JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Terpenoid Volatiles from Cultivars of Eastern Hemlock (*Tsuga canadensis*)

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The volatile terpenoid fraction from needles in 13 cultivars of *Tsuga canadensis* L. (Carriere) was analyzed by gas chromatography with mass spectrometry (GC-MS). The results of this study are considered along with previously reported results for foliar terpenoid levels of the Asian (*T. sieboldii*, *T. chinensis*, *T. diversifolia*), western North American (*T. mertensiana*, *T. heterophylla*), and eastern North American species (*T. canadensis*, *T. caroliniana*) of hemlock to draw conclusions about the potential of cultivar host resistance to the hemlock woolly adelgid (*Adelges tsugae* Annand). It is suggested that hemlocks in eastern North America have adapted their terpenoid chemistry for protection against endemic defoliators and that this has made them vulnerable to non-native, sucking pests such as adelgids and scales. Some cultivars of *T. canadensis* have a terpenoid profile that resembles that of the resistant noneastern North American species and are candidates for biological screening for resistance. Among the cultivars, the variation in terpenoid chemistry did not absolutely correspond with the considerable differences in morphological characters observed, indicating that the terpenoid chemistry is not definitively coupled with hemlock morphology.

KEYWORDS: ***Adelges tsugae*; cultivars; GC-MS; hemlock; hemlock woolly adelgid; solid-phase microextraction; terpenoids; *Tsuga canadensis***

INTRODUCTION

The nine species of hemlock (*Tsuga*) in the world (*1*) are all attacked by a minute sucking insect, the hemlock woolly adelgid (*Adelges tsugae* Annand; HWA); however, only the two species in eastern North America, *T. canadensis* L. (Carriere) and *T. caroliniana* Engelm., are seriously damaged (*2*). The adelgid is a fairly recent introduction to eastern North America (*3*) and, hence, host resistance to the pest has not evolved as it seems to have in Asia and western North America, where the adelgid and its hemlock hosts have co-occurred for long periods.

This paper is the third in a series in which we seek linkages between volatile terpenoids in the foliage and resistance of hemlock to HWA. Initially, we reported that the foliar terpenoid composition of three Asian (*T. sieboldii, T. chinensis, T. diversifolia*), two western North American (*T. mertensiana, T. heterophylla*), and two eastern North American (*T. canadensis, T. caroliniana*) species of hemlock was related to both the alleged resistance of the species and the phylogenetic relationships within *Tsuga* (*4*). Interspecies relationships based on the terpenoid profiles correspond remarkably well to phylogenetic relationships based on morphological and anatomical characters (*5*), geography (*6*), and molecular markers (*7, 8*). Next, we reported on the spatial and temporal distribution of terpenoids in *T. canadensis* in relation to the life cycle of the hemlock woolly adelgid (*9*). Statistical analysis of foliar terpenoids in both of these studies elucidated a small subset of the total volatile emission, which may be linked by phytochemical herbivore-host interactions.

T. canadensis, commonly known as eastern hemlock and Canadian hemlock, is an important forest and ornamental species. It grows on 19 million acres of forest in the eastern United States, forming a dense, evergreen canopy that provides critical habitat for many plant and animal species (*10*). Eastern hemlock is also one of the most planted and prized landscape trees in the United States, with more than 274 named cultivars (*11*). These cultivars, for the most part, were variants selected from natural or cultivated populations and then propagated asexually through cuttings, tissue cultures, or grafting. In addition to producing morphological differences, selective propagation in cultivars can introduce measured variations in the volatile fractions of foliar terpenoids (*12–15*) as well as play a role in olfactory discrimination in herbivore host selection (*16–18*). Hemlock cultivars have been classified by Swartley into groups based upon foliage deviations, color forms, and habit of growth differences (*11*). Herein, we report the relative levels of volatile terpenoids in hemlock cultivars and interpret the results in the

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10.1021/jf071947o CCC: $37.00
Published on Web 12/04/2007

Table 1. Swartley Group and Source of the Cultivars of *Tsuga canadensis* and *T. canadensis* Used in the Data Set

code	cultivar	Swartley group	source[a]
1	'Albo-spica' Jäger u Beissn.	WHITE-TIP	MA
2	'Snowflake' Swartley	WHITE-TIP	MA
3	'Bristol Short-Leaf' (provisional name)	LITTLE-LEAF	MA
4	'Microphylla' (Lindl.) Sénéclauze	LITTLE-LEAF	LG
5	'Atrovirens' Beissn.	LARGE-LEAF	LG
6	'Macrophylla' (Beissn.) Fitschen	LARGE-LEAF	MA
7	'Bennett' Swartley ex den Ouden and Boom	SPREADING	MA
8	'Bennett' Swartley ex den Ouden and Boom	SPREADING	LG
9	'Beaujean' Hillier	SPREADING	MA
10	'Callicoon' Swartley ex den Ouden and Boom	PENDULA	MA
11	'Gable Weeping' Swartley ex den Ouden and Boom	PENDULA	LG
12	'Pendula' Hort, ex Beissn.	PENDULA	MA
13	'Pendula' Hort, ex Beissn.	PENDULA	LG
14	'Sargentii' Scott, F.J.	PENDULA	LG
15	'Barry's Dwarf'	not listed	MA
	noncultivar		
A	from Morris Arboretum		MA
B	from Longwood Gardens		LG
C	from U.S. National Arboretum		(*4*)
D	from Lake Scranton in a forest setting		(*9*)

[a] MA, Morris Arboretum; LG, Longwood Gardens.

context of our previous work on volatile emissions of resistant and susceptible hemlock species.

