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Brief Report

Monoterpene Composition of Phloem of Eastern Larch (*Larix laricina* (Du Roi) K. Koch) in the Great Lakes Region: With What Must the Eastern Larch Beetle (*Dendroctonus simplex* LeConte) Contend?

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Abstract: The eastern larch beetle (*Dendroctonus simplex* LeConte) is the only tree-killing bark beetle that colonizes tamarack, or eastern larch, (*Larix laricina* (Du Roi) K. Koch) in the Great Lakes region. Historically, outbreaks have been intermittent and of short duration, frequently following predisposing factors such as drought or defoliation. However, over the past two decades, this insect has been in a perpetual state of outbreak in parts of the U.S. Great Lakes region, a deviation from historic norms. From 2001–2021, the insect impacted 300,000 ha, or 60% of the tamarack forests in Minnesota. This activity has prompted renewed interest in the beetle's chemical ecology, including aspects of host semiochemistry. While foliar chemistry has been well documented in *L. laricina*, characterization of the monoterpene composition of the phloem has been lacking. We collected phloem samples from 56 tamarack trees across 14 locations in Wisconsin and Minnesota and assessed the relative abundances of the major monoterpenes present using gas chromatography-flame ionization detector (GC-FID). Individual terpenoid components identified included α-pinene (39.4%) and Δ-3-carene (30.0%) followed by several other components in small (<8%) amounts. This knowledge provides a basis for future testing of monoterpene synergists or antagonists in pheromone lures targeting eastern larch beetle and/or its natural enemies.

Keywords: α-pinene; Δ-3-carene; host defense; kairomones; semiochemicals

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1. Introduction

The eastern larch beetle, *Dendroctonus simplex* LeConte, is a bark beetle (Coleoptera: Curculionidae: Scolytinae) native to North America, ranging from Alaska to Newfoundland. The southern portion of its range extends to the Great Lakes region. This range is sympatric with the insect's host, tamarack or eastern larch, *Larix laricina* (Du Roi) K. Koch, which is an important component of peatlands and lowland conifer forests [1]. Adult beetles emerge in the spring from hibernal galleries or the duff at the bases of trees. Females are the host-selecting sex and release aggregation pheromones while boring into the tree, attracting conspecifics. Males enter the galleries and mate with the females [2]. Female beetles oviposit into the phloem where eclosion, larval development, and pupation occur [2]. Historically, the eastern larch beetle has colonized trees weakened by wind, flooding, drought, harvesting damage, or large-scale herbivory. Sporadic but short-term outbreaks sometimes spill over to vigorous trees for three to five years [3].

Over the past 20 years, the Great Lakes region has been experiencing a large-scale eastern larch beetle outbreak that represents a duration outside of historic norms. As

of 2021, the eastern larch beetle had affected approximately 60% of all tamarack cover type in the U.S. state of Minnesota, or approximately 300,000 ha [4]. This outbreak has been associated with multiple factors, such as predisposing defoliation by the non-native defoliator larch casebearer (*Coleophora laricella* (Hübner)) and warming temperatures that may be altering the demography of eastern larch beetle, larch casebearer, and the larch casebearer's biological control complex [5–7]. The severity and duration of the current outbreak have prompted renewed interest in the chemical ecology of eastern larch beetle and its host.

One of the interests in elucidating eastern larch beetle chemical ecology is assessing host monoterpene composition [8]. Host terpenoid chemicals are constituitively present within the tree, with additional induced responses elicited when the beetles tunnel into the phloem tissues [9–11]. Terpenoid compounds play important defensive roles against beetle and/or fungal challenge [8,9,12]. These defensive compounds also affect beetle behavior such as host selection and gallery excavation [11,13].

While several studies have helped characterize pheromone production and response of eastern larch beetle, e.g., [14–17], knowledge of the chemical composition of the phloem tissues of *L. laricina* has been lacking especially in the midcontinental range of this tree. The chemical composition of *L. laricina* twig and leaf oil and resin have been characterized in northwestern Canada and New York, but with small sample sizes that limit wider inference [18,19]. Moreover, some bark beetles exhibit geographic differences in responses to terpenes [20–22]. This study aims to characterize the chemical composition of tamarack phloem in the Great Lakes region. Knowledge of potential regional differences in host monoterpene composition could help optimize lure blends to enhance sampling of bark beetles and their natural enemies or attempt tree protection schemes [14–16].

