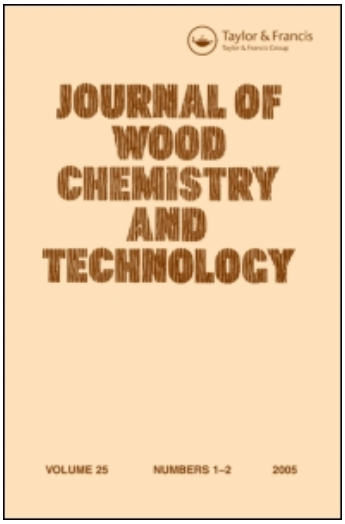

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Monoterpene Emissions from Lodgepole and Jack Pine Bark Inoculated with Mountain Pine Beetle-Associated Fungi

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Abstract: Relative monoterpene levels were analyzed from bark samples of lodgepole pine, jack pine, and their hybrids inoculated with mountain pine beetle (MPB)–associated fungi (*Leptographium longiclavatum*, *Grosmannia clavigera*, and *Ophiostoma montium*). Lodgepole pine showed the largest changes in relative emissions as a result of fungal inoculation. The relative emission of β-phellandrene increased with fungal inoculation, making it the most abundant monoterpene for inoculated samples. Relative emissions of limonene and α-pinene decreased in inoculated lodgepole pine. Lodgepole (5.6) and jack (146) pine differed in the ratio of α-pinene to myrcene; two monoterpenes involved in pheromone synthesis by the MPB. These differences may contribute to the attractiveness of the two species as hosts for MPB; with jack pine potentially less attractive than lodgepole pine. Fungal inoculation reduced α-pinene:myrcene ratios in lodgepole pine, which suggests one possible mechanism by which the fungi benefit the beetle.

Keywords: Blue stain fungi, inoculation, jack pine, lodgepole pine, monoterpene, mountain pine beetle, VOC

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INTRODUCTION

Western Canada is currently experiencing the worst mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins) epidemic in recorded history. By 2005, this epidemic was responsible for the death of pine, mainly lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), across 8.7 million hectares of forest in British Columbia (BC).[1,2] It is estimated the beetle will have killed 80% of mature pine in BC by 2013. In Alberta, the current major concern is that MPB will invade boreal forests and attack jack pine (*Pinus banksiana* Lamb.) that could be used as a conduit for continued eastward range extension.[3] In Alberta, there is a large zone of hybridization between jack and lodgepole pine, and these hybrids have already been successfully attacked.[4] It is expected that MPB will eventually invade jack pine forests.

The MPB successfully attacks its hosts with the aid of several symbiotic species of ophiostomatoid, blue stain fungi. As beetles bore through the bark of a tree they inoculate the phloem and outer sapwood with fungal spores. Upon germination, fungal hyphae spread through the phloem and sapwood and contribute to the shut down of translocation. These fungi color the sapwood blue, dehydrate the tree, and reduce terpene production, ostensibly increasing the likelihood that the beetle will overcome the tree's natural defenses.[5] The precise nature of the relationship between the beetles, fungi, and host trees is poorly known, but an improved understanding of these relationships can help predict the outcomes of beetle infestation of jack pine, thereby contributing to risk modeling.

Oleoresin, a mixture of terpenes and resin acids, serves as a physical and chemical defense mechanism for trees against insects and pathogens.[6] Differences in the physical and chemical properties of oleoresin contribute to variance in tree resistance.[7] Volatile organic compounds (VOCs) from live-standing and harvested trees show varied terpene levels and content depending on tree species and health. The major VOC from live-standing lodgepole pine[8] and the boles of harvested lodgepole pine[9] is β-phellandrene whereas α-pinene is the major monoterpene present in the foliage of harvested lodgepole pine.[9] Changes in monoterpene emissions associated with ophiostomatoid fungal infection have not been examined in lodgepole pine, but infection-associated changes have been observed in other species.[10,11]

In this study, the relative levels of monoterpenes were investigated for lodgepole pine, jack pine, and their hybrids inoculated with three species of blue stain fungi that are carried by the MPB. Within each tree species, terpene emissions were compared among each of the fungal treatments and to un-inoculated controls to test the hypothesis that fungal infection changes relative terpene emissions of bark. Relative terpene emissions were also compared between tree species.

