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ARTICLE

Short- and long-term cold storage of jack pine bolts is associated with higher concentrations of monoterpenes and nutrients[1]

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Abstract: Studies with conifer-infesting bark beetles commonly use bolts cut from trees to evaluate the effects of host tree quality on various aspects of insect biology. Yet, whether host quality changes between live trees and bolts cut from these trees has not been assessed. Particularly, changes in concentrations of defense chemicals (such as monoterpenes) and nutrients (such as nitrogen and carbon) have not been compared between live trees and their cut bolts. To determine whether monoterpene and nutrient concentrations differ after cutting, jack pine (*Pinus banksiana* Lamb.) trees in Lac La Biche (Alberta) were selected and sampled for phloem tissue. Then, these trees were harvested into two bolts per tree and stored at 4 °C for 3 and 6 months. Phloem was sampled from both live trees and bolts 3 and 6 months after storage. We found that major monoterpenes of jack pine were higher in phloem from bolts than from live trees. Storage time did not affect the results. Furthermore, some nutrients including nitrogen were also higher in bolts and varied between storage times. We conclude that researchers should be aware of the observed changes in the host quality that may have positive or negative effects on the development and behavior of bark beetles under observation.

Key words: mountain pine beetle, *Dendroctonus ponderosae*, *Pinus banksiana*, terpenes, macronutrients, micronutrients, host tree quality.

Résumé : Les études portant sur les scolytes qui s'attaquent aux conifères utilisent généralement des billes prélevées sur des arbres pour évaluer les effets de la qualité des arbres hôtes sur divers aspects de la biologie de l'insecte. Par contre, on n'a pas évalué si la qualité des arbres vivants et celle des billes coupées chez ces arbres est la même. Plus particulièrement, les changements dans la concentration des composés chimiques impliqués dans les mécanismes de défense (tels que les monoterpènes) et des nutriments (tels que l'azote et le carbone) n'ont pas été comparés chez les arbres vivants et les billes coupées provenant de ces arbres. Pour déterminer si la concentration des monoterpènes et des nutriments diffère après la coupe, des tiges de pin gris (*Pinus banksiana* Lamb.) ont été sélectionnées au lac La biche (Alberta) et des échantillons de tissus ont été prélevés dans le phloème. Ensuite, ces arbres ont été récoltés et deux billes par arbre ont été entreposées à 4 °C pendant trois et six mois. Le phloème a été échantillonné chez les arbres vivants et les billes trois et six mois après le début de l'entreposage. Nous avons trouvé que les principaux monoterpènes du pin gris étaient plus abondants dans le phloème des billes que dans celui des arbres vivants. La durée de l'entreposage n'a pas influencé les résultats. De plus, certains nutriments, incluant l'azote, étaient aussi plus abondants dans les billes et variaient selon la durée de l'entreposage. En conclusion, les chercheurs devraient être conscients des changements observés dans la qualité de l'hôte lesquels peuvent avoir des effets positifs ou négatifs sur le développement et le comportement des scolytes sous observation. [Traduit par la Rédaction]

Mots-clés : dendroctone du pin ponderosa, *Dendroctonus ponderosae*, *Pinus banksiana*, terpènes, micronutriments, macronutriments, qualité de l'arbre hôte.

Introduction

Mountain pine beetle (MPB) (*Dendroctonus ponderosae* Hopkins, 1902; Coleoptera: Curculionidae, Scolytinae) has killed millions of hectares of pine trees, mainly lodgepole pine (*Pinus contorta* Douglas ex Loudon), over the past decade in western North America (Safranyik et al. 2010). In Canada, MPB outbreaks have expanded to the novel jack pine (*Pinus banksiana* Lamb.) forests in northern Alberta (Erbilgin et al. 2014). Multiple techniques have been utilized to evaluate the effects of host tree quality on MPB in laboratory experiments, including the use of rearing tubes (e.g., Ishangulyyeva et al. 2016), phloem sandwiches (e.g., Therrien et al. 2015), and bolts (e.g., Erbilgin et al. 2014). Of these, bolts that are cut from trees to a designated length, usually 40–50 cm, are commonly used to determine the impacts of host quality on beetle host preference, pheromone production, brood production, and development because they can be easily transported, stored, and maintained in the laboratory. Despite the wide use of bolts in bark beetle research, it is unknown how host quality changes in bolts after cutting live trees.

