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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

Ectomycorrhizal fungal species differentially affect the induced defensive chemistry of lodgepole pine

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Abstract

Plants interact simultaneously with multiple organisms, including ectomycorrhizal (EM) fungal symbionts which benefit plants by facilitating resource acquisition. Yet, their role in induced plant defenses that rely on the allocation of plant resources has received little attention. We investigated whether EM fungi can affect the induction of defense-related monoterpenes in greenhouse-grown lodgepole pine (*Pinus contorta* var. *latifolia*) seedlings, and whether such effects differed between EM fungal species occurring alone or in combination. Fungal interactions on growth media were also assessed to complement the greenhouse study. Our study revealed that the production of certain monoterpenes is influenced by the fungal species colonizing pine roots. Furthermore, pine seedlings did not necessarily benefit from having associations with multiple EM fungi, as we found contrasting effects of single vs. multiple species of fungi on induced monoterpene responses. Finally, monoterpene responses were altered when early-colonizing species inhibited the colonization or development of later-arriving species. We conclude that the presence of EM fungi can impact host susceptibility to insect and pathogen attack, suggesting that seedlings establishing in areas lacking fungi that promote the induction of tree defense chemicals may suffer from increased susceptibility to future pest damage.

Keywords *Cenococcum geophilum* · *Dendroctonus ponderosae* · *Laccaria bicolor* · *Pinus contorta*

Introduction

Plants are regularly attacked by pathogens and herbivores. These selective pressures have in part led plants to evolve sophisticated defense systems involving an array of secondary chemicals that are present in plant tissues prior to attack (i.e., constitutive) or are produced in response to attack (i.e., induced) (Franceschi et al. 2005; Raffa et al. 2005, 2017). In addition to directly affecting the attackers, these chemicals can also help protect plants by attracting the natural enemies of attackers as well as help plants physiologically tolerate adverse environmental conditions (Agrawal 2011; Moore et al. 2013; Raffa et al. 2017; Erbilgin et al. 2017a, b). The protection afforded by these compounds as well as the diversity of chemicals employed can vary widely with plant species, resulting from adaptive radiation and selective pressures from adverse abiotic and biotic factors (Sequeira et al. 2000; Huber et al. 2004; Howe and Jander 2008; Moore et al. 2013; Raffa et al. 2017). Whether beneficial biotic factors, such as the presence of symbiotic microbes, can affect the production of defense-related secondary chemicals in plants is poorly understood (Gershenzon 1994; Smith and Read 2008; Karst et al. 2015).

Mycorrhizal fungi—root-inhabiting fungi that in part exchange plant-inaccessible forms of nitrogen and phosphorus for photosynthate—are symbionts critical to the survival of many globally important plants, such as pines (*Pinus* spp.). Pine species are dominant forest trees in the Northern Hemisphere (Henry 2005) whose establishment and long-term growth and development depend on symbioses formed with a wide variety of ectomycorrhizal (EM) fungi, such as *Laccaria bicolor* and *Cenococcum geophilum* (Simard et al. 1997; Bradbury et al. 1998; Karst et al. 2014). These

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fungi can support pine health by improving mineral nutrition and water uptake as well as serving as a barrier to infection by root pathogens (Marschner and Dell 1994; Kernaghan et al. 2002; Bennett et al. 2006; Lehto and Zwiazek 2011). Furthermore, EM fungi can influence constitutive defense chemicals in lodgepole pine (*Pinus contorta* var. *latifolia*). The richness and amount of chemicals such as monoterpenes can respond to variation in the composition of soil fungal communities due to mountain pine beetle (*Dendroctonus ponderosae*; MPB)-induced mortality of mature lodgepole pine (Karst et al. 2015). The magnitude of these effects on pine monoterpenes may vary with individual mycorrhizal species as well as the occurrence of other colonizing species (Karst et al. 2015). Furthermore, priority effects may also occur whereby early-arriving/colonizing fungi have a greater impact on host chemistry than later-arriving/colonizing fungi. However, whether mycorrhizal fungi can also influence monoterpene induction is unknown. Elucidating such a relationship is an important component in understanding the broader roles that soil microbes play in forest health, as induced defenses are a critical aspect of tree resistance and thus survival.

Mountain pine beetle is one of the greatest threats to North American pine forests, having killed millions of lodgepole pine trees in the past decade (Erbilgin et al. 2014). Secondary chemicals in lodgepole pine are strongly influenced by and important determinants of MPB colonization (Phillips and Croteau 1999; Keeling and Bohlmann 2006; Erbilgin et al. 2017a, b). Indeed, variation in these chemicals has been primarily attributed to selective pressures imposed by the beetle and the community of phytopathogenic fungi it vectors (Erbilgin et al. 2017b; Raffa et al. 2017). Among these secondary chemicals, toxic monoterpenes are likely the primary defense against MPB and other bark beetles (Franceschi et al. 2005; Raffa et al. 2017). While constitutive monoterpenes in pine phloem afford immediate resistance, persistent beetle attacks elicit induced monoterpene responses that afford pines greater protection due to increased concentrations of toxic compounds (Franceschi et al. 2005; Raffa et al. 2005). For example, within a few days of beetle attacks, monoterpene levels can exceed the physiological tolerance thresholds of beetles, inhibit or repel later-arriving beetles, and alter the growth of their associated fungi (Raffa et al. 2005; Cale et al. 2017). Whether the induction of monoterpenes associated with pine resistance to MPB and other bark beetles is influenced by ectomycorrhizal fungi is unknown.