EXPERIMENTAL

Study Sites

Three sites across central Alberta were chosen for tree inoculations. The pine forests at each site were mature (at least 50 years), and inoculated trees appeared healthy and had a diameter at breast height of at least 20 cm. Lodgepole pine trees were inoculated near the Berland River between Hinton and Grande Cache (53°45.361′ N, 118°20.207′ W), a montaine site with well-drained soil and a closed canopy of lodgepole pine. Hybrid pines were inoculated at a site northeast of Blue Ridge (54°13.127′ N, 115°16.456′ W), a boreal site with well-drained soil, and an open canopy of hybrid pines. Jack pines were inoculated near Tawatinaw (54°16.647′ N, 113°28.171′ W), a boreal site with sandy soils and a closed canopy of jack pine. Inoculation of the tree species at a single site is not possible because their geographic ranges do not overlap in natural forests. For this study, two trees were examined at each of the three sites.

Inoculation

The fungi used in this study were isolated from the sapwood of MPB-infested lodgepole pine trees harvested from the Willmore Wilderness Area, Alberta in January 2006. The isolates are deposited as live cultures at the Northern Forestry Centre Culture Collection (NOF). One isolate each of *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Wingfield) Zipfel, Z.W. de Beer & M.J. Wingf. (NOF 2948), *Leptographium longiclavatum* Lee, Kim & Breuil (NOF 2954), and *Ophiostoma montium* (Rumbold) von Arx (NOF 2951) was used in this study. Inoculation followed Rice et al.[12] Holes (5 mm diameter, 10 mm deep) were drilled through the bark and phloem at least 10 cm apart in a ring around the bole at breast height on each tree. Inoculum, consisting of active mycelium growing on 2% malt extract agar (MEA; 20 g Difco malt extract (Difco Laboratories, Detroit, MI), 15 g agar (Fisher Scientific, Fair Lawn, NJ), 1 L dH_2O, was inserted into three holes using a flame-sterilized probe and placed on the surface of the sapwood. A sterile wood dowel (5 mm diameter, 5–7 mm long) was placed into each hole to cover the inoculum. One control hole per tree did not receive any media and was plugged with a dowel. Parafilm® strips (American National Can, Neenah, WI) were wrapped around the trees at the inoculation sites to reduce contamination and desiccation. Trees were inoculated in August 2006, corresponding with the time when most beetles complete host colonization in Alberta.[13] Trees were harvested six weeks after inoculation in September 2006. The trees were felled, and bolts (>1.2 m long) were cut from around the inoculation site (with at least 50 cm above and below the inoculation points) and transported to the laboratory. Bark and phloem were

stripped, aseptically, from the bolts within 36 hours of harvesting and bark from above each of the inoculation sites was kept separate and placed immediately in zip-tight plastic bags. Bark from above the empty holes was used as the control. Fungi were recovered from their respective lesions.[12]

Terpene Analysis

Twenty-four bark samples [four samples per tree (control, *L. longiclavatum*, *G. clavigera*, *O. montium*), two trees per species] were stored in zip-tight plastic bags at -20°C until analyzed. Samples ranged from 3–26 g in mass. A Grab Air sample pump (1 L/min) and Anasorb 747 (200 mg sorbant) were used for each sample analyzed, both manufactured by SKC Inc. (Eighty Four, PA). A hole was made in the sample bag and the sorbant tube was inserted halfway into the hole. Parafilm® was used to seal the tube to the bag. A second hole was placed on the other side of the bag to maintain atmospheric pressure inside the sample bag. The bags were sampled for 48 hours at 22°C. Controls without bark samples monitored the background VOCs of the experimental apparatus. No compounds were found in the chromatogram where the 9 terpenes appear (data not shown). VOCs were eluted with $\sim$2 mL CH_2Cl_2, concentrated, and subjected to GC analysis. A Varian CP-3380 gas chromatograph (He carrier gas) and Phenomenex ZB-Wax (polyethylene glycol) GC Column (30 m $\times$ 0.25 mm, 1.00 μm film) were used with a temperature program of 75°C for 4 min, ramp 4°C/min to 200°C, and hold 5 min. Compounds were confirmed based on comparison of relative retention indices published.[14–16] All compounds except α-, β-phellandrene, and sabinene were also confirmed from the injection of known standards. Retention times and indices of analyzed monoterpenes are found in Table 1. Relative terpenes percentages were calculated from the

