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Ectomycorrhizal fungi mediate indirect effects of a bark beetle outbreak on secondary chemistry and establishment of pine seedlings

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Summary

- *Dendroctonus ponderosae* has killed millions of *Pinus contorta* in western North America with subsequent effects on stand conditions, including changes in light intensity, needle deposition, and the composition of fungal community mutualists, namely ectomycorrhizal fungi. It is unknown whether these changes in stand conditions will have cascading consequences for the next generation of pine seedlings.
- To test for transgenerational cascades on pine seedlings, we tested the effects of fungal inoculum origin (beetle-killed or undisturbed stands), light intensity and litter (origin and presence) on seedling secondary chemistry and growth in a glasshouse. We also tracked survival of seedlings over two growing seasons in the same stands from which fungi and litter were collected.
- Fungal communities differed by inoculum origin. Seedlings grown with fungi collected from beetle-killed stands had lower monoterpene concentrations and fewer monoterpene compounds present compared with seedlings grown with fungi collected from undisturbed stands. Litter affected neither monoterpenes nor seedling growth. Seedling survival in the field was lower in beetle-killed than in undisturbed stands.
- We demonstrate that stand mortality caused by prior beetle attacks of mature pines have cascading effects on seedling secondary chemistry, growth and survival, probably mediated through effects on below-ground mutualisms.

Introduction

Over the last decade > 47 million ha of mainly lodgepole pine (*Pinus contorta*) forests in western North America have sustained some degree of damage by mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Raffa *et al.*, 2013). Though this species is native to western North America, range expansion during the most recent outbreak has resulted in it spreading into novel pine habitats (Cullingham *et al.*, 2011; de la Giroday *et al.*, 2012). The extent of the current outbreak is unprecedented in recorded history, and is one of the few clear examples of a 'native-invader'(Simberloff, 2011). Within weeks of attack by this phloem-feeding insect, photosynthate production declines, and mortality of heavily attacked trees ensues in the months to year following (Safranyik *et al.*, 2010). The widespread mortality of pines has reduced carbon sequestration and storage of forests (Kurz *et al.*, 2008), altered watershed hydrology (Bearup *et al.*, 2014), and reduced the abundance of fungal mutualists, namely ectomycorrhizal fungi (Treu *et al.*, 2014). The composition of communities of mycorrhizal fungi, and soil fungi in general, is often altered by herbivory of host plants (Gehring & Bennett, 2009; Stursova *et al.*, 2014) and in some cases, these community shifts have been shown to influence secondary chemistry and growth of the succeeding plant generation (Kostenko *et al.*, 2012; Bezemer *et al.*, 2013). Our objective was to test whether changes in the community composition of ectomycorrhizal fungi can mediate transgenerational cascades in pine, that is, changes in the secondary chemistry and establishment of the next generation of seedlings.

Plants produce an array of secondary compounds, which mediate interactions with insect herbivores and diseases (Agrawal, 2011). Of these secondary compounds, monoterpenes are low-molecular-weight volatiles produced by terpenoid metabolism in gymnosperms, including pines (*Pinus*) (Phillips & Croteau, 1999). These compounds are critical for (direct) defense against herbivores and pathogens, attract natural enemies of herbivorous insects (indirect defense), and mediate interactions with pollinators (Moore *et al.*, 2014). Variation in secondary compounds

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between species and/or individuals within a species is often considered to be a result of coevolution with enemies (Moore *et al.*, 2014). Pines, however, have also coevolved with ectomycorrhizal fungi, mutualists responsible for soil mineral uptake in exchange for carbon. Colonization by ectomycorrhizal fungi modifies the nitrogen, phosphorus and carbon status of hosts, and fungal species vary in their effect on these properties (Smith & Read, 2008). Importantly, these resources are necessary for monoterpene synthesis (Gershenzon, 1994). The presence and identity of mycorrhizal fungi colonizing plants have been increasingly recognized as an important factor modifying host secondary chemistry and herbivore performance, yet the underlying mechanisms remain unclear (Bennett *et al.*, 2006; Gehring & Bennett, 2009; Koricheva *et al.*, 2009). Unlike the link among ectomycorrhizas, plant secondary compounds and herbivores, the effect of ectomycorrhizas on seedling establishment (growth and survival) is well documented (Smith & Read, 2008); increased seedling growth is a common response to ectomycorrhizas (Karst *et al.*, 2008). Even small gains in seedling growth can provide early competitive advantages that result in improved survival and possibly large differences in tree biomass at maturity (Oliver & Larson, 1996). Allocation to defenses such as monoterpenes, however, may compete with growth, depending on resource availability (Gershenzon, 1994; Lerdau *et al.*, 1994, 1995). Taken together, whether changes in the community composition of ectomycorrhizal fungi link the fate of adult trees to the subsequent generation of pine through effects on seedling secondary chemistry and establishment is unknown.

A variety of other factors affect monoterpenes and seedling establishment, including light and nutrients, which are also known to change as canopies degrade in beetle-killed stands. Light increases as needles and twigs fall from dead trees (Edburg *et al.*, 2012) and abscising needles from beetle-killed trees have higher nitrogen concentrations than those from regular abscission (Griffin *et al.*, 2011; Cigan *et al.*, 2015). Both changes to light and nutrients derived from litter can alter ectomycorrhizal fungal community composition (Smith & Read, 2008), pine seedling growth (Burns & Honkala, 1990; Northup *et al.*, 1995), and secondary chemistry of plant tissues (Langenheim, 1994). Past studies located in western Canada have tested the influence of seed-source availability, seedbed substrate, time since beetle infestation, overstory structure and condition on pine seedling establishment (Astrup *et al.*, 2008; Teste *et al.*, 2011a,b; Hawkins *et al.*, 2013; McIntosh & Macdonald, 2013), but none have addressed the importance of ectomycorrhizal fungi in stand regeneration following mountain pine beetle outbreaks. We set up a glasshouse experiment with the following objectives: to confirm that the community composition of ectomycorrhizal fungi differs between stands experiencing high and low rates of beetle-induced tree mortality; and to test the importance of these community changes in comparison to light- and needle-mediated effects with regard to the secondary chemistry and growth of pine seedlings. We also validate the relevance of results from this glasshouse experiment in a supplementary field experiment which shows a decline in pine seedling survival in stands with high beetle-induced tree mortality.