Host quality is critical for both adult and immature stages of bark beetles and usually characterized by the concentrations of

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defense chemicals and nutrients. For example, host monoterpenes can act as a precursor for MPB pheromones, as anti-attractants, or as anti-feedants (Erbilgin et al. 2017). Beetle development also depends on the available host phloem nutrients. For example, nitrogen is used by insects for the formation of proteins, nucleic acids, and hormones, and thus, its low content in plant tissues can be a major limiting factor for the growth and reproduction of bark beetles (Goodsman et al. 2012). In addition, metal ions obtained from plant tissues play important roles as cofactors of several enzymes and are involved in the osmoregulation, cold tolerance, and purine metabolism in insects (Dow 2017).

Approaches comparing the quality of beetles' hosts using bolts may not truly account host quality effects on MPB, as this may be substantially altered when trees are cut. To date, how cutting trees affects host quality has not be thoroughly evaluated under laboratory conditions, although such understanding could improve our knowledge about how laboratory approaches used can emulate live tree conditions. Thus, our goal was to determine the changes in the concentrations of monoterpene and macronutrients and micronutrients before and after cutting live jack pine trees. We expect that the results obtained through this study should lead us to a better understanding of consequences of cutting trees on bark beetle biology and ecology.

Methods and materials

Experimental design and sampling

We selected 30 apparently healthy jack pine trees (i.e., no signs of biotic or abiotic stress) in two forest stands in Lac la Biche, Alberta, Canada, in June 2016. Fifteen trees (diameter at breast height (1.3 m) = 27.7 ± 0.84 cm (mean ± standard error)) were selected per stand (55°04′13.8″N, 111°59′48.2″W; 54°55′10.2″N, 111°28′05.6″W). Two phloem samples (3 cm × 3 cm) from the north and south aspect of each tree were taken at 1.3 m height. Samples were kept in liquid nitrogen in the field and then stored at −40 °C in the laboratory. Two bolts (each 35 cm long) were cut from each tree sampled above and below the 1.3 m height (total of 60 bolts). Bolts were sealed with melted wax in both ends, transported to the laboratory, and stored at 4 °C. After 3 months of storage, half the bolts representing the 30 trees were sampled for phloem tissue, using the aforementioned method. The remaining bolts were similarly sampled 6 months after cutting. This sampling scheme yielded 30 phloem samples for each live tree or the 3-month-old and 6-month-old bolts. All samples were stored at −40 °C until chemical analysis. The phloem moisture of the bolts was calculated only after 6 months of storage using the following equation: ((wet weight − dry weight)/wet weight) × 100.

Defense chemical analysis

To extract defense compounds, mainly monoterpenes, the two samples taken from each tree or bolt in the different time periods were pooled and ground in liquid nitrogen. Samples were prepared and analyzed using similar procedures as those described by Erbilgin et al. (2017). Briefly, ground tissue (100 mg) was extracted twice with 0.5 mL of pentane and 0.004% tridecane as an internal standard in 1.5 mL microcentrifuge tubes. Extracts were vortexed for 30 s at 3000 rpm, sonicated for 10 min, centrifuged at 13 000 rpm for 15 min at 4 °C, and placed in a freezer for at least two hr. Extracts were then transferred into gas chromatograph (GC) vials and 1 μL were injected at a split ratio of 10:1 in a coupled GC − mass spectrometer (GC–MS) (7890A-5062C, Agilent Tech., Santa Clara, CA, USA) equipped with an HP Innowax column (inner diameter, 0.25 mm; length, 30 m; product ID: 19091N-233, Agilent Tech.) with a helium carrier gas flow of 1.1 mL·min^{-1}, temperature of 55 °C for 1 min, then 40 °C·min^{-1} to 65 °C (held for 1 min), then 40 °C·min^{-1} to 75 °C (held for 0.5 min), then 7 °C·min^{-1} to 130 °C, and then 20 °C·min^{-1} to 250 °C (held for 0.5 min). Compounds were identified by comparing retention times and mass spectra with the commercial standards and quantified through calibration curves using the standards. Monoterpenes were reported as ng·mg^{-1} of fresh weight tissue.