Making direct linkages with a pest's host plant preferences and plant chemistry is complicated by the environmental and temporal influences on the production of chemicals and the natural genetic variation in a species. Because living collections of cultivars occur in a common location and often consist of asexually propagated examples of type or variety, experimental designs will have less variance in environmental and genetic factors. For example, commercial orchards often consist of several varieties planted as grafted saplings that have direct linkage between terpene hydrocarbons emitted by specific cultivars in mixed apple tree orchards to the apple blossom weevil, *Anthonomus pomorum* (L.) (*16*). Indirect linkages are provided by the variation in volatile aldehydes with known antimicrobial activity both seasonally and among cultivars of olive (*13*). Because our earlier study found seasonal variation in hemlock volatiles (*9*), this study was restricted to sampling during the season when HWA is mobile and settling at a fixed feeding site for the remainder of its life.

MATERIALS AND METHODS

Plant Material. In June 2005, branch clippings of hemlock cultivars were obtained from Longwood Gardens (Kennett Square, PA) and Morris Arboretum of the University of Pennsylvania (Philadelphia, PA), which are a distance of 60 km apart. The *T. canadensis* cultivars sampled and their assigned Swartley group are shown in **Table 1**. Samples were obtained by clipping foliage from healthy, visually uninfested trees. The clippings were immediately placed in polyethylene bags in insulated containers on dry ice and transported to the laboratory at Villanova University, where they were kept at −80 °C until analysis. The complete data set for statistical analysis included 13 cultivars with two duplicates that were present at both arboreta, nonvarietal *T. canadensis* growing at the arboreta, *T. canadensis* growing wild in a forest near Lake Scranton, PA (*9*), and seven *Tsuga* species (*4*). All 25 foliage samples in the data set were the previous year's growth and were harvested in June.

SPME. A 100 μm PDMS fiber and a manual SPME holder (Supelco, Bellefonte, PA) were used for all SPME samplings of volatile compounds. For each sample, five nonadjacent, individual needles from randomized locations on the previous-year growth segment of the branch were analyzed individually. The previous year's growth was examined as this is the preferred feeding site of the adelgid when the samples were taken (*2*). Each needle was manually removed from the branch and allowed to reach ambient temperature. The needles were manually cut perpendicular to the long axis of the needle using stainless steel scissors into sections of <1 mm. Clippings were collected directly into five 4 mL screw-top vials and capped with PTFE/silicone septa (VWR Scientific, Pittsburgh, PA). The sample vials were placed in an aluminum block maintained at 50 °C by an Omega CN132 controller (Omega Engineering, Stamford, CT) that regulated the output of two 50 W cartridge heaters imbedded in the aluminum block. Each sample was maintained at 50 °C for 1 h to allow the volatiles to equilibrate in the headspace. Following the equilibration period, the sample vial remained in the aluminum block while the SPME fiber was exposed to the headspace for 15 min under static conditions.

GC-MS Analysis. Samples were analyzed on a Star software (Varian, Walnut Creek, CA) computer-controlled Varian 3900 gas chromatograph. The Varian 1177 injector was fitted with a Merlin Microseal septum. The injector temperature was maintained at 220 °C, and a 20:1 split ratio was used for all samples. The SPME was inserted into the injection port for 2 min for sample desorption. Chromatographic separation was accomplished using a Varian Factor 4 VF5-MS column (30 m, 0.25 mm i.d., 0.25 μm phase thickness). The column temperature program was from 60 °C (0 min hold) to 240 °C (0 min hold) at 3 °C/min. The helium carrier gas was electronic pressure controlled at a constant flow of 1.0 mL/min. The Varian 2100T ion-trap mass spectrometer was operated in EI+ mode (ionization energy = 70 eV, *m*/*z* 45–400 range).

Compound Identification and Relative Quantification. The compounds were tentatively identified using a mass spectrum database search (Varian NIST MS database, 1992, and IMS terpene library, 1992) and on the basis of their measured retention indices as compared to the retention indices reported using an equivalent DB-5 column (*19*). The experimental retention indices and mass spectra are compared to authentic samples (Aldrich, Milwaukee, WI) for 14 of the 24 compounds in **Table 2** (α-pinene, camphene, β-pinene, α-phellandrene, *o*-cymene, limonene, β-phellandrene, terpinolene, camphor, borneol, piperitone, isobornyl acetate, β-caryophyllene, and α-humulene); the identities of the other compounds are tentative. The area under an identified peak was integrated using the most intense *m*/*z* fragment (**Table 2**) from the mass spectrum for each compound. Relative quantity (area percent) is calculated by the ratio of the peak area for an individual terpenoid, relative to the total peak area for all identified terpenoids in a chromatogram. We have demonstrated that relative terpenoid peak areas in the chromatogram are especially useful in defining interspecies relationships (*4*), although the method precludes comparisons of absolute compound amounts.

Statistical Analysis. All statistical analyses were performed using SPSS (v. 14.2, Chicago, IL) on the matrix of the terpenoid profile of each specimen in the data set. To examine the terpenoids in the cultivars within the context of the terpenoid profiles of hemlock species that are resistant to HWA, the cultivar data were consolidated with our previously reported interspecies data (*4*) and data for *T. canadensis* from a natural forest (*9*). To avoid listwise exclusions and provide a complete matrix, terpenoids that were detected but below the threshold of 0.01 were assigned a value of 0.005. The data set is complex with a 5000-fold overall range in the values and considerable autocorrelation; hence, the data were examined closely for normality, correlations, covariance, and influence of ranking and several transformations of the data. Data were standardized prior to cluster and factor analysis. For hierarchical cluster analysis (HCA), proximities were computed from the Euclidean squared distance and average linkage between groups. The data set was log transformed prior to computation of the covariance matrix for principal component analysis (PCA).