Materials and Methods

Collection of fungal inoculum from soils

We modified fungal communities of potted pine seedlings through the addition of soil, a standard methodology in plant–soil feedback studies (Klironomos, 2002; Reinhart *et al.*, 2003; Callaway *et al.*, 2004; Nunez *et al.*, 2009). Fresh soil containing spores and vegetative propagules of ectomycorrhizal fungi, along with other microorganisms, is collected from multiple locations at focal sites. Often samples are then pooled to capture representative soil biota from focal sites for use as inoculum (Kardol *et al.*, 2007; Ayres *et al.*, 2009; Rodriguez-Echeverria *et al.*, 2009; Gundale *et al.*, 2014). Added in small amounts to substrates (e.g. $< 5\%$ of total soil), soil inoculum can be used to modify the fungal community composition, without changing overall properties of soil chemistry and structure. Our previous work demonstrated compositional differences in fungal communities along a gradient of beetle-induced tree mortality (Treu *et al.*, 2014); our approach here was to test whether differences in fungal communities occurring at the extremes of that gradient have functional consequences for pine seedling secondary chemistry and establishment.

In 2011, we collected fresh soil from 10 locations within a 900 m^2 plot situated within each of four stands. Each stand was dominated ($\geq 80\%$) by even-aged (120 ± 0.4 yr old, mean $\pm$ SE) lodgepole pine (*Pinus contorta* Doulg. ex Loud. var. *latifolia* Engelm.) and located in west central Alberta, Canada (54°39′N, 118°59′W; elevation 1027 m). These stands are typical of lodgepole pine forests occurring in the Upper Foothills subregion of west-central Alberta (Beckingham *et al.*, 1996). Two stands had experienced high beetle-induced tree mortality (*c.* 80% pine basal area killed) and two stands were unattacked by mountain pine beetle at the time of soil collection (hereafter referred to as 'undisturbed'). Details on survey methods for tree attack status, stand structure and composition are described in Treu *et al.* (2014). Beetle attacks were first reported in 2006 in this region and continued until 2012. Previous to this event, this region (northern Rocky Mountains) had not been considered part of the insect's historical distribution (de la Giroday *et al.*, 2012). Approximately 500 cm^3 of mineral soil (to a depth of 12 cm) was collected from each location, pooled by stand type (i.e. beetle-killed or undisturbed), and transported to the University of Alberta. Soil samples were kept on ice until sifting with a 4 mm sieve and storing at 4°C. Homogenizing soil samples by stand type ensures the same treatment (fungi representative of each stand type) was applied to all pots; however, this practice inevitably reduces the within-stand variation of fungal community composition.

Litter collection

Using similar methods for testing whether soils collected from the extremes of the beetle-induced tree mortality gradient differed in their effects on seedling secondary chemistry and growth, we collected litter and included its origin and presence as a treatment in the glasshouse experiment. Needles on the ground without visible decay were collected from the same locations as soil

samples. Litter was kept on ice during shipment to the university. Upon arrival, needles were oven-dried at 60°C for 48 h, sorted to remove nonpine particles, weighed and separated into 12 g portions, and returned to cold storage at 4°C. Both nitrogen and phosphorus concentrations in abscised needles from trees increase along gradients of beetle-induced tree mortality and time since beetle attack (Cigan *et al.*, 2015).

Glasshouse experiment

We filled 1 l pots with 700 ml of a sterilized 70 : 30 coarse sand : topsoil mixture. Sterilization was achieved by autoclaving soils at 121°C for 1 h, repeated 24 h later. Pots were inoculated with 20 ml (3% of soil volume) of forest soil from beetle-killed or undisturbed stands. Noninoculated pots received 20 ml of the sterilized potting mixture. Twenty surface-sterilized (soaked in 10% bleach solution for 20 min) seeds of lodgepole pine were sown into each pot after cold stratification, and thinned to one individual per pot within 4 wk after germination. Seeds were provided by Smoky Lake Forest Nursery, Alberta (seedlot number: NWB1 64-8-6-1981 PL) and were sourced from the same provenance from which soils were collected. Overlaid on the three fungal treatments were additional manipulations: litter addition to the surface of pots and light. Twelve grams of field-collected litter from either beetle-killed or undisturbed stands were added to pot surfaces, in addition to a no litter control. Light intensities in the experiment were based on measurements taken in the understory of stands from where soil and litter were collected. Light was reduced by 61% with shade cloth or left as ambient (supplemented from metal halide lamps for 12 h daily); shaded conditions measured, on average, 337 μmol photon m^{-2} s^{-1} and ambient conditions measured 846 μmol photon m^{-2} s^{-1}, determined monthly during the afternoon. Average monthly daytime temperature ranged from 22 to 25°C. Seedlings were watered daily; fertilizer was not applied.

Treatments were replicated eight times in a full factorial (three soil fungal inoculum treatments × three litter treatments × two light intensities), completely randomized block design, yielding a total of 144 pots. Each of the eight blocks was divided in half and randomly assigned a light treatment. Within each light treatment, one replicate of each treatment combination (fungal inoculum, three levels × litter treatment, three levels) was positioned. After 9.5 months, seedlings were excised at the root collar, and stem and foliar tissues were separated, and stored at −40°C. Needles and stems were freeze-dried for 48 h in preparation for chemical analyses (see later) and afterwards they were weighed. Roots of each seedling were rinsed under tap water to remove adhering soil, cut into *c.* 1 cm fragments, and 100 root tips per seedling were assessed for ectomycorrhizal colonization and coarsely morphotyped based on color, tip shape, branching pattern, texture, and features of emanating hyphae when present (Goodman *et al.*, 1998).

