment, time or their interaction (Table 4).

[1] Only the S-enantiomer of limonene was detected in pinyon pine seedlings.

Hence, the insignificant effects on total monoterpene concentration were likely driven by the lack of response of its major constituent, (+)-α–pinene (Table 4; Fig. 12b). Similar to total monoterpenes, the individual compounds (+)-α–pinene, camphene, carene, β–myrcene and β–phellandrene were not impacted by damage treatment, time or the interaction (Table 4; Fig. 12b, Fig. 14b, Fig. 15b, Fig. 17b and Fig 18b).

Root concentrations of (-)-α–pinene, β–pinene and S-limonene were, however, significantly affected by the damage treatment (Table 4). Specifically, (-)-α–pinene (5.6% of the root total concentration; Table 2) in MeJA treated trees was significantly higher than that in trees in the control and mechanically damaged treatments, but not significantly different from MeJA+M treated trees (Fig. 11b). Second, β–pinene (5.6% of the root total concentration; Table 2) concentrations in both MeJA and MeJA+M treated seedlings were significantly higher than control and mechanically damaged seedlings (Fig. 13b). Third, S-limonene (5.25% of the total root monoterpene content; Table 2) exhibited a significantly *lower* concentration in MeJA and MeJA+M treated plants than in mechanically damaged plants, but was not significantly different from trees in the control treatment. Time and the interaction of time and treatment showed significant effects on only one root monoterpene compound, S-limonene (Table 3).

Comparison of Needle and Root Monoterpene Responses

Overall, the magnitude and directional effects of damage treatment, time and their interaction on needle and root monoterpenes were different. Damage treatment had more dramatic effects on needle (+)-α–pinene, β–myrcene and β–phellandrene concentrations.

Furthermore, damage treatment, time and their interaction affected different root monoterpene compounds relative to their foliar counterparts. Root (-)-α–pinene and β–pinene were impacted by damage treatment, while root S-limonene was affected by damage treatment, time and interaction between time and treatment.

Discussion

Overall, my results showed that simulated foliar herbivory significantly induced monoterpene production in both needles and roots. However, the specific compounds induced differed between needles and roots, and the direction and magnitude of these changes resulted in a significant damage effect on total needle monoterpenes, but not on total root monoterpenes. Damage treatment, rather than time, was the important factor affecting induction in both needles and roots, with the application of MeJA eliciting increased concentrations for most individual monoterpene compounds. Out of the eight monoterpenes investigated, time and the interaction of time and treatment only impacted one compound in roots and none in needles.

Following attack by herbivores or pathogens, a number of plant species have been reported to exhibit induced responses, or changes in traits and processes that are used in defense against future attacks (Bezemer and van Dam, 2005). The benefit of employing induced responses is that the plant can tailor its response to the herbivore that is feeding, as the hormones produced depend on the herbivore species and determine which specific genes are upregulated and the specific defenses that are produced (Lankau, 2007). Thus, the magnitude and direction of induced responses can be quite complex, and may depend

not only on the species of herbivore, but on where plants produce secondary metabolite compounds, constitutive levels of defense compounds present, the frequency and intensity of herbivory, nutrient availability, environmental conditions, and the likelihood that particular tissue will be attacked (Kaplan et al., 2008; Lewinsohn et al., 1991).

Several studies have shown that induced responses to insect attack are systemic, where the damaged tissue produces a signal that is transmitted throughout undamaged parts of the plant causing an induced response in another plant organ (van Dam and Heil, 2011; Rasmann et al., 2009; Erb et al., 2009). Such induced responses in undamaged tissues can be caused by belowground organisms and/or aboveground organisms and several different signals (e.g., chemical and electrical) have been proposed as transporters of information (Bezemer and van Dam, 2005). For example, several studies have shown that components of herbivore salivary gland secretions are required to trigger defense production pathways involving signaling by methyl jasmonate (Reymond et al., 2004; Maffei et al., 2007). In fact, even without actual mechanical damage, MeJA can act as the signal that induces changes in plant defense compounds (Martin et al., 2003; Gómez et al., 2010; Pauwels et al., 2009). For example, several studies have shown that application of MeJA to leaves or stems resulted in increases in localized defenses (i.e., traumatic resin ducts in *Picea abies* stems, Martin et al., 2002; glandular trichomes in *Lycopersicon esculentum* leaves, Boughton et al., 2005; volatile emissions in *Iva frutescens* leaves, Degenhardt and Lincoln, 2006). My study found similar results, where the exogenous application of MeJA alone elicited a significant increase in the local foliar concentrations of (+)-α–pinene, β–myrcene and β-phellandrene. Furthermore, foliar application of MeJA on pinyon pines, resulted in a systemic increase in the level of *different* chemical

defenses in the roots relative to needles, particularly (-)-α–pinene, β–pinene and S-limonene. While this phenomenon of MeJA application to aboveground tissues eliciting changes in belowground organs has been described in a number of agricultural systems (i.e., *Zea Mays* L., Feng et al., 2012; *Brassica campestris*, Ludwig-Müller et al., 1997), this response has been rarely investigated in tree species and the mechanistic underpinnings driving this differential induction of compounds between organs remains to be elucidated.

Because monoterpenes are volatile, mechanical damage to needles results in a significant release of these compounds to the atmosphere, causing some difficulty in assessing potential increases in needle monoterpene production in response to mechanical damage due to concomitant release to the atmosphere. Furthermore, mechanical damage alone may cause a weaker induced response than actual herbivore damage because it is missing some stimulus that herbivores provide (Litvak and Monson, 1997). Several studies have measured monoterpene emission rates as well as monoterpene concentrations in the needles and monoterpene cyclase (enzymes involved in monoterpene production) activity to determine the net effect of herbivore-induced responses in conifers. By combining these three measurements, the data showed an increase in cyclase activity but no increase in concentration due to high emissions in ponderosa pine, lodgepole pine and Norway spruce (Litvak and Monson, 1997; Martin et al., 2002). In the current study, MeJA application without physical damage to leaf tissues allowed direct analysis of changes in monoterpene production without mechanical damage influencing emissions by exposing the resin directly to the atmosphere. My results showed that total monoterpenes, (+)-α–pinene, β–myrcene and β–phellandrene in

pinyon pine *needles* were significantly higher in MeJA treated seedlings than in MeJA+M treated seedlings. *Root* (-)-α–pinene and β–pinene concentrations were also affected by MeJA application, similarly to those in needles; specifically, *root* (-)-α–pinene and β–pinene concentrations were significantly higher in MeJA treated seedlings than in M treated and control seedlings.

The analyses in this study showed that time rarely had an effect on pinyon pine needle and root monoterpenes (only for S-limonene). One reason for this may be because I analyzed individual trees at each time interval and individual trees may, themselves, show substantial variation in monoterpene content. Such individual variation and the relatively small sample size at each time interval may have made it difficult to detect a significant effect of time over the 20 days of the experiment (see Fig. 1-9). In addition, Sjödin et al. (1996) and Persson et al. (1996) also found that the relative amounts of individual monoterpenes in *Pinus sylvestris* and *Picea abies* exhibited great variation between and within trees. In another system of an herbaceous weed, *Plantago lanceolata* (Plantaginaceae), plants were harvested at different times following damage by caterpillars and an induced response was only evident at certain time periods (Fuchs and Bowers, 2004). This may also be the case with pinyon pines. Another explanation for the lack of time effect could be because *Pinus* trees contain relatively high levels of constitutive defense, store these defensive compounds over long period of time, and thus show no substantial induction following herbivory or experience a lag in their induced response that we failed to capture (Lewinsohn et al., 1991). However, more detailed analyses (i.e., cyclase activity, sustained damage, larger sample sizes, longer sampling

periods, etc.) are required in order to better test for the timing of induced responses in this system.

From this study, my results show how simulated aboveground herbivory can alter both above and belowground chemical defenses in pinyon pines. However, the ecological role of these altered levels of monoterpenes against herbivores/pathogens remain to be elucidated. In order to predict the susceptibility of plants to their antagonists (natural enemies) and gain a more comprehensive understanding of the cascading effects of conifer defenses throughout an ecosystem, future work should focus on the effectiveness of the induced responses in both needles and roots in defense against herbivores, belowground pathogens, and root mycorrhizae. MeJA treatment may be used to apply to conifer roots as a pest management strategy, as MeJA has been shown to enhance conifer seedlings defense against pests and pathogens (Huber et al., 2005). As suggested by Huber, the effects of belowground MeJA treatment in pinyon pine are important to be able to develop a better strategy that can reduce mortality of pinyon pine. Furthermore, knowledge of aboveground plant-herbivore interactions and their influence on belowground processes is essential to be able to effectively manage the pinyon-juniper ecosystem.

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Tables

Table 1. Correlation and *P* values between total and individual monoterpenes in needles and roots.

Monoterpene Compounds	Correlation	*P*
(-)-α–pinene	-0.0146	0.889
(+)-α–pinene	0.181	0.0814
β–pinene	0.307	**0.00276**
camphene	-0.0485	0.642
carene	0.191	0.0655
S-limonene	0.0934	0.370
β–myrcene	-0.0391	0.708
β–phellandrene	-0.0258	0.805
total monoterpenes	0.132	0.203

Table 2. Percent of the total monoterpene concentration for each compound observed in pinyon pine seedling needles and roots from trees exposed to all damage types over a 20 day sampling period. Values are the means (n=94).

Monoterpene Compounds	Percent Total Needle Monoterpene Concentration	Percent Total Root Monoterpene Concentration
(+)-α–pinene	45.9	45.9
(-)-α–pinene	19.2	5.60
β–pinene	18.4	2.44
β–myrcene	7.39	5.72
carene	3.16	26.4
β–phellandrene	2.84	7.06
S-limonene	2.31	5.24
camphene	0.744	1.79

Table 3. *F* statistics and *P* values for differences in concentrations of each needle monoterpene compound and total monoterpenes in needles due to treatments, days post treatments and treatments per days post treatment.

Monoterpene compounds in needles	Treatments		Time (Days Post Treatments)		Treatment x Time	
	F	*P*	*F*	*P*	*F*	*P*
(-)-α–pinene	2.16	0.101	0.244	0.972	0.695	0.822
(+)-α–pinene	4.01	**0.0113**	0.674	0.693	1.129	0.345
β–pinene	1.29	0.286	0.469	0.853	0.819	0.687
camphene	1.83	0.151	1.98	0.0713	0.775	0.737
carene	0.856	0.469	1.30	0.266	1.22	0.264
S–limonene	1.74	0.169	0.447	0.869	0.859	0.640
β–myrcene	3.01	**0.0366**	0.911	0.504	0.964	0.517
β–phellandrene	3.92	**0.0126**	1.73	0.118	0.857	0.642
Total Monoterpenes	3.77	**0.0150**	0.314	0.945	0.698	0.818

Table 4. *F* statistics and *P* values for differences in concentrations of each root monoterpene compound and total monoterpenes in roots due to treatments, days post treatments and treatments per days post treatment.