Table 2. Mean Terpenoid Percentages (± SD) of Individual Needles ($n = 5$) for *Tsuga canadensis*[a]

	m/z	*T. canadensis* 'Albo-spica' code 1	*T. canadensis* 'Snowflake' code 2	*T. canadensis* 'Bristol Short Leaf' code 3	*T. canadensis* 'Microphylla' code 4	*T. canadensis* 'Atrovirens' code 5	*T. canadensis* 'Macrophylla' code 6	*T. canadensis* 'Bennett' code 7	*T. canadensis* 'Bennett' code 8	*T. canadensis* 'Beaujean' code 9
tricyclene	93	5.53 ± 0.64	5.39 ± 1.22	7.42 ± 1.47	7.82 ± 1.07	5.86 ± 0.76	7.44 ± 0.58	4.39 ± 0.49	4.76 ± 0.83	3.09 ± 0.63
α-pinene[b]	93	14.77 ± 1.46	16.60 ± 2.53	22.25 ± 2.47	15.52 ± 1.78	19.20 ± 1.32	22.71 ± 1.80	22.02 ± 1.79	15.67 ± 1.23	11.62 ± 1.96
camphene[b]	93	14.15 ± 0.87	12.33 ± 2.66	12.72 ± 1.78	11.77 ± 1.24	12.66 ± 1.19	15.86 ± 1.43	11.55 ± 1.44	10.55 ± 0.52	10.04 ± 1.84
sabinene	93	0.65 ± 0.07	0.46 ± 0.15	nd	0.13 ± 0.18	0.15 ± 0.14	nd	0.05 ± 0.07	nd	0.08 ± 0.06
β-pinene[b]	93	1.35 ± 0.13	1.17 ± 0.16	3.39 ± 0.39	3.31 ± 0.35	2.42 ± 0.11	2.35 ± 0.27	4.33 ± 0.43	2.65 ± 0.48	1.65 ± 0.23
myrcene	93	1.97 ± 0.36	2.24 ± 0.50	0.98 ± 0.63	1.47 ± 0.38	1.68 ± 0.20	0.70 ± 0.17	1.49 ± 0.16	1.82 ± 0.07	1.19 ± 0.22
α-phellandrene[b]	91	0.60 ± 0.07	nd	3.18 ± 1.26	2.36 ± 0.34	1.02 ± 0.15	0.41 ± 0.09	3.33 ± 0.47	2.12 ± 0.49	0.98 ± 0.22
o-cymene[b]	119	nd	nd	0.27 ± 0.24	1.25 ± 0.05	0.48 ± 0.10	0.45 ± 0.28	0.93 ± 0.63	1.61 ± 0.12	0.22 ± 0.16
limonene[a]	67	0.73 ± 0.11	1.29 ± 0.20	2.25 ± 1.43	3.43 ± 0.34	2.83 ± 0.27	1.45 ± 0.52	4.41 ± 0.74	5.55 ± 0.19	3.55 ± 0.47
β-phellandrene[b]	93	0.57 ± 0.09	0.35 ± 0.12	2.35 ± 1.60	3.86 ± 0.23	2.20 ± 0.40	1.28 ± 0.32	3.73 ± 0.67	4.43 ± 0.14	2.40 ± 0.37
cis-ocimene	93	0.40 ± 0.07	0.22 ± 0.10	1.52 ± 0.62	2.56 ± 0.42	1.28 ± 0.17	0.93 ± 0.18	2.71 ± 0.43	3.46 ± 0.23	1.70 ± 0.30
γ-terpinene	93	nd	nd	0.05 ± 0.09	0.08 ± 0.10	0.20 ± 0.05	nd	0.08 ± 0.09	0.11 ± 0.11	0.09 ± 0.08
terpinolene[b]	93	0.04 ± 0.05	0.19 ± 0.21	0.28 ± 0.30	0.33 ± 0.17	0.33 ± 0.12	0.07 ± 0.10	0.49 ± 0.21	0.58 ± 0.18	0.56 ± 0.28
camphor[b]	95	nd	0.29 ± 0.10	nd	0.03 ± 0.04	1.49 ± 0.11	1.77 ± 0.02	0.17 ± 0.13	0.24 ± 0.15	0.13 ± 0.11
borneol[b]	95	nd	nd	0.27 ± 0.22	0.03 ± 0.06	0.16 ± 0.11	0.51 ± 0.18	0.14 ± 0.09	0.17 ± 0.13	0.20 ± 0.12
piperitone[b]	82	0.04 ± 0.04	nd	3.83 ± 0.91	5.14 ± 0.53	3.36 ± 0.31	1.45 ± 0.24	3.53 ± 0.34	4.67 ± 0.52	1.85 ± 0.38
isobornyl acetate[b]	95	46.92 ± 5.51	44.37 ± 3.91	35.21 ± 3.39	35.95 ± 3.29	37.35 ± 2.34	34.36 ± 0.42	32.75 ± 3.06	33.33 ± 3.84	38.95 ± 2.43
β-caryophyllene[b]	91	3.08 ± 0.16	2.82 ± 0.51	1.35 ± 0.21	1.31 ± 0.09	1.66 ± 0.22	1.43 ± 0.14	2.72 ± 0.31	4.55 ± 0.25	6.01 ± 0.90
α-humulene[b]	93	2.62 ± 1.08	3.34 ± 0.70	1.59 ± 0.25	nd	3.28 ± 0.39	2.72 ± 0.47	4.15 ± 0.65	2.85 ± 1.28	9.16 ± 1.70
γ-muurolene	161	0.37 ± 0.07	0.31 ± 0.14	0.05 ± 0.04	0.44 ± 0.32	0.32 ± 0.07	0.39 ± 0.12	0.43 ± 0.18	1.61 ± 0.45	1.39 ± 0.15
germacrene D	161	6.43 ± 0.48	4.77 ± 1.79	nd	1.28 ± 0.32	0.47 ± 0.03	nd	0.17 ± 0.14	0.43 ± 0.25	1.25 ± 0.09
viridiflorene	189	0.05 ± 0.03	0.07 ± 0.03	nd	0.06 ± 0.05	0.09 ± 0.05	0.04 ± 0.03	0.07 ± 0.05	0.14 ± 0.09	0.30 ± 0.04
γ-cadinene	161	0.49 ± 0.25	0.64 ± 0.24	0.19 ± 0.14	0.31 ± 0.22	0.60 ± 0.06	0.62 ± 0.14	0.35 ± 0.20	0.85 ± 0.18	1.44 ± 0.19
δ-cadinene	161	1.24 ± 0.12	1.25 ± 0.50	0.42 ± 0.12	0.94 ± 0.09	0.98 ± 0.09	1.26 ± 0.22	0.85 ± 0.08	1.89 ± 0.48	2.57 ± 0.49

