

Terpene Chemistry of Eastern Hemlocks Resistant to Hemlock Woolly Adelgid

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Abstract Recent studies have identified a small number of individual eastern hemlock trees that demonstrate relative resistance to the introduced sap-feeding insect, the hemlock woolly adelgid. Using gas chromatography, we compared concentrations of twenty-two terpenoids in susceptible and relatively resistant trees, both in the forest and in propagated cuttings in a common-garden setting. Terpenoid concentrations were higher in twig tissue of resistant *versus* susceptible trees, across six sampling dates and at both sites. These changes may be particularly important because the hemlock woolly adelgid feeds on twig tissue. Because the common-garden cuttings were free of herbivores, the higher terpenoid concentrations are interpreted as a constitutive defense. Increased levels of monoterpenes and sesquiterpenes imply an

overall increase in the input of carbon precursors to both terpenoid synthesis pathways.

Keywords *Tsuga canadensis* · *Adelges tsugae* · Terpene chemistry · Host resistance · Invasive species · Growth-differentiation balance

Introduction

The discovery, development, or maintenance of herbivore-resistant genetic lineages can play a key role in the conservation of plant species attacked by introduced pests (Bentz et al. 2002; Burdon 2010; Ingwell and Preisser 2011; Mattson 1986; Reis et al. 2004; Schoettle et al. 2012). Here, we addressed factors that may be linked to resistance to the hemlock woolly adelgid (*Adelges tsugae* Annand, abbrev. HWA) in rare individuals of eastern hemlock (*Tsuga canadensis*). *Adelges tsugae* is a sessile, piercing-sucking insect introduced to the eastern United States from Japan (Havill et al. 2006; McClure 1991). While HWA causes limited damage to *Tsuga* hosts in its native range of East Asia and the American Pacific Northwest (Lagalante and Montgomery 2003; Montgomery et al. 2009; Oten et al. 2012), HWA poses a serious threat to the host species, eastern hemlock (*T. canadensis*) and Carolina hemlock (*T. caroliniana*), in its introduced range (McClure 1991; Orwig and Foster 1998; Orwig et al. 2002). Eastern and Carolina hemlock also are colonized by a second introduced piercing-sucking insect, the elongate hemlock scale (EHS, *Fiorinia externa*), which contributes to hemlock decline, but may deter later co-infestation by HWA (Gomez et al. 2012).

Hemlock woody adelgid feeds on nutrients in the xylem ray parenchyma cells, and prefers the most recent flush of growth (McClure 1989; Young et al. 1995). Infestation by HWA has been shown to alter xylem growth and water

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relations in eastern hemlock (Domec et al. 2013; Gonda-King et al. 2012). This effect is associated with a hypersensitive response (Radville et al. 2011), reduced photosynthetic productivity (Domec et al. 2013), and mobilization of nitrogen to new-growth tissues (Gomez et al. 2012). In addition, infested hemlocks display a local increase in aromatic compounds (Pezet et al. 2013), and a simultaneous increase in monoterpene volatilization and decrease in stored mono- and sesquiterpenes (Broeckling and Salom 2003; Pezet et al. 2013). A 10- to 100-fold increase in methyl salicylate, a molecule involved in the salicylic acid signaling pathway for systemic acquired resistance (Vlot et al. 2009), also has been detected in adelgid-infested eastern hemlocks (Pezet et al. 2013). In other systems, some of these responses have been linked to plant defense against herbivores or pathogens (Wu and Baldwin 2010); in eastern and Carolina hemlock, however, infestation by HWA leads to tree decline and eventual mortality (McClure 1991; Paradis 2011).

Resistance to HWA has been observed in rare individuals of eastern hemlock found growing vigorously in otherwise adelgid-devastated hemlock stands (Caswell et al. 2008; Ingwell and Preisser 2011). Heritable resistance was assessed by propagating cuttings from these trees and control (*i.e.*, HWA-susceptible) eastern hemlocks. Once the cuttings were established, both control and resistant cuttings were inoculated with HWA, and adelgid settlement and survival was assessed. Adult HWA densities were significantly lower on the resistant cuttings than on the susceptible cuttings (Ingwell and Preisser 2011), supporting the hypothesis that some rare eastern hemlock individuals possess a degree of HWA resistance, and implying that this resistance is manifest as antibiosis.