Here, a greenhouse experiment was conducted to examine the potential influence of EM fungal species on defense-related monoterpene induction in lodgepole pine. More specifically, lodgepole pine seedlings were grown in a greenhouse using soil inoculated with *L. bicolor*, *C. geophilum*, or both species in several combinations. These seedlings were then treated with one of two defense-related hormones to trigger monoterpene induction. Fungal colonization of pine roots, pine biomass, and monoterpene concentrations were quantified. A laboratory experiment was then conducted using artificial growth media inoculated with *L. bicolor* and *C. geophilum* to determine how these fungi may interact when co-colonizing pine roots. These results were used to help explain those of the greenhouse experiment. Overall, this approach was used to address two hypotheses: (1) colonization by EM fungi affects the production of defense-related monoterpenes and (2) this effect can differ between seedlings colonized by individual EM fungal species and those colonized by more than one species.

Materials and methods

Greenhouse experiment

Experimental setup and mycorrhizal treatment application

Lodgepole pine seedlings were grown in the greenhouse from seeds that were provided by the Tree Improvement Branch, Kalamalka Forestry Centre (Vernon, BC, Canada). Seeds were stratified for 28 days prior to sowing. Stratification included a surface sterilization by soaking seeds in 5% bleach for 15 min, followed by rinsing with distilled water and soaking for 24 h in distilled water. Excess water was drained, and seeds were surface dried and stored in the dark for 28 days at 4 °C. Seeds were then sown into 400 ml pots filled with potting material (70:30 sterile sand:top soil). Four seeds were sown into each pot. Seedlings were thinned to one seedling 1 week after germination began; the most vigorous seedling was retained in each pot. Seedlings were grown for 1 year at 21 °C under natural light–dark regime. After 5 months, seedlings were placed in dormancy conditions of 4 °C and a 12:12 h light:dark regime for 6 weeks (after an acclimation period of a gradual temperature decline from 21 to 4 °C during a 2-week period). After this period, seedlings were reconditioned to warmer temperatures reflecting growing conditions over 1 week when temperatures were gradually returned to 21 °C. Pots were fertilized with a 8:20:30 (*N*:*P*:*K*) formulation 1 week prior to (50 ppm) and twice (125 ppm) during dormancy to avoid phosphorus deficiency. A 10:52:10 fertilizer (400 ppm) was applied immediately following dormancy, whereas a 10:20:10 fertilizer (100 ppm) was otherwise applied three times a week until 3 weeks prior to defense-related hormone application (described below). Iron chelate (17.5 ppm) was added to post-dormancy latter fertilizations. Pots were rotated in the greenhouse once a week to ensure equal sunlight exposure.

Seedlings were harvested by carefully uprooting them from the pots. Roots were cleaned of potting mixture using

a gentle brush, then wrapped in aluminum foil, and stored at 4 °C. Seedling foliage, stems, and roots were separated and weighed fresh to measure their biomass. Stem and foliage tissues were combined for later chemical analysis.

Seedling inoculations with EM fungi

Immediately after seeds were sown, their pots were inoculated with 10 ml of a liquid culture representing one of six EM fungal treatments: (1) *Cenococcum geophilum* (isolate UAMH 5512) alone, (2) *Laccaria bicolor* (isolate UAMH 8232) alone, (3) *C. geophilum* plus *L. bicolor* combined, (4) *C. geophilum* followed by *L. bicolor* (CG priority), (5) *L. bicolor* followed by *C. geophilum* (LB priority), and (6) non-inoculated as a control. The number of replications used was 31–44, depending on treatment (Table 1). The isolates of *C. geophilum* and *L. bicolor* used were originally collected from lodgepole pine forests in Alberta and provided by the University of Alberta Microfungus Collection and Herbarium. Liquid cultures of the fungi were used as inoculum and prepared by growing fungal cultures in modified liquid Melin-Norkrans modified media starting from 30 culture plugs (8 mm dia) taken from the margins of actively growing cultures on potato dextrose agar. The hyphal densities of 2-week-old liquid cultures were quantified using a hemocytometer and standardized among treatments and applications by dilution, as needed. For the combined treatment, 5 ml of liquid cultures of each fungus were mixed immediately before application. After the initial application, fungal inoculations were reapplied every 15 days for a total of six applications. For the CG priority and LB priority treatments, the first fungus was used for inoculations 1–3 and the second fungus for inoculations 4–6. For example, for pots of the CG priority treatment, *C. geophilum* was inoculated when seeds were sown and the next two applications, but *L. bicolor* inoculations were made for the last three applications.