2. Materials and Methods

2.1. Field Collection

Phloem samples were acquired from four trees at each of 14 sites (Table 1), totaling 56 samples. These trees ranged in diameter from approximately 18–30 cm DBH (diameter at breast height, 1.4 m) and did not display signs of insect or pathogen infection. An arc punch (2.54 cm diameter) was hammered into the tree at breast height and phloem was carefully removed with a scalpel. Phloem core samples were placed in 20 mL scintillation vials and kept in a cooler with dry ice before transfer to a −80 °C freezer for 24–48 h. Sampling was conducted 15–16 July 2021.

Table 1. The 14 locations from which phloem cores of *Larix laricina* (Du Roi) K Koch were sampled across Minnesota and Wisconsin on 15–16 July 2021.

Site	State	Site Coordinates (Latitude, Longitude)
1	WI	45.64609, −89.69925
2	WI	46.08356, −89.10286
3	WI	46.06834, −89.05433
4	WI	45.90623, −89.32976
5	WI	46.16631, −90.90850
6	MN	47.03354, −92.56989
7	MN	47.06933, −92.61704
8	MN	47.13486, −92.61704
9	MN	46.99555, −93.13276
10	MN	46.97377, −92.99593
11	MN	47.33794, −94.08489
12	MN	47.23245, −94.63135
13	MN	46.54460, −94.30573
14	MN	44.97318, −93.32852

2.2. Chemical Analysis

Phloem samples were ground in a ceramic mortar and pestle (Coors) after freezing with liquid nitrogen. Then, samples were transferred to a 20 mL beaker. From there, the approximately 1 g sample was covered in hexane (0.3 mL hexane/0.6 g phloem 1:5–1:10 phloem to solvent ratio). The sample was left for 15–20 min to thaw, and the contents were then filtered through a small cotton batting plug.

We focused on identification of nine major monoterpenes identified in previous literature as major compounds in tamarack leaf/twig oil [19]. These monoterpenes from the phloem samples were identified (and further quantified) by GC-FID via retention time comparison with a synthetic mixture solution of the authentic monoterpene compounds (1 mg/mL each in hexane) under the same GC-FID conditions (see below for details). Compounds selected included α-pinene, β-pinene, Δ-3-carene, camphene, sabinene, myrcene, terpinolene, D-limonene, and β-phellandrene. A combined mixture was made with all nine analytes in hexane (approximately 1 mg/mL concentration for each); each analyte was sourced from Berjé Inc., Cartaret, NJ, USA except β-phellandrene (Synergy Semiochemicals Corporation, Delta, BC, Canada). GC inlet and oven parameters were tuned to get the best separation of the nine peaks. Samples were analyzed on an Agilent 7890A GC-FID with autosampler. The column used was an Agilent DB-1 column (length: 30 m, i.d.: 0.32 mm, column film thickness: 1.00 µm, nonpolar) with a hydrogen carrier gas. Operating conditions were as follows: Injection size per sample was 2 µL with a split ratio of 20:1. The inlet temperature was 250 °C and FID temperature was 325 °C. The column started at 40 °C and was held at this temperature for 1 min. Temperature was ramped up to 90 °C at a rate of 20 °C/min and held for 5 min. From there, the temperature was ramped at a rate of 25 °C/min up to 325 °C and held for 3 min before finishing the run.

First, individual standards for each analyte in hexane (approximately 1 mg/mL each) were run using the optimized GC parameters in order to identify the retention time of each analyte under the conditions outlined. Then after determining the approximate concentration of analytes in the phloem samples (0.01–0.1 mg/mL), a combined standard solution, made with known concentrations of each analyte in hexane ranging from 0.04–0.17 mg/mL on the same order of magnitude as the samples, was run under the same conditions. These data constituted a single level calibration table that was used to quantitate the 9-monoterpenes (% wt/v) in *Agilent OpenLab Magic GC* software (Version A.01.02, Santa Clara, CA, USA). No internal standard within samples was used, but examination revealed consistency between samples in the FID response of each peak. D-limonene and β-phellandrene were reported as a combined peak due to difficulty in separation of peaks with the GC column used.

2.3. Statistical Analysis

We analyze and report proportions of each monoterpene component from the phloem samples instead of amount per gram dry phloem, as phloem dry weight information was not retained due to a data curation error. To calculate mean proportions of each monoterpene in the samples, we fit a cell means model of the component proportion (response variable, asin(sqrt(y)) transformed) as a function of compound type (a categorical variable; each of nine monoterpenes and an 'unidentified' category) in a mixed-effects ANOVA carried out in R [23] (R Core Team 2020, Version 3.5.0, Vienna, Austria). We fit the term for compound type as a fixed effect and terms for tree nested within sampling site as random effects to contend with geographic variation. This analysis yielded the mean proportion of each compound with an associated standard error. The standard error was multiplied by $Z = \pm 1.96$ and then added/subtracted from the mean estimate to obtain 95% confidence intervals about each mean once back-transformed.