Molecular identification of ectomycorrhizal fungi

The identity of fungi colonizing roots was determined by Sanger sequencing. For each seedling, we sampled one root tip of each morphotype. DNA was extracted from a single fresh root tip by incubating it in 10 μl of Sigma Extract-N-Amp Extraction Buffer (Sigma-Aldrich) at 65°C for 10 min and then at 95°C for 10 min, followed by addition of 30 μl of Sigma Extract-N-Amp Neutralization Buffer (Sigma-Aldrich). Amplification of the internal transcribed spacer (ITS) region of fungal nuclear rDNA was performed in 16 μl reactions using primers NSI1 and NLB4 (Martin & Rygiewicz, 2005) at 10 μM concentration each, 1 μl of DNA extract diluted to 1 : 100 concentration, and 8 μl of Sigma RedExtract-N-Amp PCR ReadyMix (Sigma-Aldrich Inc.). Thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, 40 cycles of denaturation (95°C for 1 min 30 s), annealing (57°C for 1 min), and extension (72°C for 1 min 30 s), followed by a final extension step of 10 min at 72°C. Amplified products were verified on a 1.5% agarose gel and were cleaned enzymatically using ExoSAP-IT (USB/Affymetrix Inc., Santa Clara, CA, USA). Cycle sequencing was performed in 10 μl reactions containing either the forward primer NSI1 or the reverse primer NLB4 at 0.5 μM concentration, 1 μl of cleaned PCR product, 0.5 μl of BigDye Terminator v3.1 Ready Reaction Mix, and 1.5 μl of 5× Sequencing Buffer (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions for cycle sequencing reactions were as follows: initial denaturation at 96°C for 1 min, and 35 cycles of denaturation (96°C for 30 s), annealing (50°C for 15 s), and extension (60°C for 2 min). Sequencing reactions were cleaned using EtOH precipitation and run on an ABI 3730 DNA analyzer (Applied Biosystems).

Sequences were first edited manually using Geneious software (Biomatters Ltd, Auckland, New Zealand) using the approach of Taylor & Houston (2011). In brief, bases with phred scores < 20 were converted to Ns and sequence ends trimmed using a 3% error probability limit. Trimmed sequences with > 2% Ns remaining were deleted from further analysis using BioEdit 7.2.5 (Hall, 2005). Sequences were grouped into operational taxonomic units (OTUs) at 97% sequence identity using CAP3 (Huang & Madan, 1999) with the following nondefault settings: maximum overhang percentage length = 60; match score factor = 5; overlap percentage identity cutoff = 96; clipping range = 6. Consensus sequences from each OTU were queried against the GenBank databases using nucleotide BLAST. BLAST results from the query dataset were additionally examined for anomalies (Nilsson *et al.*, 2012) and sequences with likely assembly chimeras were discarded. Taxonomic matches were based on ≥ 97% sequence similarity, which is shown to be a reasonable approximation for fungal species (Taylor *et al.*, 2014). A second round of identification was performed through querying the UNITE (Koljalg *et al.*, 2013) and EMERENCIA (Nilsson *et al.*, 2005) databases. Sequences of all ectomycorrhizal fungal OTUs were submitted to the GenBank database (Table 1).

Chemical analysis

We separately analyzed stems and needles for stored monoterpene composition. All tissues were ground in a Cryogenic Grinder (Spex CertiPrep 6770 Freezer Mills, Fisher Scientific, Ottawa, ON, Canada). Monoterpenes were extracted using

Table 1 GenBank accession numbers of operational taxonomic units (OTUs) of fungi identified on roots of *Pinus contorta* growing in soils collected from undisturbed and beetle-killed stands in west-central Alberta, Canada

OTU	Best GenBank match	Best GenBank match accession number	% identity	Query coverage (%)
Unidentified fungus 1	Uncultured fungus clone IH_Tag067_0506	EU292346	98.1	100.0
Helotiales 1	Uncultured Helotiales clone	KF879473	99.1	100.0
Tuber 1	*Tuber pacificum* isolate FFP977	JQ712002	100.0	100.0
	Tuber pacificum isolate FFP739	JQ711993	100.0	100.0
	Tuber pacificum isolate FFP756	JQ711955	100.0	100.0
	Tuber sp. P-11-10-13	KF742755	100.0	100.0
Unidentified fungus 2	Uncultured fungus clone N 147	JF3000746	99.0	99.0
Sebacina vermifera	*Sebacina vermifera* isolate FFP337	JQ711843	97.0	100.0
Sebacinaceae 1	Uncultured mycorrhiza (Sebacinaceae) 4078	AY634132	99.0	94.0
Pezizaceae 1	Uncultured Pezizaceae clone 1211a	FJ788776	95.4	89.2
Pyronemataceae 1	Uncultured Pyronemataceae clone UBCOFE363A	GU452518	99.0	99.0

% identity, percentage of identical bases in the alignment for which all sequence are identical; query coverage, percentage of the query sequence covered by hit.

similar methods reported in Erbilgin *et al.* (2014). Briefly, 100 mg of ground tissue was transferred to a 1.5 ml microcentrifuge tube. All samples were extracted twice with 0.5 ml dichloromethane and 0.01% tridecane as surrogate standard. After adding the solvent, the samples were vortexed for 30 s, sonicated for 10 min, subsequently centrifuged at 18 506 ***g*** and 0°C for 15 min, and placed in a freezer for at least 2 h to freeze the pellet. Extracts were transferred into an amber GC vial and stored at −40°C before chemical analysis by GC/MS. Extracts (1 μl) were analyzed using a GC/MS (Agilent 7890A/5062C; Agilent Tech, Santa Clara, CA, USA) equipped with a chiral column (HP Innowax-20B column (ID 0.25 mm, length 30 m); Agilent Tech) with helium as the carrier gas flow set to 1.1 ml min^{-1}. Each analysis began at an initial temperature of 75°C for 15 min, followed by an increase in 5°C min^{-1} until 230°C was reached. Peaks were identified using the following standards: borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol, camphor, 3-carene, terpinolene, (−)-α-pinene, (+)-α-pinene, racemic α-pinene, (−)-β-pinene, (*S*)-(−)-limonene, (*R*)-(+)-limonene, (−)-camphene, (+)-camphene, myrcene, bornyl acetate, cis-ocimene (SAFC Supply Solutions, St Louis, MO, USA), and β-phellandrene (Glidco Inc., Jacksonville, FL, USA). Chemical purity of all these compounds was > 99%. Compounds were identified by comparing retention times and mass spectra with those of the standard chemicals. Quantity of chemicals was calculated using response curves generated from analyses of a dilution sequence of known quantities of standards. Calibration with these standards allowed for analysis of quantitative differences on volatile samples among treatments. The concentration of monoterpenes was reported as ng mg^{-1} fresh plant mass. The threshold detection was pg for all monoterpenes. From this chemical analysis, we measured three properties of seedling secondary chemistry: the amount, richness, and composition of monoterpenes. Amount was measured as the total concentration of all monoterpenes summed, and richness was the number of individual compounds detected in each seedling. Composition was the profile of monoterpenes present in a seedling, that is, a multivariate measure of the amount of each individual compound found in a seedling. All these properties are known to determine the direct and indirect defenses of pine seedlings (Raffa *et al.*, 2005, 2013).