Monoterpene compounds	Treatments		Time (Days Post Treatments)		Treatment x Time	
	F	*P*	*F*	*P*	*F*	*P*
(-)-α–pinene	6.16	**<0.001**	0.503	0.829	0.988	0.490
(+)-α–pinene	0.461	0.711	1.57	0.160	0.443	0.980
β–pinene	14.6	**<0.001**	0.802	0.589	0.561	0.929
camphene	1.42	0.245	1.16	0.336	1.24	0.249
carene	0.242	0.867	0.742	0.637	0.833	0.670
S-limonene	4.86	**0.00423**	2.92	**0.0105**	2.53	**0.00251**
β–myrcene	1.65	0.186	1.17	0.334	0.715	0.801
β–phellandrene	1.82	0.154	1.18	0.324	0.925	0.562
Total Monoterpenes	0.445	0.722	1.49	0.187	0.624	0.885

Figures

a.

b.

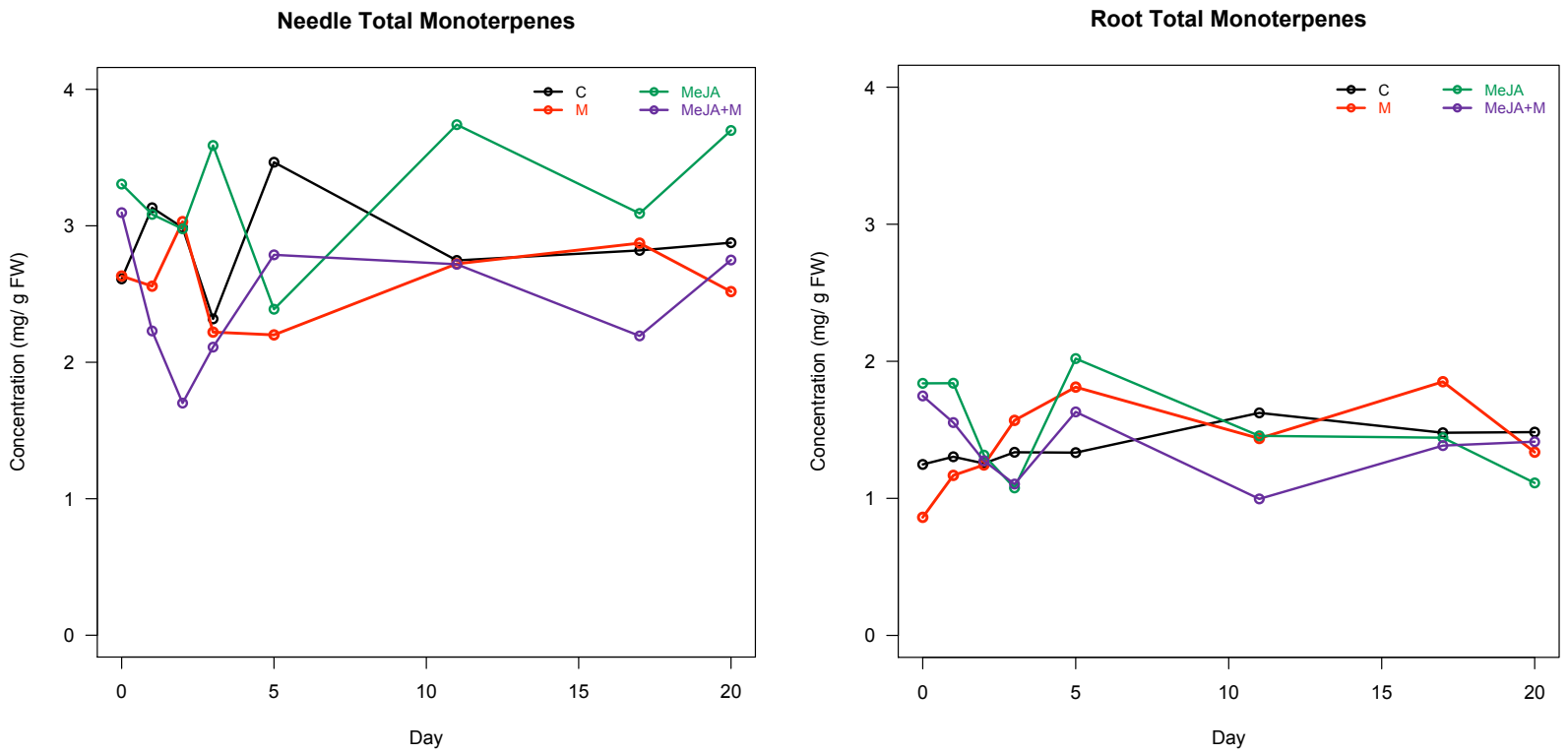

Fig. 1. Changes in the concentration of total monoterpenes in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

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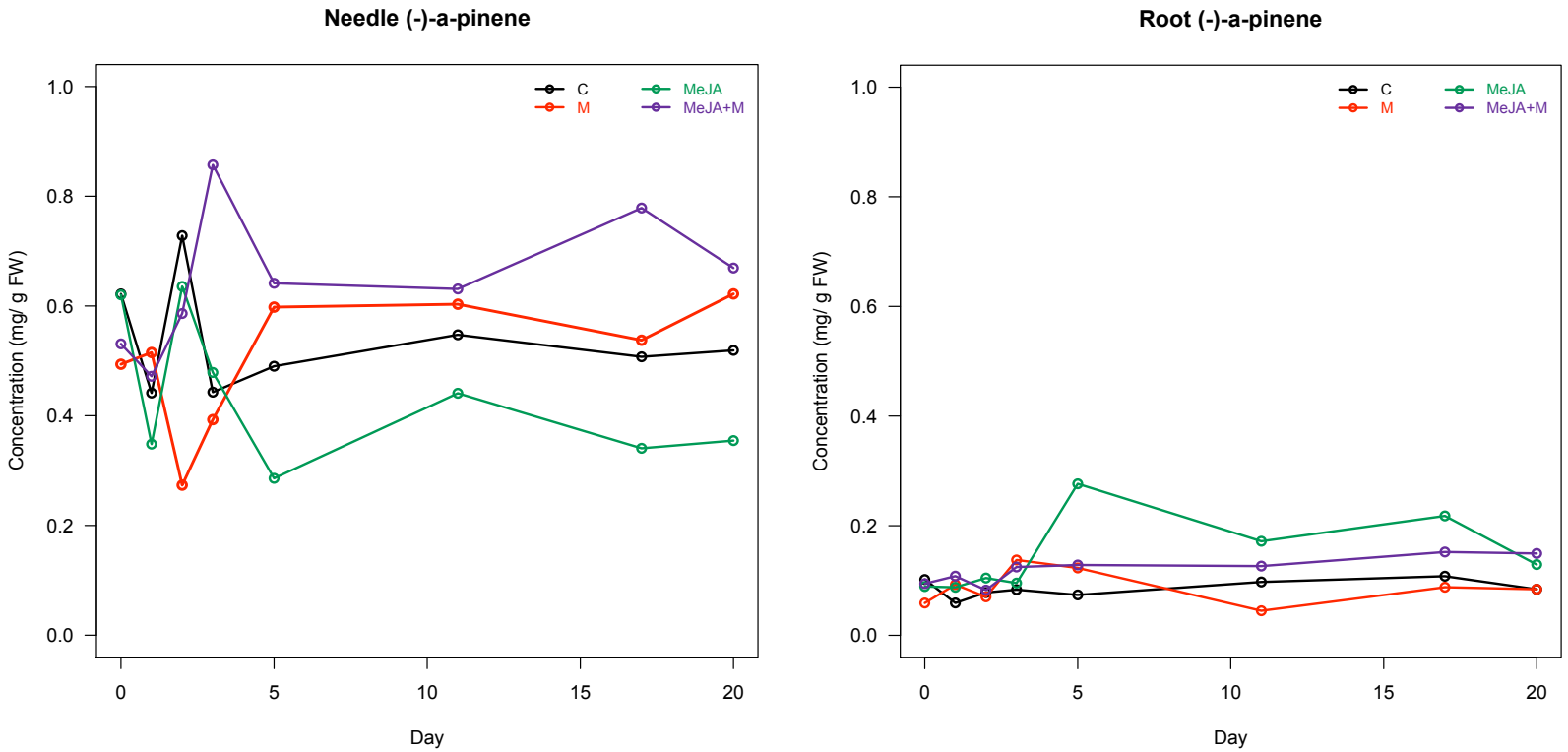

Fig. 2. Changes in the concentration of (-)-α-pinene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

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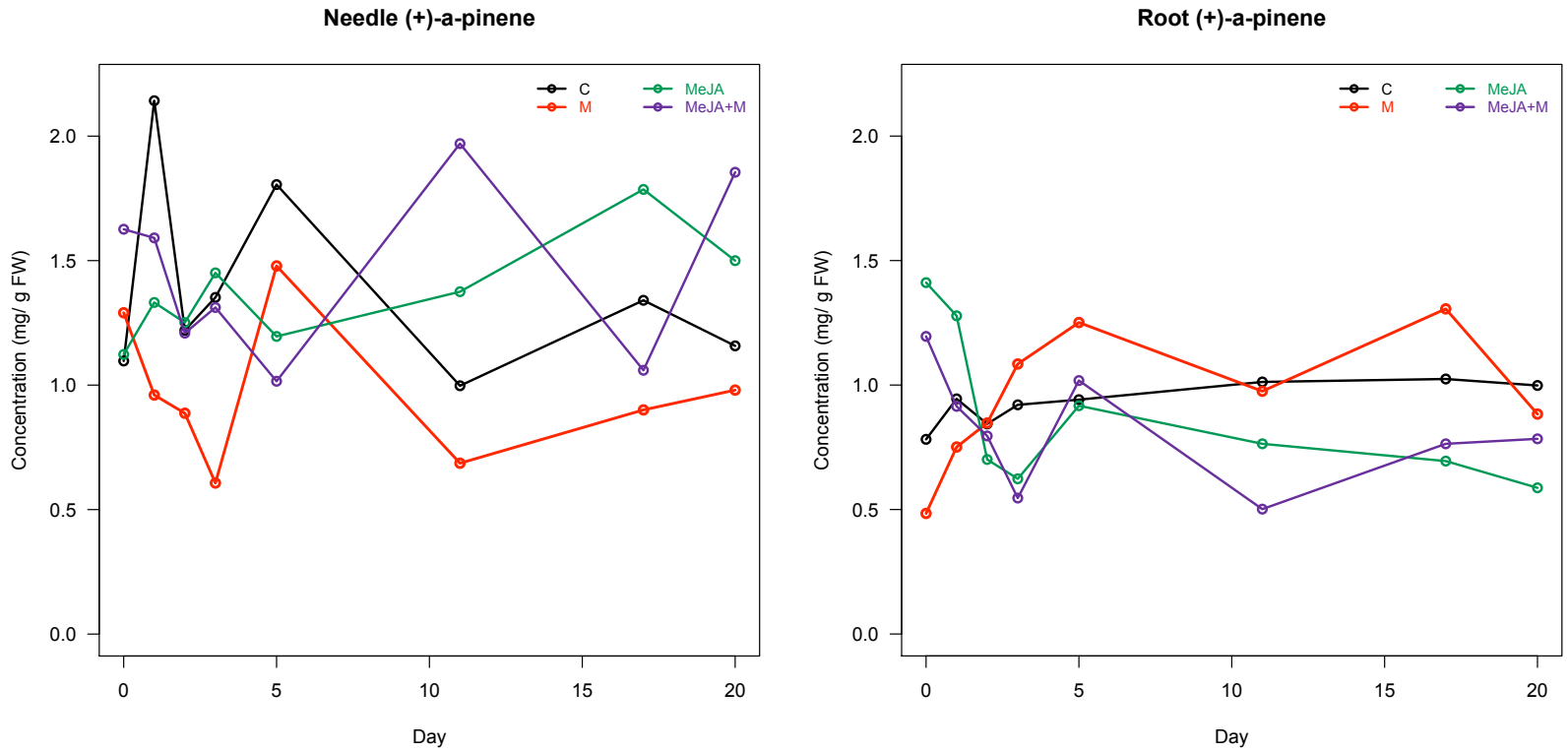

Fig. 3. Changes in the concentration of (+)-α-pinene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

b.

