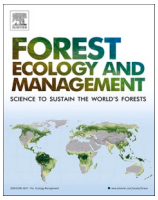
Forest Ecology and Management 545 (2023) 121258

Contents lists available at ScienceDirect

Forest Ecology and Management

journal homepage: www.elsevier.com/locate/foreco



Defensive response of evolutionarily naïve *Pinus sylvestris* to the mountain pine beetle fungal associate *Grosmannia clavigera* in comparison to *Pinus ponderosa*

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ARTICLE INFO

Keywords:
Mountain pine beetle
Novel interaction
Phytochemistry
Terpenoids
Range expansion

ABSTRACT

Mountain pine beetle (MPB, *Dendroctonus ponderosae*) is a destructive pest of pine forests in western North America. This insect is currently expanding its range across the Canadian boreal forest towards eastern North America, where a suite of novel pine species will be encountered. One species of pine without prior association with MPB is *Pinus sylvestris* (Scots pine), which is native to Europe and naturalized in parts of central and eastern North America. Here, we take advantage of a unique opportunity in the Black Hills of South Dakota where an isolated, planted, and mature stand of *P. sylvestris* and native *Pinus ponderosa* (ponderosa pine) co-exist within the range of MPB. We conducted a punch-inoculation experiment to determine the chemical response of *P. sylvestris* from a blue-stain fungus associated with MPB, *Grosmannia clavigera*, and compared the response to that of *P. ponderosa*. We found that *P. sylvestris* had a higher localized monoterpene response than *P. ponderosa* in response to inoculation, but a lower sesquiterpene response. Among the significant monoterpenes associated with MPB behavior, limonene, 3-carene, and myrcene had a larger localized response in *P. sylvestris* than *P. ponderosa*; lower levels of 4-allylanisole were found in *P. sylvestris*. Fungal inoculation did not induce a stronger terpenoid response than mechanical wounding without inoculation, indicating that *P. sylvestris* responds to mechanical damage similarly as to fungal inoculation. *Pinus sylvestris* may provide one alternative plantation species for timber production in the Great Lakes Region following mountain pine beetle incursion, however, more evaluation is needed to determine the role of this species in future plantings.

1. Introduction

Mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins) is arguably the most destructive arthropod disturbance agent in North America (Safranyik and Carroll, 2007). In the past two decades, MPB has breached the historical geo-climatic barrier of the Rocky Mountains and threatens to expand its range eastward across the northern boreal forest of Canada to eastern North America (Safranyik et al., 2010; Clark et al., 2014; Cullingham et al., 2011; Erbilgin et al., 2014; Cooke and Carroll 2017). A hybrid zone of the MPB's historic host, lodgepole pine (*Pinus contorta* Douglas) and an evolutionary naïve host, jack pine (*Pinus banksiana* Lamb.) has been hypothesized to serve as an ecological bridge across the boreal forest during range expansion (Lusebrink et al., 2013; Erbilgin et al., 2014; Trowbridge and Keefover-Ring 2017). Because MPB preferentially reproduces in live trees but is not yet found in areas of eastern North America such as the Great Lakes region, forecasting suitability and susceptibility to pine species of the region such as *Pinus resinosa* Sol. ex Aiton, *Pinus strobus* L., *Pinus sylvestris* L., and *P. banksiana* has proved challenging. Lab and field studies using cut logs instead of live trees have shown that MPB can successfully colonize and reproduce in cut logs of all major *Pinus* species present in the Great Lakes region (Cale et al., 2015; Rosenberger et al. 2017, 2018; Erbilgin et al. 2020). Outside of reports of attacks of specimens in arboreta, however, it remains largely unknown how defenses of live trees of pine species without historic coexistence with MPB would fare in the case of an eventual range overlap (Cook and Martinez, 2018; Rosenberger et al. 2019).

Classification of tree defense compounds can be split into two categories: constitutive and induced (Keeling and Bohlmann 2006). Constitutive compounds are always present while induced compounds

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https://doi.org/10.1016/j.foreco.2023.121258
Received 22 February 2023; Received in revised form 4 July 2023; Accepted 6 July 2023
Available online 19 July 2023

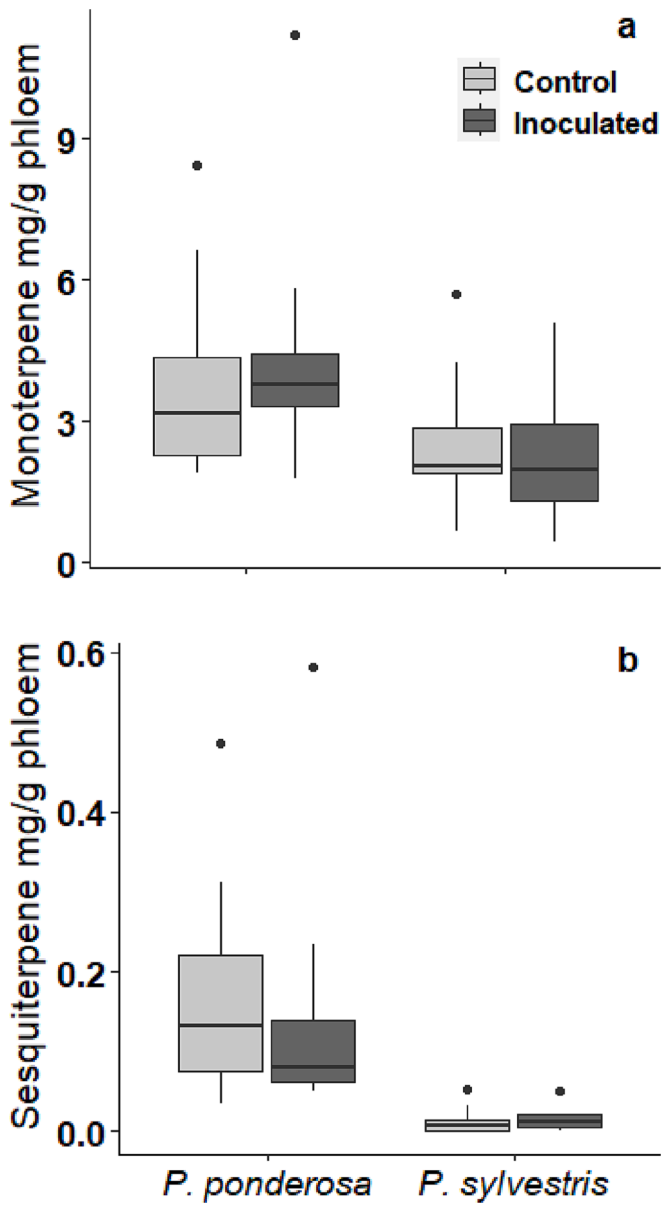

Fig. 1. Constitutive levels of (a) monoterpenes and (b) sesquiterpenes for *Pinus ponderosa* and *P. sylvestris* in mock-inoculated trees and inoculated trees. Darker lines within the boxplot represent the median, the upper and lower limits of the boxplot denote the upper and lower quartiles, respectively, and the 'whiskers' denote 1.5 times the interquartile range limits. Points above the boxplots represent values outside these ranges.