One previous study (Ingwell et al. 2009) examined eastern hemlocks that were tentatively identified as HWA-resistant. Although potassium levels were higher in HWA-resistant *vs.* HWA-susceptible eastern hemlocks, there were no other differences in nutritional content (Ingwell et al. 2009). Various studies have examined traits that correlate with HWA susceptibility both within and among *Tsuga* species. In Carolina hemlock, lower levels of the lipid hexacosanol may be associated with decreased HWA susceptibility (Kaur 2009). Across *Tsuga* species, thicker epicuticular wax at the point of HWA stylet insertion (Oten et al. 2012), higher levels of the terpenes α -pinene, α -humulene, β -caryophyllene, and germacrene D, and lower levels of the terpene isobornyl acetate (Lagalante and Montgomery 2003), are associated with decreased HWA susceptibility. A comparable degree

of intraspecific variation in terpene profiles has been observed among ornamental varieties of eastern hemlock (Lagalante et al. 2007), suggesting that natural populations of eastern hemlock also may vary in their terpene profiles.

We explored one potential mechanism of HWA resistance in eastern hemlock by conducting an across-season study of terpene profiles in the identified resistant parent trees and their clonal sapling offspring, *vs.* those of mature and sapling HWA-susceptible eastern hemlocks. Because terpenes act as toxins and semiochemicals in the complex oleoresin that serves as the primary defense of conifers against herbivory, they are likely candidates for allelochemical resistance to HWA. Phenolics, also key in conifer defense chemistry, were not addressed in this study as they appear at very low concentrations when measured by the following method of solvent extraction, filtration, and gas chromatography. Lagalante and Montgomery (2003) suggested that different terpenoids, acting either individually or in combination, may participate directly in plant resistance by serving as HWA feeding stimulants or deterrents. Alternatively, other processes in the resistant trees may influence monoterpene and sesquiterpene concentrations, so that our observed terpene chemistry would provide indirect evidence about the resistance mechanism. Our study intended to provide a thorough profile of monoterpenes and sesquiterpenes present in each season and detect both univariate and multivariate correlations to resistance status.

Methods and Materials

Resistant and control mature trees in a New Jersey forest (NJ), and resistant and control sapling trees (*i.e.*, rooted cuttings) in a common garden at the University of Rhode Island (URI), were sampled at six intervals from May 2012 to June 2013. Solvent extraction and gas chromatography (GC) were used to measure the concentration of each identified terpene in each tree. Terpene profiles were analyzed for concentration differences in single terpenes, as well as for multivariate differences across all terpenes present.

Study Site 1: New Jersey “Bulletproof Stand.” Previous research (Ingwell and Preisser 2011) identified eight putatively HWA-resistant eastern hemlocks growing on state-owned land adjacent to the Delaware Water Gap National Recreation Area in northern New Jersey, USA, of which trees #1–5 were tested for resistance by bioassay of clonal saplings (Ingwell and Preisser 2011). Trees #1–5 were sampled at all collection

Table 1 Collection dates and trees sampled at (a) the New Jersey forest site (mature trees) and (b) the University of Rhode Island common garden site (sapling trees)

(a) New Jersey forest site		
Collection Date	Resistant trees	Control trees
May 2012	#1–5	5 total; three locations 1–5 km away
June 2012	#1–5	6 total; three locations 1–5 km away
October 2012	#1–7	21 total; 4 within the resistant stand, 5 on the slope, 12 at two locations 5 km away
December 2012	#1–7	16 total; 3 within the resistant stand, 6 on the slope, 7 at 5 km away
April 2013	#1–7	17 total; 3 within the resistant stand, 7 on the slope, 7 at 5 km away
June 2013	#1–7	15 total; 3 within the resistant stand, 6 on the slope, 6 at 5 km away, 8 at 6.5 km away, 7 at 1 km away
(b) University of Rhode Island common garden site		
Collection Date	Resistant trees	Control trees
July 2012	5 clones each for resistant #1–5	10 Massachusetts saplings, 9 Pennsylvania saplings
September 2012	5 clones each for resistant #1–5	10 Massachusetts saplings, 9 Pennsylvania saplings
December 2012	5 clones each for resistant #1–5	10 Massachusetts saplings, 9 Pennsylvania saplings

dates, and trees #6 and #7 were sampled beginning in October 2012 (Table 1). All of the sampled individuals are mature trees growing within a 0.25 km radius in a hemlock, white pine, and mixed hardwood forest.

Control trees were selected within a 5 km radius of the resistant trees, to control for microclimate and soil conditions, as well as genetic variation expected over longer distances. Trees in moderate to good current health, as observed by presence of current season growth, were selected to control for effects of tree health decline on terpene chemistry. To ensure that the trees were in fact HWA-susceptible, we used only trees that were infested with HWA or had evidence of needle loss due to previous infestations. We initially used five control trees for balanced replication with the five resistant trees we initially sampled. Beginning in October 2012, 15–21 control trees were used to improve statistical power. Different control trees were used across collection dates (Table 1).