Field experiment

At the same time as soils and litter were collected, we sowed seeds of lodgepole pine within each of six stands. Four of these stands were the same as those used for soil and litter collection, another was categorized as 'undisturbed' (< 10% basal area of lodgepole pine killed by mountain pine beetle), and the other had experienced beetle-induced tree mortality at the same rate as the other two beetle-killed stands described previously. Distance between stands ranged from 1 to 50 km. These stands made up a larger field study with the objective of testing the influence of common mycorrhizal networks on seedling establishment following the beetle outbreak. As part of this larger study, we dug 10 holes randomly distributed throughout a 900 m^2 plot within each stand. The holes were three times the volume of the pots used in the glasshouse and were backfilled with field soil. In October 2011, we sowed the surface of each backfilled hole with 20 seeds from the same seedlot as used in the glasshouse experiment. The following spring (May 2012), we found that germination was non-existent, and reseeded with an additional 20 seeds. Survival of germinants was assessed in May 2013 in each of the 10 locations across the six stands.

Statistical analysis

We used linear mixed models in IBM SPSS Statistics Version 21 (IBM Corp., Armonk, NY, USA) to test the main effects and interactions among light, litter, and soil fungal inoculation on log-transformed seedling mass, total monoterpenes and $\log_e$-transformed richness of monoterpenes present in needles and stems. Fixed factors included light, litter and inoculum; to account for variance linked to the split-plot arrangement of the light treatments within the glasshouse, we included a random block × light interaction term. Post hoc tests on differences based on inoculum origin were done with least significant difference

tests ($\alpha = 0.05$). Differences in seedling monoterpene composition based on soil fungal inoculum origin were tested using canonical correspondence analysis with the 'candisc' package (Friendly & Fox, 2013) in R 2.15.3 (R Core Team, 2013). To test for differences between fungal communities colonizing potted seedlings grown with inoculum from undisturbed and beetle-killed stands, a permutational multivariate analysis of variance was run in R 2.15.3 (R Core Team, 2013) using the adonis() function in the 'vegan' package (Oksanen *et al.*, 2013) with permutations set to 999 and all other parameters as default. We used the adonis() function because it is a more robust version of permutational MANOVA (Anderson, 2001), able to handle multiple variables and less sensitive to dispersion effects (Oksanen *et al.*, 2013). A *t*-test was performed to assess differences in seedling survival rates in the field between stand types, that is, undisturbed and beetle-killed.

Results

We identified ectomycorrhizal fungi colonizing roots of pine seedlings and found that seedlings grown without soil fungal inoculum were free of ectomycorrhizas. From 100 successful amplifications submitted across 69 ectomycorrhizal seedlings, 97 yielded informative sequences and sequence clustering yielded eight OTUs, two of which did not match sequences accessioned in GenBank. The other six OTUs were identified as *Tuber* 1, *Sebacina vermifera*, Helotiales 1, Sebacinaceae 1, Pezizaceae 1, and Pyronemataceae 1 (Table 1). Subsequent searches on the UNITE (Koljalg *et al.*, 2013) and Emerencia (Nilsson *et al.*, 2005) databases did not provide additional taxonomic information on the unidentified fungi. Fungal communities differed between seedlings inoculated by soil from beetle-killed vs undisturbed stands (Supporting Information Table S1). Most seedlings were colonized by a single OTU; 13% were colonized by two or three fungal OTUs. Eight OTUs were present across seedlings inoculated with soils from undisturbed stands, two more than seedlings inoculated with soil from beetle-killed stands (Fig. 1). Sebacinaceae 1 and 'unidentified fungus 2' were found exclusively on seedlings grown with inoculum from undisturbed stands, but at low frequencies precluding tests of significance (Sebacinaceae 1, seven of 32 seedlings; unidentified fungus 2, one of 32 seedlings). Root colonization did not differ based on inoculum origin (mean ± SE, 25 ± 2.6%; $t(94) = 1.54$, $P = 0.13$).

Three seedlings died over the course of the glasshouse experiment. Of these, all three were shaded, two did not receive fungal inoculum and one received inoculum from the undisturbed stands. Approximately half of the seedlings which did not receive fungal inoculum yielded too little tissue (i.e. < 50 mg) for monoterpene analysis. Thirteen and 19 seedlings receiving fungal inoculum from undisturbed and beetle-killed stands, respectively, also yielded too little tissue for monoterpene analysis. For both needles and stems, soil fungal inoculum was the only factor explaining variation in total amount and richness of monoterpenes (Fig. 2; Tables S2–S5). Total concentration of monoterpenes was highest in pine seedlings grown with soil fungal inoculum from undisturbed stands, followed by beetle-killed stands, and lowest in seedlings without inoculum. In particular, colonization by Helotiales 1 alone coincided with lower total concentration of monoterpenes in stems (4.2 ± 0.32 ng mg^{-1} fresh plant mass) than in seedlings colonized by other OTUs co-occurring with or in isolation of Helotiales 1 (5.2 ± 0.42 ng mg^{-1} fresh plant mass ($t(43) = -1.99$; $P = 0.05$)). The same trend was evident in total monoterpenes of needles but was not significant ($t(40) = -1.53$; $P = 0.14$). The richness of monoterpenes in tissues was higher in seedlings grown with soil fungal inoculum from undisturbed stands than in seedlings grown with inoculum from beetle-killed stands. This trend was more pronounced in stems than in needles (Fig. 2). The number of OTUs colonizing roots of a seedling did not correlate with total amount or richness of monoterpenes in stems or needles (minimum $P = 0.06$). Neither light nor litter affected seedling secondary chemistry (Tables S2–S5).

