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Volatile emissions of eastern hemlock, *Tsuga canadensis*, and the influence of hemlock woolly adelgid

Corey D. Broeckling*, Scott M. Salom

Department of Entomology, Virginia Tech, 216 Price Hall, Blacksburg, VA 24061, USA

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Abstract

The volatile emissions of eastern hemlock, *Tsuga canadensis* Carriere, were identified and quantified using standard and chiral gas chromatography and mass spectrometry. All of the identified compounds were monoterpenes, and included α-pinene, myrcene, tricyclene, camphene, α-phellandrene, β-pinene, limonene, β-phellandrene, terpinolene, and bornyl acetate. α-Pinene, myrcene, and camphene comprised greater than 75% by mass of the total release. Infestation by the exotic insect, hemlock woolly adelgid (HWA, *Adelges tsugae* Annand), resulted in an increased release rate of monoterpenes from branch tips. Release rate was negatively correlated to the amount of the branch tip sample that was new growth, suggesting that release rate is greater from previous-year foliage. Additionally the percent composition of the volatile profile is slightly altered by infestation, with α-pinene comprising 57% of volatiles from infested foliage and 66% from uninfested foliage.

Keywords: *Tsuga canadensis*; Pinaceae; Eastern hemlock; *Adelges tsugae*; Homoptera; Monoterpenes; Volatile emission

1. Introduction

The exotic hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, has been recognized as a major pest of eastern hemlock and the less frequently encountered Carolina hemlock, *Tsuga caroliniana* Engelm. (McClure, 2001). HWA is thought to have been introduced to western North America from Asia (McClure, 2001) in 1924 (Annand). It was later discovered in the eastern United States in the early 1950′s in Richmond, VA and has spread as far north as Massachusetts and south and west to North Carolina and West Virginia (McClure, 2001). HWA damages hemlock by depleting nutrient stores from ray parenchyma cells via long stylets. This depletion causes reduced growth and ultimately needle loss, bud mortality, and branch and tree mortality (McClure, 1991; Young et al., 1995). In the forest setting, classical biological control is the most promising technique for controlling the spread of HWA.

Several predator species have been imported from HWA's native range for the purpose of classical biological control. Currently *Pseudoscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) is being mass reared and released and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *Scymnus* spp. (Coleoptera: Coccinellidae) are nearing the release stage (McClure, 2001; Salom et al., 2001). Successful biological control requires the ability to accurately track the population dynamics of the predator and prey species. If we understand how the predator finds its host, we can utilize that host-finding mechanism to develop an efficient trap-based sampling procedure. It is our hypothesis that these specialist predators employ olfactory cues in host-finding.

Additionally, specialist predators are often able to detect the presence of herbivores through the effect of that herbivore on the volatile release of the plant (Dickens, 1999). We are interested in the emissions of hemlock foliage infested and uninfested with HWA.

Studies of foliar and twig chemistry have been conducted to reveal both intrinsic and released secondary metabolites in several conifer species, however *Tsuga*

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* Corresponding author at current address: Plant Biology, Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA. Tel.: +1-580-224-6713; fax: +1-580-224-6692.

E-mail address: cdbroeckling@noble.org (C.D. Broeckling).

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spp. are relatively poorly studied. McClure and Hare (1984) analysed foliar terpenes of *Tsuga canadensis* and *Tsuga sieboldii* Carr., which resulted in the identification and quantification of 15 compounds through steam distillation of plant material. No significant defensive wound response to infestation by two scale insect species, *Fiorinia externa* Ferris and *Nucalaspis tsugae* Marlatt (Homoptera: Diaspididae) was observed. However, fecundity of the scale species was affected by changes in concentration of specific terpenes, suggesting an adaptive advantage of increasing production. von Rudloff's (1975) summary of his work on the chemosystematics of conifers, including analysis of *T. canadensis*, *T. heterophylla* (Raf.) Sarg. and *T. mertensiana* (Bong.) Carr., is the most comprehensive published analysis of hemlock terpenes. Although these studies provide a valuable background to the present study, the profiles were obtained through intensive foliar extraction methods, and *Tsuga* spp. volatile profiles have not been described. As it is specifically the volatile emissions of hemlock and/or HWA that predators might utilize in long-range host-location, we have identified and quantified the volatiles associated with these two trophic levels and determined whether a *T. canadensis* wound response is expressed in the volatile profile.