The success of EM fungal treatments was assessed by measuring percent colonization of each fungus as determined by morphotyping a subset of 100 randomly selected root tips per seedling. Root tips were cut into 1–2 cm lengths and put into a Petri dish containing distilled water and evaluated for morphotypes and other characteristics indicative of *C. geophilum*, *L. bicolor*, or other/non-colonized types (Goodman 1996; Martin and Selosse 2008).

Hormone treatment application

To investigate how the mycorrhizal treatments influence defense-related induced monoterpenes, induction was elicited using two phytohormones: methyl jasmonate (MJ) and methyl salicylate (MS). Ten days before harvest, half of the seedlings received the MJ treatment while the other half received MS. Fifty microliter solutions of MJ or MS in 0.1% (v/v) Tween 20 were applied to seedling stems using a foam brush. Seedlings were not watered for 24 h to ensure that solutions were absorbed and kept in separate greenhouse rooms for 24 h to avoid cross-elicitation between treatments. Fertilizer was not applied after the hormone application to avoid potential nutrient-defense feedbacks.

Monoterpene extraction and chromatographic analysis

Monoterpenes were extracted from the aboveground tissues of each seedling. Needle and stem tissues were ground together in liquid nitrogen using a mortar and pestle, and monoterpenes were extracted using the methods described by Erbilgin et al. (2017b). Briefly, 100 mg of ground tissue was extracted twice with 0.5 ml of dichloromethane and 0.019% tridecane as an internal standard. Extractions were vortexed for 30 s, sonicated for 10 min, and centrifuged at 18,400 rcf at 0 °C for 15 min. Centrifuged extractions were stored at − 40 °C for an hour to encourage further separation between supernatant and ground sample. The supernatant was collected and transferred to a 2 mL glass gas chromatography vial through a glass-wool filter, and stored at − 40 °C until chromatographic separation.

Extractions (1 µl) were injected into a gas chromatograph/mass spectrometer (Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with a HP-Chiral-20B column (I.D. 0.25 mm, L 30 m) (Agilent Tech.) with a helium carrier gas flowing at 1.1 ml min^{-1}, and a temperature program of 50 °C for 1 min, increased to 65 °C for 2 min by 40 °C min^{-1}, then to 85 °C for 2 min by 40 °C min^{-1} and then to 240 °C for 1 min by 10 °C min^{-1}. To identify individual compounds (mainly monoterpenes), the following standards

Table 1 The number of replications in each fungal treatment

	Fungal treatments						
	Cenococcum geophilum	*Laccaria bicolor*	Combined	CG priority	LB priority	Control	Total
Number of replications	41	36	40	31	44	38	230

CG, *Cenococcum geophilum* was inoculated alone; LB, *Laccaria bicolor* was inoculated alone; combined, Both the fungi were inoculated together; CG priority, *C. geophilum* was inoculated first and then *L. bicolor*; LB priority, *L. bicolor* was inoculated first and then *C. geophilum*; and control, no fungi were inoculated and served as control

were used: (−)-α- and (+)-α-pinene, (−)- and (+)-β-pinene, (−)- and (+)-camphene, myrcene, (−)- and (+)-limonene, 3-carene, terpineol (chem purity > 90%), (+)-cymene, sabinene, terpinolene, *p*-cymene, β-thujone (enantiomeric ratio 92.5/7.5), pulegone, terpinolene (> 90%), borneol, 4-allylanisole (Fluka, Sigma-Aldrich, Buchs, CH), γ-terpinene, α-terpinene (Sigma-Aldrich, St. Louis, MO, USA), *cis*-ocimene (> 90%), bornyl acetate (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (> 85%). Where chemical purity was not noted, the purity was 97%. Compounds were identified by comparing retention times and mass spectra to those of the standard chemicals. Quantity of chemicals was calculated using calibrated curves generated from analyses of a serial of dilution of known quantities of standards, and calculated as μg of compound per mg of wet tissue.

Laboratory experiment

A laboratory experiment was conducted to understand how *C. geophilum* and *L. bicolor* may be interacting on seedling roots in the above greenhouse experiment. Fungal growth on artificial media was compared among six treatments reflecting the mycorrhizal treatments used in the greenhouse experiment: (1) *C. geophilum* alone as control, (2) *L. bicolor* alone as control, (3) both fungi on separate halves of a partitioned plate (partitioned), (4) both fungi on opposite ends of a non-partitioned plate (combined), (5) *C. geophilum* grown on an established *L. bicolor* culture (LB priority), and (6) *L. bicolor* grown on an established *C. geophilum* culture (CG priority). Each treatment was replicated 15 times.

Fungal cultures were prepared by first growing master cultures on potato dextrose agar. After 15 days, master cultures were sub-cultured; with the culture plug (8 mm dia.) being placed onto either the center (priority and individual-fungus treatments) or equidistant locations (combined treatment) of 100 mm dia. Petri dishes of potato dextrose agar. Cultures were then grown in total darkness at room temperature (22 °C) for a length of time dependent on treatment (15-day period for *L. bicolor* and a 30-day period for *C. geophilum*) as each fungus had different growth rates. This was done to allow growth measurements to be made before cultures covered the entire plate surface. To measure the growth response to *C. geophilum* to an established *L. bicolor* culture (i.e., the LB priority treatment), *L. bicolor* was inoculated 8 days prior to *C. geophilum*. Similarly, to measure the growth response of *L. bicolor* to an established *C. geophilum* culture (i.e., the CG priority treatment), *C. geophilum* was inoculated 15 days prior to *L. bicolor*.