	m/z	*T. canadensis* 'Callicoon' code 10	*T. canadensis* 'Gable Weeping' code 11	*T. canadensis* 'Pendula' code 12	*T. canadensis* 'Pendula' code 13	*T. canadensis* 'Sargentii' code 14	*T. canadensis* 'Barry's Dwarf' code 15	*T. canadensis* (Morris Arboretum) code A	*T. canadensis* (Longwood Gardens) code B	*T. canadensis* (U.S.N.A.) (*4*) code C	*T. canadensis* (Lake Scranton) (*9*) code D
tricyclene	93	7.50 ± 0.17	4.04 ± 0.44	4.97 ± 0.50	3.30 ± 0.65	3.69 ± 0.89	5.21 ± 0.74	5.96 ± 1.02	6.04 ± 0.76	4.32 ± 0.37	5.72 ± 1.80
α-pinene[b]	93	19.02 ± 0.80	12.21 ± 1.31	15.86 ± 1.75	14.51 ± 1.64	16.54 ± 1.49	16.02 ± 1.78	16.76 ± 2.56	19.24 ± 2.35	13.19 ± 0.55	15.25 ± 0.66
camphene[b]	93	14.03 ± 1.87	9.92 ± 0.84	12.19 ± 1.14	10.91 ± 1.19	10.09 ± 1.42	12.10 ± 1.60	10.76 ± 0.86	13.08 ± 1.46	7.79 ± 0.76	10.01 ± 1.15
sabinene	93	0.61 ± 0.24	0.31 ± 0.03	0.25 ± 0.14	0.50 ± 0.18	0.31 ± 0.11	0.46 ± 0.14	0.44 ± 0.09	0.10 ± 0.14	0.15 ± 0.05	0.49 ± 0.21
β-pinene[b]	93	1.89 ± 0.27	1.86 ± 0.15	2.24 ± 0.21	1.93 ± 0.71	3.39 ± 0.57	1.82 ± 0.17	2.39 ± 0.52	2.51 ± 0.42	2.44 ± 0.06	2.56 ± 0.62
myrcene	93	2.02 ± 0.55	1.56 ± 0.07	1.07 ± 0.27	1.70 ± 0.11	0.99 ± 0.17	2.18 ± 0.10	1.97 ± 0.16	1.63 ± 0.23	1.65 ± 0.56	2.38 ± 0.41
α-phellandrene[b]	91	1.66 ± 0.38	1.77 ± 0.38	1.49 ± 0.27	2.00 ± 0.27	3.32 ± 0.18	0.40 ± 0.31	2.17 ± 0.61	1.35 ± 0.49	1.45 ± 0.58	1.99 ± 0.46
o-cymene[b]	119	nd	0.25 ± 0.06	0.21 ± 0.10	0.19 ± 0.10	0.86 ± 0.16	nd	0.38 ± 0.19	0.33 ± 0.20	1.63 ± 0.19	0.48 ± 0.12
limonene[a]	67	1.11 ± 0.21	3.10 ± 0.29	2.41 ± 0.39	4.72 ± 0.20	4.29 ± 0.62	0.74 ± 0.32	2.28 ± 0.13	2.99 ± 0.26	1.96 ± 0.18	2.42 ± 0.17
β-phellandrene[b]	93	1.00 ± 0.11	2.65 ± 0.26	2.28 ± 0.33	2.20 ± 0.27	5.20 ± 0.85	1.21 ± 0.06	1.43 ± 0.40	2.69 ± 0.30	3.06 ± 0.64	1.56 ± 0.19
cis-ocimene	93	0.62 ± 0.30	1.81 ± 0.15	1.34 ± 0.25	2.63 ± 0.05	3.17 ± 0.39	0.24 ± 0.22	2.88 ± 0.30	1.56 ± 0.33	1.91 ± 0.37	2.80 ± 0.28
γ-terpinene	93	nd	0.18 ± 0.05	0.07 ± 0.05	0.06 ± 0.07	0.11 ± 0.06	nd	0.10 ± 0.02	0.11 ± 0.05	0.18 ± 0.05	0.12 ± 0.03
terpinolene[b]	93	0.04 ± 0.07	0.61 ± 0.10	0.32 ± 0.25	0.41 ± 0.28	0.77 ± 0.10	0.11 ± 0.13	0.42 ± 0.07	0.33 ± 0.17	0.13 ± 0.01	0.50 ± 0.08
camphor[b]	95	nd	0.50 ± 0.10	0.67 ± 0.21	0.72 ± 0.06	0.45 ± 0.12	nd	nd	1.19 ± 0.22	na	na
borneol[b]	95	nd	0.26 ± 0.08	0.33 ± 0.14	0.07 ± 0.07	0.10 ± 0.11	nd	2.86 ± 0.87	0.46 ± 0.26	2.99 ± 0.38	1.65 ± 1.25
piperitone[b]	82	0.09 ± 0.10	3.17 ± 0.42	2.57 ± 0.48	1.05 ± 0.50	1.43 ± 0.28	nd	4.69 ± 0.84	2.81 ± 0.76	3.56 ± 0.18	3.97 ± 0.74
isobornyl acetate[b]	95	43.06 ± 4.62	50.70 ± 2.86	41.55 ± 3.69	46.48 ± 5.27	36.01 ± 2.37	44.68 ± 4.61	37.75 ± 5.83	38.29 ± 3.47	42.86 ± 1.28	37.10 ± 2.02
β-caryophyllene[b]	91	3.38 ± 0.38	2.60 ± 0.32	2.31 ± 0.49	4.03 ± 0.65	2.98 ± 0.39	3.00 ± 0.33	1.30 ± 0.19	1.74 ± 0.16	1.37 ± 0.12	1.53 ± 0.15
α-humulene[b]	93	5.28 ± 0.17	0.19 ± 0.26	5.44 ± 0.77	5.91 ± 1.17	3.45 ± 0.58	5.19 ± 0.81	3.26 ± 0.56	3.10 ± 0.11	3.26 ± 0.47	3.51 ± 0.44
γ-muurolene	161	0.08 ± 0.07	0.22 ± 0.06	0.23 ± 0.11	0.19 ± 0.15	0.18 ± 0.12	0.08 ± 0.06	0.09 ± 0.08	0.33 ± 0.05	0.70 ± 0.11	0.60 ± 0.42
germacrene D	161	0.30 ± 0.21	0.61 ± 0.13	0.44 ± 0.23	3.75 ± 0.60	0.43 ± 0.24	3.15 ± 0.34	0.10 ± 0.11	0.39 ± 0.09	0.66 ± 0.29	1.86 ± 1.53
viridiflorene	189	0.07 ± 0.04	0.09 ± 0.03	0.10 ± 0.04	0.09 ± 0.09	0.08 ± 0.05	nd	0.08 ± 0.10	0.10 ± 0.02	0.48 ± 0.07	0.13 ± 0.03
γ-cadinene	161	0.44 ± 0.19	0.34 ± 0.07	0.57 ± 0.07	0.49 ± 0.10	0.47 ± 0.15	0.45 ± 0.21	0.52 ± 0.13	0.50 ± 0.16	2.17 ± 0.53	0.77 ± 0.17
δ-cadinene	161	1.10 ± 0.17	0.53 ± 0.11	0.65 ± 0.29	1.10 ± 0.17	0.65 ± 0.29	1.32 ± 0.22	0.73 ± 0.19	1.14 ± 0.21	3.23 ± 0.79	1.19 ± 0.30