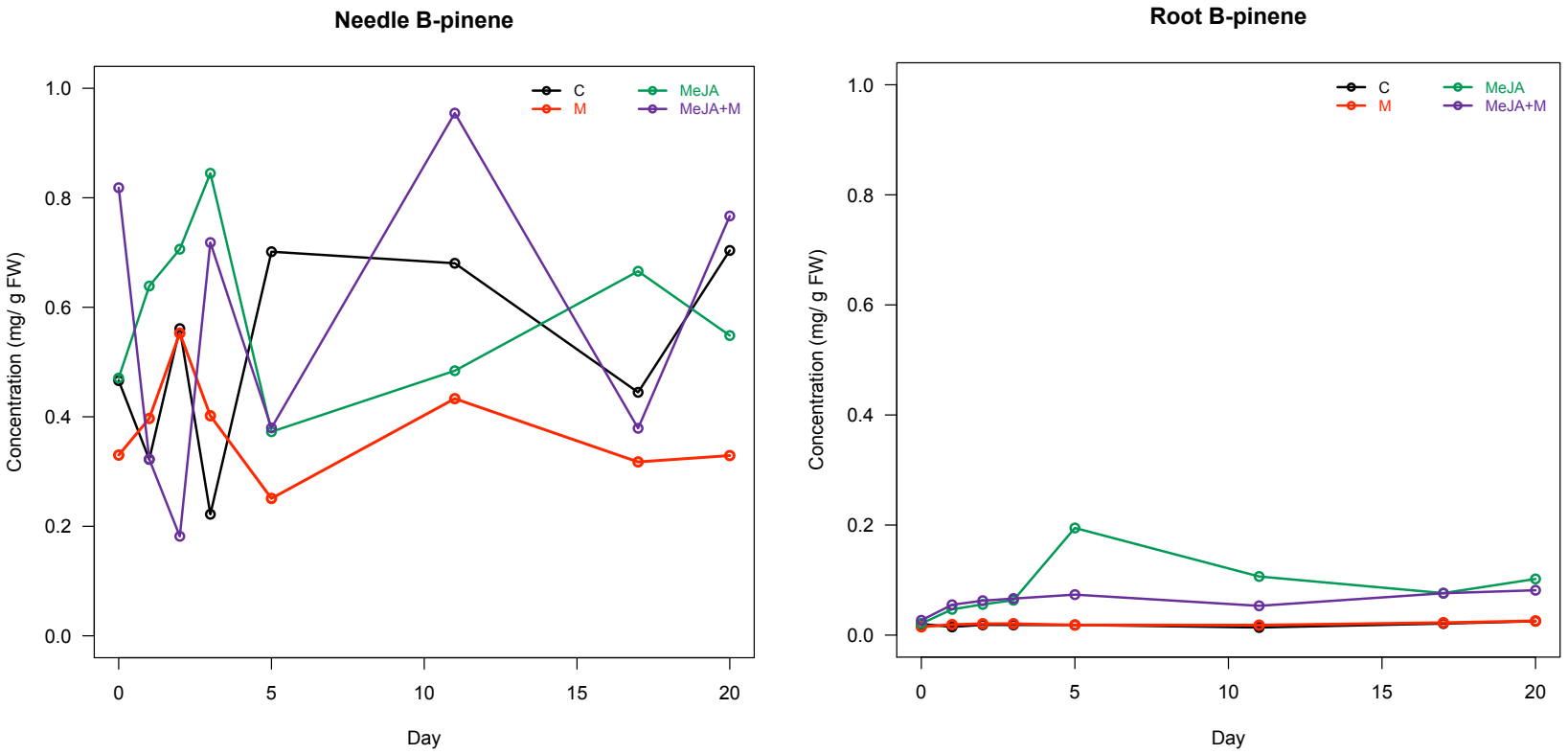

Fig. 4. Changes in the concentration of β-pinene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

b.

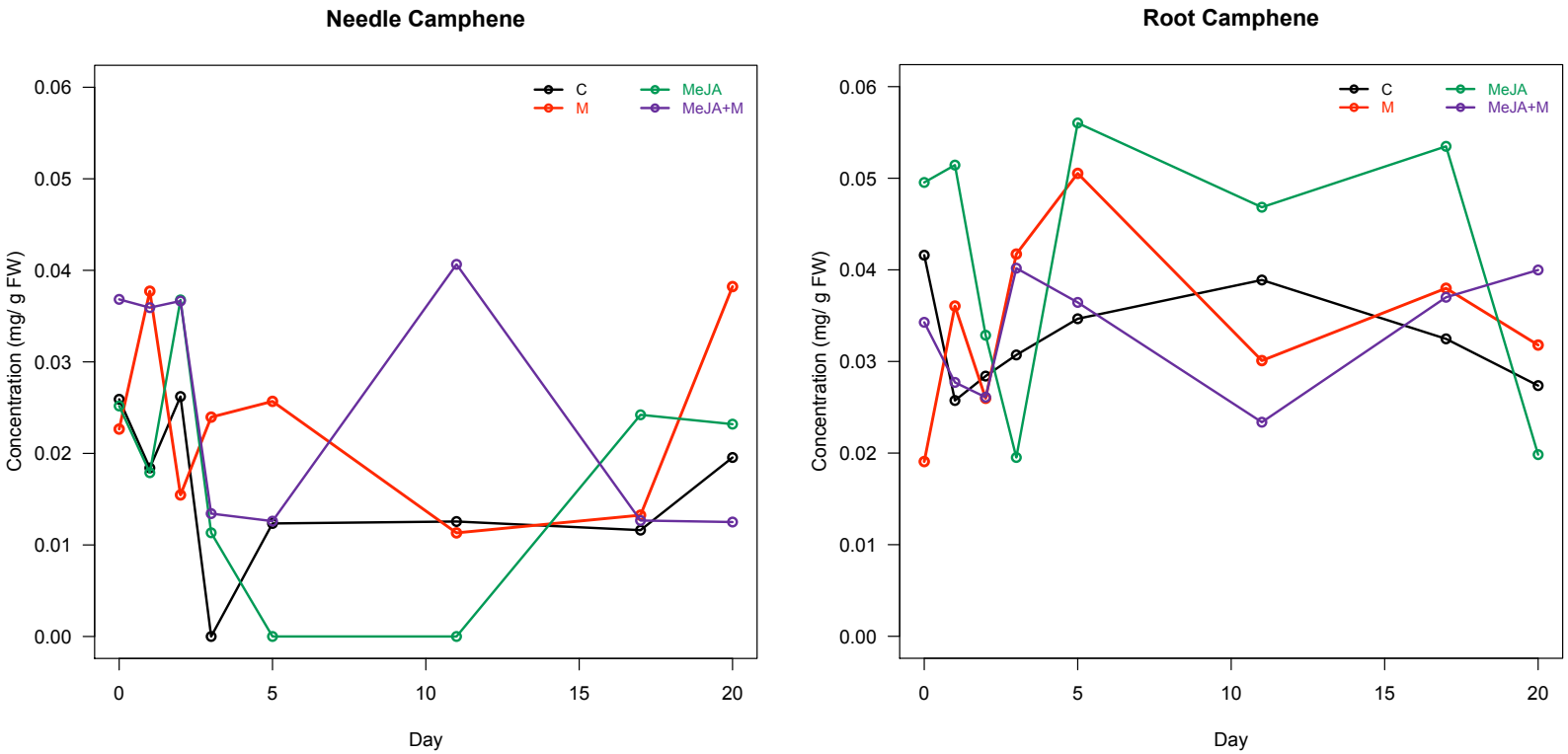

Fig. 5. Changes in the concentration of camphene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

b.

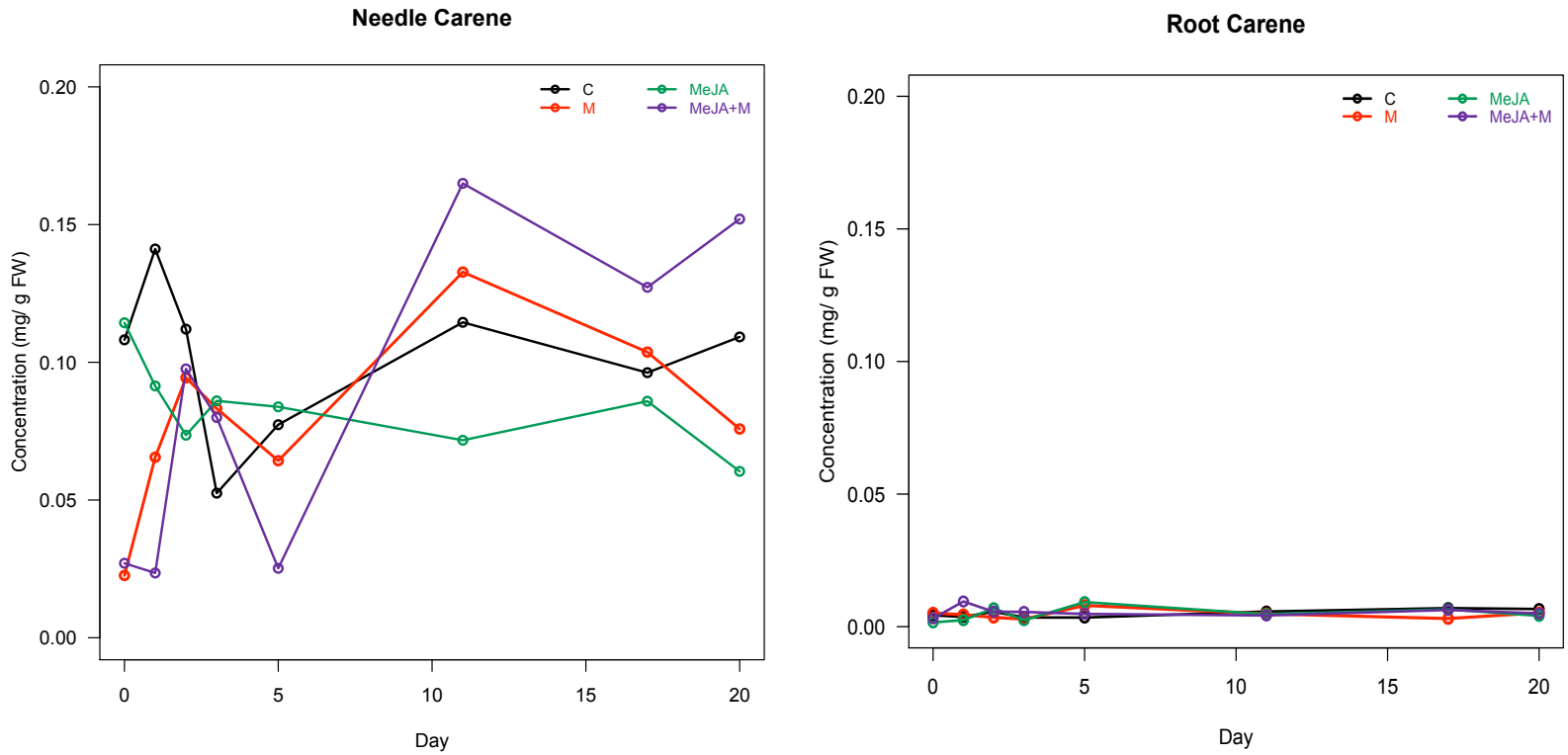

Fig. 6. Changes in the concentration of carene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

b.