Culture growth (area) measurements were made using image analysis techniques. Images were taken using a Nikon D7100 camera mounted on a stand 50 cm above the culture plates. The camera was set to ISO: Auto, *F*:5.3, *A*:40. A ruler was placed in frame to scale and measure image elements. Images were taken at 0, 9, 15, and 30 days post-inoculation, except *L. bicolor* which was not measured at day 30 since it entirely covered the media before this time. Images were quantified using the Image J (National Institutes of Health, Bethesda, MD, USA) (Abramoff et al. 2004). The final culture area and per-day growth rate were calculated and used for data analysis.

Data analysis

For the greenhouse experiment, the development of mycorrhizal roots in each treatment, and thus treatment application success, was determined by assessing the percent colonization of each fungus on seedling roots. Differences in percent colonization among treatments for each fungus were separately tested for by one-way ANOVA. One-way ANOVA was also used to if the effect of mycorrhizal treatments on seedling biomass of seedlings differed among treatments. Separate models were run for aboveground, belowground, and total biomass (g) response variables. The effects of EM treatments on the composition of seedling monoterpenes (proportion of total monoterpenes) and total monoterpene concentration (ng/mg fresh weight) were tested for statistical significance using one-way ANOVA tests separately for MJ- and MS-treated seedlings. Separate tests were performed for these hormone groups because these hormones can elicit different and antagonistic metabolomic responses from pine seedlings (Erbilgin and Colgan 2012). Tukey's honest significant difference (HSD) tests were performed following all significant ANOVA models.

For the laboratory experiment, one-way ANOVA was used to test differences in the culture areas (mm^2) and growth rates ($mm^2\ day^{-1}$, calculated from the final culture area) of *C. geophilum* and *L. bicolor* interaction treatments followed by Tukey HSD tests, as needed.

All statistical analyses were performed in the R software version 3.3.2 (R Core Team 2016). Data were log-transformed to satisfy model assumptions of normality and homogeneity of variance, as needed. Figures were generated using non-transformed data.

Results

Greenhouse experiment

Root colonization by EM fungi

The ectomycorrhizal treatments were successful as morphotyping indicated that *L. bicolor* and *C. geophilum* colonized roots of seedlings in their respective treatments. However, the percent of roots colonization by *C. geophilum*

($F_{3,152} = 13.52$, $P < 0.001$) or *L. bicolor* ($F_{3,148} = 16.98$, $P < 0.001$) significantly varied among treatments (Fig. 1). For both fungi, the highest root colonization occurred when each fungus was inoculated alone, and percent colonization declined when they were inoculated together. For *C. geophilum*, percent colonization was 28% lower when both *C. geophilum* and *L. bicolor* were inoculated at the same time, 32% lower when *C. geophilum* was inoculated prior to *L. bicolor* (CG priority), and 42% lower when *L. bicolor* was inoculated prior to *C. geophilum* (LB priority), relative to the *C. geophilum* alone treatment (Fig. 1a). Similarly, the percent colonization of seedling roots by *L. bicolor* was 25% for the LB priority, 37% lower for the CG priority, and 38% lower for the combined treatments, relative to the *L. bicolor* alone (Fig. 1b). *Cenococcum geophilum and L. bicolor* were not observed on the roots of the control seedlings. However, 15% (±1 SE) on average of these roots were colonized by an unknown fungus that was not observed on the roots of treated seedlings.

Response of seedling biomass to fungal treatments

Total seedling biomass tended to be higher in the individual-species treatments compared to the combination treatments, but overall difference was non-significant. However, the total biomass of treated seedlings tended to be 2–30% greater than that of control seedlings without any fungal inocula. Seedling aboveground, stem, foliar, and root biomasses did not significantly respond to fungal treatments.