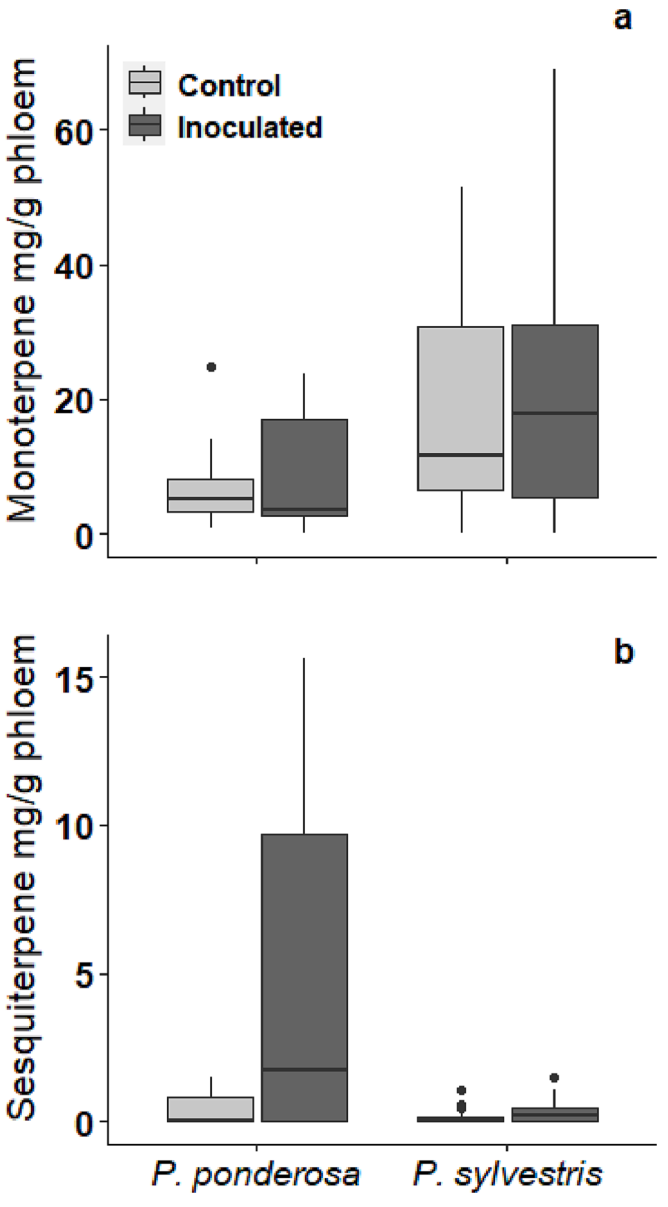

Fig. 2. Chemistry of localized response samples. Changes from initial constitutive samples in (a) monoterpene and (b) sesquiterpene levels of *Pinus ponderosa* and *P. sylvestris* in mock-inoculated trees (light boxplots) and inoculated trees (dark boxplots) at wound sites after three weeks. Darker lines within the boxplot represent the median, the upper and lower limits of the boxplot denote the upper and lower quartiles, respectively, and the 'whiskers' denote 1.5 times the interquartile range limits. Points above the boxplots represent values outside these ranges.

become activated when an herbivore attacks. If the physical barriers of trees (e.g., bark) are breached, constitutive compounds such as oleoresin in resin ducts (Tisdale et al. 2003) and phenolics (Brignolas et al. 1995) form the next line of chemical defense against an attacker (Franceschi et al. 2005; Huang et al. 2020). Oleoresin is comprised of monoterpenes, diterpenes acids, and sesquiterpenes that can be toxic to an invader (Himejima et al., 1992). The amount of oleoresin and the terpenoid diversity varies between pine species, as well as geographically and seasonally (Nerg et al., 1994; Yazdani and Nilsson, 1986; Keeling and Bohlmann 2006; West et al., 2016; Davis et al. 2018). The viscous oleoresin can also serve as a secondary physical barrier. At the site of attack, a localized, induced chemical response may also occur where higher levels of compounds are synthesized and aggregate (Huber et al., 2004; Langenheim, 1994; Martin and Bohlmann, 2005; Raffa and Berryman, 1983). A recent *meta*-analysis found that a general positive correlation exists between concentrations of constitutive monoterpenes and inducible monoterpenes in conifer species (Howe et al. 2020). But instances of negative associations do exist (Koricheva et al. 2004), especially when nutrients are limited (Sampedro et al. 2011). Increased resin flow and necrotic lesions may also form around the site of attack. Induced tree responses from MPB attack can cause a 100-fold or more increase in monoterpene levels in some *Pinus* species (e.g., Boone et al., 2011).

When MPB attacks a tree, the beetle introduces a suite of microorganisms (Mercado et al., 2014), including fungi (Paine et al., 1997; Six, 2003), bacteria (Adams et al., 2013; Cardoza et al., 2009; Hulcr et al., 2011; Therrien et al., 2015), and yeasts (Hunt and Borden, 1990). Arguably, the most important microorganism against which an MPB attacked-tree must defend itself is the fungus *Grosmannia clavigera*