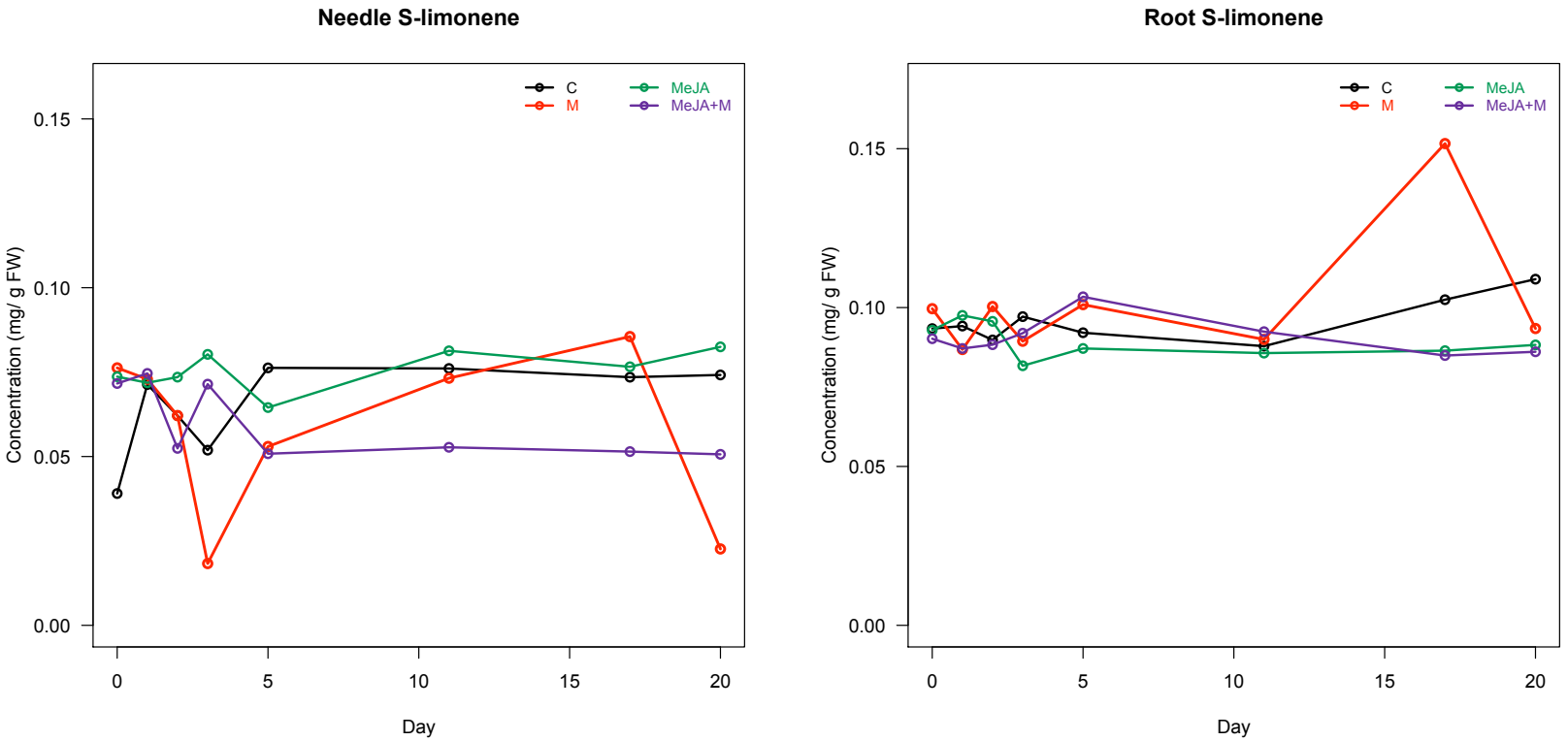

Fig. 7. Changes in the concentration of S-limonene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

b.

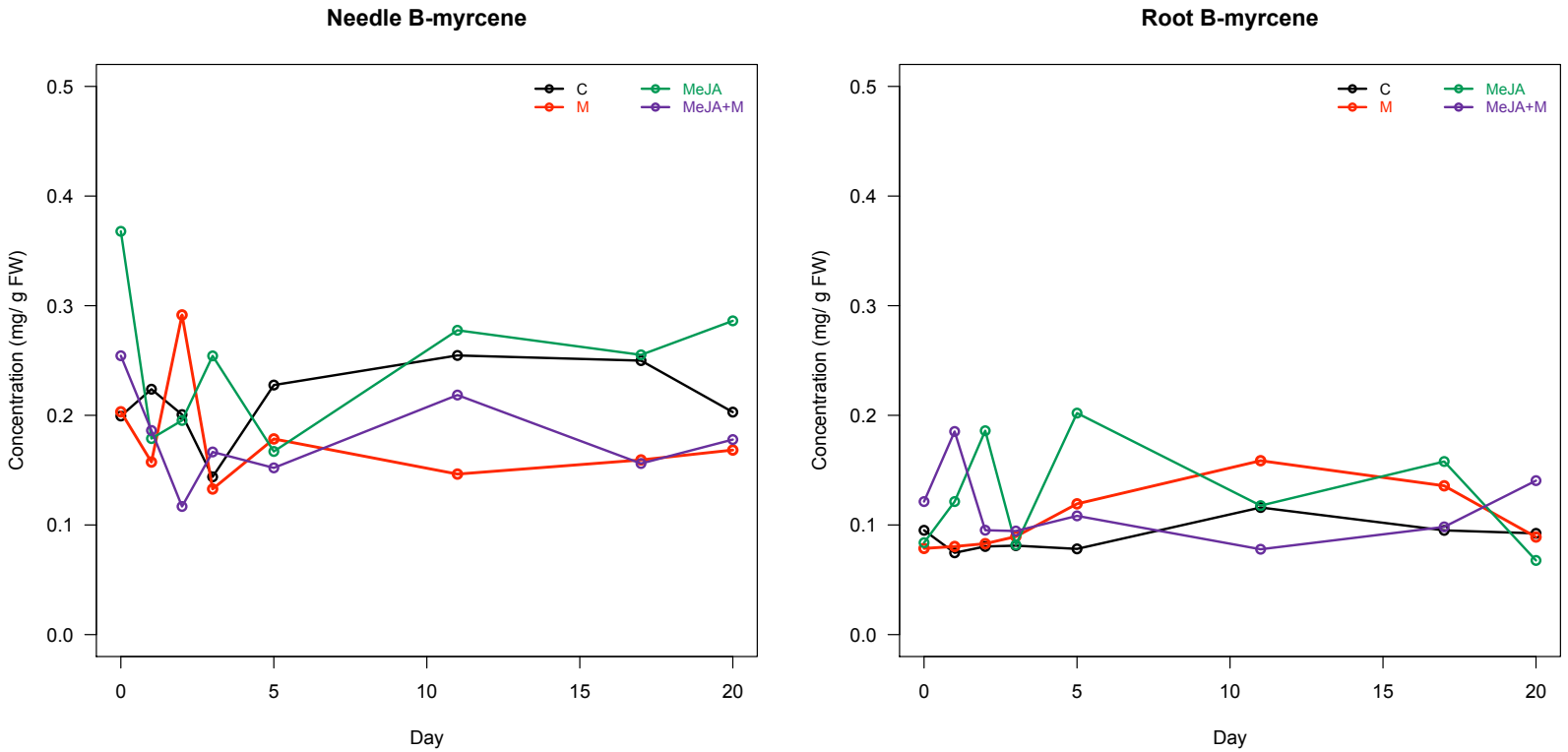

Fig. 8. Changes in the concentration of β-myrcene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

b.

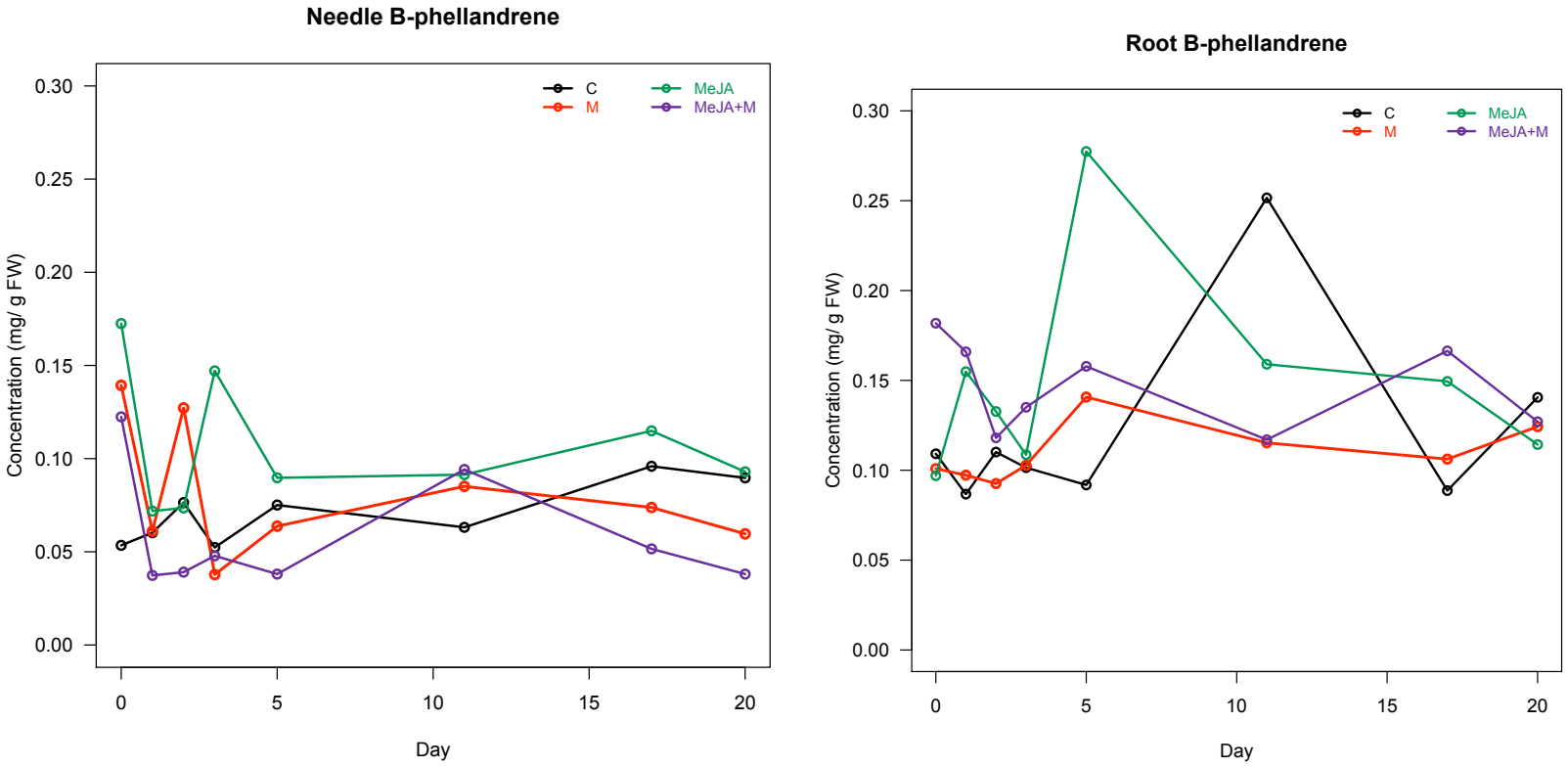

Fig. 9. Changes in the concentration of β-phellandrene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to each damage treatment sampled over a 20 d period. Points represent the mean concentration (n=3).

a.