Table 1. Results from linear mixed model analyses comparing monoterpene and nutrient concentrations of live jack pine (*Pinus banksiana*) trees with stored bolts.

	Wald χ^2 (df = 2)	*P* value
Monoterpenes (ng·mg^{-1} of fresh weight phloem tissue)		
α-Pinene	232.67	<0.001
β-Pinene	123.45	<0.001
Camphene	119.60	<0.001
β-Phellandrene	82.27	<0.001
Limonene	73.14	<0.001
Myrcene	68.63	<0.001
Terpinolene	18.54	0.008
3-Carene	7.11	0.028
Nutrients (percentage in dry weight phloem tissue)		
Nitrogen	128.16	<0.001
Zinc	90.62	<0.001
Calcium	69.73	<0.001
Copper	53.62	<0.001
Magnesium	33.80	<0.001
Potassium	37.88	<0.001
Sulfur	31.68	<0.001
Manganese	23.03	<0.001
Phosphorus	20.31	<0.001
Carbon	4.06	0.262
Iron	0.43	0.804

Note: Bolts were stored at 4 °C for 3 and 6 months after cutting and half of the bolts were sampled at each storage time. *N* = 30 per treatment. df = degrees of freedom.

Nutrient analysis

Ground tissue pooled (40 mg) from each tree and bolt was dried at 40 °C during 24 hr in the oven and transported to Natural Resources Analytical Laboratory at the University of Alberta for macronutrient and micronutrient analyses. Samples were analyzed in a Thermo FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Bremen, Germany). Thirty samples were run twice to corroborate the results. Nutrients are reported in percentage of dry weight.