[a] Each terpenoid was quantified using the *m/z* value listed (nd, not detected; na, not analyzed). [b] The experimental retention indices and mass spectra were compared to authentic samples; identity of the other compounds is tentative.

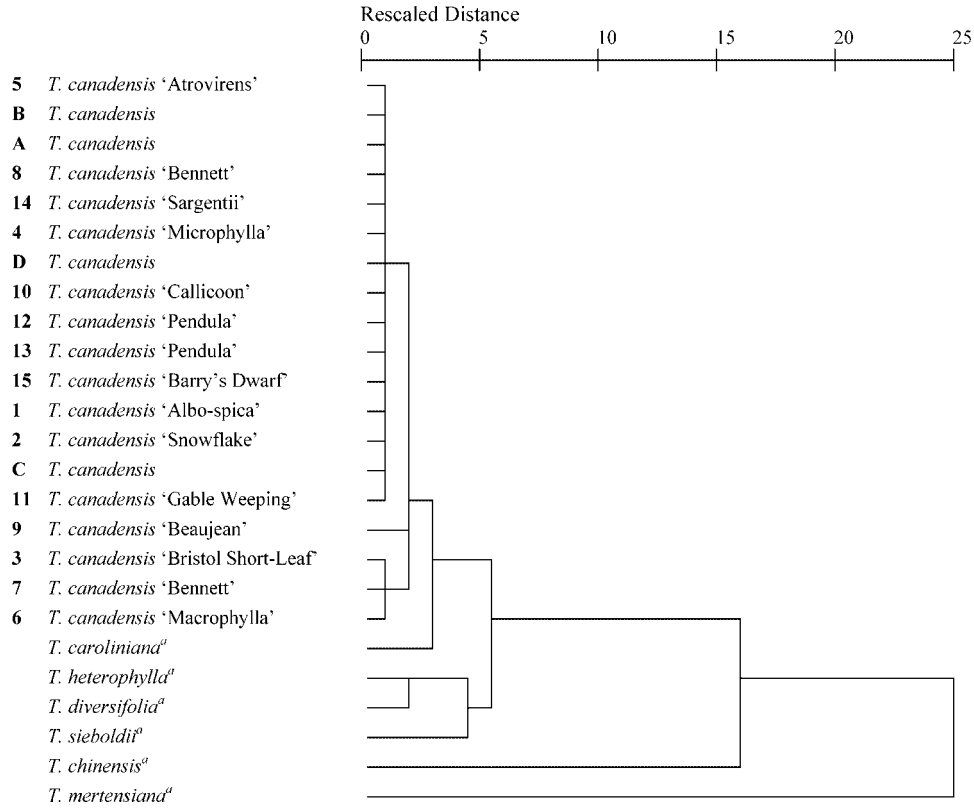

Figure 1. Dendrogram from the hierarchical cluster analysis with *T. canadensis* and its cultivars labeled using codes listed in **Table 1** and [a] ref *4*.