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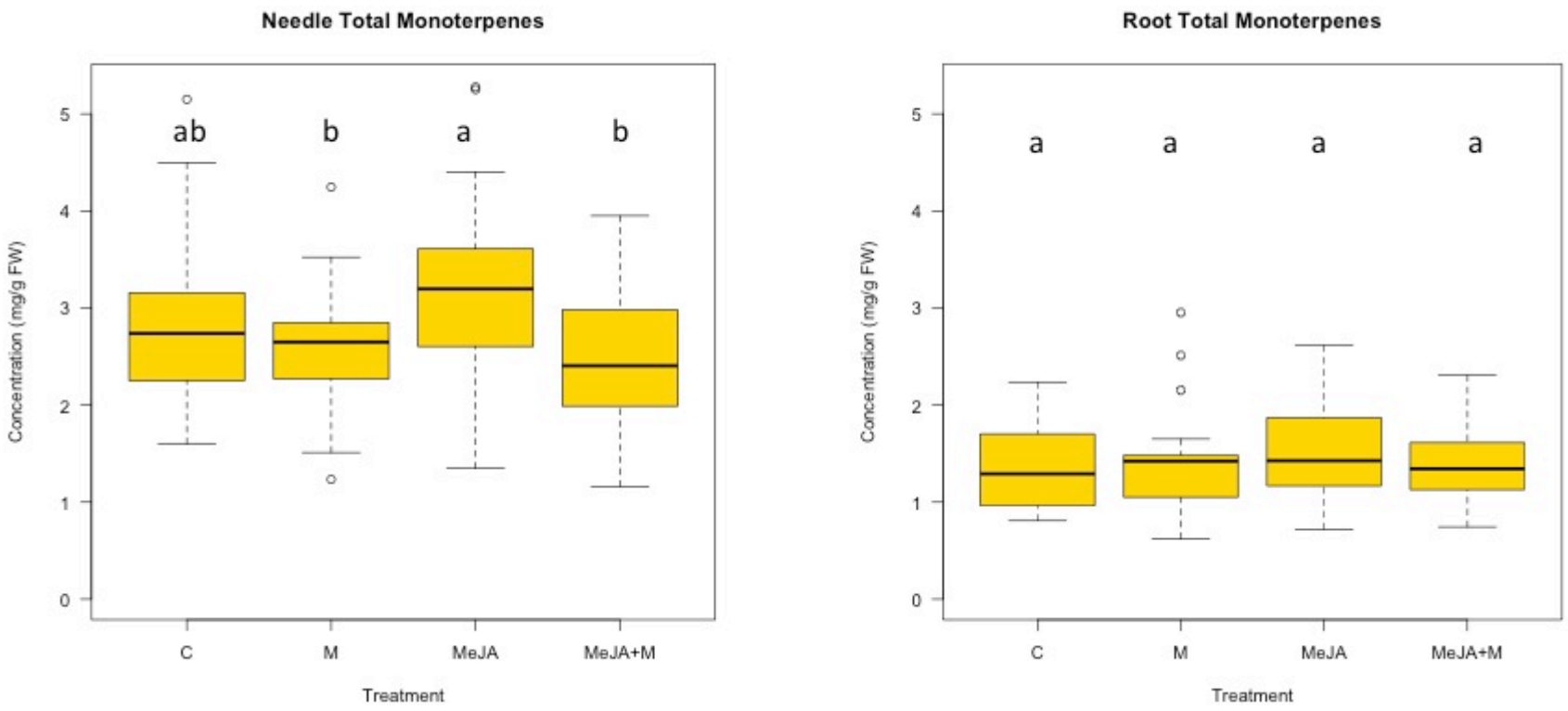

Fig. 10. The concentration of total monoterpenes in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

a.

b.

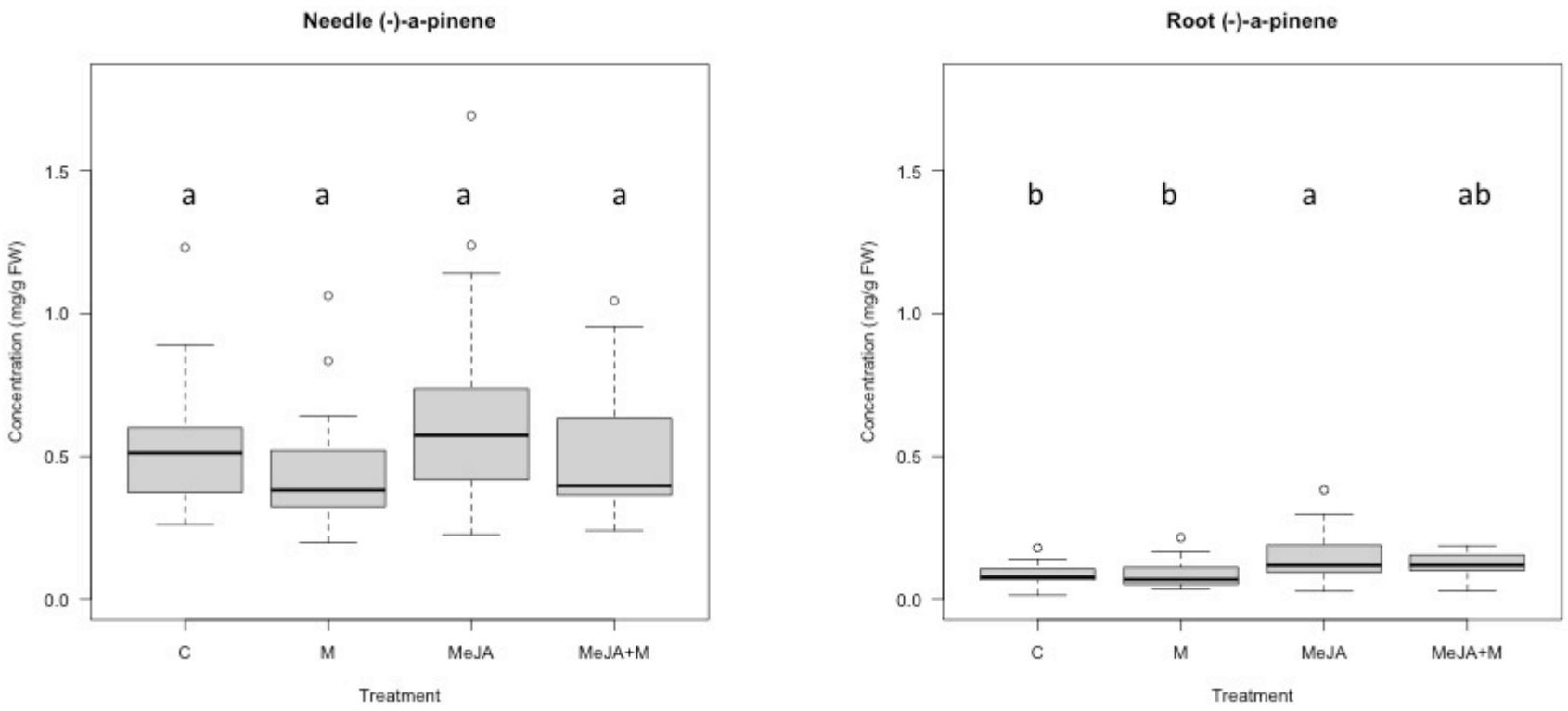

Fig. 11. The concentration of (-)-α-pinene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

a.

b.

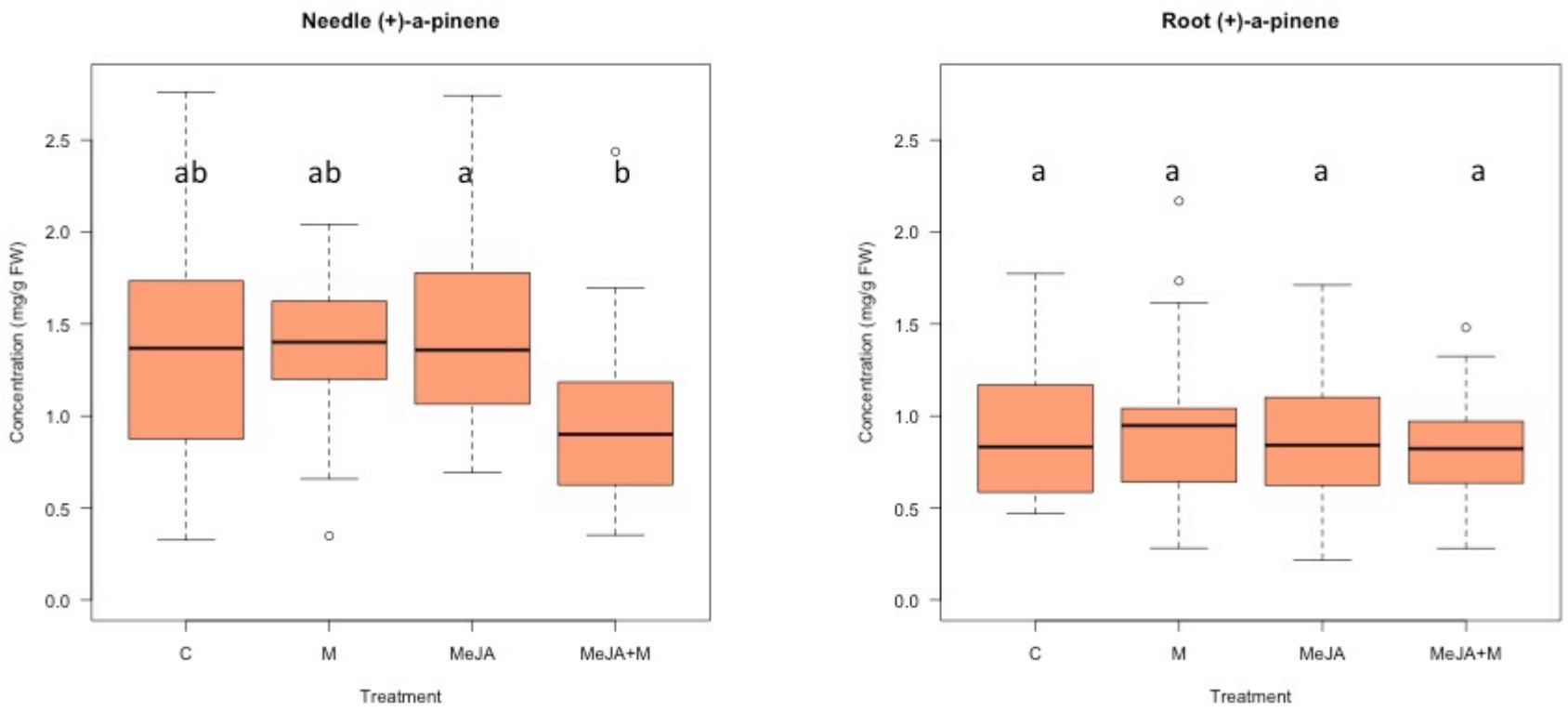

Fig. 12. The concentration of (+)-α-pinene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

a.

b.