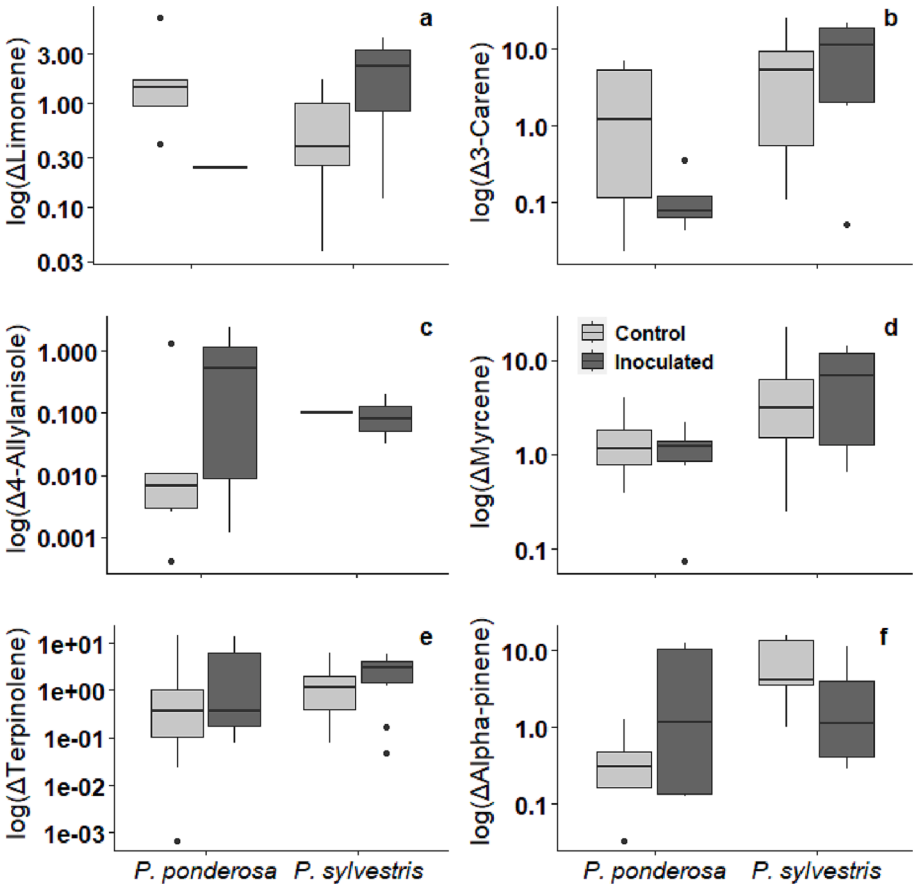

Fig. 3. Changes in localized levels of monoterpenes and 4-allylanisole present at mock-inoculation (light boxes) and fungal inoculation (dark boxes) with *Grosmannia clavigera* sites after three weeks post-inoculation in both *Pinus ponderosa* and *P. sylvestris*. Darker lines within the boxplot represent the median, the upper and lower limits of the boxplot denote the upper and lower quartiles, respectively, and the 'whiskers' denote 1.5 times the interquartile range limits. Points above the boxplots represent values outside these ranges.

Zipfel, de Beer and Wingfield, due to its virulence (Robinson-Jeffrey and Davidson 1968). This fungus is beneficial to MPB because it can metabolize oleoresin terpene compounds into relatively benign products (DiGuistini et al., 2011), exhaust carbohydrate reserves of the host (Goodsman et al., 2013; Roth et al., 2018), and supplement nutrients to developing beetle larvae that may not be present in the phloem (Bleiker and Six, 2007). Although the relationship of western *Pinus species* to *G. clavigera* is well studied (Alamouti et al., 2011; Arango-Velez et al., 2016), no studies to date have investigated the response of pines at risk from range expansion of MPB to *G. clavigera*, such as *P. sylvestris*.

Pinus sylvestris is the most widely distributed pine species in the world, spanning across Europe and northern Asia, naturalized throughout the Great Lakes and New England regions of North America, and planted in various locations across the southern hemisphere. Interestingly, one study found that *P. sylvestris* is less susceptible to mortality from MPB than *P. ponderosa* (Rosenberger et al., 2019), although chemical defenses were not quantified. To better predict the potential impacts of MPB on *P. sylvestris* forests within and outside of North America, we compared the chemical response of *P. sylvestris* and *P. ponderosa* (*Pinus ponderosa* Douglas ex. Laws) to the MPB fungal associate *G. clavigera*. We conducted a punch-inoculation experiment (Christiansen et al. 1999; Krokene et al. 1999) in a mixed stand of both species in the Black Hills of South Dakota, a region of the USA where MPB and *G. clavigera* is endemic.

In this study, we focus on two major terpenoid groups, monoterpenes and sesquiterpenes, which have well-known roles in pine defense against bark beetles and other organisms (Langenheim, 1994; Franceschi et al., 2005; Huber et al., 2004; Raffa and Berryman, 1983). Specifically, we measured the localized mono- and sesquiterpene response of *P. sylvestris* and *P. ponderosa* that were wounded with a small punch to simulate attack by MPB and then inoculated with *G. clavigera* or left as a control. We hypothesized that higher levels of defensive compounds would be localized in *P. ponderosa* than *P. sylvestris*, because of its long evolutionary association with the beetle-fungal complex. *Pinus sylvestris* shares no evolutionary history with MPB and no primary pest of *P. sylvestris* exists in its native range, although *Ips acuminatus* has been involved in killing alpine *P. sylvestris* in Europe (Wermelinger et al., 2008).

2. Materials and methods

2.1. Field design

We selected 32*P. sylvestris* and 32*P. ponderosa* with little or no prior beetle attack (visually determined from lack of pitch tubes and frass) from a single stand in the Black Hills National Forest, South Dakota, USA 44°16′00.0″ N 103°37′17.0″ W. *Pinus ponderosa* is native to the Black Hills while *P. sylvestris* is non-native but has become naturalized over the past century. The total basal area of the stand, which is dominated by ponderosa pine (~79%), was 18.24 m^2/hectare. The mean age of sampled *P. sylvestris*, determined by tree coring, was 71.1 (±2.8 standard error of mean) years and the mean age of *P. ponderosa* was 71.5 (±2.7) years, indicating that both species were planted soon after the last time *P. ponderosa* were harvested from this stand. The mean diameters at breast height of sampled *P. sylvestris* and *P. ponderosa* were 31.6 (±17.1) cm and 32.5 (±13.1) cm, respectively.

For both *Pinus* species, we inoculated the phloem layer of 16 trees with a 1.19 cm plug of the fungus *G. clavigera* at approximately breast height (1.4 m above ground level) on the south side of the tree. The strain of *G. clavigera*, 'L3b4b-b' (Genbank accession number: OR074397), originated from a mountain pine beetle in the Black Hills of South Dakota, USA that had colonized a billet of lodgepole pine transported from the Bighorn Mountains in Wyoming, USA. The fungus was grown on malt extract agar for 14 days and confirmed to have fruiting

Table 1
Mean +/- SE mono- and sesquiterpene response to inoculation of *Grosmannia clavigera* in mock-inoculated and inoculated trees, Black Hills of South Dakota, USA, summer 2016.