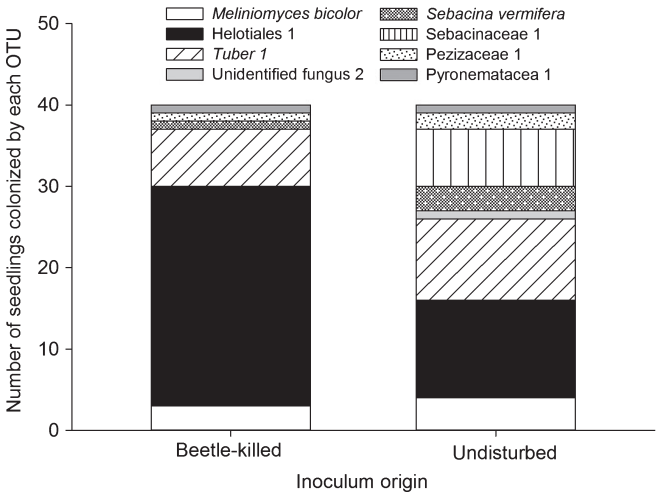

Fig. 1 Differences in the presence of ectomycorrhizal fungi by inoculum origin (beetle-killed (> 80% tree mortality caused by mountain pine beetle, *Dendroctonus ponderosae*) and undisturbed stands in west-central Alberta, Canada). Fungi represented by eight operational taxonomic units (OTUs) colonize lodgepole pine (*Pinus contorta*) seedlings in isolation of, or in combination with, other OTUs; the numbers of seedlings grown with fungal inoculum from beetle-killed and undisturbed stands are 37 and 32, respectively. Fungi recovered from seedlings grown under different light and litter treatments were pooled.

Stem and needle monoterpene composition differed by soil fungal inoculum origin (Tables S6, S7). Stems of seedlings grown with inoculum from undisturbed stands were dominated by high concentrations of β-phellandrene (Fig. 3a; Table S8); the majority of compounds decreased in concentration when seedlings were grown with inoculum from beetle-killed stands or no inoculum (Fig. 3a; Tables S6, S7). Similar patterns of monoterpene profiles were observed in needles as in stems of seedlings, but were not as pronounced (Fig. 3b; Table S8).

The effect of inoculum origin on seedling mass depended on light intensity (Fig. 4; Table S9). Seedlings grown with inoculum from beetle-killed stands had lower mass (22% less) than those with inoculum from undisturbed stands, but only under ambient light intensities. Under both shade and ambient light, seedlings without ectomycorrhizal associations grew the least. Seedlings

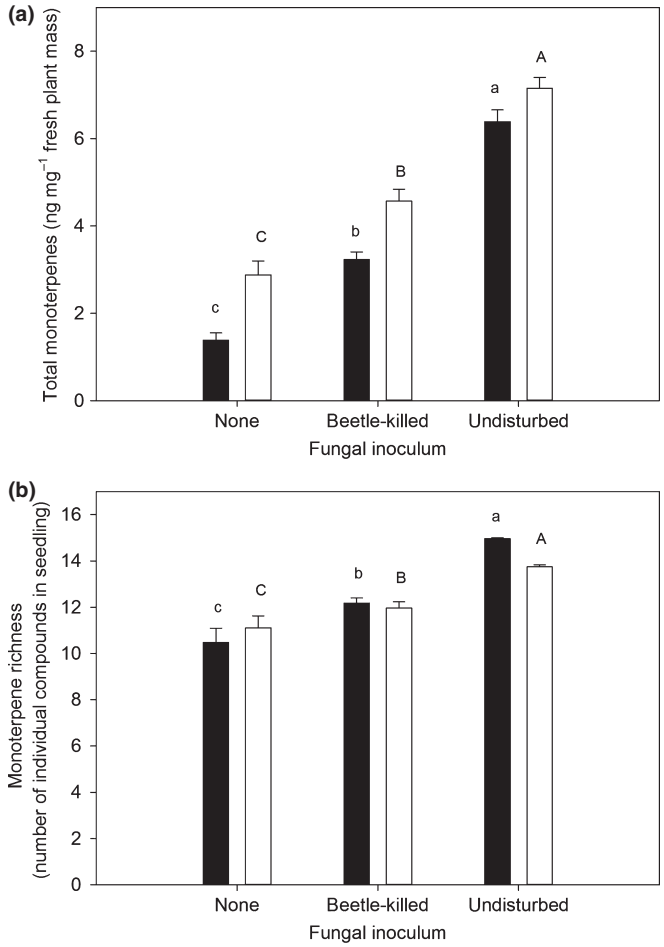

Fig. 2 (a) Mean total monoterpene concentrations (± SE) of pine (*Pinus contorta*) by fungal inoculum (none, or originating from beetle-killed (> 80% tree mortality caused by mountain pine beetle, *Dendroctonus ponderosae*) or undisturbed stands in west-central Alberta, Canada) in stems (closed bars) and needles (open bars). (b) Mean monoterpene richness (± SE) (number of individual compounds in a seedling) in pine by fungal inoculum (none, or originating from beetle-killed or undisturbed stands in west-central Alberta, Canada) in stems (closed bars) and needles (open bars). For a tissue type, means labeled with different letters are significantly different at $P < 0.05$.

colonized by Helotiales 1 alone had lower total seedling mass than that of seedlings colonized by other OTUs co-occurring with or in isolation of Helotiales 1, but this effect was not significant ($t(67) = -1.71$; $P = 0.09$). In the field, seedling survival was 27% (± 0.036) in undisturbed stands and 1.1% (± 0.003) in beetle-killed stands ($t(48) = 5.94$; $P < 0.001$).