Study Site 2: University of Rhode Island Common Garden - Sapling clones of resistant trees #1–5 were established at the University of Rhode Island by propagating cuttings from the mature trees (Caswell et al. 2008; Ingwell and Preisser 2011). Cuttings of 8 cm of terminal growth were collected from the parent trees in January 2007 and rooted by using a treatment of Dip-N-Grow plant hormone solution (Griffin Greenhouse Supplies, Tewksbury, MA, USA). Saplings were established outdoors in three planting boxes, each 3.8 m² and 30 cm deep, filled with a 1:1 mix of soil and compost, supplemented with soil from an established hemlock stand. Planting boxes were exposed to full sun and sheltered from wind by proximity to a low building on the south and east sides. Five saplings were successfully

established from each of five parent trees, yielding 25 resistant saplings in total (Table 1). All saplings were free of herbivores.

For control trees, 19 genetically individual saplings were established adjacent in the planting boxes, with equal sun exposure and no herbivores (Table 1). Ten saplings were collected from the Quabbin forest (New Salem, MA, USA) in 2009. An additional nine saplings were purchased from a Michigan nursery (Van Pine's Nursery, West Olive, MI, USA), originally grown from seed collected in Indiana County, Pennsylvania, in autumn 2009.

Extraction of Terpenes from Plant Material Samples of hemlock tissue for chemical analysis were collected at seasonal intervals from May 2012 to June 2013 at both sites (Table 1). Each month's collection at a site was performed on a single day in the afternoon daylight hours. Each sample was collected by cutting the terminal flush of growth (current-year growth) from 10–15 tips scattered over 2–4 mature branches or circularly around one sapling tree, totaling approximately 75 cm of tissue. Samples were promptly flash-frozen in liquid nitrogen and transported on dry ice until storage at –80 °C in the laboratory.

From each sample, a total of 18 cm of growth was selected at random for extraction. HWA on the sample were counted (HWA cm⁻¹), and EHS was rated categorically for density (0=no EHS cm⁻¹, 1=0–1 EHS cm⁻¹, 2=1–10 EHS cm⁻¹, 3=11–100 EHS cm⁻¹). Needles were separated from twigs with tweezers. Approximately 1 ml volume of needles, or ca. 30 needles, were selected at random and placed in a pre-weighed vial. Twigs were ground under liquid nitrogen and placed in a second pre-weighed vial. Tissue dry weight was determined following extractions and 2–6 wk in a 75 °C drying oven.

One milliliter of methyl tert-butyl ether (MTBE) was applied to each vial as an extraction solvent. Tissue was extracted for 16–19 h with continuous agitation. Extracts were treated with 0.3 ml of 0.1 M aqueous ammonium carbonate, filtered on silica gel, activated carbon, and magnesium sulfate (3:1:2 ratio), and eluted with 0.5 ml hexanes. Filtered eluates were stored at $-20\text{ }^{\circ}\text{C}$ in glass vials capped with PTFE/silicone septa.

Quantification of Terpenoid Compounds Terpene compounds in samples were quantified by gas chromatography with flame ion detection (Hewlett-Packard Agilent 6890, running Agilent ChemStation software). Separations were performed on an Agilent HP-5 capillary column, non-polar with crosslinked 5 % phenyl / 95 % methyl siloxane, 0.25 μm film thickness, 0.32 mm diam, 30 m length. The column was trimmed by 8 cm during previous use. The helium carrier gas was in constant flow mode at 2.2 ml min^{-1} and average velocity 36 cm sec^{-1} ; sample was injected with split ratio of 3:1, split flow of 6.5 ml min^{-1} , and total flow of 11.1 ml min^{-1} . The injection volume was 1 μl at inlet temperature of 250 $^{\circ}\text{C}$. The GC oven temperature was programmed to start at 60 $^{\circ}\text{C}$ and rise to 158 $^{\circ}\text{C}$ with holds at 64 $^{\circ}\text{C}$, 100 $^{\circ}\text{C}$, and 126 $^{\circ}\text{C}$ to improve separation of compounds, followed by a high-temperature bake out at 200 $^{\circ}\text{C}$ to maintain instrument cleanliness (Table 2). The flame-ion detector was set at 300 $^{\circ}\text{C}$, with hydrogen flow at 30 ml min^{-1} , air flow at 300 ml min^{-1} , and nitrogen makeup flow at 25 ml min^{-1} . The detector began data collection after 3 min of solvent cut-time.

Raw quantity (pA sec) was calculated for each terpene by software integration of peak area on the chromatogram (Hewlett-Packard ChemWare). Concentration ($\mu\text{g ml}^{-1}$) was calculated using experimentally-determined calibration curves. Calibration curves were determined individually for those compounds that were available as analytical standards (Sigma-Aldrich, Saint Louis, MO, USA): α -pinene, camphene, sabinene, β -pinene, myrcene, α -phellandrene, *p*-cymene, limonene, eucalyptol, camphor, piperitone, bornyl acetate, β -caryophyllene, and α -humulene. Averaged

calibration curves of structurally similar terpenoids were used when no commercial standard was available: tricyclene, γ -muurolene, germacrene D, γ -cadinene, δ -cadinene, and unknown compounds A and B. Tissue concentration ($\mu\text{g g}^{-1}$ dry weight) of each terpene was calculated by dividing terpenoid concentration by the sample's tissue dry weight.