Compound	Type[Ø]	Mean Constitutive Levels (mg terpene/g phloem) (±SEM)				Mean Localized Levels (mg terpene/g phloem) (±SEM)			
		Pinus ponderosa		*Pinus sylvestris*		*Pinus ponderosa*		*Pinus sylvestris*	
		Mock-Inoculated	Inoculated	Mock-Inoculated	Inoculated	Mock-Inoculated	Inoculated	Mock-Inoculated	Inoculated
α-pinene	M	0.420 ± 0.131	0.485 ± 0.115	0.456 ± 0.105	0.466 ± 0.111	0.369 ± 0.099	1.579 ± 1.030	2.441 ± 1.266	0.987 ± 0.742
α-terpinene	M	0.020 ± 0.014	0.007 ± 0.001	0.005 ± 0.002	0.008 ± 0.001	0.055 ± 0.052	0.082 ± 0.041	0.028 ± 0.009	0.068 ± 0.016
α-terpineol	M	0.011 ± 0.001	0.016 ± 0.004	0.058 ± 0.017	0.047 ± 0.013	0 ± 0	0 ± 0	0.035 ± 0.023	0 ± 0
β-pinene	M	0.135 ± 0.029	0.170 ± 0.031	0.343 ± 0.131	0.225 ± 0.070	0.804 ± 0.259	0.072 ± 0.026	4.025 ± 1.548	4.782 ± 2.265
p-cymene	M	0.013 ± 0.001	0.015 ± 0.002	0.022 ± 0.002	0.023 ± 0.002	0.014 ± 0.004	0.015 ± 0.004	0.040 ± 0.016	0.011 ± 0.007
γ-terpinene	M	0.009 ± 0.001	0.010 ± 0.001	0.009 ± 0.001	0.009 ± 0.001	0.0368 ± 0.026	0.311 ± 0.111	0.086 ± 0.026	0.155 ± 0.033
3-carene	M	0.541 ± 0.094	0.556 ± 0.094	0.273 ± 0.046	0.276 ± 0.039	1.915 ± 0.670	0.152 ± 0.052	4.633 ± 1.834	5.785 ± 2.159
Bornyl acetate	M	0.089 ± 0.027	0.077 ± 0.017	0.006 ± 0.001	0.006 ± 0.001	0.064 ± 0.019	0.427 ± 0.132	0.046 ± 0.026	0.317 ± 0.186
Camphene	M	0.025 ± 0.007	0.019 ± 0.003	0.027 ± 0.003	0.027 ± 0.003	0.019 ± 0.007	0.280 ± 0.104	0.511 ± 0.161	0.841 ± 0.312
Camphor	M	0 ± 0	0 ± 0	0.004 ± 0.002	0.006 ± 0.002	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Gernyl acetate	M	0.112 ± 0.030	0.064 ± 0.028	0.003 ± 0.001	0.002 ± 0.001	0.075 ± 0.037	0.060 ± 0.029	0 ± 0	0 ± 0
Limonene	M	0.955 ± 0.263	1.029 ± 0.351	0.070 ± 0.012	0.074 ± 0.011	0.955 ± 0.455	0.167 ± 0.081	0.537 ± 0.147	1.374 ± 0.389
Myrcene	M	1.327 ± 0.124	1.757 ± 0.098	1.085 ± 0.236	1.035 ± 0.266	2.338 ± 0.255	1.095 ± 0.337	5.087 ± 1.464	4.362 ± 1.436
Ocimene	M	0.004 ± 0.002	0.006 ± 0.002	0 ± 0	0 ± 0	0 ± 0	0.027 ± 0.011	0.007 ± 0.004	0.012 ± 0.006
Pulegone	M	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sabinene	M	0.024 ± 0.009	0.021 ± 0.005	0 ± 0	0.001 ± 0.001	0.017 ± 0.006	0.003 ± 0.002	0.192 ± 0.081	0.203 ± 0.076
Terpinolene	M	0.085 ± 0.028	0.034 ± 0.013	0.026 ± 0.007	0.024 ± 0.004	0.274 ± 0.083	3.577 ± 0.351	1.386 ± 0.432	2.819 ± 0.0487
Terpinyl acetate	M	0.027 ± 0.007	0.026 ± 0.004	0.016 ± 0.003	0.017 ± 0.002	0.041 ± 0.014	0.539 ± 0.214	0.130 ± 0.055	0.389 ± 0.134
α-Cedrene	S	0.002 ± 0.001	0.002 ± 0.001	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
α-Humulene	S	0.004 ± 0.002	0.002 ± 0.001	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
α-Longipinene	S	0.005 ± 0.001	0.005 ± 0.001	0.002 ± 0.001	0.003 ± 0.002	0.037 ± 0.013	0.353 ± 0.138	0.070 ± 0.042	0.088 ± 0.050
β-Cedrene	S	0 ± 0	0.001 ± 0.000	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
β-Elemene	S	0 ± 0	0.001 ± 0.001	0.002 ± 0.001	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.012 ± 0.006
Caryophyllene	S	0.003 ± 0.001	0.002 ± 0.001	0 ± 0	0 ± 0	0.002 ± 0.001	0.008 ± 0.005	0.018 ± 0.013	0.029 ± 0.010
Longifolene	S	0.037 ± 0.006	0.051 ± 0.007	0.004 ± 0.001	0.007 ± 0.002	0.359 ± 0.114	4.187 ± 1.265	0.076 ± 0.036	0.239 ± 0.087
4-allylanisole	P	0.013 ± 0.003	0.009 ± 0.002	0.003 ± 0.001	0 ± 0	0.012 ± 0.004	0.332 ± 0.171	0.006 ± 0.006	0.015 ± 0.013

[Ø] M = monoterpene; S = sesquiterpene; P = phenylpropanoid.

bodies. Prior to inoculation, we used a 1.19 cm plug cutter (Harbor Freight, Calabasas, CA) attached to a battery-operated hand drill (DeWalt, Baltimore, MD) to remove the bark and expose the phloem layer. We used a separate 1.19 cm plug cutter to obtain the fungal plug and sterilized the plug cutter by dipping it in 90% Ethanol and then placing it over a lighter between each inoculation. We then placed the inoculum into the wound and replaced the 1.19 cm outer bark plug over the inoculation site of all trees and covered the wound with duct tape to reduce likelihood of airborne fungal infection. Sixteen *P. sylvestris* trees and sixteen *P. ponderosa* trees served as experimental mock-inoculation controls where we drilled 1.19 cm holes into the tree, but no fungal inoculation was conducted. We chose trees at random, but alternated between inoculated trees and mock-inoculated trees within a species. Immediately after wounding/inoculating trees, constitutive phloem samples were taken on the opposite (i.e., north) side of each tree at breast height to quantify defenses at time of experiment initiation. All inoculations and mock inoculations were conducted during 28 and 29 July 2016.