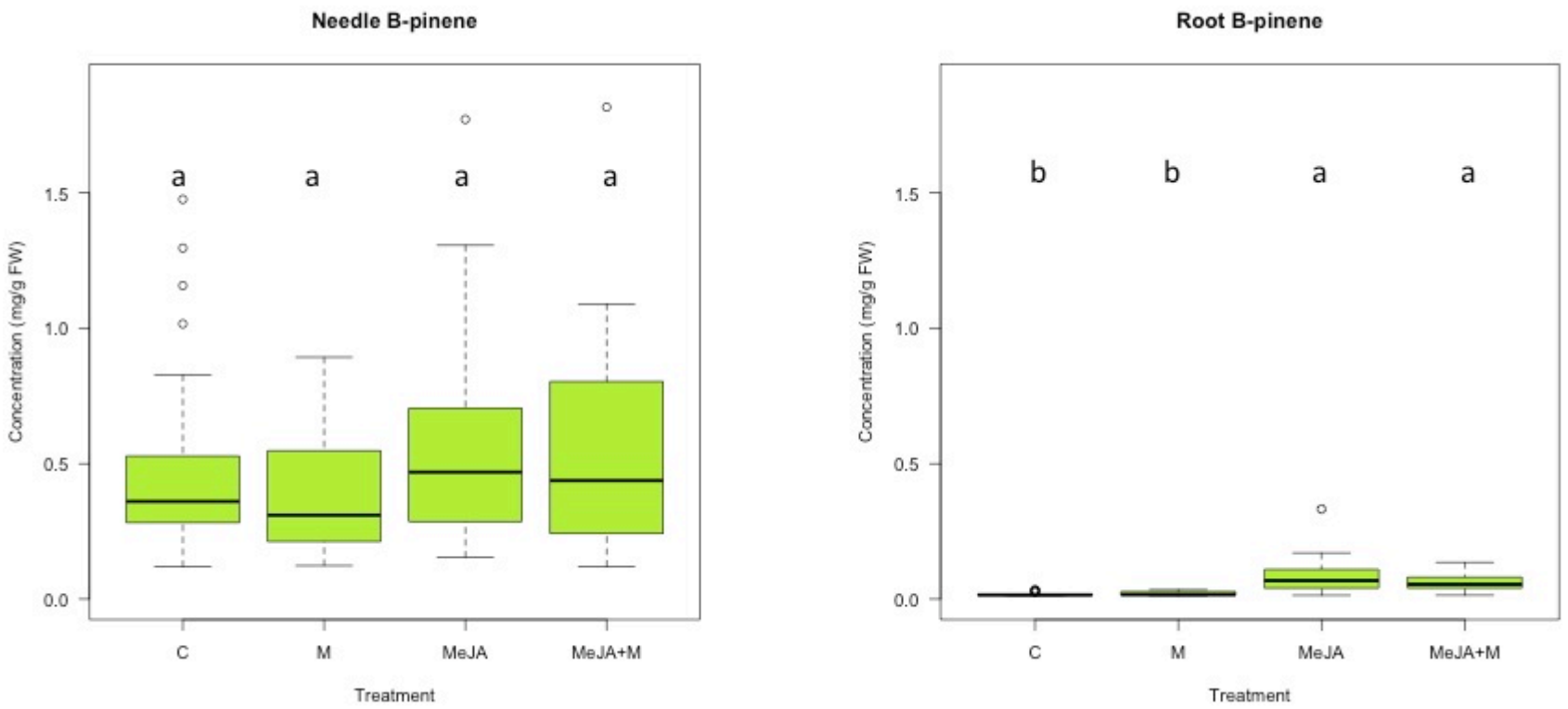

Fig. 13. The concentration of β-pinene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

a.

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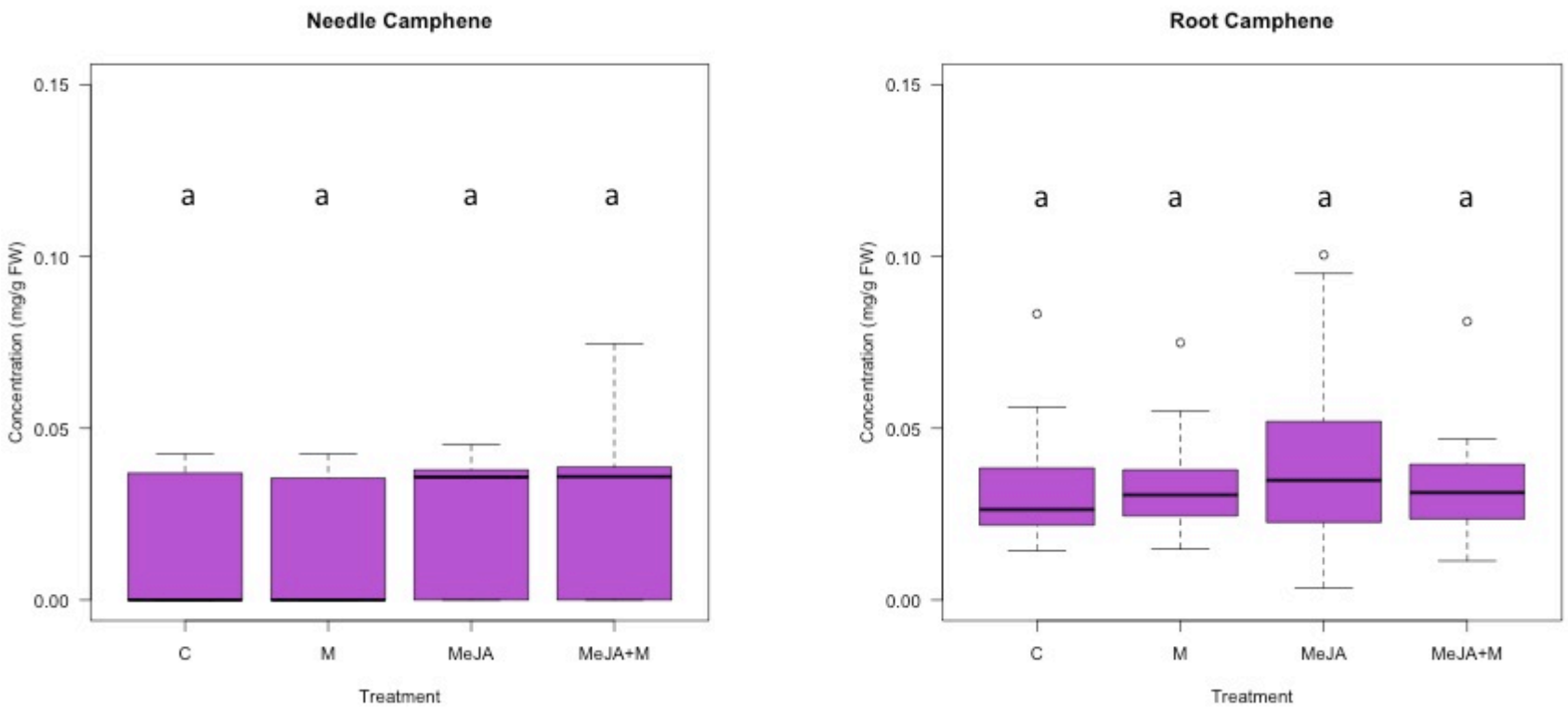

Fig. 14. The concentration of camphene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

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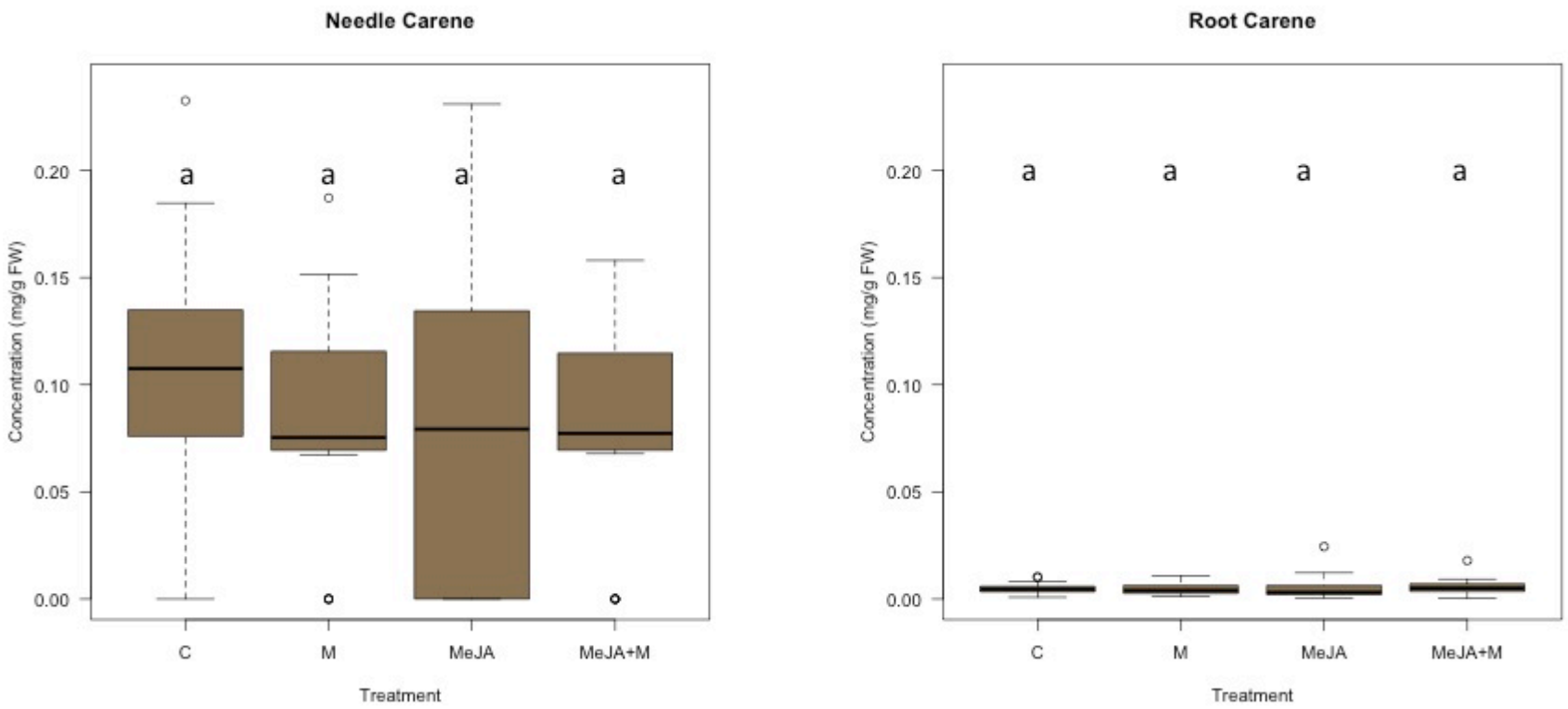

Fig. 15. The concentration of carene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

a.

b.