Compounds were identified based on previous work (Pezet et al. 2013), retention time comparison to analytical standards on the GC-FID, and comparison to chromatograms from gas chromatography–mass spectrometry on select samples (GCMS; Shimadzu GC-2010 and GCMS-QP2010 Plus with an HP-5 column). References for mass spectrometry included a software library (Stein 2005), published reference (Adams 2009), and analytical standards (Sigma-Aldrich, Saint Louis, MO, USA).

While previous research has described monoterpenes, sesquiterpenes, small phenolics, and green leaf volatiles in eastern hemlock (Lagalante and Montgomery 2003; Pezet et al. 2013), the present method captured only mono- and sesquiterpenes and their derivatives. Using analytical standards, we determined that our filtration step almost completely removed aromatic compounds, including benzyl alcohol and methyl salicylate. While quantification of these compounds was desirable, a consistent and clean filtration method was necessary for maintenance of the GC instruments over numerous samples.

Statistical Analyses All statistical analyses were performed in R (version 2.15.2, R Development Core Team 2012). Current-year needle and twig tissue were analyzed separately, and each site was analyzed separately. Prior to analysis, outliers were identified and removed. Univariate outliers were defined as being over four standard deviations away from the mean in any one compound, and multivariate outliers were over 10x further from the centroid than 90 % of the observations, based on Mahalanobis distance (McCune et al. 2002). Compounds were removed from the analysis as insufficiently sampled if they were undetected in more than 20 % of samples or if the raw quantity median was 10 pA sec or less.

The resulting datasets contained 22 compounds, of which 10 were present in twigs (Table 3a) and 21 present in needles (Table 3b). Two more variables were added, “total monoterpenes” and “total sesquiterpenes,” representing the sum of concentrations of the fourteen monoterpenes and six sesquiterpenes in each sample, respectively. A logarithmic transformation of ($\log_e(x+1)$) was applied to all concentration data and all HWA density data to improve normality.

Differences between resistant and control trees in concentrations of individual terpenoid compounds were identified using repeated measures ANOVA, followed

Table 2 Programmed heat ramp for GC-FID and GCMS ovens

Time (min.)	Rate ($^{\circ}\text{C min}^{-1}$)	Target ($^{\circ}\text{C}$)	Hold (min.)
–	–	60	0
0	2	64	3
5	2	68	0
7	20	100	3
11.6	3	126	5
25.3	3	158	0
35.9	80	200	5
40.9	–	–	–

by ANOVA of each month separately to identify seasonal patterns of resistance-correlated terpene chemistry. Multivariate differences in terpene profile were identified using MANOVA within each month. To test whether insect densities of HWA and elongate hemlock scale (EHS) confounded these results, HWA density and EHS categorical density rating were included as covariates in MANCOVA.

To further clarify the degree of confounding among explanatory variables, partitioning of variance was used to produce Venn diagrams displaying the percent of total variance explained uniquely by resistance status, HWA density, and EHS categorical density; the percent of total variance jointly explained by two or all three of the explanatory variables; and the percent of total variance not explained by the explanatory variables. Prior to partitioning of variance analysis, high collinearity among some terpenes was treated by removing terpenes from analysis if they were correlated with another terpene at Pearson's $r > 0.70$. Selection of which correlated terpene to remove was based on AIC value and previous ANOVA results. Twelve compounds were retained for partitioning of variance in needle samples and seven in twig samples.

Results

Single-Terpene Differences Between Resistant and Susceptible Trees Twigs from resistant trees tended to contain higher terpenoid concentrations, with the statistical significance of this trend differing by collection month and site (Table 3a). In the URI September and December twig collections, 9 of 10 and 10 of 10 compounds, respectively, were found at significantly higher concentrations (ranging from 1.1–4.2-fold) in resistant trees vs. control trees (ANOVA, $P < 0.05$, Table 3a). Similarly, in the NJ December twig collection, 5 of 10 compounds had significantly higher concentrations (1.5–3.5-fold) in resistant trees (ANOVA, $P < 0.05$, Table 3a).

Monoterpenes and sesquiterpenes in twigs appear equally likely to appear at high concentrations in resistant trees (Fig. 1a, b). The twig collections noted above, URI September, URI December, and NJ December, show that the total concentration of monoterpenes and total concentration of sesquiterpenes were significantly higher (1.6–3.1-fold, respectively) in resistant vs. control trees (ANOVA, $P < 0.05$, Table 3a). In other twig collections, total monoterpenes and total sesquiterpenes are not significantly different between resistant and control trees (ANOVA, $P > 0.10$), although there is a consistent trend towards higher concentrations in resistant trees (Table 3a).