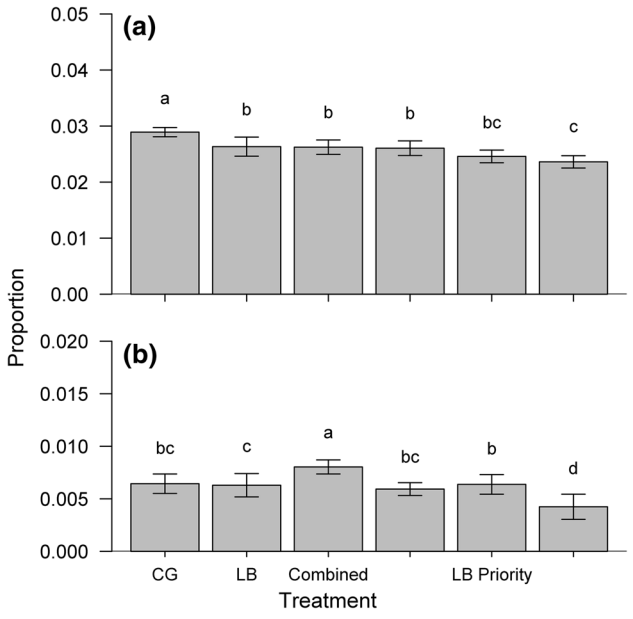

Fig. 1 Mean (±SE) percent colonization of lodgepole pine (*Pinus contorta* var. *latifolia*) seedlings by *Cenococcum geophilum* (**a**) and *Laccaria bicolor* (**b**) in five inoculation treatments: *C. geophilum* inoculated alone (CG; $N = 41$), *L. bicolor* alone (LB; $N = 36$), the fungi inoculated together (combined; $N = 40$), *L. bicolor* inoculated after *C. geophilum* (CG priority; $N = 31$), *C. geophilum* inoculated after *L. bicolor* (LB priority; $N = 44$), or no inoculation by either fungus (control; $N = 38$). Bars with different letters were statistically different as indicated by Tukey HSD tests

Fungal treatment effects on seedling defense chemistry

Fungal treatments affected the induced monoterpenes of seedlings treated with defense-related hormones MJ and MS. For MJ-treated seedlings, we detected a significant effect of mycorrhizal treatment on the proportions of (−)-α-pinene ($F_{5,116} = 5.19$, $P < 0.001$), (+) α-pinene ($F_{5,116} = 2.68$, $P = 0.020$), and myrcene ($F_{5,116} = 3.13$, $P = 0.01$) (Fig. 2). The proportion of (−)-α-pinene in treated seedlings ranged from 11% less (for *L. bicolor* alone) to 28% greater (*L. bicolor* and *C. geophilum* combined) than the controls (Fig. 2a). For (+)-α-pinene, the proportion of this compound in treated seedlings ranged from 28% less (*C. geophilum* alone) to 2% greater (GC priority) than controls (Fig. 2b). The proportion of myrcene in treated seedlings ranged from 15% less (*L. bicolor* and *C. geophilum* combined) to 5% greater (LA priority) than the controls (Fig. 2c). The trend in all compounds in treated and control seedlings is shown in Supplementary Table 1 for compound proportions and in Supplementary Table 2 for compound concentrations.

For MS-treated seedlings, we detected significant effects of mycorrhizal treatments for myrcene ($F_{5,86} = 2.63$, $P = 0.030$) and (+)-limonene ($F_{5,86} = 2.48$, $P = 0.042$) (Fig. 3). The proportion of myrcene in treated seedlings ranged from 10% greater (CG priority) to 22% greater (*C. geophilum* alone) than that in control seedlings (Fig. 3a), whereas the proportion of (+)-limonene in treated seedlings ranged from 39% greater (CG priority) to 89% greater (*L. bicolor* and *C. geophilum* combined) than the controls (Fig. 3b). The trend in all compounds in treated and control seedlings is shown in Supplementary Table 3 for compound proportions and in Supplementary Table 4 for compound concentrations..

Laboratory experiment

The total growth of *C. geophilum* (30-day growth) and *L. bicolor* (15-day growth) differed among treatments (Fig. 4). For *C. geophilum*, total mean culture area of the combined and LB priority treatments were 54 and 100% lower, respectively, than the *C. geophilum* control (Fig. 4a; $F_{2,42} = 335.84$, $P < 0.001$). There was no growth in LB priority treatment and thus was not included in the statistical analysis. Similarly, mean total area of *L. bicolor* cultures of the combined and CG priority treatments was 48 and 95% lower, respectively, than the *L. bicolor* control (Fig. 4b; $F_{2,42} = 240.80$, $P < 0.001$).