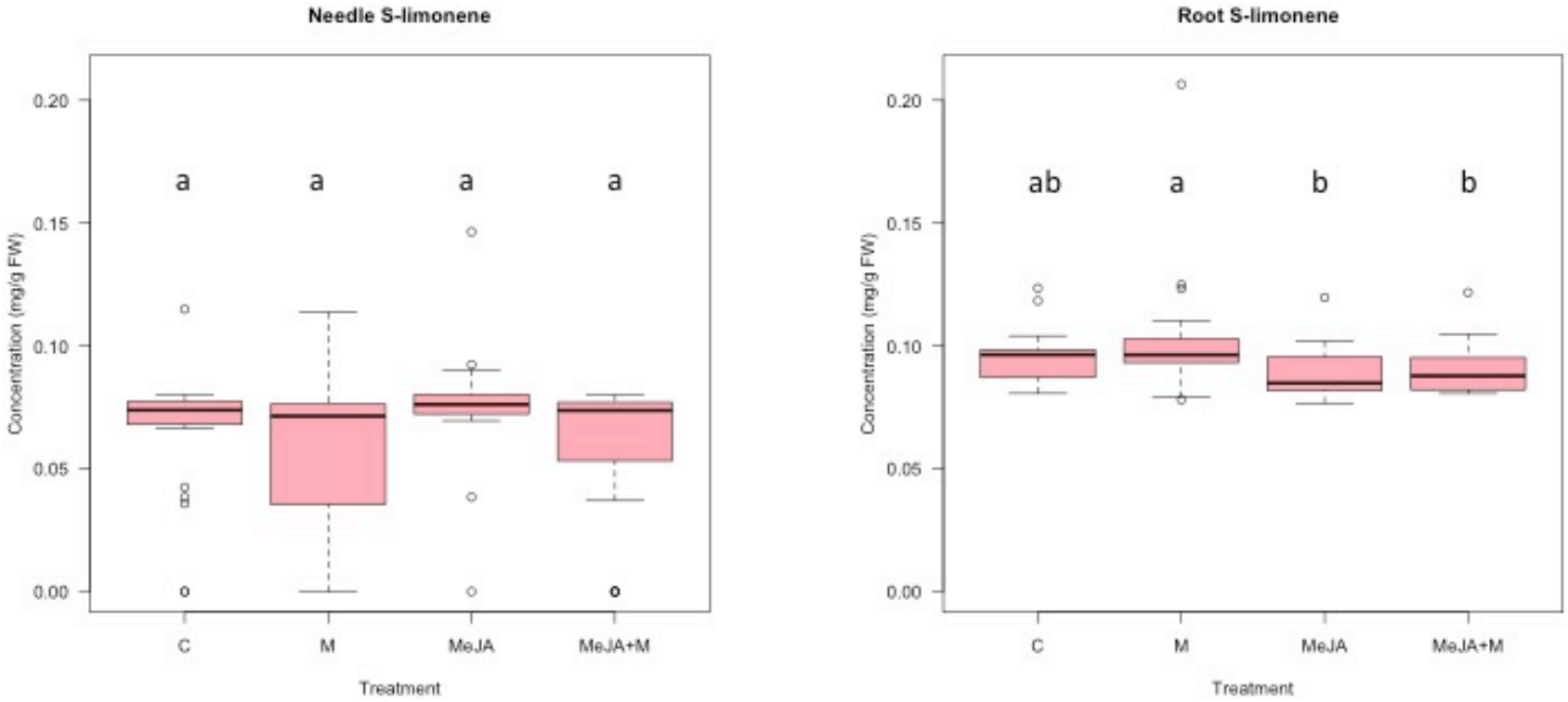

Fig. 16. The concentration of S-limonene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

a.

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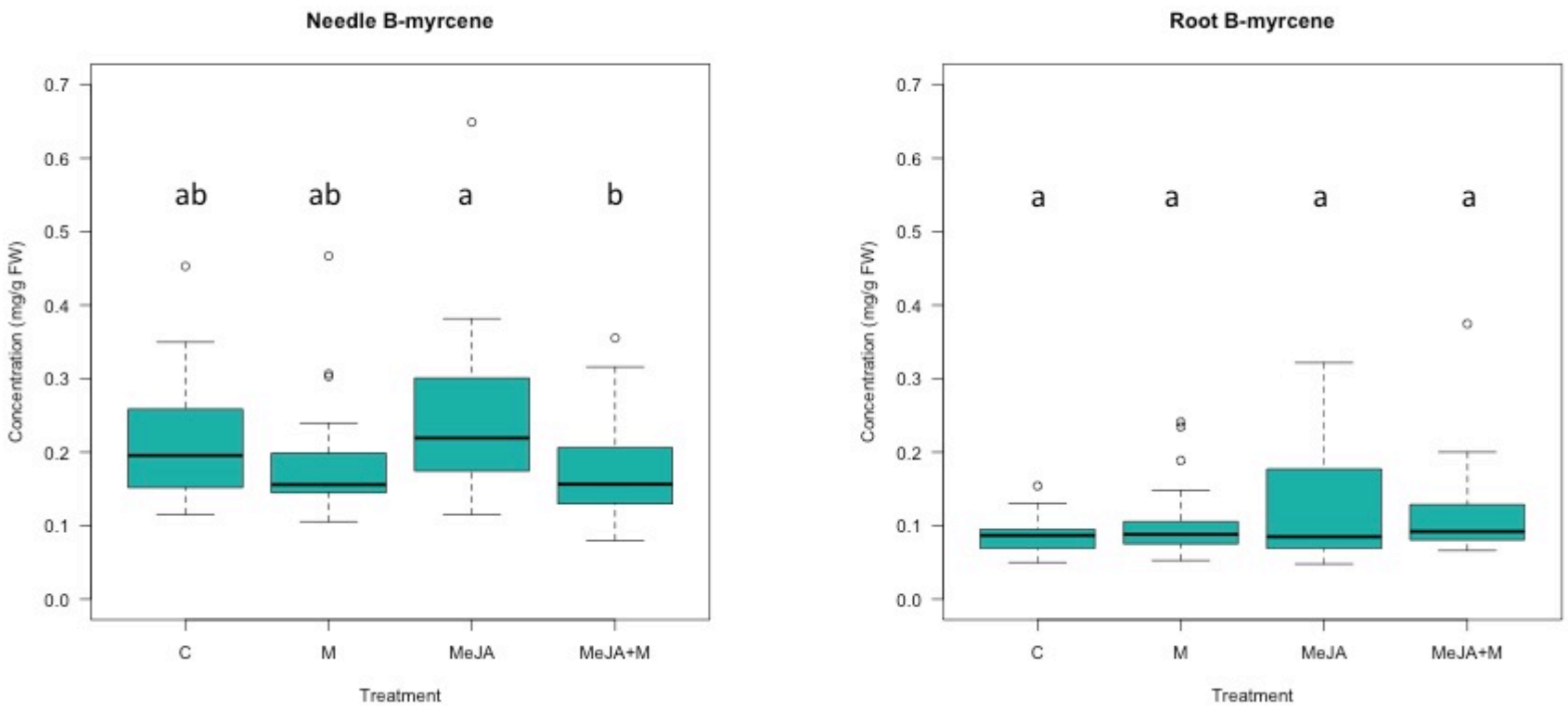

Fig. 17. The concentration of β-myrcene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

a.

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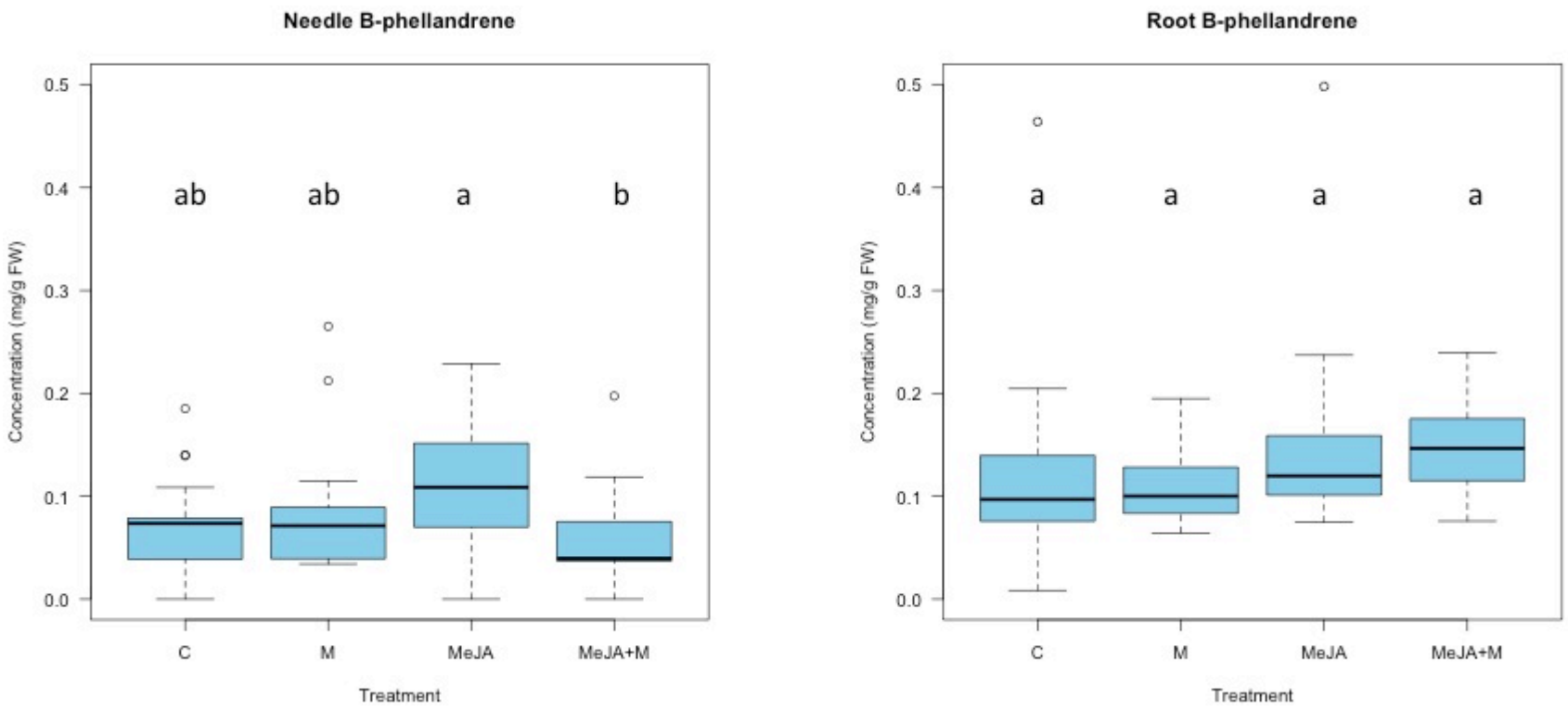

Fig. 18. The concentration of β-phellandrene in the needles (left, a) and roots (right, b) of pinyon pine seedlings exposed to no treatment (C), mechanical damage only (M), MeJA application only (MeJA), or mechanical damage + MeJA application (MeJA+M). Dashed lines represent the sample minimum and maximums, the boxes represent the lower and upper quartiles, the solid black line is the median, and the open circles represent potential outliers.