RESULTS

Twenty-four terpenoids were measured in the 13 cultivars and nonvarietal *T. canadensis* growing at the arboreta (**Table 2**). The standardized terpenoid data set was first analyzed using HCA to identify inter- and intraspecies similarities. A dendrogram from the HCA based on the terpenoid profiles is shown in **Figure 1**. The agglomeration schedule indicates that there are four well-separated clusters among the species. These clusters contain, in descending order of similarity, (1) *T. mertensiana*, 2) *T. chinensis*, 3) the two Japanese species (*T. sieboldii*, *T. diversifolia*) with *T. heterophylla* from western North America, and (4) the eastern North American species, *T. caroliniana* and *T. canadensis*, including its cultivars. *T. mertensiana* and *T. chinensis* reside in distinct clusters and are unique in terpenoid volatiles in comparison to the other specimens analyzed. With respect to the cultivar groupings in the eastern North American cluster, four of the cultivars are weakly separated from the main *T. canadensis* group, three in one group and one by itself.

The dependence of the specimen volatiles on environment was examined through the two cultivars ('Pendula' and 'Bennett') and noncultivar *T. canadensis* analyzed from both arboreta. The noncultivar *T. canadensis* and both provenances of the 'Pendula' cultivar are placed in the same dendrogram grouping, indicating a lack of environmental influence on the volatile terpenoid profile. However, the duplicate 'Bennett' cultivars are placed in separate groupings of close proximity. According to Swartley, 'Bennett' is a confused cultivar and plants arising in seed beds may be indistinguishable from the clone, which originated from a shipment of hemlock from Japan. Thus, although there may be some environmental influence in the 'Bennett' cultivars, it appears to be minimal.

The HCA of the volatile terpenoid fraction supports the notion of segregation both within and between species; however, it provides little insight into chemical differences between the groupings. We therefore used principal component analysis (PCA) to identify compounds associated with the segregation of the cultivars among the species. A PCA was executed on a covariance matrix treating the 25 specimens as variables and the 24 terpenoids as cases. Untransformed terpenoid values produced a two-component solution with segregation similar to the groupings in **Figure 1**. The terpenoid factor scores (**Table 3**) indicate that isobornyl acetate is highly associated with component 1 and α-pinene, germacrene D, and β-phellandrene are highly associated with component 2. This untransformed PCA was dominated by the variance in the major terpenoids (isobornyl acetate and α-pinene), and thus a log transformation of the data set was used to examine the role of the minor terpenoids.

The PCA on the log-transformed data produced three components that accounted for 87.8% of the total variance, with 69.1, 13.1, and 5.6% for components 1, 2, and 3, respectively (**Figure 2**). The most remarkable difference from the PCA of the untransformed data is that *T. caroliniana* is now grouped apart from *T. canadensis* and its cultivars and resides in close proximity to *T. diversifolia*. Four of the cultivars, 'Albo-spica', 'Snowflake', 'Callicoon', and 'Barry's Dwarf', are also grouped in proximity to the Asian and western North American species. The scores of these cultivars in component 1 are low, and a clear resistance/susceptible dichotomy is not apparent because *T. caroliniana*, which is susceptible, is grouped with the Asian and western species. Component 2 shows a strong separation of *T. canadensis* and its cultivars from the other species. Three of the above cultivars have the highest scores in component 3, whereas the scores of the other cultivars are distributed in the range of the species. Although the PCA clearly separates out three to four of the cultivars from the others, the relative susceptibility of these cultivars is less clear.

The factor scores (**Table 3**) identify which of the terpenoids are associated with the components. Germacrene D and sabinene

Table 3. Values of Factor Scores from the Principal Component Analysis Using Untransformed and Log-Transformed Values for the Terpenoids in the 25 Specimens

	untransformed		log transformed		
	component 1	component 2	component 1	component 2	component 3
isobornyl acetate	4.404	−0.131	1.730	0.600	1.233
α-pinene	0.474	3.683	1.234	0.907	0.682
camphene	0.949	−0.304	1.223	0.067	1.122
tricyclene	0.243	−0.482	0.879	−0.072	0.801
α-humulene	−0.121	0.423	−0.095	0.734	0.729
β-caryophyllene	−0.243	0.304	0.049	0.604	0.607
β-phellandrene	−0.628	1.359	0.436	0.846	−0.852
germacrene D	−0.766	1.578	−1.618	0.994	1.026
β-pinene	−0.301	0.229	0.399	0.455	0.005
limonene	−0.061	−0.526	0.612	−0.135	0.266
δ-cadinene	−0.423	0.366	−0.373	0.975	−0.099
myrcene	−0.144	−0.456	0.015	0.445	0.220
α-phellandrene	−0.332	0.069	0.534	0.612	−1.425
piperitone	−0.062	−0.702	1.689	−2.532	−0.496
cis-ocimene	−0.127	−0.621	0.565	−0.141	−0.355
γ-cadinene	−0.376	−0.125	−0.660	0.802	−0.284
γ-muurolene	−0.399	−0.181	−0.955	0.581	−0.383
o-cymene	−0.287	−0.615	0.206	−0.369	−2.074
borneol	−0.258	−0.703	−0.116	−1.048	−1.140
terpinolene	−0.296	−0.641	−0.473	−0.483	−0.343
camphor	−0.286	−0.658	−1.039	−2.842	1.928
sabinene	−0.313	−0.595	−2.007	−0.221	1.152
viridiflorene	−0.325	−0.616	−1.242	−0.124	−0.820
γ-terpinene	−0.320	−0.657	−0.995	−0.655	−1.498