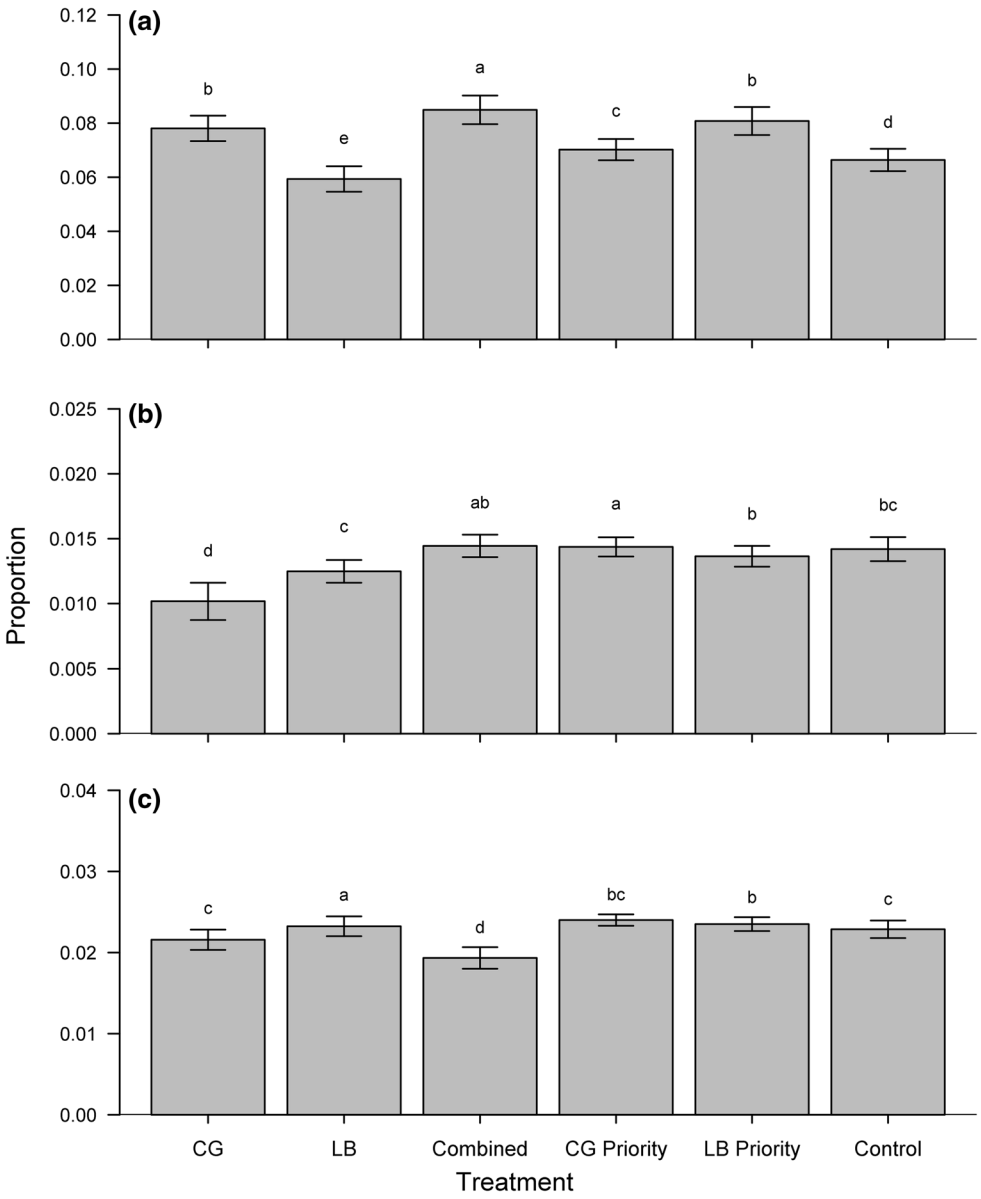

Fig. 2 Mean (±SE) proportion of (−)-α-pinene (**a**), (+)-α-pinene (**b**), and myrcene (**c**) in lodgepole pine (*Pinus contorta* var. *latifolia*) seedlings treated with defense-related hormone methyl jasmonate and inoculated with six treatments of ectomycorrhizal fungi: *Cenococcum geophilum* inoculated alone (CG; $N=41$), *Laccaria bicolor* alone (LB; $N=36$), the fungi inoculated together (combined; $N=40$), *L. bicolor* inoculated after *C. geophilum* (CG priority; $N=31$), *C. geophilum* inoculated after *L. bicolor* (LB priority; $N=44$), or no inoculation by either fungus (control; $N=38$). Bars with different letters are statistically different as indicated by Tukey HSD tests

The growth rate of the fungi was compared among treatments and controls separately analyzed for each fungus (Fig. 4). For *C. geophilum*, growth rate significantly varied among treatments (Fig. 4c; $F_{1,28,}=51.82$, $P<0.001$). The mean growth rate of this fungus in the combined treatment was 63% lower than the *C. geophilum* control. This fungus did not grow in the LB priority treatment (Fig. 4c). Similarly, for *L. bicolor*, the mean culture growth rate significantly differed among treatments (Fig. 4d; $F_{2,42}=115.62$, $P<0.001$). The growth rate of *L. bicolor* cultures was lower when *C. geophilum* was present: 64% lower in the combined treatment and 93% lower in the CG priority treatment (Fig. 4d).

Discussion

The presence of EM fungi can affect the induced chemistry of pine trees. Our greenhouse experiments showed that induced monoterpene response in pines varied with EM fungal species, inter-fungal interactions, and the order in which roots were colonized by each fungus (i.e., priority effects). These results are in agreement with those of others showing that constitutive monoterpenes vary when lodgepole pine seedlings are grown with different communities of soil fungi (Karst et al. 2015). However, not all monoterpenes were affected by EM fungi, supporting