Table 1. Retention times and retention indices of monoterpenes analyzed

Terpene	Retention time (min)	Retention index
α-Pinene	4.2	1050
Camphene	5.1	1093
β-Pinene	6.1	1135
Sabinene	6.4	1147
α-Phellandrene	7.2	1175
Myrcene	7.4	1182
Limonene	8.7	1226
β-Phellandrene	9.0	1236
p-Cymene	11.1	1297

summation of the area counts from the 9 terpenes studied. All terpenes and *n*-alkanes were purchased from Aldrich Chemical Co. (Milwaukee, WI). One way ANOVA with a Bonferonni post hoc test (Statistica 7.0) was used to test for mean significant difference.

RESULTS AND DISCUSSION

Compared with controls, inoculated jack and hybrid pines showed fewer changes in VOCs than inoculated lodgepole pine. All inoculated jack pine showed a 28% average relative reduction of both camphene and limonene (Figure 1). Other effects on jack pine were specific to fungal species. Samples inoculated with *L. longiclavatum* showed a 33% reduction in β-pinene compared with controls, whereas inoculation with *G. clavigera* and *O. montium* caused lesser reductions of 24% and 21%, respectively. Myrcene, accounting for only 0.6% of total analyzed emissions, increased by 110% for the *O. montium* samples, with lesser increases of 22% and 16% for *L. longiclavatum* and *G. clavigera*, respectively.

In hybrid pine, limonene decreased 66% overall following fungal inoculation compared to controls (Figure 2). The three fungi differed in their effects on relative myrcene and β-phellandrene levels in hybrid pines. Inoculation with *L. longiclavatum* was associated with decreases of 21% in levels of both monoterpenes and *O. montium* was associated with decreases of 58% and 67% in levels of myrcene and β-phellandrene, respectively. In contrast,

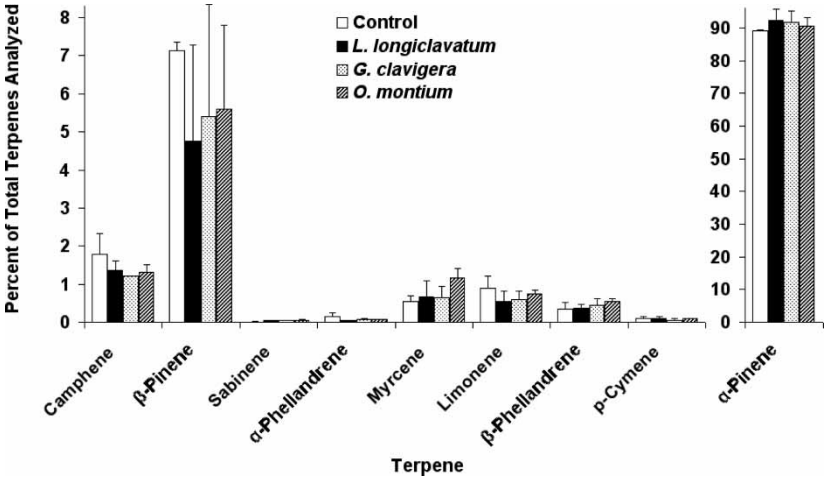

Figure 1. Mean (SD) relative monoterpene emissions from jack pine bark inoculated with three species of ophiostomatoid fungi associated with MPB, and from uninoculated controls ($n = 2$).