had the largest negative loadings for component 1. The cultivars 'Albo-spica', 'Snowflake', and 'Barry's Dwarf' have elevated levels of germacrene D and in this respect are similar to the resistant species. As observed in our previous studies, isobornyl acetate, which has a high, positive factor loading, appears to be associated with the susceptible species. Although there is a clear pattern for isobornyl acetate, which has elevated levels in *T. caroliniana* and *T. canadensis* and its cultivars and lower levels in the resistant species, this terpenoid is not especially low in the three identified cultivars. Whereas component 2 did not appear to define resistance, piperitone had a high, negative factor score for component 2 and is extremely low in the species (<0.04%), being >1% for *T. canadensis* and most of its cultivars with the exceptions being the above three cultivars.

Considering whether a compound is a monoterpene or sesquiterpene and the factor scores of component 1, the predominant monoterpenes (isobornyl acetate, α-pinene, camphene) that are associated with susceptibility have high positive factor scores, whereas the predominant sesquiterpenes that are associated with resistance have low or negative factor scores (germacrene D, β-caryophyllene, α-humulene). Camphor and sabinene are examples of monoterpenes that appear to have high PCA loadings due to a high variability among the samples, and meaningful associations with either species or resistance is not apparent.

DISCUSSION

Using statistical procedures, the 13 eastern hemlock cultivars were placed within the greater context of inter- and intraspecies association on the basis of the measured *Tsuga* terpenoid profiles. In one scenario, a single terpenoid, isobornyl acetate, appears to be correlated to resistance to the HWA. In another scenario, hemlock phylogeny seems to be related to a complex group of several terpenoids. Geographic isolation from the HWA may have resulted in the two phylogenetically, unrelated species in eastern North America developing a secondary chemical composition that affords protection from endemic foliage-chewing herbivores (Lepidoptera), but left it to vulnerable to sucking herbivores (Hemiptera). Among the cultivars, which

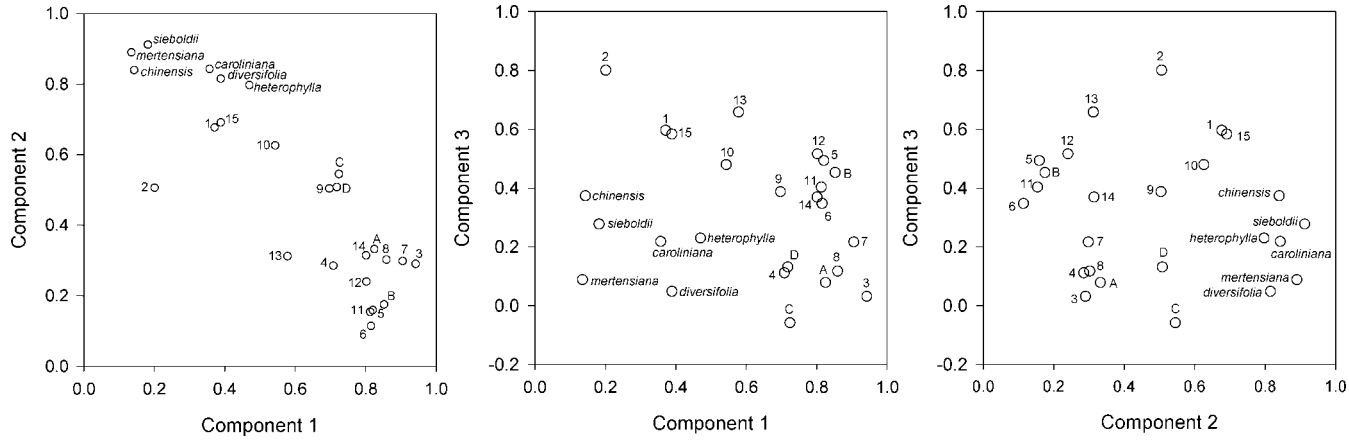

Figure 2. Rotated component loadings from the principal component analysis for the log-transformed terpenoid data with *T. canadensis* and its cultivars labeled using codes listed in **Table 1**.

are artificially selected on the basis of morphological peculiarities rather than by reaction to natural stressors, there may be a regression to the volatile composition observed in the western North American and Asian species, which have coevolved with the HWA.

The groupings based on the HCA conform to the accepted systematic separation of the genus into two sections, with *T. mertensiana* by itself in section Micropeuce and the section Hesperopeuce including all other species of *Tsuga*. *Tsuga mertensiana* is very different from the other species in terpenoid composition, with the highest or lowest levels of 9 of the 25 terpenoids measured. Recognizing this, we removed it from the analysis, but found that the associations among the remaining specimens were minimally altered. With the exception of the distant separation of *T. mertensiana* from the other western North American species, *T. heterophylla*, the hemlocks co-occurring in the same region are placed in close proximity to each other by the HCA on untransformed values. A recent molecular phylogenetic study indicates that *T. caroliniana* is more closely related to the Asian species, and in particular *T. diversifolia*, than to *T. canadensis*, whereas *T. canadensis* is not closely related to any species (*8*). This is the placement of the species using the log-transformed PCA. Thus, the relatively high levels of isobornyl acetate in *T. caroliniana* and *T. canadensis* appear to reflect biogeography more than phylogeny.