Discussion

Through our glasshouse experiment, we show that in the absence of any direct signal between mature and juvenile pines, prior attacks by the mountain pine beetle on mature pines indirectly influence the secondary chemistry and growth of seedlings. Through our field experiment, we show that pine seedling survival is drastically reduced in beetle-killed stands compared with that in undisturbed stands. These cascading effects on the succeeding generation of pine are most probably mediated by the disruption of below-ground mutualists, ectomycorrhizal fungi.

Little research has been done on understanding the relationship among secondary chemistry, insect herbivores, and ectomycorrhizas (Hartley & Gange, 2009). Consequently, there is a gap in our knowledge regarding feedbacks among insect outbreaks, establishment of ectomycorrhizal seedlings, and seedling resistance to future insect and pathogen attacks. This gap is a major oversight, as economic and ecological benefits gained from pine forests are derived from trees dependent on ectomycorrhizal fungi. Several studies have shown that colonization by arbuscular mycorrhizal fungi can alter terpene production (stored and/or emitted), depending on tissue and compound (Rapparini *et al.*, 2008; Asensio *et al.*, 2012). We demonstrate that in the absence of ectomycorrhizas, there are striking decreases in constitutive defenses of above-ground tissues of seedlings. Moreover, community context is pivotal; changes in the composition of ectomycorrhizal fungal communities driven by beetle-induced tree mortality result in decreases in the amount and richness of monoterpenes in seedlings. In our glasshouse experiment, changes in communities comprising disturbance-tolerant (i.e. able to survive sieving) ectomycorrhizal fungi were observed between soils from attacked and undisturbed stands. Beetle-induced tree mortality in the field has caused more pronounced changes in ectomycorrhizal fungal communities involving nearly 100 species (Treu *et al.*, 2014). In particular, both changes in the composition of fungal communities and loss of ectomycorrhizal fungi in beetle-killed stands may result in establishing pine seedlings with reduced constitutive defenses.

In our field experiment, seedling survival was greatly reduced in beetle-killed stands. Compared with undisturbed stands, beetle-killed stands in this region have been shown to have increased soil moisture, soil nitrate, and litter deposition (Cigan *et al.*, 2015), in addition to changes in the composition of fungal communities (Treu *et al.*, 2014). Together or in part, these changes in stand conditions following beetle-induced tree mortality may affect the survival of pine seedlings establishing in the understory. Results from our glasshouse experiment indicate that the presence of specific taxa of ectomycorrhizal fungi (i.e. Helotiales) can modify the quantity of stored monoterpenes in stems. To further investigate the link between the presence of this particular group of fungi in soils and seedling survival in the field, we sequenced DNA extracted from roots cored at multiple locations from the six stands (see Methods S1). We found that the frequency of Helotiale taxa (Table S10) (measured by the proportion of sequence reads belonging to species of this order) increased from 44 ± 0.10 to 72 ± 0.022% between soils from undisturbed and beetle-killed stands (Table S11). Although we do not show causation, taken together, results from the glasshouse and field experiment indicate that the presence of Helotiales taxa reduce the amount of monoterpenes in pine seedlings and, possibly, their survival. Whether fungal-mediated reductions in seedling monoterpenes underlie rates of mortality in the field deserves further study.

Although performed on roots, results of earlier studies support our findings that ectomycorrhizas increase the production of