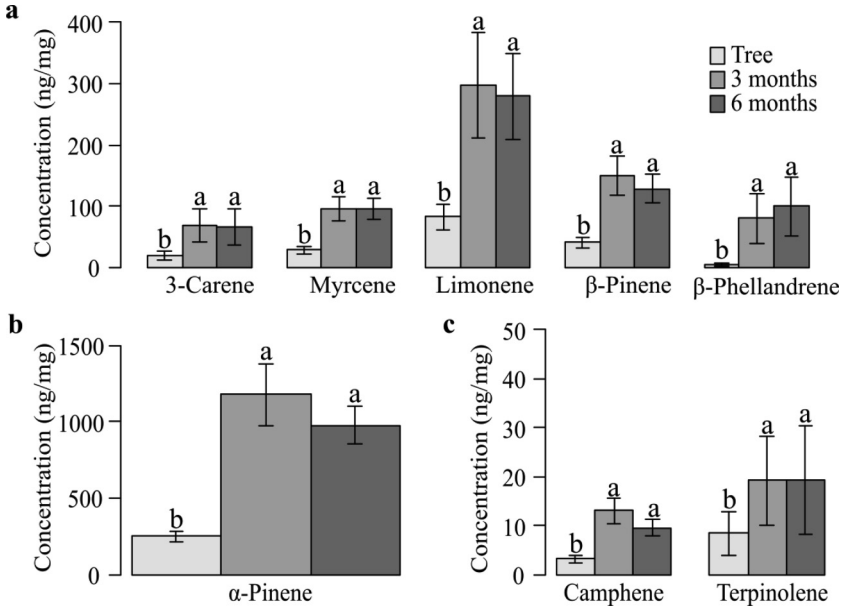
Statistical analyses

To compare live trees and bolts, linear mixed models were performed for each monoterpene and nutrient concentration in each of tissue type (live tree, 3 and 6 month storage) using the function lme from the nlme package in R. We used time as a fixed effect (tree: tissue taken from live trees, and time 3 and 6: tissues taken from bolts after 3 and 6 months of storage, respectively) and trees and sites as random effects. Data were natural log transformed to meet model assumptions of normality and homoscedasticity. Significance of the fixed effect in the model was determined using the Wald chi-square test from the car package in R. Post-hoc Tukey's honest significance difference test was carried out with the general linear hypothesis function (glht) in the package multcomp using an alpha value of 0.05 to indicate significant differences between times. The statistical significance of the *P* values was assessed through the Holm's adjustment method for an experiment-wise error rate of 0.05. Raw data are shown in the figures.

Results and discussions

Both 3-month-old and 6-month-stored bolts had higher concentrations of monoterpenes than live trees (Table 1; Figs. 1*a*–1*c*). Specifi-

Fig. 1. Mean (± standard error) monoterpene concentrations in phloem tissues sampled from live jack pine (*P. banksiana*) trees (tree) and bolts after three (3 months) and six (6 months) months of storage. Bars with different lowercase letters indicate statistical difference on a particular chemical (e.g., 3-Carene) among treatments. *N* = 30 per treatment.

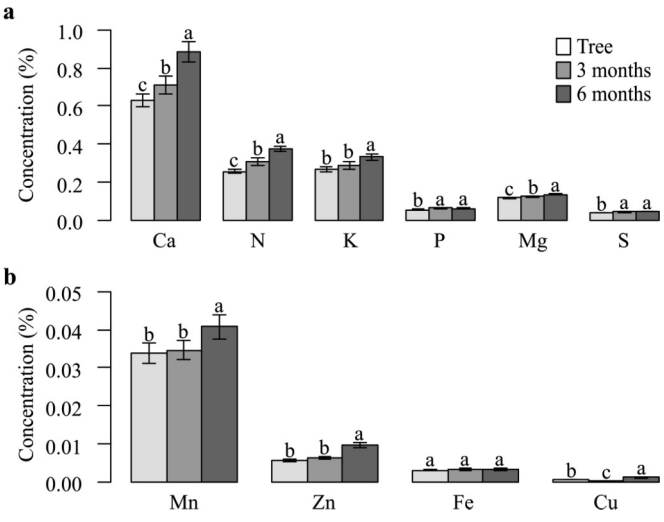


cally, α-pinene (3.73× after 3 months and 2.93× after 6 months) and β-pinene (2.69× after 3 months and 2.17× after 6 months), camphene (3.06× after 3 months and 1.98× after 6 months), limonene (2.63× after 3 months and 2.41× after 6 months), and myrcene (2.63× after 3 months and 2.42× after 6 months) were significantly higher in bolts and were present in almost all trees and bolts sampled. β-Phellandrene, terpinolene, and 3-carene were mostly detected in bolts and were significantly higher in bolts (β-phellandrene 21.9× after 3 months and 27.48× after 6 months; terpinolene 1.28× after 3 months and 1.29× after 6 months; 3-carene 2.57× after 3 months and 2.46× after 6 months) than trees. No statistical differences were found between storage times. We did not conduct any statistical analysis for the remaining monoterpenes, as they were either below detection limit or present in very few samples collected.

The higher concentration of monoterpenes in bolts is partially explained by a progressive loss of water from bolts. Although the reduction of moisture is not quantified in the present study, Redmer et al. (2001) stored red pine (*P. resinosa*) bolts at 10 °C and reported that bolts lost about 20% of phloem water after 3 months of storage and about 50% after 6 months of storage. In the current study, after 6 months of storage, the phloem moisture content in bolts was 64% ± 4.38% (mean ± standard error). Because our quantification methods for monoterpenes were based on the fresh weight of phloem tissues, loss of moisture likely reduced the overall fresh weight of tissues quantified, thus increasing their concentration. However, because monoterpene concentrations did not differ between 3-month and 6-month storage periods and a larger portion of moisture remained in the phloem, we suspect that water loss cannot fully explain the changes observed in the bolts. One likely explanation is that most of the changes observed in the bolts occurred during the first 3 months, and storage beyond this point did not influence monoterpenes. In this study, monoterpenes were quantified based on fresh tissues, as they represent a more realistic proxy to what beetle larvae are facing under wood than under dry tissues.