HWA is a recent introduction to eastern North American, but recent genetic studies suggest that it has been present on all the other hemlock species for some time (*20*). Our data suggest that the eastern North American hemlocks, in the absence of HWA, synthesize a complex mixture of terpenoids dominated by isobornyl acetate. We suggest that hemlocks in eastern North America have adapted their terpenoid chemistry for protection against defoliators, and this has made them vulnerable to non-native sucking pests such as adelgids and scale insects. In eastern North America, pressure on hemlock from native herbivores is primarily from several Lepidoptera defoliators, especially the hemlock looper, *Lambdina fiscellaria* (Guenee). As far as we know, terpenoids have not been extensively examined in eastern hemlock in relation to looper defoliation; however, relationships between defoliators and foliar terpenoids have been extensively examined for other members of the Pinaceae. Douglas fir [*Pseudostsuga menziesii* (Mirb.) Franco], which is resistant to the western spruce budworm (*Choristoneura occidentalis* Freeman), had higher levels of camphene and bornyl acetate, whereas susceptible trees had higher levels of β-pinene and an unidentified terpene (*21*). An earlier study reported that high foliar levels of camphene and bornyl acetate were highly toxic to the budworm (*22*). The latter paper concluded that terpene production was genetically controlled and that host races of the budworm evolve in response to local variation. Fraser fir [*Abies fraseri* (Pursh) Poir] provides another example of a possible link between volatile terpenoids and host resistance to a hemipteran insect. The monoterpenes 3-carene and β-pinene as well as high levels of sesquiterpenes were linked to resistance to the balsam woolly adelgid (*23*). This adelgid, unlike HWA, feeds on the trunk of the tree or at the shoot internodes.

There are two other hemipteran insects in addition to HWA that have been introduced from Japan that are pests of hemlocks native to eastern North America, *Fiorinia externa* Ferris and *Nuculaspis tsugae* (Marlatt). The fecundity of these scale insects on *T. canadensis* and *T. sieboldii* was negatively related to relative concentration of α-phellandrene and positively to limonene and myrcene (*24*). In that study, current-year needles, <2 months old, were examined, whereas we examined previous-year's growth, which is >10 months old. In our analysis, these same three terpenoids seem to be more related to species than to resistance. In Japan, where these two scale insects and HWA are native, *T. sieboldii* may be using different sets of terpenoids in young and old foliage to ward off the scale insects, which prefer young tissue, whereas HWA prefers mature tissue. Our previous study of temporal and spatial variations in terpenoid production in *T. canadensis* found that germacrene D is a highly variable compound in the current-year's growth leaf cushion, and its production may be correlated with the settling period of the HWA sistens generation. Additionally, limonene and α-phellandrene are much higher in immature foliage than in mature hemlock needles (*9*).

There is some evidence of segregation according to the Swartley groups in the PCA, particularly in component 3. Plots of component 3 versus either component 1 or 2 provide some degree of clustering with respect to the five Swartley groups in **Table 1**. Unlike PCA, the observed HCA groupings do not conform to groupings based upon the peculiar morphological characteristics of the cultivars, with the exception of isolating 'Beaujean'. This is the only cultivar we tested that Swartley (*11*) indicates is a witches' broom, an abnormal congested growth caused by the development of the adventitious buds present at the internode. Normally, the adventitious buds remain dormant, but if the growing buds are lost because of late frost, drought, or animal feeding, they are stimulated to break dormancy. We (M.E.M.) have observed development of adventitious buds later in the year following the normal spring bud flush in severely infested eastern hemlocks and a witches' broom as the only remaining lush foliage on a large hemlock tree in severe decline from HWA infestation. 'Beaujean' is distinguished from the other *T. canadensis* specimens in much higher levels of α-humulene and β-carophyllene, which are characteristic of the resistant Asian species. Additionally, the HCA groups the cultivar growth characteristic of slow growth or dwarfism. The cultivars 'Bennett', 'Callicoon', 'Barry's Dwarf', 'Gable Weeping', and 'Beaujean' are dwarf. Both witches' broom and dwarfism are derived from genetic bud mutations.

In conclusion, the minor terpenoids appear to be strongly linked to speciation in *Tsuga*, whereas the major terpenoids in *T. canadensis* and *T. caroliniana* are associated with the susceptibility of these species to HWA. The composition of minor components in some cultivars is similar to the resistant species and provides a rationale to evaluate enhanced resistance among cultivars of *T. canadensis*. Unfortunately, the cultivars are present in ornamental settings, and although we can artificially challenge these with the adelgid, we have found that we cannot be assured that these are free of residual effects of pesticides. We can, however, use the chemical analysis to screen living plant collections to select specimens with suspected resistance and culture these in a common, pesticide-free environment for bioassay. For example, a trial with only the cultivars 'Albo-spica', 'Callicoon', 'Pendula', and 'Bennett' would permit comparison of the effects of the high and low levels of isobornyl acetate, germacrene D, piperitone, and limonene on the hemlock woolly adelgid.

ACKNOWLEDGMENT

We acknowledge Casey Sclar (Longwood Gardens) and Tony Aiello (Morris Arboretum) for their assistance in providing the *Tsuga* samples used in this work.

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Received for review July 1, 2007. Revised manuscript received October 20, 2007. Accepted October 21, 2007. F.C.C. acknowledges Villanova University for an Undergraduate Research Award. This work was funded by the USDA, U.S. Forest Service (05-JV-11242328-096).

JF071947O