To determine differences in post inoculation chemical response between *P. ponderosa* and *P. sylvestris*, trees were revisited approximately three weeks later (17–19 August 2016). Then, we collected "localized response samples" by removing 1.19 cm diameter phloem plugs within the fungal lesion if present but not greater than 3 cm above where we inoculated. Phloem plugs were also collected on the north side of each tree horizontal and outside of the lesion zone to the constitutive samples, which we term the "systemic response samples" by comparing with the

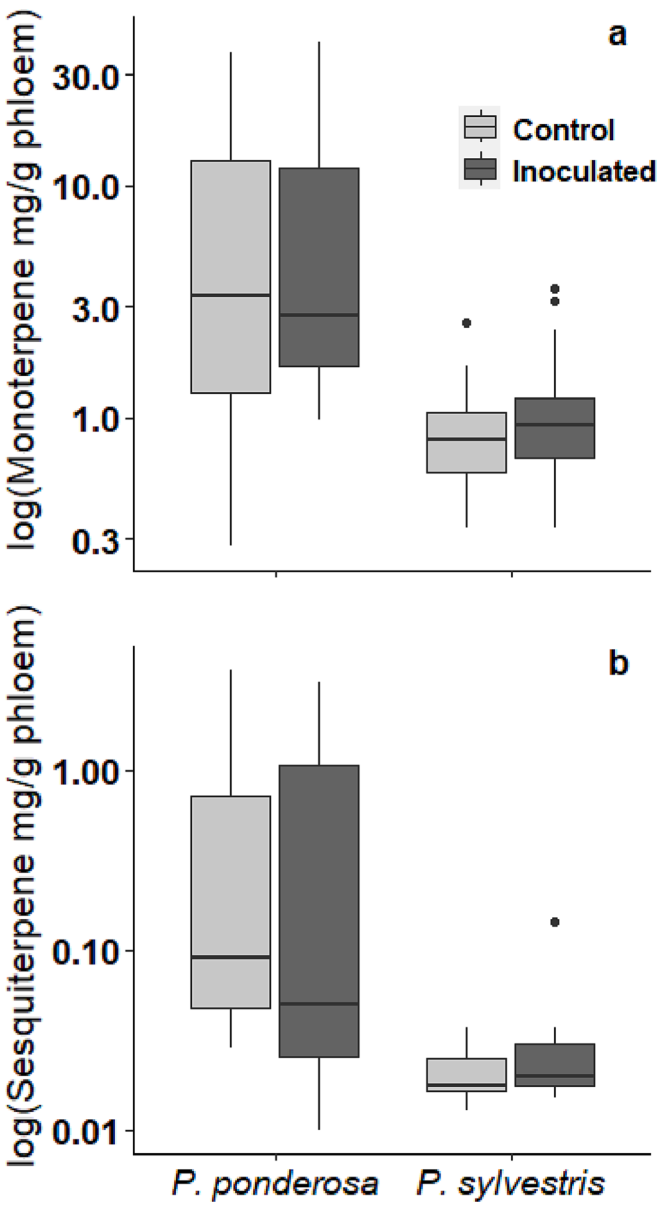

Fig. 4. Systemic increases (Δ: systemic levels – constitutive levels) in (a) monoterpene and (b) sesquiterpene levels of ponderosa pine and Scots pine. Levels were calculated by subtracting constitutive levels of chemicals taken at experiment initiation from those measured from the opposite side of the tree from wounding sites three weeks later. Darker lines within the boxplot represent the median, the upper and lower limits of the boxplot denote the upper and lower quartiles, respectively, and the 'whiskers' denote 1.5 times the interquartile range limits. Points above the boxplots represent values outside these ranges.

localized inoculation samples (terpene response conditions) (Keefover-Ring et al. 2016). All phloem samples were immediately frozen with either ice packs (initial constitutive samples) or dry ice (localized response and systemic response samples) in the field and then stored in the laboratory at −25 °C. Prior to collecting the latter phloem samples, we also measured the lesion length response (if present) by removing the outer bark until the total lesion was revealed around wounding site, which can be further evidence of fungal uptake and localized defensive response (Berryman, 1972; Christiansen et al., 1987).

2.2. Chemical analysis

Processing and analysis of phloem samples followed a modified protocol from Rosenberger et al. (2017). All phloem samples were stored at −20 °C prior to extraction. Samples, approximately 0.56 cm^2, were flash frozen in liquid nitrogen prior to being finely chopped using a scalpel with a #22 blade. The blade was washed with gas chromatography (GC) grade hexane or changed in-between samples. The phloem samples were extracted twice with 0.75 mL GC grade hexane containing 0.025 mM heptyl acetate (extraction solvent). The extractions were performed in 4 mL screw top glass vials at room temperature without agitation. Agitation saw less extraction of terpenes and the materials were large enough that centrifugation was not necessary to remove the eluent from the extraction. The first 0.75 mL extraction was removed after 8 h, and 0.75 mL fresh extraction solvent were added to the phloem. The second extraction was allowed to incubate for 16 h for a total of 24 h of extraction time. The phloem was rinsed with 0.5 mL extraction solvent, which was added to the previous two extractions for a final volume of 2 mL.

The remaining phloem pellet was dried in an oven at 60 °C for a minimum of three days before being weighed to normalize the terpene extracts. One milliliter of the extraction solution was transferred to an autosampler vial (Restek, Bellafonte, PA) using a 3 mL syringe fitted with a 0.45 μm polyvinylidene fluoride syringe filter. With later extractions, particularly systemic samples from *P. ponderosa*, 250 μL limited volume glass inserts (Restek) were used in the GC vials to conserve extraction solution allowing for later injections at higher or lower injection volumes as necessary. Gas chromatography-mass spectrometry (GC–MS) analysis was carried out using a Shimadzu QP2010S equipped with a Restek Rxi-5 ms column (30 m × 0.25 mm) using helium as the carrier gas at a column flow rate of 0.60 mL/min. Initial oven temperature was 55.0 °C which was held for five minutes before being increased at 1 °C per minute to 60.0 °C. The temperature was then held for five minutes before it was increased at 1 °C per minute to 70.0 °C. Then increased at 10 °C per minute to 160.0 °C followed by 2 °C per minute to 175.0 °C. Finally, the temperature was increased at 30 °C per minute until 250.0 °C where it was held for 6 min for a total GC program of 46 min.