2. Results and discussion

HWA density on infested trees was extremely variable, with densities on individual twigs ranging from 0.00 to 7.27 HWA/cm. The range of values of mean HWA-density per sample (all twigs from a given tree) was smaller, from 0.00 to 1.01 HWA/cm of twig. Although two samples from the infested category did not contain HWA on the twigs examined for density estimates, the twigs were taken from trees supporting HWA populations. We therefore continue to consider them infested samples. None of the twigs sampled from the uninfested category contained HWA.

Of the total twig samples from individual trees, samples from infested trees ranged from 93–100% new growth (mean ± S.D. = 98.34 ± 2.24), while those from uninfested samples contained 98–100% new growth (mean ± S.D. = 99.64 ± 0.68). Despite the seemingly small difference between the two values, there was a significant negative relationship between HWA population density and percent of sample that is current year growth ($r^2 = 0.3023$, $\beta = -0.0779$, $P = 0.0080$). This result is to be expected, as HWA-feeding on eastern hemlock results in a reduction in growth at branch tips (McClure, 1991).

Ten compounds were identified consistently as components of the hemlock volatile profile (Table 1). Among these compounds, it appears that α- and β-pinene, camphene, limonene and α- and β-phellandrene are present both as their plus and minus isomers, although one β-pinene and one α-phellandrene isomer coeluted, making definitive identification and quantification impossible. In addition, a peak immediately following the limonene peak had a retention time and mass spectrum suggestive of an optical isomer; however the spectrum of the second peak possessed ions not in the standard likely due to a small co-eluting peak, thus only the first peak was considered. That is, only the isomers confidently identified are reported. Isomeric composition was not significantly altered by HWA for any identified compound ($P > \chi^2$: α-pinene = 0.289, camphene = 0.618).

Our results are qualitatively in agreement with the results of solvent extracts of *T. canadensis* foliage (McClure and Hare, 1984; von Rudloff, 1975), although expectedly fewer compounds were encountered at quantifiable levels. The percent composition is altered in this study as compared to those examining extracts, with oxygenated monoterpenes representing a much lower proportion in the volatile samples. For example, McClure and Hare (1984) found that bornyl acetate comprised approximately 33% of the extracted terpenoids, whereas the volatile profile is only 3% bornyl acetate by mass.

Infested hemlock tended to have a higher release rate for all compounds (Table 1a). Due to high variability many compounds failed to demonstrate a statistically significant change at the 0.05 level. However, several were significant at the 0.10 level. Inclusion of HWA density and/or percent new growth as covariates in the pre-determined statistical model did not clarify the treatment effect. However, when release rate for individual compounds was regressed against percent new growth of each sample, nearly every compound demonstrated a significant negative regression (Table 2). The combined results of these tests suggest that the altered release rate explained by HWA presence and that explained by percent new growth is largely redundant. This suggestion is supported by the significant regression of HWA density vs. percent new growth. Despite high variability, the trend is clear: infested foliage tends to release monoterpenes at a higher rate, often more than double than that from uninfested foliage.