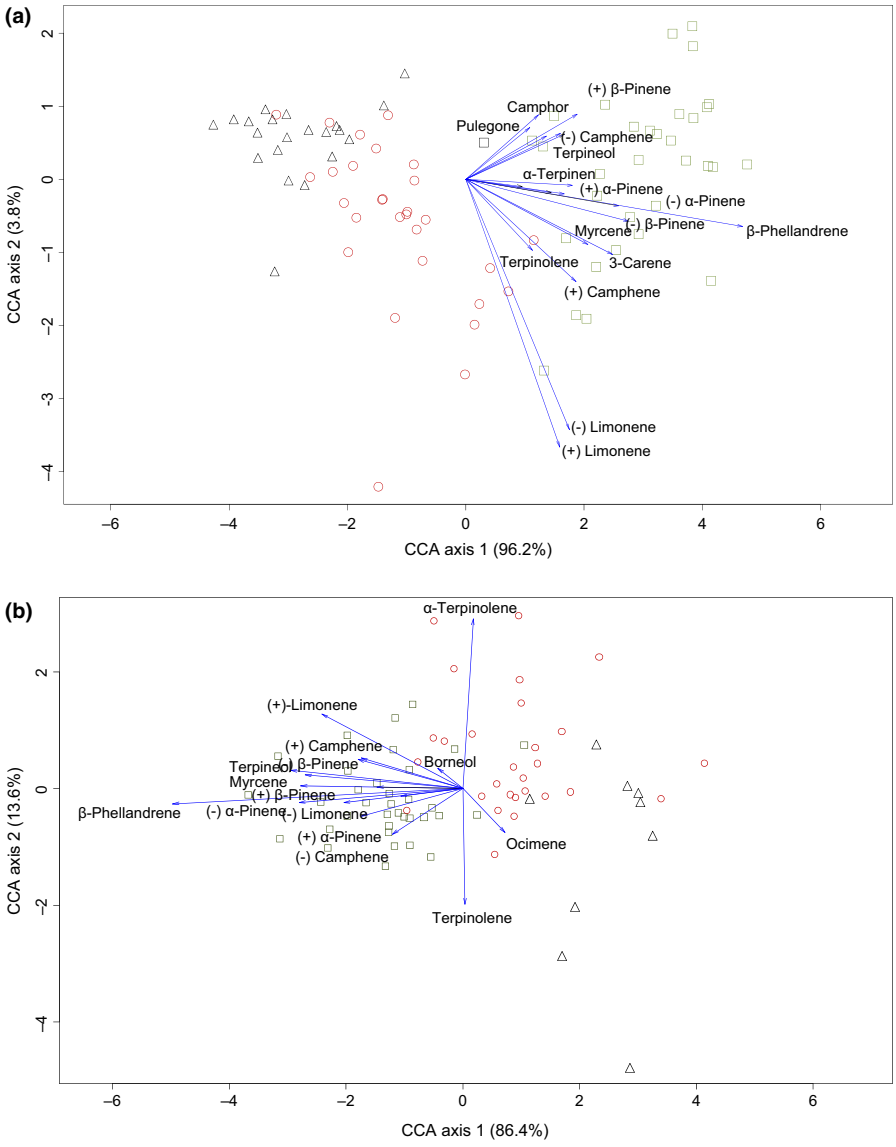

Fig. 3 Chemical constituents of lodgepole pine (*Pinus contorta*) seedlings. Results of canonical correspondence analysis (CCA) of stems (a) and needles (b) by soil fungal inoculum origin. Triangles, pine seedlings grown without soil inoculum; circles, seedlings inoculated with fungi from stands with > 80% tree mortality caused by mountain pine beetle (*Dendroctonus ponderosae*); squares, seedlings inoculated with fungi from stands free of beetle-induced tree mortality. Length of vector indicates the strength of the relationship between chemical compound and axis.

monoterpenes, and that changes in the community composition of ectomycorrhizal fungi alter the amount of specific constituents. Earlier research shows that ectomycorrhizal roots contain similar volatile compounds to those of nonmycorrhizal pine seedlings; however, they occur in much greater concentrations (Krupa & Fries, 1971; Krupa *et al.*, 1973). The number of chemicals identified in our study is higher than that detected in earlier studies, and thus probably underlies our ability to detect changes in the richness of monoterpenes in addition to their abundance. Of the monoterpenes, α-pinene, 3-carene and terpinolene all increased in concentration when *Pinus sylvestris* was colonized by *Boletus variegatus* (Krupa & Fries, 1971). Different species of ectomycorrhizal fungi, however, elicited specific monoterpene responses (Krupa *et al.*, 1973). Of the little research performed on assessing the response of stored foliar monoterpenes to ectomycorrhizal colonization, total monoterpenes were unaffected by the presence of *Cenococcum geophilum* colonizing roots of *P. sylvestris* seedlings, and the presence of ectomycorrhizas did not affect plant host susceptibility to a polyphagous insect herbivore (Manninen *et al.*, 1998). We found that seedlings singularly colonized by Helotiales 1 had lower total monoterpenes than those colonized by other fungi in the absence or presence of this particular species. This finding suggests that the effects of this particular fungal species on monoterpene production (and seedling growth) may be offset by co-colonization of other ectomycorrhizal fungi. In our sampling, we selected roots colonized by putative ectomycorrhizal fungi; however, Helotiales 1 and the taxa belonging to this order retrieved from sequencing DNA extracted from soil cores may be root endophytes rather than fungi forming ectomycorrhizas (Tedersoo *et al.*, 2009). These taxa were absent from sporocarp surveys of these sites (Treu *et al.*, 2014). Our results indicate that the role these fungi play in host defense and growth warrants further investigations.

The presence of ectomycorrhizal fungi may affect plant secondary chemistry through alterations of carbon to nutrient ratios, allowing for increased investment in carbon-based defenses, such

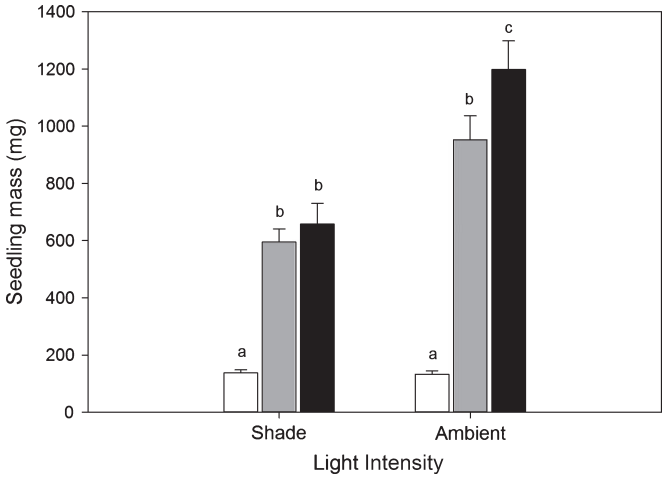

Fig. 4 Lodgepole pine (*Pinus contorta*) seedling mass by fungal inoculum type and light intensity. Mean total masses (± SE) of pine seedlings without fungal inoculum (white bars), inoculated with soil fungi from stands with > 80% tree mortality caused by mountain pine beetle (*Dendroctonus ponderosae*) (gray bars), and free of beetle-induced tree mortality (black bars) are presented at two light intensities: ambient (846 μmol photon m^{-2} s^{-1}) and shade (337 μmol photon m^{-2} s^{-1}). Within a light intensity, bars labeled with different letters are significantly different at $P < 0.05$.

as monoterpenes (Jones & Last, 1991), or through the acquisition of resources allowing mycorrhizal plants to increase allocation to both growth and defense, that is, a relaxed tradeoff (reviewed in Bennett *et al.*, 2006). Increasing light intensities amplified the effect of fungal origin on seedling growth, indicating a hierarchy of energy allocation; differences in biomass occurred at higher resource (light) levels than those where differences in defense emerged. Specifically, origin of fungi affected seedling growth only at ambient light intensities, whereas greater monoterpene richness and abundance were found in seedlings grown with inoculum from undisturbed stands regardless of light intensity. Thus, allocation to growth may be secondary to defense in young seedlings (Erbilgin & Colgan, 2012). This finding also suggests that potential growth benefits gained from increased light with the loss of canopy cover may be partially offset through changes in below-ground mutualisms and monoterpene production in seedlings establishing in beetle-killed stands.