The samples were injected at either 2, 4, or 8 μL depending on the level of the analytes in question. We produced seven-point calibration curves (R^2 greater than 0.99) and response factors were generated in triplicate using analytic standards of α-pinene, camphene, sabinene, β-pinene, myrcene, 3-carene, α-terpinene, p-cymene, limonene, ocimene, γ-terpinene, terpinolene, α-thujone, camphor, borneol, α-terpineol, 4-allylanisole, pulegone, boryl acetate, terpinyl acetate, α-longipinene, gernyl acetate, β-elemene, longifolene, α-cedrene, caryophyllene, β-cedrene, α-humulene, and linalool. All standards were purchased from Sigma Aldrich (St. Louis, MO). These calibration curves and response factors were used to determine final levels (mg of terpene /g of phloem dry weight) and ratios for each compound in the phloem extracts.

2.3. Data analysis

We examined three different response variables: individual terpenoids, total terpenoids, and lesion length. We used a 2 × 2 factorial design to examine how these responses varied with tree species (*P. sylvestris* or *P. ponderosa*) and treatment (inoculated or mock-inoculated), and their interaction. Where the response variable concerned either localized or systemic levels, we analyzed the net change between post-inoculation levels and constitutive levels. Where an interaction was not significant in the model, we removed the interaction term. Model assumptions of equal variance and normal distribution of residuals were examined with residual plots. Non-normal data were log transformed. Data was analyzed using *R* version 3.4.3 (R Development Core Team, 2016, Vienna, Austria) with a threshold of $\alpha = 0.05$ for

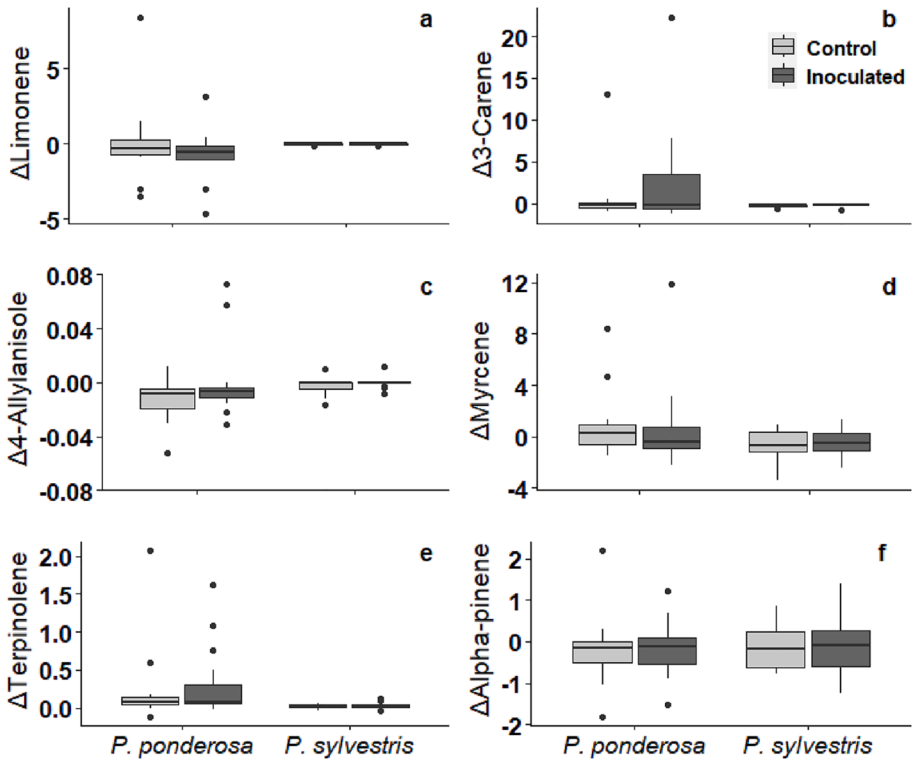

Fig. 5. Changes in systemic levels of various monoterpenes and 4-allylanisole of *Pinus ponderosa* and *P. sylvestris* in response to inoculation with the fungus *Grosmannia clavigera*. Darker lines within the boxplot represent the median, the upper and lower limits of the boxplot denote the upper and lower quartiles, respectively, and the 'whiskers' denote 1.5 times the interquartile range limits. Points above the boxplots represent values outside these ranges.

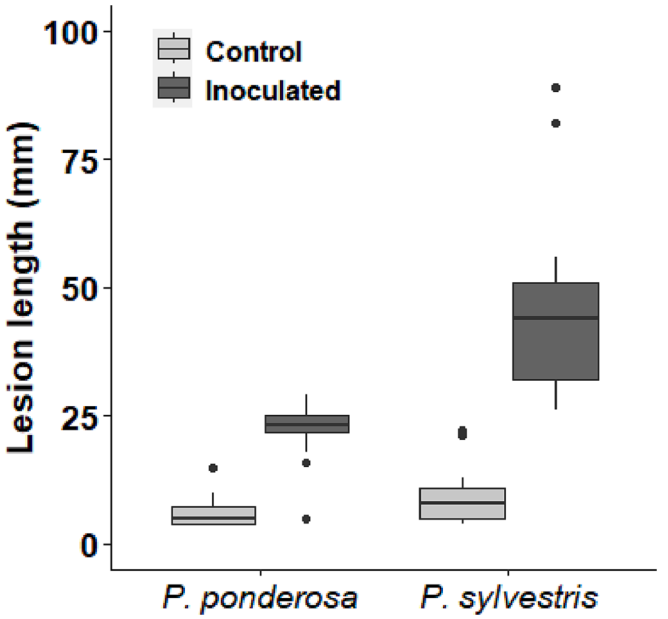

Fig. 6. Comparison of *Pinus ponderosa* and *P. sylvestris* lesion length response three weeks after mock-inoculations and inoculation with the fungus *Grosmannia clavigera*. Darker lines within the boxplot represent the median, the upper and lower limits of the boxplot denote the upper and lower quartiles, respectively, and the 'whiskers' denote 1.5 times the interquartile range limits. Points above the boxplots represent values outside these ranges.

statistical significance. We used the R package 'ggplot2' to construct all figures (Wickham, 2009).