Tissue age has been shown to affect both the absolute quantity and the proportion of conifer foliage secondary metabolites (Persson et al., 1993). Our data suggest that the release rate of monoterpenes from the previous-year foliage is higher than that from new growth. As HWA populations increase and new growth decreases, the monoterpene profile is altered as a byproduct of a higher proportion of old growth. Additionally, within a given day the treatment effect (HWA*block interaction) was significant for minus and total α-pinene and myrcene release rates (Table 1a), suggesting that the altered release rate is dependent in part on daily variability. These two compounds also showed a similar relative increase with total α-pinene increasing by a factor of 1.61 and myrcene by a factor of 1.73 as a result of HWA

Table 1
A. Mean release rate and B. mean proportions of volatile emissions from eastern hemlock foliage infested with and uninfested with HWA

A. Compound	Mean release rate (ng/g wet wt/h)				Statistical results			
	Infested foliage		Uninfested foliage		HWA main effect		HWA*Block	
	Mean	S.E.	Mean	S.E.	F	$P>F$	F	$P>$F
(−)-α-Pinene	239.02	64.19	163.02	35.56	2.49	0.148	3.47	0.045
(+)-α-Pinene	355.18	87.99	205.03	22.57	3.12	0.108	1.50	0.273
Total α-pinene	594.20	145.55	368.04	53.07	4.08	0.071	2.96	0.068
Myrcene	115.16	26.46	66.71	12.14	5.38	0.043	2.99	0.067
Tricyclene	39.12	13.16	10.36	1.76	3.05	0.111	0.56	0.732
(−)-Camphene	115.99	39.36	31.86	4.97	3.03	0.113	0.58	0.715
(+)-Camphene	8.59	2.54	2.66	0.78	3.12	0.108	0.45	0.807
Total camphene	124.58	41.85	34.52	5.62	3.05	0.112	0.57	0.722
α-Phellandrene	60.08	16.23	26.89	3.14	4.59	0.058	1.60	0.246
(−)-β-Pinene	33.18	8.89	16.58	3.50	3.38	0.096	1.62	0.242
Limonene	9.49	2.25	5.42	1.17	3.16	0.106	1.86	0.189
β-Phellandrene	46.29	10.72	20.34	6.66	5.05	0.048	1.51	0.269
Terpinolene	2.03	0.78	0.00	0.00	5.32	0.044	0.73	0.617
Bornyl acetate	24.21	4.64	14.22	2.37	2.35	0.157	0.56	0.732
Total terpenes	1048.33	261.91	563.09	79.41	4.41	0.062	2.04	0.158

B. Compound	Mean proportion of total monoterpenes				Statistical results			
	Infested foliage		Uninfested foliage		HWA main effect		HWA*Block	
	Mean	S.E.	Mean	S.E.	χ^2	$P>\chi^2$	χ^2	$P>\chi^2$
(−)-α-Pinene	0.23	0.02	0.28	0.02	3.52	0.061	4.57	0.510
(+)-α-Pinene	0.35	0.01	0.38	0.02	2.53	0.112	8.07	0.242
Total α-pinene	0.57	0.02	0.66	0.02	11.35	0.001	6.51	0.260
Myrcene	0.12	0.01	0.12	0.01	0.00	0.965	4.78	0.443
Tricyclene	0.03	0.01	0.02	0.00	2.18	0.140	0.94	0.967
(−)-Camphene	0.09	0.02	0.06	0.01	2.69	0.101	0.69	0.984
(+)-Camphene	0.01	0.00	0.00	0.00	0.99	0.320	1.76	0.881
Total camphene	0.10	0.02	0.06	0.01	2.62	0.105	0.67	0.984
α-Phellandrene	0.06	0.01	0.05	0.00	0.99	0.321	4.31	0.505
(−)-β-Pinene	0.03	0.00	0.03	0.01	0.17	0.683	7.08	0.215
Limonene	0.01	0.00	0.01	0.00	0.68	0.410	5.16	0.397
β-Phellandrene	0.05	0.01	0.03	0.01	3.37	0.096	3.19	0.671
Terpinolene	0.00	0.00	0.00	0.00	23.03	0.000	10.86	0.054
Bornyl acetate	0.03	0.00	0.03	0.00	0.07	0.789	2.27	0.802