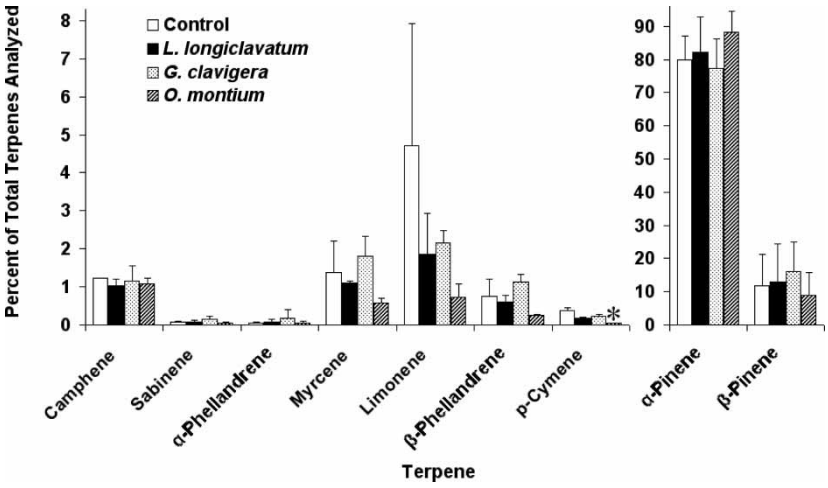

Figure 2. Mean (SD) relative monoterpene emissions from hybrid pine bark inoculated with three species of ophiostomatoid fungi associated with MPB, and from un-inoculated controls ($^*p < .01$, $n = 2$).

inoculation with *G. clavigera* was associated with increased levels of both compounds (30% for mycene and 50% for β-phellandrene). Interspecific differences in effect on host plant VOC chemistry could provide a possible mechanism for differential effects of the fungi on the beetle.

Lodgepole pine showed the greatest changes in bark VOCs following fungal inoculation. Inoculation resulted in a 61% and 55% average decrease in camphene and limonene, respectively (Figure 3). The reduction in limonene is similar to that observed in hybrid pine. α-Pinene, which accounts for 17% of terpenes analyzed in the control samples, decreased by an average of 64% for inoculated samples. *p*-Cymene decreased by 47% following inoculation of *G. clavigera*, but both *L. longiclavatum* and *O. montium* had lesser influence on concentration. The most abundant terpene in the controls was β-pinene, accounting for 36% of total terpenes analyzed. However, relative concentrations of this terpene were lowered to 27%, 32%, and 20% for samples inoculated with *L. longiclavatum*, *G. clavigera*, and *O. montium*, respectively. Increases in emissions with fungal inoculation were observed for sabinene, α-phellandrene, and β-phellandrene, which showed average increases of 93%, 15%, and 80%, respectively. The increase in β-phellandrene for inoculated samples brought its overall average concentration to 54% of the total terpenes analyzed, making it the most abundant terpene. β-Phellandrene, was one of 17 bark volatiles that elicited an antennal response for the MPB.[17] Volatiles emanating from the abdomens of female MPB include hydrated compounds of β-phellandrene, that are believed to be metabolized via a specific pathway to detoxify the large

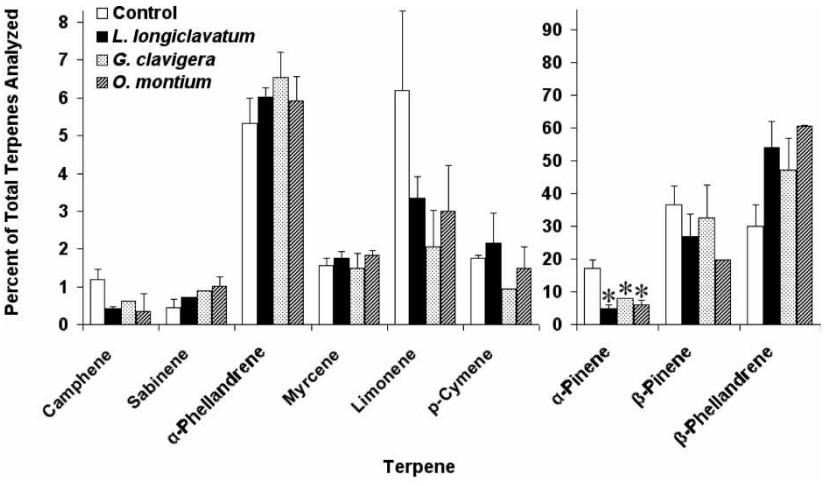

Figure 3. Mean (SD) relative monoterpene emissions from lodgepole pine bark inoculated with three species of ophiostomatoid fungi associated with MPB, and from un-inoculated controls ($^*p < .01$, $n = 2$).

amounts of β-phellandrene encountered by beetles during host colonization.[18] Thus, β-phellandrene may function as a kairomone for MPB host detection, but likely has little other value to the beetle.