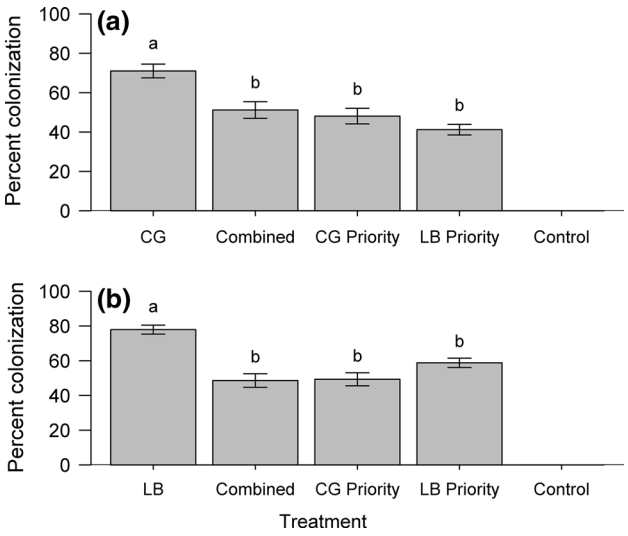

Fig. 3 Mean (±SE) proportion of myrcene (**a**) and (+)-limonene (**b**) in lodgepole pine (*Pinus contorta* var. *latifolia*) seedlings treated with defense-related hormone methyl salicylate and inoculated with six treatments of ectomycorrhizal fungi: *Cenococcum geophilum* inoculated alone (CG; $N=41$), *Laccaria bicolor* alone (LB; $N=36$), the fungi inoculated together (combined; $N=40$), *L. bicolor* inoculated after *C. geophilum* (CG priority; $N=31$), *C. geophilum* inoculated after *L. bicolor* (LB priority; $N=44$), or no inoculation by either fungus (control; $N=38$). Bars with different letters are statistically different as indicated by Tukey HSD tests

the general idea that the levels of some pine monoterpenes may be strongly controlled by genetics, while others may be sensitive to changes in growing conditions (Forrest 1981; Ott et al. 2011; Erbilgin et al. 2017a).

Ectomycorrhizal fungi differentially affect monoterpene induction in pine seedlings, as can occur also in other plant–mycorrhizae systems (Bennett et al. 2009). We showed that the proportion of (−)-α-pinene [one of the most abundant monoterpenes in lodgepole pine phloem (Erbilgin et al. 2017b)] in seedlings differed with the species of colonizing EM fungus. For example, seedlings grown with *C. geophilum* alone had proportionally more (−)-α-pinene than seedlings grown with *L. bicolor* alone. Similar interspecific differences were observed for other monoterpenes such as myrcene, (+)-limonene, and (+)-α-pinene. Changes in monoterpene composition can likely be explained by the relative contributions of each fungus to seedling nutrition, as monoterpene production is affected by both carbohydrates (Goodsman et al. 2013) and nitrogen (Gershenzon 1994; Karst et al. 2015). In addition, mycorrhizal fungi have been shown to alter resource allocation among different plant tissues (Bennett and Bever 2007), suggesting that differential allocation of resources within seedlings may be an alternative explanation for our results. Currently, we do not know the relative effects of each fungus on seedling resource allocation and nutrition (Bennett et al. 2006, 2009; Bennett and Bever 2007).

The effects of multiple EM fungi on the induced monoterpenes of pine seedlings can vary with the individual compound. For example, seedlings colonized by *C. geophilum* and *L. bicolor* together had lower myrcene levels than seedlings colonized by either fungus alone. However, in other cases some monoterpenes were higher in seedlings colonized by both fungi together. For example, both enantiomers of α-pinene were higher in seedlings colonized by both fungi than in seedlings colonized by either fungus alone. These results support earlier studies indicating that plants do not necessarily nutritionally benefit from forming associations with multiple mycorrhizal fungi (Baxter and Dighton 2001; Bennett and Bever 2007; Kennedy et al. 2007). Indeed, this has been shown for several arbuscular mycorrhizal systems (Bennett and Bever 2007; Bennett et al. 2009). Thus, less carbon and/or nitrogen could be available for secondary chemical production in seedlings colonized by both of the fungi.

The influence of EM fungi on induced monoterpenes in pine seedlings can be subject to priority effects when early-arriving/colonizing species inhibit the colonization or development of later-arriving/colonizing species. Indeed, we showed that proportions of (−)-α-pinene in seedlings where *C. geophilum* was applied to seedlings before *L. bicolor* (i.e., CG priority treatment) were lower than in those seedlings treated with both fungi simultaneously or *C. geophilum* alone. This effect on monoterpenes may be an indirect consequence of interspecific competition between fungi, which commonly occurs among EM fungi (Wu et al. 1999; Kennedy et al. 2009; Kennedy 2010). Our laboratory experiment supported this by showing that both *C. geophilum* and *L. bicolor* can inhibit the growth of the other species. This growth was lowest when the fungi colonized media where the other species had already established. While the outcome of inter-fungal competition in the presence of seedling roots may differ from our findings using artificial media, our results suggest that there is an associated cost in plant-induced chemicals when early- and late-arriving/colonizing EM fungi compete with one another on the same host plant. We hypothesize that the resources the fungi would otherwise provide the plant may be diverted to competition in the presence of other co-colonizing EM species, thereby reducing the availability of nutrients to support the induction of secondary chemicals.