Table 2
Summary of regression analyses of percent-new-growth of eastern hemlock samples vs. compound release rate

Compound	β	r^2	$P<t$
(−)-α-Pinene	−68.098	0.4621	0.0005
(+)-α-Pinene	−70.931	0.2901	0.0097
Total α-pinene	−139.028	0.3987	0.0016
Myrcene	−25.465	0.3759	0.0024
Tricyclene	−7.771	0.1500	0.0749
(−)-Camphene	−23.952	0.1606	0.0646
(+)-Camphene	−1.802	0.2027	0.0355
Total Camphene	−25.754	0.1637	0.0618
α-Phellandrene	−14.504	0.3469	0.0039
(−)-β-Pinene	−7.962	0.3340	0.0048
Limonene	−1.867	0.2711	0.0130
β-Phellandrene	−9.863	0.2924	0.0094
Terpinolene	−0.612	0.2489	0.0181
Bornyl acetate	−1.952	0.0673	0.2436
Total terpene	−234.781	0.3470	0.0039

infestation. Nearly all other compounds showed a higher factor of increase, with increases greater than 3× relative to uninfested foliage.

In addition to the increased volatile release in response to HWA, there was a significant change in the relative proportion of certain compounds (Table 1b). The portion of the terpene profile represented by total α-pinene and its minus isomer was lower for infested foliage as compared to uninfested foliage. Proportions of several other compounds were somewhat higher for infested foliage. As with release rate, the inclusion of covariables failed to improve differentiation of infested and uninfested foliage. However, proportional α-phellandrene and terpinolene content demonstrated significant regression against HWA density, and α-pinene and β-phellandrene were significant at the 0.10 level (Table 3). Our results suggest that the altered relative

Table 3
Summary of regression analyses of HWA density vs. proportional composition of total monoterpenes released by eastern hemlock foliage

Compound	β	r^2	$P < t$
(−)-α-Pinene	−0.0849	0.1033	0.1448
(+)-α-Pinene	−0.0189	0.0128	0.6158
Total α-pinene	−0.1039	0.1631	0.0623
Myrcene	−0.0058	0.0059	0.7330
Tricyclene	−0.0005	0.0001	0.9661
(−)-Camphene	0.0160	0.0106	0.6484
(+)-Camphene	0.0044	0.0732	0.2233
Total Camphene	0.0205	0.0149	0.5889
α-Phellandrene	0.0351	0.2670	0.0138
(−)-β-Pinene	0.0108	0.0589	0.2766
Limonene	0.0041	0.0680	0.2411
β-Phellandrene	0.0374	0.1743	0.0532
Terpinolene	0.0026	0.2431	0.0197
Bornyl acetate	−0.0002	0.0000	0.9843

proportions are more directly influenced by HWA density than by the age of the foliage, although HWA density and percent new growth are not independent of each other.

3. Concluding remarks

HWA elicits an increased release in monoterpenes from eastern hemlock branch tips, which may be driven indirectly through a reduction in new growth. We implicate percent new growth as the driving factor contributing to the observed changes. However, percent new growth is dependent on HWA density, resulting in an indirect effect of HWA on the volatile profile.

McClure (1991) found that new growth was severely stunted by HWA densities above 2.0 HWA/cm of branch. Our data suggests that more subtle effects on the reduction in new growth can be found at densities much lower than this. The observed increase in release rate of monoterpenes from branch tips was highly correlated with the percent new growth of the sample. The increase in release rate may be a passive consequence of HWA feeding. Alternatively, it is possible that eastern hemlock actively responds to HWA damage with an increase in monoterpene production, which potentially serves a defensive function. HWA was found to have a lower survival rate on previous-years growth as compared to current-year growth (McClure, 1991), but is still able to cause mortality in eastern hemlock following several years of infestation despite high mortality on older foliage.