In needles, no consistent trend could be identified in individual or grouped terpenoid compounds. Although monoterpenes tended to appear at higher concentrations in resistant trees (Table 3b), neither total monoterpenes nor total sesquiterpenes differed significantly between resistant and control trees (ANOVA, $P > 0.10$, Fig. 1c, d). In the URI September needles collection, a trend of lower terpenoid concentrations in needles of resistant trees appeared, with 4 of 21 compounds having a significantly lower mean in resistant trees than in control trees (ANOVA, $P < 0.05$, Table 3b). However, the trend was not repeated in other months and sites.

Repeated measures ANOVA analysis confirmed the connection between terpene concentration and resistance status. In twigs, total sesquiterpene concentration varied significantly with resistance at both sites, and total monoterpene concentration did so at the New Jersey forest site only (Table 4a). In twigs, 6 of 10 compounds varied significantly with resistance at both sites (Table 4a). In needles, results for total concentrations were inconsistent between sites. In needles, 6 of 21 compounds varied significantly with resistance at both sites (Table 4b). Terpene concentrations also varied significantly with month, especially in needles (Table 4b).

Multivariate Terpene Profiles with Resistance Status, HWA Density, and EHS Density Resistance status explained a significant amount of variance in twig terpenoid concentrations from URI September and December collections (MANOVA, $P < 0.001$ in both cases). When HWA and EHS densities were included as cofactors in the analysis, resistance status remained a significant explanatory variable in these collections. In collections of twigs and needles from other months, resistance status explained only a significant part of variance of terpenoid concentrations when HWA and EHS densities were included as cofactors; we believe this is due to interactions between HWA, EHS, and resistance status. Notably, in most months, forest site resistant trees have zero or very low density infestations of HWA and EHS, while forest site control trees have a range of infestation densities; this creates a statistical interaction among HWA, EHS, and resistance status.

The partitioning of variance analysis separately displayed the unique and confounded explanatory power of these three variables in each collection (Online Supplement). Resistance uniquely explained between 1 % to 16 % of the total variance in each month's collection, with HWA uniquely explaining 2 % to 44 % and EHS uniquely explaining 0.5 % to 34 % of the total variance. Confounded variance was greatest between resistance

and HWA, ranging from 0 % to 23 % of the total variance in each month's collection. Interactions, displayed as negative percentages, were observed between resistance and HWA in 6 of 15 collections, and between resistance and EHS in 7 of 15 collections. Residual, unexplained variance was greater than 50 % of total variance in all collections except needles of NJ June 2012. Results of partitioning of variance were not notably different between needle and twig collections.

Discussion

Higher terpene levels in the twigs of HWA-resistant eastern hemlocks provide insight into possible mechanisms for observed resistance to adelgid infestation, although no evidence exists yet that the terpenes are the cause of resistance. The trend of 1.1–4-fold higher terpenoid concentrations in twigs of resistant trees, across all terpenoids and all seasons (Table 4a), suggests that the resistance mechanism does not rely on any

changes or up-regulation within the separate biosynthesis pathways for monoterpenoids or sesquiterpenoids (Bernard-Dagan et al. 1982) or among individual terpene synthases (Franceschi et al. 2005), but rather in the availability of their shared precursors, dimethylallyldiphosphate (DMADP) and isopentenylidiphosphate (IDP), or total availability of carbon. Although lack of herbivory on the resistant trees could cause greater carbon availability, the fact that the herbivore-free saplings in the URI common garden display an even stronger resistant-control difference suggests that herbivory is not the cause of the observed difference. The observation of increased terpenoid concentrations in twigs but not in needles of resistant hemlock is interesting because HWA feeds on nutrients in storage and transportation cells in the twig, the xylem ray parenchyma (Franceschi et al. 2005; Young et al. 1995). Previous studies have focused on terpene chemistry and nutrient content of the needles; following Pezet et al. (2013), this study included the twigs as HWA's direct feeding site.

There are two possible and non-mutually exclusive explanations for the higher terpene concentrations in the