The effects of EM colonization on pine monoterpenes may feedback to affect EM fungi in soil. While our work supports that of others (Karst et al. 2015) showing that the colonization of lodgepole pine roots by EM fungi can stimulate the production of monoterpenes in pine, these compounds can affect the activities of EM fungi. Pine litter emits monoterpenes at levels comparable to those present in living

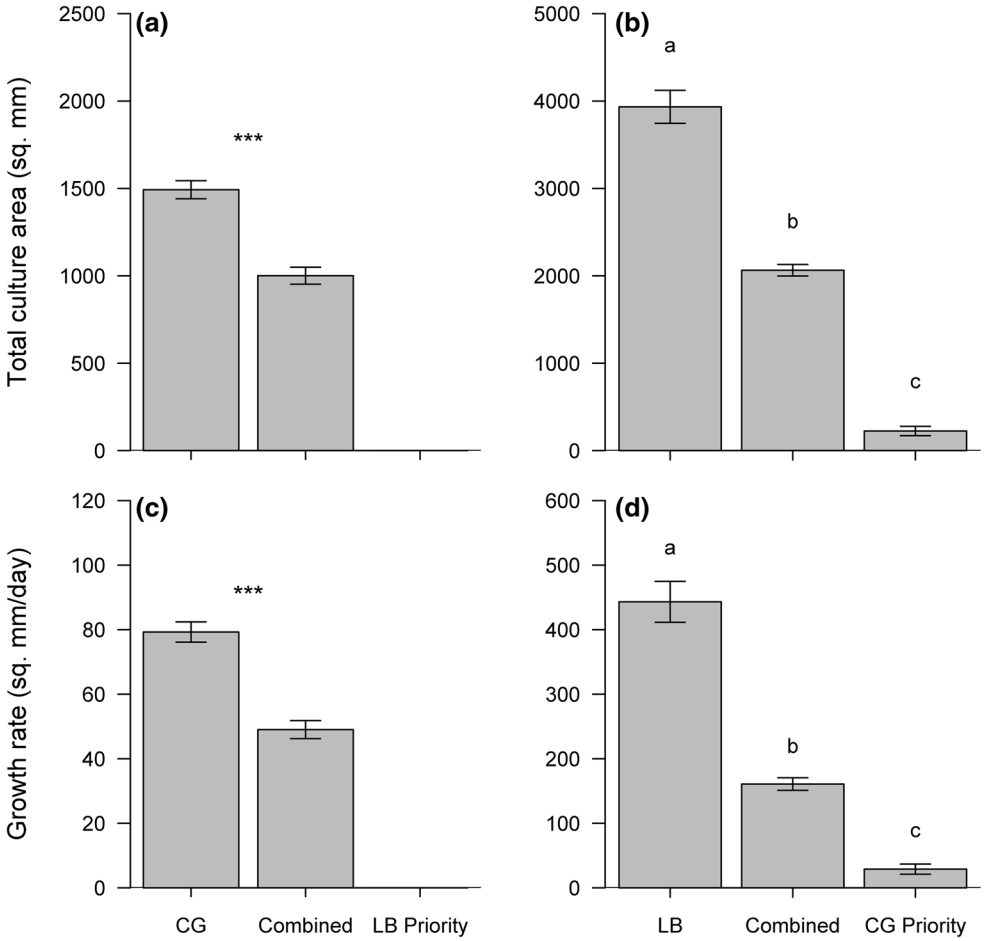

Fig. 4 Mean (±SE) differences in total growth (sq. mm; **a**, **b**) and growth rate (sq. mm/day; **c**, **d**) for *Cenococcum geophilum* (**a**, **c**) and *Laccaria bicolor* (**b**, **d**) cultures grown on artificial media for 30 or 15 days, respectively. Cultures were grown in three treatments: each fungus alone (CG and LB), both fungi together (combined), and one fungus before the other (LB priority and CG priority). All treatments were replicated 15 times. Bars with different letters are statistically different as indicated by Tukey HSD tests

pine tissue into the upper horizons of forest soils (Ludley et al. 2009a). Exposure to monoterpene vapors can increase the rate at which certain EM fungi colonize conifer roots and can inhibit the growth of many EM fungi, including *C. geophilum* and *Laccaria* species (Ludley et al. 2008, 2009b).

In conclusion, the complexity of plant interactions with an array of beneficial and antagonistic organisms makes it difficult to predict the evolution of plant defense responses without first incorporating the effects of these organisms into theories of plant–insect/pathogen co-evolution. Ectomycorrhizal fungi can influence the production of secondary compounds in plants, likely by directly providing nutrients (e.g., nitrogen), or indirectly by promoting plant growth, which in turn accelerates the photosynthetic carbon uptake as carbon-based defense compounds need nitrogen and other minerals in their production. These results demonstrate first that EM fungi should also be considered a part of host plant co-evolution against antagonistic organisms (Karst et al. 2015). Second, the presence of EM fungal species that affect the constitutive and induced chemistry of pine seedlings has important implications for the successful establishment of pine in various habitats. Pine seedlings establishing in areas that lack the fungal species or communities that promote defense chemical induction may quickly succumb to pest damage. Therefore, soil microbes play important roles in forest health and development. To our knowledge, this study is the first to examine tree defensive induction in response to colonization by EM fungi. Additional studies are needed to determine how particular EM fungi or communities alter aboveground interactions with pest insects and pathogens in both spatial- and species-specific contexts.

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Author contribution statement SSK, JAC, and NE conceived and designed the experiments. SSK performed the experiments and SSK and JAC analyzed the data. SSK, JAC, and NE wrote the manuscript.

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