This study does not allow us to distinguish an active induced defense from a passive byproduct of the depletion of nutrient reserves. However the regression analyses suggest that reduced growth is an important factor contributing to the altered release rate. These results suggest that the changes in release rate and composition could be used as indicators of the presence of HWA by specialist predators that use olfactory cues. In related experiments, the specialist biological control agent *Laricobius nigrinus* demonstrated a behavioral response to HWA-infested hemlock odors and possesses several types of antennal sensory structures suggestive of olfactory capabilities (Broeckling, 2002). The results offer promise that we may be able to utilize innate host-finding mechanisms to develop a more accurate sampling procedure for biological control agents of HWA.

4. Experimental

4.1. *Volatile collection*

Twenty-four eastern hemlock trees were selected from a nursery plot at Price's Fork Research Center, Blacksburg, VA (UTM 17- 545,275 E; 411,844 N). Half of the trees were treated systemically with Thiamethoxam® via soil injection to eliminate HWA populations 2 years previous to the study. The remaining trees were left untreated. All trees selected were 2–3 m tall and apparently of sound health, having sent out new shoots during the previous spring despite infestation with HWA. Samples were collected between 11:00 and 13:00 hours from 20 to 28 August 2001. External environmental conditions were recorded with a Hobo® data logger. These data are not reported as they did not play a role in interpretation of collected volatile data. At this time of year, HWA is settled at the base of needles on current year growth in a state of summer dormancy.

Volatile collection was performed by cutting 24 hemlock branch tips, approximately 10.0 cm each, from each tree. The collective volatile release of the 24 branch tips from each tree comprised one sample. One sample was taken from each of 24 trees. To eliminate potential variability due to aspect or relative crown position, two tips were removed from the upper-crown, two from mid-crown, and two from the lower-crown from each of four cardinal directions. As a result of the stringent nature of tip selection and the patchy in-tree distribution of HWA, not all branch tips were infested. Priority was given to branch tip location.

Once removed from the tree, the cut end was immediately inserted into a disc of floral foam (Oasis® floral products: 2 cm, 5 cm diameter) saturated with distilled water. As terpenes are of low polarity and generally highly hydrophobic, the contribution of volatiles extruded from the cut twig, through the water-saturated floral foam, and into the collection air-flow would be minute. Lewinsohn et al. (1993) demonstrated a dramatic increase in monoterpene release rate in wounded saplings of grand fir. However, the method of volatile collection allowed the wound to be exposed to the air flow and collection trap. The same study revealed increased (1.9 times) monoterpene cyclase activity 7 days following wounding. Cut ends were inserted into floral foam immediately following removal from the tree. Collection period terminated 1 h after wounding. While this procedure cannot substitute for sampling an intact twig, preliminary results obtained prior to the experiment suggested that alternative methods were flawed as well. For example, volatile sampling of intact potted saplings introduces volatile components from the soil and litter, and assumes that the volatile profile for a sapling will not differ from that of a more mature tree. Developmental state, however, has been repeatedly shown to affect volatile release (Agelopoulos et al., 2000; Kuzma and Fall, 1993; Scutareanu et al., 1997; Takabayashi et al., 1994). Another alternative was to utilize a portable bag-based collection system such as that utilized by Bernasconi et al. (1998). Attempts were made to employ this type of system; however, to form an airtight seal around the base of the stem virtually ensures some level of mechanical damage to the stem near the point of the seal, introducing both a wound effect and sample-to-sample variability. A compromise between sample integrity and analytical reproducibility was thus reached.