Table 3 Relative terpenoid concentration^{a,b} in (a) twigs and (b) needles

(a) Twigs: ratios for Resistant trees to Control trees

TWIGS	NJ ^c June 2012	NJ ^c Oct 2012	NJ ^c Dec 2012	NJ ^c Apr il 2013	NJ ^c June 2013	URI ^d Sept 2012	URI ^d Dec 2012
Monoterpenes							
Tricyclene	0.75 °	1.28	2.00	1.19	1.92	1.70*	1.09 *
α-Pinene	1.06	1.20	1.49 *	1.49	1.07	1.92 *	1.49 *
Camphene	0.78	1.45	1.94	1.46	1.58 °	2.80 *	2.26 *
β-Pinene	1.14	1.41 °	1.67 °	2.40	1.18	2.55 *	2.24 *
Myrcene	0.96	1.16	2.18 *	2.28	1.13	3.79 *	1.95 *
Limonene	1.12	1.49 *	1.25 °	1.28 *	1.15 °	1.65 *	1.23
Bornyl Acetate	0.77 °	1.07	1.04	0.82 °	1.51 °	2.04 *	1.84 *
Sesquiterpenes							
β-Caryophyllene	0.96	2.00	3.50 *	1.86	0.97	2.20 *	2.63 *
α-Humulene	0.93	1.83 °	2.63 *	1.65	0.98	2.08 *	2.34 *
Germacrene D	n/a	3.18 °	3.22 *	1.93	1.06	1.65 *	4.18 *
Totals							
Total Monoterpenes	0.94	1.22	1.57 *	1.49	1.13	2.25 *	1.59 *
Total Sesquiterpenes	0.94	2.45 °	3.11 *	1.82	1.02	1.98 *	2.91 *

Table 3 (continued)

(b) Needles: ratios for Resistant trees to Control trees

NEEDLES	NJ ^c May 2012	NJ ^c June 2012	NJ ^c Oct 2012	NJ ^c Dec 2012	NJ ^c April 2013	NJ ^c June 2013	URI ^d July 2012	URI ^d Sept 2012	URI ^d Dec 2012
Monoterpenes									
Tricyclene	1.08	1.02	1.09	0.96	1.06	1.14	1.17	0.98	1.12
α-Pinene	1.23	1.17	1.17 *	1.03	1.11	1.09	1.13	0.95	1.07
Camphene	1.04	1.13	1.11	0.99	1.05	1.14	1.15	0.96	1.07
Sabinene	1.16	1.04	1.21 °	1.15	1.37	1.10	1.15	0.87	0.93
β-Pinene	1.20	0.98	1.18	1.13	1.22	1.12	1.10	0.96	1.11
Myrcene	1.17	1.08	1.10	0.98	0.82	1.07	0.97	0.74 °	1.02
α-Phellandrene	1.36	1.23	1.30 °	1.18 °	1.30	1.78	1.25 *	0.89	1.19
p-Cymene	1.48	0.94	1.18 *	1.09	1.07	1.35	1.10	0.93	0.95
Limonene	1.04	0.89	1.12	1.05	1.10	1.08	0.89	0.88	1.02
Eucalyptol	0.90	0.32 *	1.04	1.21	1.71 °	0.38	0.64	0.73	0.75
Camphor	1.27	0.57 *	0.84	0.90	0.96	4.08 *	1.09	0.85	1.04
Piperitone	0.91	0.57 *	1.16	1.12	0.87	n/a	0.74	0.73	0.99
Bornyl Acetate	1.00	1.12	1.10	1.00	1.06	1.11	1.12	0.94	1.04
Unknown A (suspected monoterpene)	n/a	n/a	0.93	0.82	0.76 *	0.94	n/a	0.76 *	0.89 *
Unknown B (suspected monoterpene acetate)	n/a	n/a	0.44 °	0.79	0.60	0.37	0.50 °	0.37 *	0.57
Sesquiterpenes									
β-Caryophyllene	1.24	1.07	1.06	1.03	1.03	1.07	1.03	0.84	0.94
α-Humulene	1.23	1.05	1.04	1.03	1.02	1.06	1.03	0.84	0.94
γ-Murolene	n/a	n/a	1.18 °	1.13	1.06	0.97	0.90	0.89	1.04
Germacrene D	n/a	n/a	1.39 °	3.36 *	2.44 *	0.90	1.21	0.92	1.11
γ-Cadinene	n/a	n/a	1.09 °	0.97	0.93	0.93	0.85	0.79 *	0.92
δ-Cadinene	n/a	n/a	1.10 °	1.03	1.01	0.93	1.06	0.81 *	0.96
Totals									
Total Monoterpenes	1.05	1.01	1.12	1.02	1.06	1.11	1.08	0.93	1.05
Total Sesquiterpenes	1.24	1.05	1.07 °	1.06	1.04	1.03	1.05	0.84 °	0.95

^a Number indicates ratio of Resistant trees to Control trees' average concentrations. Dark gray shading indicates higher concentration in Resistant, light gray indicates lower concentration in Resistant, and white indicates no difference ± 0.05 fold

^b Marginal significance ($P < 0.10$) by ANOVA is indicated by a circle, full significance ($P < 0.05$) by an asterisk

^c NJ New Jersey forest mature trees

^d URI University of Rhode Island common-garden saplings

resistant trees. First, the growth-differentiation balance (Herms and Mattson 1992) in the resistant hemlocks may be altered, leading these trees to allocate more carbon toward constitutive defenses at the expense of either growth or storage of carbohydrates. Second, the resistant hemlocks may maintain greater or more available total carbon resources than susceptible hemlocks, resulting in more carbon available for both growth and defense, leading to the observed elevated concentrations of terpenoids in resistant trees.