There were large differences in bark VOCs among the three pine species. Overall, the emissions from the hybrid pines used in this study were similar to those found for jack pine, which is consistent with the observation that these hybrids are morphologically more similar to jack pine than lodgepole pine. However, hybrid pines showed relative levels of myrcene and limonene resembling those found in lodgepole pine, rather than jack pine. Jack pine emissions, on average, consisted of 91% α-pinene, whereas the average amount of α-pinene from all lodgepole pine samples comprised only 8.9% of the measured monoterpenes. Female MPB produce *trans*-verbenol, an aggregation pheromone, by metabolizing α-pinene.[19] For *trans*-verbenol to be an attractant for the MPB, it needs to be combined with other monoterpenes.[20] The most effective monoterpene synergist for *trans*-verbenol is myrcene.[21] Although α-pinene is ineffective as a synergist for *trans*-verbenol, α-pinene can be autoxidized to verbenone, a potent anti-aggregation pheromone of the MPB.[22] The relative amounts of α-pinene and myrcene may play a key role in controlling beetle behavior. Relative α-pinene emissions were 10-fold higher, on average, in jack pine than in lodgepole pine bark samples. Meanwhile, relative myrcene emissions were 2.2-fold lower in jack than lodgepole pine. These large differences in α-pinene and myrcene result in the vastly different ratios of the two monoterpenes (Table 2). Lodgepole pine had an overall average α-pinene:myrcene ratio of

Table 2. Relative α-pinene:myrcene ratios of bark samples

Pine species	Fungus	α-Pinene:myrcene ratio
Jack	None	167 ± 42
Jack	*L. longiclavatum*	171 ± 10
Jack	*G. clavigera*	164 ± 85
Jack	*O. montum*	80.0 ± 20
Hybrid	None	71.0 ± 50
Hybrid	*L. longiclavatum*	75.6 ± 14
Hybrid	*G. clavigera*	45.3 ± 18
Hybrid	*O. montum*	158 ± 46
Lodgepole	None	11.0 ± 2.9
Lodgepole	*L. longiclavatum*	2.75 ± 0.99
Lodgepole	*G. clavigera*	5.54 ± 1.6
Lodgepole	*O. montum*	3.25 ± 1.0

5.6 whereas jack pine had an average ratio of 146. Inoculation with MPB-associated fungi altered these ratios. Interestingly, inoculation caused the greatest decrease in ratio in lodgepole pine samples. Thus, the lower relative α-pinene:myrcene ratio in lodgepole pine may make this species a more attractive host than jack pine for MPB. The lower α-pinene:myrcene ratio in lodgepole pine would lead to a lower relative concentration of verbenone, an oxidized product of α-pinene, and a higher relative amount of aggregation pheromone *trans*-verbenol and its synergist myrcene after an initial female beetle attack and colonization by associated fungi.

CONCLUSIONS

There were fewer changes in monoterpene emissions associated with fungal inoculation in jack and hybrid pine samples than observed in lodgepole pine. The greatest change in jack pine was a 110% increase in myrcene caused by the inoculation of *O. montium*. Lodgepole pine showed relative camphene, limonene, and α-pinene emissions decrease from the presence of ophiostomatoid fungal pathogens, whereas relative β-phellandrene levels increased. Overall monoterpene emissions from hybrid pine bark closely resembled those found for jack pine. VOC emission profiles of lodgepole pine and jack pine differed, with considerably lower α-pinene:myrcene ratios in lodgepole pine. These differences may contribute to the attractiveness of the two species as hosts for MPB; with jack pine potentially less attractive than lodgepole pine. Inoculation with MPB-associated fungi decreased α-pinene:myrcene ratios in lodgepole pine, suggesting one biochemical avenue of benefit for the beetle.

This study represents preliminary work as an initial assessment of monoterpenes from jack, hybrid, and lodgepole pine bark. The analysis of absolute terpene emissions from live-standing pines during the time of beetle flight under different levels of stress, including fungal inoculation and beetle attack, would provide a more accurate representation of VOCs that a beetle encounters during host selection. Future work could offer insight into the likelihood of the MPB successfully invading jack pine forests.

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