The floral foam disc containing 24 twigs was brought into the laboratory, placed into a one-pint glass jar, and sealed with a rubber stopper containing an inlet and outlet port of glass pipette. The containment apparatus was connected via Teflon® tubing to a flow of compressed air (0.5 l/min), which was passed through a charcoal filter, over the foliage, and through a glass pipette containing 0.6 g of Porapak® Q adsorbent backed on either side by glass wool. The adsorbent trap was exposed to the airflow for 60 min at 23–24 °C under ambient room lighting. At the end of the collection period, volatile compounds were extracted from the trap with 2.5 ml of 0.01 mg/ml (+)-longifolene in hexane applied in 0.5 ml aliquots. The total 2.5 ml of extract was pooled into a single vial and the solvent extract was reduced to 0.1 ml under a gentle nitrogen flow and stored at −60 °C until analyses were performed. To test for trap breakthrough, a second adsorbent trap was placed in-line following the sample trap. No trap breakthrough was detected utilizing this collection regimen. The use of the slightly less volatile sesquiterpene, (+)-longifolene, as an internal standard likely resulted in a slight underestimation of monoterpene loss while concentrating under nitrogen flow. However, it was necessary to choose a compound that did not interfere with hemlock volatiles on either of two GC columns.

Branch tips were weighed immediately following volatile collection for determination of wet weight. Although the weight of the twig may have been slightly altered during the volatile collection period due to water uptake, the immediate insertion of the twig into a water source was considered more important in preserving the integrity of the sample. Additionally, the weight would likely be altered consistently in both infested and uninfested samples. We randomly selected 12 of the 24 branch tips and recorded branch tip length and number of adelgids present to estimate adelgid density and percent of the sample represented by current year's growth.

4.2. *Separation and identification*

A 2.0 μl splitless injection was made on a Perkin-Elmer® Autosytem GC; standard separation was executed using a DB-5 column (30 m, 0.32 mm O.D., 0.25 um film, J and W Scientific®) with an initial temperature of 50 °C (8 min) ramped at 2 °C/min to 70 °C (0 min) and then to 200 °C (20 min) at 20 °C/min. Chiral separation was performed on a β-Dex 120 capillary GC column (30 m, 0.25 mm O.D., 0.25 um film, Supelco®). Oven temperature was initially set to 70 °C (5 min) and then ramped at 3 °C/min to 175 °C and held for 15 min. Column pressure was set to 35 psi. All samples were run within eight weeks of collection. Concentration was determined through comparison of peak areas in collected samples to a dilution series of known concentration, with adjustments made based on the loss of internal standard to calculate release rate. Tricyclene concentration was calculated using the α-pinene dose–response curve, as high purity tricyclene is commercially unavailable. This is reasonable because flame ionization detector response is dependent on carbon number and saturation level (McNair and Miller, 1997). α-Pinene and tricyclene are structurally very similar, with identical molecular weight and similar retention times.

Compounds were tentatively identified on a HP 5790A Series gas chromatograph coupled to a VG Analytical Mass Spectrometry system utilizing electron impact ionization (70 keV) and magnetic sector mass separation. Confirmation was achieved by comparing retention times of unknowns to commercially available standards (Aldrich, Milwaukee, WI, USA; Hercules, Wilmington DE, USA; Rodney Croteau, Washington State University, USA) on DB5 and β-Dex 120 columns. We were unable to obtain optically pure α-phellandrene for isomeric identification, thus we report the pooled plus and minus isomers simply as α-phellandrene. β-phellandrene was unobtainable, thus our identification is reliant on the mass spectrum and the work by von Rudloff (1975) on hemlock constituent terpenes.

4.3. *Statistical design and analysis*

A generalized randomized block design was used, with two samples of each treatment (HWA) being taken during each of 6 days (blocks), to examine the HWA main-effect as well as the HWA*day interaction. Analyses were executed in PROC MIXED (SAS® v 6.12) to compare the release rate of infested and uninfested trees for each compound and total monoterpene emission rate. Each individual compound was converted to a mass proportion of total monterpene emission and treatment vs control trees were compared by conducting χ^2 tests in PROC MIXED. Isomeric proportions were compared in the same fashion. Simple linear regressions were performed using PROC REG.

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