3. Results

Baseline constitutive levels of total monoterpenes ($F_{1,61} = 14.48$, $P = 0.0003$) and total sesquiterpenes ($F_{1,61} = 35.33$, $P < 0.0001$) were higher in *P. ponderosa* trees than *P. sylvestris* trees (Fig. 1). As expected, baseline levels of both monoterpenes ($F_{1,61} = 0.13$, $P = 0.72$) and sesquiterpenes ($F_{1,61} = 0.57$, $P = 0.45$) were similar between both treatment groups (i.e., mock-inoculated and inoculated) at the start of the experiment.

Twenty-one days after inoculation, the net change between localized levels and constitutive levels of *P. sylvestris* monoterpenes were higher than *P. ponderosa* ($F_{1,61} = 12.36$, $P = 0.0008$; Fig. 2a), but the opposite trend was observed for total sesquiterpene levels ($F_{1,61} = 9.17$, $P = 0.0036$; Fig. 2b). Localized increases in monoterpene levels were similar between mock- and fungal-inoculated treatments for each species ($F_{1,61} = 0.43$, $P = 0.52$; Fig. 2a). In contrast, a pronounced sesquiterpene accumulation was observed in the fungal-inoculated trees for both species ($F_{1,61} = 8.92$, $P = 0.0041$) — an 11.6- and a 2.2-fold increase from constitutive levels in sesquiterpenes was observed for *P. ponderosa* and *P. sylvestris*, respectively (Fig. 2b).

While overall induced monoterpene levels were similar at wounding sites for both species, we noted differences in individual monoterpenes (Fig. 3). The localized response of limonene ($F_{1,61} = 11.07$, $P = 0.0015$) and 3-carene ($F_{1,61} = 9.75$, $P = 0.0027$) were greater in *P. sylvestris* than *P. ponderosa* (Fig. 3a,b). However, accumulation of the phenylpropanoid 4-allylanisole ($F_{1,61} = 4.68$, $P = 0.0477$) was higher in *P. ponderosa* than *P. sylvestris* (Fig. 3c). Increases in myrcene ($F_{1,61} = 12.21$, $P = 0.0009$) were higher in *P. sylvestris* than *P. ponderosa* (Fig. 3d). Terpinolene ($F_{1,61} = 0.03$, $P = 0.87$) and α-pinene ($F_{1,61} = 0.8$, $P = 0.38$) had similar concentrations between pine species (Fig. 3e,f). The localized responses were similar between the mock- and fungal-inoculations for all six

monoterpenes presented in Fig. 3. The mean constitutive and localized levels of all other compounds analyzed can be found in Table 1.

The changes in systemic levels of total monoterpenes ($F_{1,61} = 12.06$, $P = 0.001$) and total sesquiterpenes ($F_{1,61} = 9.75$, $P = 0.0027$) following inoculation (i.e., increases in phloem chemicals taken from phloem tissues on the opposite side of the tree) were higher in *P. ponderosa* samples than *P. sylvestris* (Fig. 4). Systemic levels of monoterpenes ($F_{1,61} = 0.05$, $P = 0.83$) and sesquiterpenes ($F_{1,61} = 0.08$, $P = 0.72$) within a tree species were similar between mock- and fungal-inoculations (Fig. 4).

Changes in systemic levels of monoterpenes were larger in *P. ponderosa* than *P. sylvestris* for 3-carene ($F_{1,59} = 4.57$, $P = 0.0368$; Fig. 5b), myrcene ($F_{1,59} = 5.38$, $P = 0.0238$; Fig. 5d), and terpinolene ($F_{1,59} = 7.21$, $P = 0.0094$; Fig. 5e). No pine species level effects were observed for limonene ($F_{1,59} = 0.54$, $P = 0.46$; Fig. 5a), 4-allylanisole ($F_{1,59} = 1.95$, $P = 0.17$; Fig. 5c), or α-pinene ($F_{1,59} = 0.09$, $P = 0.77$; Fig. 5f). Like the localized responses at wounding sites, no treatment effects were observed for any of the six compounds presented in Fig. 5.

We observed a significant species-treatment interaction on the lengths of lesions produced in response to *G. clavigera* inoculation ($F_{1,60} = 8.77$, $P = 0.0044$). *Pinus sylvestris* lesion length was longer in both inoculated and mock-inoculated trees than *P. ponderosa*, but lesion lengths of both species were greater in inoculated trees than mock-inoculated trees (Fig. 6).

4. Discussion

Although *P. sylvestris* is evolutionarily naïve to MPB and *G. clavigera*, this pine species is not naïve to insect and fungal attack in its native range (Lieutier et al., 1995). Our findings that *P. sylvestris* induces localized responses of total monoterpene levels (Fig. 2) and some individual terpenoids (Fig. 3) in response to fungal inoculation with *G. clavigera* suggests that the evolutionary naïve *P. sylvestris* may be better able to defend itself against MPB than the co-evolved *P. ponderosa* (Boone et al. 2011). This finding aligns with earlier field observations in the Black Hills, South Dakota, where numerous pitch tubes have been observed on *P. sylvestris* individuals, but no mortality occurred – even amid surrounding *P. ponderosa* mortality (Rosenberger et al. 2019).

The effect of specific terpenoids on MPB behavior and attack success is well documented for pine species native to western North America (Boone et al., 2011; Chiu et al., 2017; Reid et al., 2017). When volatiles of the terpenes α-pinene, terpinolene, and myrcene are released by *Pinus* species, they can attract more MPB (Borden et al., 2008; Gray et al., 2015). Additionally, α-pinene is the precursor for the female-produced aggregation pheromone of MPB, *trans*-verbenol. When females synthesize *trans*-verbenol, a mass attack event can occur and the trees defenses may be overwhelmed (Pitman et al., 1968). Erbilgin et al. (2020) found that MPB was able to produce these aggregation pheromones when feeding on *P. sylvestris*.

Compounds such as 4-allylanisole, limonene, and 3-carene are known to have either deterrent or defensive properties for *Picea* species and *Larix* species against spruce beetle (*Dendroctonus rufipennis* Kirby) and eastern larch beetle (*Dendroctonus simplex* LeConte), respectively (Werner, 1995).Higher levels of 4-allylanisole is associated with MPB repellence (Emerick et al., 2008). Recently, Sullivan et al. (2022) found that 4-allylanisole enhanced the attraction of southern pine beetle (*Dendroctonus frontalis* Zimmerman) when combined with attractive semiochemicals. Limonene has repeatedly been found to be the most toxic terpenoid to MPB and other bark beetles (Reid and Purcell, 2011; Manning and Reid; 2013; Chiu et al., 2017). Low levels of 3-carene can attract MPB (Clark et al., 2014; Taft et al., 2015) but high levels can be toxic (Reid et al., 2017). In high enough levels, most monoterpenes appear to be toxic to MPB (Reid et al., 2017), and can have negative impacts on beetle development (Manning and Reid, 2013).