The fact that we found the same trend of higher terpene concentrations in resistant trees both in forest trees and in the common garden saplings increases our confidence in our results. Specifically, having the common garden data allows us to reject the hypothesis that the observed differences are the result of variation in herbivory. Given only the forest site, it is plausible that greater herbivory on the control trees might deplete the trees' carbon resources and decrease terpene concentrations. Since all of the common garden saplings are herbivore-free and display the same trend, however, this idea cannot explain our results. In addition, environmental variables such as sunlight may influence terpene levels even between branches on a single forest tree (EA McKenzie and JS Elkinton, unpublished data), likely explaining some of the noise found by partitioning of variance analysis (Online Supplement). Because all of the common garden control and resistant saplings are located adjacent to each other within planting

boxes, however, they are exposed to essentially identical environmental conditions. The consistency between the NJ forest and the URI common garden in repeated measures ANOVA results (Table 4a) and concentration trend (Table 3a) strongly support the correlation of increased terpenoid concentration in twigs with HWA-resistant status.

The difference between resistant and susceptible hemlock individuals appears to be a pattern across many terpenoids in twig tissue, rather than relying on one or several specific terpenoids. Such a broad change may be due possibly to greater carbon availability or allocation of carbon toward defense rather than toward growth or storage. Future research could distinguish these two options by comparing growth rate, water usage, and carbon assimilation between resistant and susceptible eastern hemlocks, and by determining whether phenolic defenses are also increased in the resistant trees.

Without evidence of terpenoids directly affecting HWA health, we cannot conclude that the observed constitutive increase in twig terpenoid concentrations in twigs represents the resistance mechanism directly. Future research might determine the effect of specific terpenes on HWA, perhaps through induction of terpene production in susceptible eastern hemlocks or addition of terpenes to an artificial diet for HWA. The correlation of terpene concentrations with resistance status may assist individuals in developing cultivars of eastern hemlock resistant to HWA by providing a quicker and

Fig. 1 Relative total concentrations of terpenes^{a,b,c,d}. (a & b) Total monoterpenes and total sesquiterpenes in twigs; (c & d) total monoterpenes and total sesquiterpenes in needles. ^aBar displays average concentration in Control (dark) or Resistant (light) trees. Units are natural log of concentration. ^bError bars display standard error. ^cMarginal significance ($P < 0.10$) by ANOVA is marked by a gray circle, full significance ($P < 0.05$) by a black asterisk. ^dSite is indicated as New Jersey forest mature trees or University of Rhode Island (URI) common garden saplings

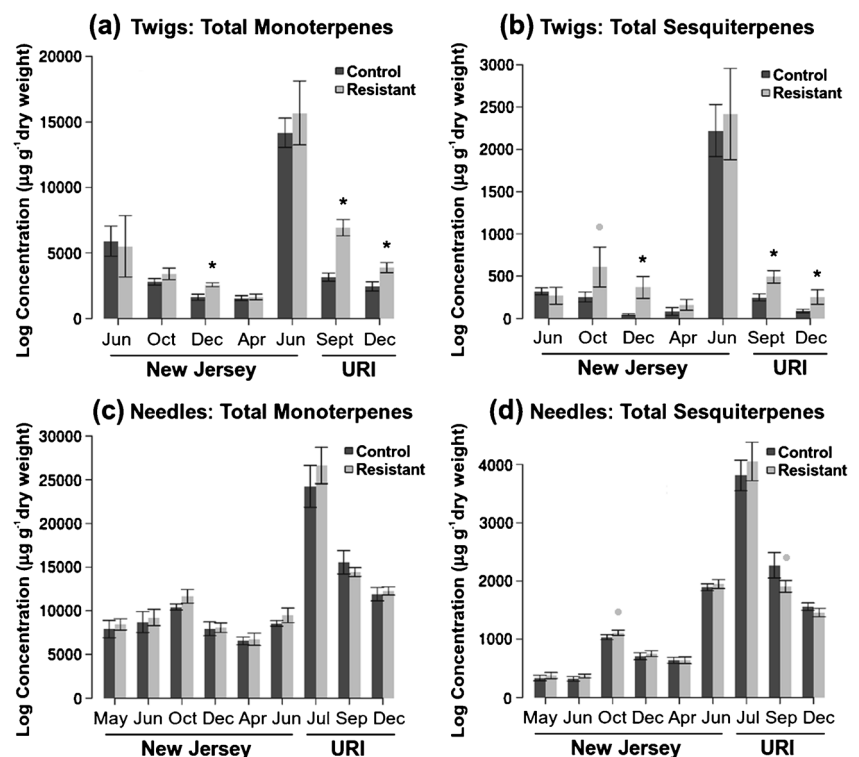


Table 4 Repeated measures anova of terpenoid concentrations against resistance status^{a,b} in (a) twigs and (b) needles