While the relevance of our findings for understanding plant–insect interactions should be interpreted cautiously, because monoterpene composition varies ontogenetically (Barton & Koricheva, 2010; Erbilgin & Colgan, 2012), and replication by disturbance type should be increased, there are at least three mechanisms by which plants can benefit from having rich and abundant secondary compounds. First, plants with these properties can undergo the greatest chemical changes that are the least suitable for herbivores and diseases, an idea supported by within-species comparisons of resistant and susceptible pines (Raffa & Berryman, 1982; Boone *et al.*, 2011). Second, plants with rich and abundant secondary compounds may have better indirect defenses by attracting natural enemies of herbivores (Mumm & Hilker, 2006). Many parasitoids, and some predators, orient towards plant odors, including specific chemical signals released following feeding by herbivores to locate their prey. In particular, high amounts of β-phellandrene characterized seedlings grown with inoculum from undisturbed stands. This compound, a major constituent monoterpene in lodgepole pine, is a synomone (i.e. a volatile benefiting both the receiver and the emitter) for a variety of insects, including major predators of mountain pine beetle (Miller & Borden, 2000). Third, orientation by insect herbivores to their pheromones is often reduced by release of host-plant volatiles, including monoterpenes (but see Erbilgin *et al.*, 2003, 2007). This has been shown in a wide variety of insects, including Lepidoptera (Landolt & Heath, 1989), Coleoptera (Erbilgin & Raffa, 2000), and Diptera (Bartelt *et al.*, 1986). Our results demonstrate that seedlings inoculated by fungi from beetle-killed stands or without ectomycorrhizas express defense characteristics of stressed trees, suggesting they may be prone to successful insect attacks.

Although light influenced growth of pine seedlings, it did not affect amount or richness of monoterpenes stored in plant tissues. Processes related to carbon supply mediated by light intensity may be decoupled from monoterpene synthesis (Langenheim, 1994). Alternatively, it is possible that seedlings grown under higher light had higher terpene synthesis, but the resulting compounds were subsequently released as volatiles. Monoterpene emissions often increase with light (Staudt & Bertin, 1998; Tarvainen *et al.*, 2005; Dindorf *et al.*, 2006), though less is known about the effects of light on stored monoterpenes. Similar to light, litter had no effect on secondary chemistry of pine seedlings. Although the quality of needle litter differed between undisturbed and beetle-killed stands (Cigan *et al.*, 2015), for several reasons these differences may have not affected secondary chemistry or seedling growth. The placement of needle litter on soil surfaces of potted seedlings may have delayed decomposition; most breakdown occurs in mineral horizons rather than on soil surfaces (Prescott *et al.*, 2004). The potted soils were autoclaved, probably killing many microbes necessary for decomposition. In addition to the placement of litter and treatment of soils, the length of time for decomposition to occur may have also been much longer than the duration of our experiment. The effects of litter inputs on mineral nutrient availability may not peak until > 5 yr (Huber, 2005).

In conclusion, plant secondary compounds are a coevolutionary response to herbivore attack, and for pines, the production of terpenoids is believed to partly emerge from a long-standing relationship with bark beetles (Raffa & Berryman, 1987). Variation in total monoterpenes and the composition thereof may be the result of historical selection pressure of bark beetles (Raffa *et al.*, 2013) and current abiotic and biotic environmental conditions (Langenheim, 1994; Clark *et al.*, 2012). Our results show that another coevolved interaction, that occurring between pines and ectomycorrhizal fungi, also influences the secondary chemistry of pines. Long-lived organisms such as trees may rely on assemblages of mutualists as a way to confront fast-evolving enemies (Arnold *et al.*, 2003). Thus, the ratcheting of the coevolutionary arms race between pines and bark beetles may be mitigated by a third partner, ectomycorrhizal fungi. Our results indicate that

biotic interactions, specifically mutualisms, are critical in mediating how pine seedlings establishing in beetle-killed stands with no previous history of mountain pine beetle may interact with and respond to a novel insect. Not only do ectomycorrhizal associations influence secondary chemistry of pine seedlings, but disruption of these mutualisms in beetle-killed stands also decreases seedling growth and may underlie poor survival. In many forests across western North America, epidemic populations of mountain pine beetle have left landscapes of dead pine trees. The legacy of the mountain pine beetle, however, extends far beyond this single cohort of trees; transgenerational cascading effects of this native invader can influence subsequent generations through disrupted below-ground mutualisms. Such far-reaching cascading effects emphasize the interconnectedness of seemingly discrete components of ecosystems.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Results of permutational MANOVA testing the effects of soil fungal inoculum origin, litter, light, and their interactions on ectomycorrhizal fungal community composition of *Pinus contorta* seedlings

Table S2 Results of linear mixed model testing of the effects of soil fungal inoculum, litter, light, and their interactions on total monoterpenes in stems of *Pinus contorta* seedlings

Table S3 Results of linear mixed model testing of the effects of soil fungal inoculum, litter, light, and their interactions on total monoterpenes in needles of *Pinus contorta* seedlings

Table S4 Results of linear mixed model testing of the effects of soil fungal inoculum, litter, light, and their interactions on monoterpene richness of stems of *Pinus contorta*

Table S5 Results of linear mixed model testing of the effects of soil fungal inoculum, litter, light, and their interactions on monoterpene richness of needles of *Pinus contorta* seedlings

Table S6 Summary of raw concentrations of monoterpenes (ng mg^{-1} fresh mass of plant tissue) in *Pinus contorta* stems by soil fungal inoculum

Table S7 Summary of raw concentrations of monoterpenes (ng mg^{-1} fresh mass of plant tissue) in *Pinus contorta* needles by soil fungal inoculum

Table S8 Results of canonical correlation analysis testing the relationship between seedling monoterpene profiles and soil inoculum type

Table S9 Results of linear mixed model testing of the effects of soil inoculum, light, and their interactions on log-transformed mass of *Pinus contorta*

Table S10 List of Helotiales taxa found in soils of *Pinus contorta* stands in west-central Alberta, Canada

Table S11 Abundance of Helotiales measured by proportion of total sequences representing taxa belonging to Helotiales found in soils of *Pinus contorta* stands in west-central Alberta, Canada

Methods S1 Next-generation sequencing of fungi occurring in soils across pine stands in west-central Alberta, Canada.

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