We observed that the mock-inoculated and inoculated *P. sylvestris* pine had similar levels of terpenoid response, suggesting that wounding and not *G. clavigera* infection was the cause of this defense induction. Our result is consistent with another fungal inoculation study on *P. sylvestris* in its native range: the responses of *P. sylvestris* in Sweden inoculated with *L. wingfieldii* and mock-inoculated trees showed similar terpenoid responses (Krokene et al., 2000). Likewise, Villari et al. (2012) observed that mechanically wounding *P. sylvestris* resulted in a similar induced response to fungal inoculation with the pathogens *Ophiostoma clavatum* Math.-Käärik and *Hyalorhinocladiella macrospora* Franke-Grosm. One explanation for this trend may be that *P. sylvestris* is exposed to greater levels of defoliators than *P. ponderosa* and has a more sensitive response to mechanical wounding. For example, 3-carene, myrcene, terpinolene, β-phellandrene, α-pinene, and β-pinene levels all increased in response to oviposition in needles by the sawfly *Diprion pini* (Mumm et al., 2003), and feeding by the same insect resulted in high concentrations of 3-carene (Heijari et al., 2008). Because some level of insect feeding and oviposition occur each year on *P. sylvestris*, they may be more primed to defend themselves on an annual basis than *P. ponderosa* which more often defends in cyclical patterns.

Another surprising result was the lack of a localized monoterpene response observed in *P. ponderosa* to inoculation with *G. clavigera*. Previous works on *G. clavigera* in *P. ponderosa* have been mixed. Some studies have shown a large induced response of *P. ponderosa* to *G. clavigera* and/or mechanical wounding (Keefover-Ring et al. 2016; Cale et al., 2017), while others showed similar results to the ones presented here (Gaylord et al., 2011). One possible explanation is that a prolonged MPB outbreak lasting from the early 2000′s to 2016 (Chisholm et al. 2021) across the Black Hills, SD may have reduced the annual energy reserves these trees have needed to defend themselves. The non-typical MPB outbreak lasting approximately 15 years may have left this population of *P. ponderosae* in a weakened state. Mason et al. 2019 found that historical exposure to bark beetle attacks reduced the size and number of resin ducts in following years growth rings. The same could be true of defensive compounds.

A large, localized response may not always be beneficial to the host tree. Due to the sensitivity of *P. sylvestris* to pathogenic fungi, the heightened defense response may exhaust the carbohydrate reserves of the host plant, leaving it more vulnerable to future beetle attack. High localized increases of limonene were observed in *P. sylvestris* after inoculation with *L. wingfieldii* or *O. canum* Münch, but samples collected more than 20 cm from the inoculation site had reduced responses (Fäldt et al., 2006). A large, localized response can also exhaust carbohydrate reserves required to synthesize terpenoids, which can increase the susceptibility to bark beetle infestations (Goodsman et al., 2013; Roth et al., 2018). Repeated MPB attacks could be more detrimental to *P. sylvestris* than *P. ponderosa* because of the lower total systemic monoterpene levels, assuming carbohydrate reserves are exhausted at the same rate in both species.

Pinus sylvestris appears to have some level of intrinsic defense against attack – whether that be pathogenic or mechanical. It is possible that the co-evolutionary history of *P. sylvestris* with the suite of pests in Europe occupying a similar ecological niche as MPB in North America has allowed *P. sylvestris* to defend itself against MPB and its associated fungi. Thus, any potential MPB introduction into Europe may not necessarily lead to an aggressive invasion of *P. sylvestris* forests, however further work needs to be conducted to understand if *P. sylvestris* is able to defend against repeated MPB attacks (Rosenberger et al. 2019). MPB has not yet established in countries outside of its native range, although it has been intercepted in New Zealand (Brockerhoff et al., 2006) and Australia (EPPO Global Database, https://gd.eppo.int/taxon/DENCPO/distribution/AU). An MPB invasion is concerning in countries outside of North America where *Pinus* species are major components of the ecosystem and economic infrastructure.

One limitation of our study was freezing our constitutive, localized, and systemic samples at different temperatures in the field (i.e., using ice packs for the first constitutive samples and switching to dry ice for the others, prior to placement in freezers). The different initial sub-freezing temperatures may have affected sample preservation for volatile

components such as monoterpenes, so appropriate caution should be exercised when interpreting our results. Another limitation may be indicated by the lesions measured in some controls that may be indicative of a failure to fully sterilize the plug cutters between treatments in the field (Fig. 6). Because we only did single inoculations instead of mass inoculations, the induced response of both pine species may not fully reflect infestation by MPB during periodic outbreaks. Other variables that may have influenced our results include selection of fungal isolate (see Linnakoski et al. 2017) and the amount of time between sample collections (see Soderberg et al. 2022).

Because *P. sylvestris* is not native to North America and it is not currently a dominant source of timber in North America, forest managers will need to decide if this pine species could or should become a more dominant tree in plantation forestry. Our results suggest that *P. sylvestris* could be one option for wider planting should more comprehensive silvicultural planning in North American states and provinces east of the front line of MPB become necessary in the future. However, more evaluation is needed to determine the role of *P. sylvestris* in future plantings.

CRediT authorship contribution statement

Kevin D. Chase: Visualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. **Kathryn J. Rynders:** . **Mitchell P. Maddox:** . **Brian H. Aukema:** Project administration, Data curation, Methodology, Formal analysis, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I will upload my data to Data Dryad.

Acknowledgements

This project was funded by the Minnesota Invasive Terrestrial Plants and Pests Center (Appropriation: ML 2015, Ch. 76, Art. 2, Subd. 6A) and the Minnesota Environment and Natural Resources Trust Fund 338 (Appropriation: M.L.2014 Chpt. 226, Sec. 2 subd.4e.). We thank Bob Blanchette and Benjamin Held (University of Minnesota) for providing fungal samples, Jonah Widmer and Jacob Wittman (University of Minnesota) for laboratory and field assistance, and Kurt Allen (USFS) for providing logistical support in the Black Hills National Forest. We thank Andrew Loyd (Bartlett Tree Research Laboratory) and Chad Rigsby (Bartlett Tree Research Laboratory) for early reviews of this manuscript. The generous and insightful comments of two anonymous reviewers were greatly appreciated.

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