References

Baldwin, I. T. (1989). Mechanism of damage-induced alkaloid production in wild tobacco. *Journal of Chemical Ecology, 15*(5), 1661-1680.

Baldwin, I. T. (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proceedings of the National Academy of Sciences, 95*(14), 8113-8118.

Bezemer, T., Wagenaar, R., Van Dam, N., Van Der Putten, W., & Wäckers, F. (2004). Above-and below-ground terpenoid aldehyde induction in cotton, gossypium herbaceum, following root and leaf injury. *Journal of Chemical Ecology,30*(1), 53-67.

Bezemer, T., & van Dam, N. (2005). Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology & Evolution, 20*(11), 617-624. doi: 10.1016/j.tree.2005.08.006

Boughton, A. J., Hoover, K., & Felton, G. W. (2005). Methyl jasmonate application induces increased densities of glandular trichomes on tomato, *Lycopersicon esculentum. Journal of Chemical Ecology, 31*(9), 2211-2216.

Carisey, N., & Bauce, E. (1997). Impact of balsam fir foliage age on sixth-instar spruce budworm growth, development, and food utilization. *Canadian Journal of Forest Research*, *27*(2), 257-264.

Coleman, D. C., Crossley, D. A., & Hendrix, P. F. (2004). *Fundamentals of soil ecology* Academic Press.

Degenhardt, D. C., & Lincoln, D. E. (2006). Volatile emissions from an odorous plant in response to herbivory and methyl jasmonate exposure. *Journal of Chemical Ecology, 32*(4), 725-743.

Erb, M., Lenk, C., Degenhardt, J., & Turlings, T. C. (2009). The underestimated role of roots in defense against leaf attackers. *Trends in Plant Science, 14*(12), 653-659.

Feeny, P. (1976). Plant apparency and chemical defense. *Biochemical interactions between plants and insects* (pp. 1-40) Springer.

Feng, Y. J., Wang, J. W., & Jin, Q. (2010). Asian corn borer (*Ostrinia furnacalis*) damage induced systemic response in chemical defence in bt corn (*Zea mays* L.). *Allelopathy Journal, 26*(1), 101-112.

Feng, Y., Wang, J., Luo, S., Fan, H., & Jin, Q. (2012). Costs of jasmonic acid induced defense in aboveground and belowground parts of corn (*Zea mays* L.). *Journal of Chemical Ecology, 38*(8), 984-991.

Fuchs, A., & Bowers, M. D. (2004). Patterns of iridoid glycoside production and induction in *Plantago lanceolata* and the importance of plant age. *Journal of Chemical Ecology, 30*(9), 1723-1741.

Gerber, E., HINZ, H. L., & Blossey, B. (2007). Interaction of specialist root and shoot herbivores of *Alliaria petiolata* and their impact on plant performance and reproduction. *Ecological Entomology, 32*(4), 357-365.

Gómez, S., Ferrieri, R. A., Schueller, M., & Orians, C. M. (2010). Methyl jasmonate elicits rapid changes in carbon and nitrogen dynamics in tomato. *New Phytologist, 188*(3), 835-844.

Hummelbrunner, L. A., & Isman, M. B. (2001). Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). *Journal of Agricultural and Food Chemistry, 49*(2), 715-720.

Iason, G. (2005). The role of plant secondary metabolites in mammalian herbivory: Ecological perspectives. *Proceedings of the Nutrition Society, 64*(01), 123-131.

Jacobi, W. R., & Cranshaw, W. (2009). *Pinyon pine diseases and insects* Colorado State University Extension.

Kaplan, I., Halitschke, R., Kessler, A., Sardanelli, S., & Denno, R. F. (2008). Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology, 89*(2), 392-406. doi: 10.1890/07-0471.1

Karban, R., & Baldwin, I. T. (1997). *Induced responses to herbivory.* University of Chicago Press.

Keeling, C. I., & Bohlmann, J. (2006). Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytologist, 170*(4), 657-675.

Kessler, A., Halitschke, R., & Baldwin, I. T. (2004). Silencing the jasmonate cascade: Induced plant defenses and insect populations. *Science Signaling, 305*(5684), 665.

Kong, J., Lee, S., Moon, Y., Lee, S., & Ahn, Y. (2007). Nematicidal activity of cassia and cinnamon oil compounds and related compounds toward *Bursaphelenchus xylophilus* (Nematoda: Parasitaphelenchidae). *Journal of Nematology, 39*(1), 31.

Lankau, R. A. (2007). Specialist and generalist herbivores exert opposing selection on a chemical defense. *New Phytologist, 175*(1), 176-184.

Lewinsohn, E., Gijzen, M., & Croteau, R. (1991). Defense mechanisms of conifers differences in constitutive and wound-induced monoterpene biosynthesis among species. *Plant Physiology, 96*(1), 44-49.

Lin, C., Owen, S. M., & Peñuelas, J. (2007). Volatile organic compounds in the roots and rhizosphere of *Pinus* spp. *Soil Biology and Biochemistry, 39*(4), 951-960.

Litvak, M. E., Constable, J. V., & Monson, R. K. (2002). Supply and demand processes as controls over needle monoterpene synthesis and concentration in douglas fir [*Pseudotsuga menziesii* (mirb.) franco]. *Oecologia, 132*(3), 382-391.

Litvak, M. E., & Monson, R. K. (1998). Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. *Oecologia, 114*(4), 531-540.

Ludley, K. E., Robinson, C. H., Jickells, S., Chamberlain, P. M., & Whitaker, J. (2008). Differential response of ectomycorrhizal and saprotrophic fungal mycelium from coniferous forest soils to selected monoterpenes. *Soil Biology and Biochemistry, 40*(3), 669-678.

Ludwig-Müller, J., Schubert, B., Pieper, K., Ihmig, S., & Hilgenberg, W. (1997). Glucosinolate content in susceptible and resistant chinese cabbage varieties during development of clubroot disease. *Phytochemistry, 44*(3), 407-414.

Maffei, M. E., Mithöfer, A., & Boland, W. (2007). Before gene expression: Early events in plant–insect interaction. *Trends in Plant Science, 12*(7), 310-316.

Martin, D. M., Gershenzon, J., & Bohlmann, J. (2003). Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of norway spruce. *Plant Physiology, 132*(3), 1586-1599.

Martin, D., Tholl, D., Gershenzon, J., & Bohlmann, J. (2002). Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiology, 129*(3), 1003-1018.

McDowell, N., Pockman, W. T., Allen, C. D., Breshears, D. D., Cobb, N., Kolb, T., . . . Williams, D. G. (2008). Mechanisms of plant survival and mortality during drought: Why do some plants survive while others succumb to drought? *New Phytologist, 178*(4), 719-739.

Mithöfer, A., & Boland, W. (2012). Plant defense against herbivores: Chemical aspects. *Annual Review of Plant Biology, 63*, 431-450.

Mueller, R. C., Scudder, C. M., Porter, M. E., Talbot Trotter, R., Gehring, C. A., & Whitham, T. G. (2005). Differential tree mortality in response to severe drought: Evidence for long-term vegetation shifts. *Journal of Ecology, 93*(6), 1085-1093.

Omer, A. D., Thaler, J. S., Granett, J., & Karban, R. (2000). Jasmonic acid induced resistance in grapevines to a root and leaf feeder. *Journal of Economic Entomology, 93*(3), 840-845.

Paine, T., & Hanlon, C. (1994). Influence of oleoresin constituents fromPinus ponderosa andPinus jeffreyi on growth of mycangial fungi from *Dendroctonus ponderosae* andDendroctonus jeffreyi. *Journal of Chemical Ecology, 20*(10), 2551-2563.

Paré, P. W., & Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiology, 121*(2), 325-332.

Pauwels, L., Inzé, D., & Goossens, A. (2009). Jasmonate-inducible gene: What does it mean? *Trends in Plant Science, 14*(2), 87-91.

Persson, M., Sjödin, K., Borg-Karlson, A., Norin, T., & Ekberg, I. (1996). Relative amounts and enantiomeric compositions of monoterpene hydrocarbons in xylem and needles of *Picea abies. Phytochemistry, 42*(5), 1289-1297.

Pieterse, C., Wees, S. v., Ton, J., Pelt, J. v., & Loon, L. (2002). Signalling in Rhizobacteria: Induced systemic resistance in *Arabidopsis thaliana*. *Plant Biology, 4*(5), 535-544.

Rasmann, S., Agrawal, A. A., Cook, S. C., & Erwin, A. C. (2009). Cardenolides, induced responses, and interactions between above-and belowground herbivores of milkweed (*Asclepias* spp.). *Ecology, 90*(9), 2393-2404.

Reymond, P., Bodenhausen, N., Van Poecke, R. M., Krishnamurthy, V., Dicke, M., & Farmer, E. E. (2004). A conserved transcript pattern in response to a specialist and a generalist herbivore. *The Plant Cell Online, 16*(11), 3132-3147.

Sjödin, K., Persson, M., Borg-Karlson, A., & Norin, T. (1996). Enantiomeric compositions of monoterpene hydrocarbons in different tissues of four individuals of *Pinus sylvestris*. *Phytochemistry, 41*(2), 439-445.

Strack, D., Vogt, T., & Schliemann, W. (2003). Recent advances in betalain research. *Phytochemistry, 62*(3), 247-269.

Theis, N., & Lerdau, M. (2003). The evolution of function in plant secondary metabolites. *International Journal of Plant Sciences, 164*(3), S93-S102. doi: 10.1086/374190

Tholl, D. (2006). Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology, 9*(3), 297-304.

Trowbridge, A. M. (2012). *The effects of atmospheric composition, climate, and herbivory on plant secondary metabolism and volatile organic carbon emissions.* Ph.D. Dissertation, University of Colorado at Boulder.

van Dam, N. M. (2012). Phytochemicals as mediators of aboveground–belowground interactions in plants. *The Ecology of Plant Secondary Metabolites: From Genes to Global Processes,* , 190.

van Dam, N. M., & Heil, M. (2011). Multitrophic interactions below and above ground: En route to the next level. *Journal of Ecology, 99*(1), 77-88.

van Dam, N. M., Raaijmakers, C. E., & Van Der Putten, Wim H. (2005). Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra. Entomologia Experimentalis Et Applicata, 115*(1), 161-170.

van Dam, N. M., Witjes, L., & Svatoš, A. (2004). Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. *New Phytologist, 161*(3), 801-810.

Whittaker, R. H., & Feeny, P. P. (1971). Allelochemics: Chemical interactions between species. *Science, 171*(3973), 757-770.

Wilt, F., Miller, G., Everett, R., & Hackett, M. (1993). Monoterpene concentrations in fresh, senescent, and decaying foliage of single-leaf pinyon (pinus-monophylla torr and frem - pinaceae) from the western great-basin. *Journal of Chemical Ecology, 19*(2), 185-194. doi: 10.1007/BF00993688

Zangerl, A. R., & Bazzaz, F. A. (1992). Theory and pattern in plant defense allocation. *Plant Resistance to Herbivores and Pathogens.University of Chicago Press, Chicago*, 363-391.