(a) Twigs: <i>p</i> -values for Resistant trees to Control trees						
TWIGS	NJ ^c : resistance	NJ ^c : month	NJ ^c : interaction resistance*month	URI ^d : resistance	URI ^d : month	URI ^d : interaction resistance*month
Monoterpenes						
Tricyclene	0.104	0.037*	0.070°	0.227	0.279	0.808
α-Pinene	0.001*	0.014*	0.677	0.044*	< 0.001*	0.638
Camphene	0.673	0.899	0.049*	< 0.001*	0.663	0.887
β-Pinene	0.079°	0.647	0.985	0.003*	< 0.001*	0.387
Myrcene	0.034*	0.900	0.618	0.001*	0.020*	0.005*
Limonene	< 0.001*	< 0.001*	0.790	0.109	< 0.001*	0.718
Bornyl Acetate	0.007*	0.084°	0.142	< 0.001*	0.568	0.677
Sesquiterpenes						
β-Caryophyllene	0.096°	0.788	0.794	< 0.001*	0.005*	0.004*
α-Humulene	0.009*	0.500	0.575	0.001*	< 0.001*	0.141
Germacrene D	0.220	0.009*	0.084°	0.945	0.059°	0.032*
Totals						
Total Monoterpenes	0.001*	0.187	0.355	0.114	< 0.001*	0.569
Total Sesquiterpenes	0.028*	0.342	0.864	0.003*	0.001*	0.089°
(b) Needles: <i>p</i> -values for Resistant trees to Control trees						
NEEDLES	NJ ^c : resistance	NJ ^c : month	NJ ^c : interaction resistance*month	URI ^d : resistance	URI ^d : month	URI ^d : interaction resistance*month
Monoterpenes						
Tricyclene	0.708	< 0.001*	0.029*	< 0.001*	< 0.001*	0.423
α-Pinene	0.840	< 0.001*	< 0.001*	0.007*	< 0.001*	0.410
Camphene	0.585	< 0.001*	< 0.001*	0.002*	< 0.001*	0.226
Sabinene	0.029*	0.449	0.455	< 0.001*	< 0.001*	0.122
β-Pinene	0.001*	0.013*	< 0.001*	0.003*	0.002*	0.729
Myrcene	0.980	< 0.001*	< 0.001*	0.714	< 0.001*	0.838
α-Phellandrene	< 0.001*	0.022*	0.562	0.013*	0.028*	0.798
p-Cymene	< 0.001*	0.018*	0.022*	0.020*	0.378	0.307
Limonene	< 0.001*	0.021*	0.010*	0.040*	0.002*	0.653
Eucalyptol	0.019*	0.303	0.488	0.487	0.558	0.843
Camphor	< 0.001*	0.667	0.010*	0.066°	0.023*	0.285
Piperitone	< 0.001*	< 0.001*	0.880	0.199	0.765	0.167
Bornyl Acetate	0.798	< 0.001*	< 0.001*	< 0.001*	< 0.001*	0.165
Unknown A (suspected monoterpene)	0.285	< 0.001*	< 0.001*	0.449	< 0.001*	0.827
Unknown B (suspected monoterpene acetate)	0.412	0.001*	< 0.001*	< 0.001*	0.558	0.378
Sesquiterpenes						
β-Caryophyllene	0.004*	< 0.001*	< 0.001*	0.521	< 0.001*	0.085°
α-Humulene	0.006*	0.001*	0.047*	0.545	< 0.001*	0.086°
γ-Murolene	0.323	< 0.001*	< 0.001*	0.285	< 0.001*	0.622
Germacrene D	0.103	< 0.001*	0.483	0.092°	< 0.001*	0.669
γ-Cadinene	0.217	< 0.001*	< 0.001*	0.028*	< 0.001*	0.938
δ-Cadinene	0.450	< 0.001*	< 0.001*	0.985	0.014*	0.248
Totals						
Total Monoterpenes	0.488	0.987	0.001*	0.002*	< 0.001*	0.317
Total Sesquiterpenes	0.003*	< 0.001*	< 0.001*	0.501	< 0.001*	0.094°

^a *P*-values are reported from type II ANOVA's with sample month as an interacting factor and tree identity within month as the repeated measure factor

^b Marginal significance (*P*<0.10) by ANOVA is indicated by a circle, full significance (*P*<0.05) by an asterisk

^c NJ New Jersey forest mature tree

^d URI University of Rhode Island common-garden saplings

less expensive assay for resistance than inoculation trials with the insect.

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