Higher concentrations of nutrients were generally found in bolts than in trees, and they varied between storage times (Table 1; Figs. 2*a* and 2*b*). The concentrations of calcium (1.13× after 3 months and 1.29× after 6 months), nitrogen (1.21× after 3 months and 1.46× after 6 months), potassium (1.08× after 3 months and 1.24× after 6 months),

Fig. 2. Mean (± standard error) nutrient concentrations in phloem tissue sampled from live jack pine (*P. banksiana*) trees (tree) and bolts after three (3 months) and six (6 months) months of storage. Ca, calcium; N, nitrogen; K, potassium; P, phosphorus; Mg, magnesium; S, sulfur; Mn, manganese; Zn, zinc; Cu, copper; Fe, iron. Bars with different lowercase letters indicate statistical difference on a particular nutrient (e.g., calcium) among treatments. *N* = 30 per storage time.



phosphorus (1.18× after 3 months and 1.15× after 6 months), magnesium (1.05× after 3 months and 1.21× after 6 months), sulfur (1.12× after 3 months and 1.17× after 6 months), manganese (1.03× after 3 months and 1.17× after 6 months), and zinc (1.11× after 3 months and 1.72× after 6 months) were significantly higher in bolts. The concentration of copper was lower after 3 months of storage (0.01×) but higher after 6 months of storage (1.89×).

The carbon and iron concentrations did not vary significantly during the storage times (carbon: 1.02× after 3 months and 0.98× after 6 months; iron 1.13× after 3 months and 1.08× after 6 months).

An increase in nutrient levels in bolts has been reported in other studies (Holub et al. 2002). Although the mechanism is not clear, it is likely that during the storage process, nutrients were redistributed into bolt phloem, leading to increased nutrient concentrations (Boddy and Watkinson 1995). For nitrogen, fixation by bacteria in bolts may also be a potential mechanism during storage (Graham and Cromack 1982). Because we used dried phloem for nutrient analysis, water loss likely did not influence the nutrient concentrations observed in the current study.

The results reported here are relevant for studies determining the potential effect of host pine quality on bark beetles. First, relatively higher concentrations of monoterpenes along with reduced phloem moisture content may alter pheromone production by mature bark beetles and larval development (Erbilgin et al. 2017). Presence of pheromone precursors such as α-pinene may have a positive effect on pheromone production, as there is a positive linear relationship between α-pinene concentrations and *trans*-verbenol production in MPB (Erbilgin et al. 2014). In contrast, presence of toxic compounds such as limonene may inhibit larval development, as limonene is one of the most toxic monoterpenes present in the phloem of MPB host trees (Chiu et al. 2017). Second, changes in nutrient concentrations from live trees to bolts may affect MPB fitness. For example, higher nitrogen concentration in bolts may accelerate larval development because trees tend to be limited in nitrogen (Goodsman et al. 2012). Third, even though we took many steps to minimize moisture loss from our bolts (i.e., waxed both ends of bolts in the field, stored bolts at 4 °C a few hours after cutting), apparently significant changes in monoterpene and nutrient concentrations are unavoidable. Further studies are needed to determine if storage temperatures below 4 °C should further minimize the changes observed in this study and whether the observed changes can also occur in other pine species that are used to rear bark beetles. Nevertheless, researchers should consider the consequences of changes in tree defense chemicals and nutrients on bark beetle biology when they conduct experiments with bolts or other proxy approaches in the laboratory.

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