3. Results

Our results show that α-pinene is the highest concentration monoterpene in phloem of *L. laricina* followed by Δ-3-carene, although there was a high amount of variability across the 56 samples (Figure 1). The percentage of α-pinene in the terpenoid blend within the

phloem samples ranged from 12.8% to 75.5%, for example, and the percentage of Δ-3-carene likewise varied from 1.5% to 67.4% (Figure 1). Despite this variability, the median percentages were almost identical at 34.5% for α-pinene and 33.8% for Δ-3-carene. Overall, the estimate of the population mean was approximately 10% higher for α-pinene than Δ-3-carene, at 39.4% vs. 30.0%, respectively (Table 2). All other identifiable compounds, such as camphene, sabinene, β-pinene, myrcene, and limonene + β-phellandrene, were present in low amounts collectively under 8% (Table 2). Less than 10% of compounds were left unidentified.

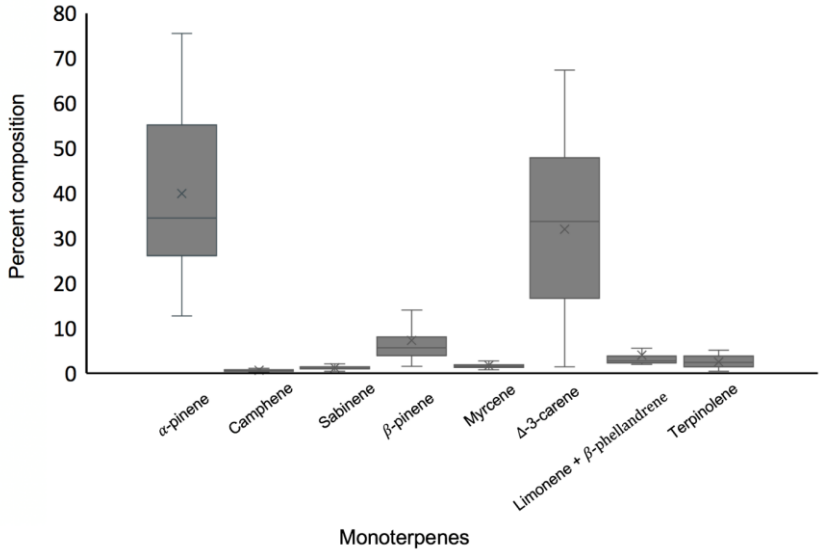

Figure 1. Percent composition of monoterpenes within 56 *Larix laricina* (Du Roi) K Koch trees throughout Minnesota and Wisconsin collected from 15–16 July 2021. The x on each box represents the mean; the midlines represent the median; and the whiskers represent the interquartile range.

Table 2. Population estimates of mean percent compositions of various monoterpenes within 56 *Larix laricina* (Du Roi) K Koch sampled from Minnesota and Wisconsin, 15–16 July 2021, with 95% CI.

Compound	Mean Composition (%)	95% CI
α-pinene	39.4	36.7, 42.1
Camphene	0.7	0.3, 1.2
Sabinene	1.2	0.7, 1.9
β-pinene	6.5	5.2, 8.0
Myrcene	1.7	1.1, 2.5
Δ-3-carene	30.0	27.5, 32.6
D-limonene + β-phellandrene	3.6	2.7, 4.8
Terpinolene	2.4	1.6, 3.3
Unidentified Compounds	10.0	8.4, 11.8

4. Discussion and Conclusions

Our finding that two monoterpenes comprise more than two thirds of the monoterpene complex in phloem samples from *L. laricina* in Minnesota and Wisconsin are similar to previous results across the range of tamarack [18,19]. Yet, the proportions of these compounds are highly variable between studies and there is geographic variation between which compounds make up this two-thirds majority. In New York, for example, Stairs (1967) found that α-pinene dominated the terpene blend (58.9%) with Δ-3-carene at only 9.1%, thus comprising 68.0% of the samples. Our study found a more even mixture, on average, between the two chemicals with 39.4% α-pinene and 30.0% Δ-3-carene (Table 2, Figure 1). Von Rudloff (1987) found a similarly even mixture of α-pinene (23.0%) and Δ-3-carene (22.2%) in twigs of trees across the Yukon Territory, Alberta, and Saskatchewan

regions of Canada, although his overall percentages were lower than those we noted. Our finding of β-pinene at only 6.5% of the terpene blend of phloem, on average, was four times lower than noted by Stairs (1967) and half that noted by Von Rudloff (1987). Collectively, results across these studies provide evidence of high variation in constitutive monoterpenes with which beetles must contend across the range of *L. laricina* in North America.

Within the Great Lakes Region, the phloem chemistry of *L. laricina* appears to be similar to that found in the foliage, although important variation exists. Ward et al. (2019), for example, found similarly high levels of α-pinene and Δ-3-carene followed by lower concentrations of β-pinene in needles of *L. laricina*, mirroring our findings in phloem monoterpenes. Additionally, Ward et al. (2019) found trace amounts of limonene, myrcene, and terpinolene within the foliage, with sabinene as another high concentration component (0–17 mg/g of foliage) [24]. Work done in Wisconsin, however, found that concentrations of α-pinene were approximately double that of Δ-3-carene in short shoot foliage of *L. laricina* and between three and four times higher in the long shoot foliage (mean concentrations between 0.67–2.61 mg/g; [25]). This study also found trace amounts of sabinene, β-phellandrene, limonene with higher percent composition of camphene (4%–8.5%) and myrcene (3.5%–6%) [25]. The composition of these monoterpenes within the host can vary seasonally as well as in response to resource availability [25].

The monoterpenes identified are volatile compounds that have a range of bioactivity [8,26–29]. Host monoterpenes play a role in antifungal defense, inhibiting pathogenic fungal growth [11], as well as repelling or killing bark beetles through resinosis [29]. Monoterpenes are frequently precursors to bark beetle pheromones [30], and some host monoterpenes, such as α-pinene, β-pinene, camphene and Δ-3-carene can also be synergists when combined with beetle pheromones [14–16,21,31–34] Some monoterpenes, such as limonene, β-phellandrene, and myrcene are antagonists to the eastern larch beetle pheromone, frontalin [33]. This antagonistic response is not surprising as limonene and myrcene are highly toxic to bark beetles, including the eastern larch beetle [32–36]. Compounds such as sabinene, terpinolene, camphene, and β-phellandrene, present here in trace amounts, may be antennally active in bark beetles such as *Ips typographus* L. [37], but we do not fully understand their ecological roles at present.

Our present work is investigating how the addition of select terpenes identified in this study changes the composition of insects such as natural enemies that are attracted to pheromones of the eastern larch beetle, as natural enemies can exhibit different responses to semiochemical cues than their prey. Clerid beetles, for example, use chemical cues such as pheromones and plant monoterpenes to locate bark beetle prey [38–40], but their responses to individual compounds may differ from that of the bark beetles. For example, while the combination of α-pinene with Pheroprax® elicits a synergistic response from *Ips typographus* L., *Thanasimus femoralis* (*Zetterstedt*) displays an antagonistic response when α-pinene is added to the pheromone [41]. Similarly, *Thanasimus dubius* (Fabricius) displays different attractancy to the chirality of its prey's pheromone such as *Ips pini* (Say) [42], and *Dendroctonus frontalis* Zimermann [43]. Selective terpenoid blends with attractive pheromone baits may help reduce natural enemy bycatch in pheromone monitoring schemes [38–44].

Within this study, we utilized a grinding technique with a mortar, pestle, and liquid nitrogen for sample maceration and homogenization prior to compound extraction with solvents [45,46]. Other methods of sample homogenization involving sonication and centrifugation may improve sample recovery and further refine quantification of compounds, especially of trace chemicals. Future research should also focus on elucidating the composition of other important chemicals such as sesquiterpenes and phenols, which was beyond the scope of this initial study.

Understanding the phloem monoterpene composition of *L. laricina* can inform the development of better monitoring lures or anti-aggregation blends to protect high-value trees. Work is currently ongoing assessing the attractancy of these compounds in field conditions within the Great Lakes region. Future work should examine variation in terpenoid profiles of *L. laricina* over a wider continental area. Populations of *Dendroctonus*

valens LeConte from the central Sierra Nevada region of the USA and Shanxi Province, China show geographic differences in attractancy to host monoterpenes, for example [20], and populations of several *Dendroctonus* spp. within North America such as *D. brevicomis* LeConte, *D. rufipennis* Kirby, and *D. frontalis* Zimmermann exhibit geographic variation in pheromone production and response [47–49]. As the eastern larch beetle has a large range spanning both sides of the North American continent [1–3], it is plausible that there could be variation in eastern larch beetle pheromone production and response to host compounds as well. Future work is necessary to explore concentrations causing attraction or repulsion in concert with pheromone components of *D. simplex*.

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Data Availability Statement: Data will be deposited into the University of Minnesota digital repository (DRUM) upon manuscript acceptance.

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