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Analysis of Terpenoids from Hemlock (*Tsuga*) Species by Solid-Phase Microextraction/Gas Chromatography/Ion-Trap Mass Spectrometry

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A sampling method for determining the volatile terpenoid composition from single needles of seven *Tsuga* species was developed using headspace solid-phase microextraction (SPME). A reproducible sampling method for the volatile components was generated by examination of sample storage, method of needle cutting, and headspace sampling duration. Following SPME collection of the volatile compounds from the seven *Tsuga* species, gas chromatography/ion-trap mass spectrometry was used to identify 51 terpenoids present in the needle headspace. A semiquantitative method was devised to express individual terpenoid amounts as a percentage of all of the identified peaks in the chromatogram. The semiquantitative results permitted facile interspecies comparison using principal component analysis. Two components were able to account for 90% of the variance and were interpreted as a "species" component and a "resistance/susceptibility" component. Three interspecies groupings were evident from the principal component analysis: (1) *Tsuga canadensis* and *Tsuga caroliniana*; (2) *Tsuga chinesnsis*, *Tsuga diversifolia*, *Tsuga heterophylla*, and *Tsuga sieboldii*; and (3) *Tsuga mertensiana*. The finding that *T. mertensiana* was grouped alone and far removed from the other species adds to the morphological evidence that this species should be segregated from other *Tsuga*.

KEYWORDS: ***Adelges tsugae*; chemosystematics; terpenoids; GC-MS; hemlock; hemlock woolly adelgid; SPME; *Tsuga canadensis*; *Tsuga caroliniana*; *Tsuga diversifolia*; *Tsuga chinensis*; *Tsuga heterophylla*; *Tsuga mertensiana*; *Tsuga sieboldii***

INTRODUCTION

The genus *Tsuga* (hemlock trees) consists of nine species, two in eastern North America, two in western North America, and five in Asia (*1*). Phylogenetic relationships between species in the genus have been based on morphological and anatomical characters (*2*), geography (*3*), and molecular markers (*4*), but there is no general agreement on phylogeny. We wish to identify relationships between species in the context of resistance/susceptibility of *Tsuga* species to the hemlock woolly adelgid (*Adelges tsugae* Annand). The Asian and western North American hemlock species are considered to be resistant to the hemlock woolly adelgid, and the eastern North American species are very susceptible, resulting in eventual tree death (*5*).

Volatile terpenoids are abundant and diverse in conifers and play a complex, vital role in relationships between plants and insects. Signals for sexual reproduction (pheromones, kariomones), for defense against herbivores (allomones), or to attract natural predators of herbivores (synomones) are conveyed through volatile terpenoids (*6*). Identifying these chemical signals and their function may suggest alternative methods to enhance resistance of plants to insect attacks. For instance, in *Tsuga*, the foliar terpenoids in *Tsuga canadensis* (L.) Carriere and *Tsuga sieboldii* Carriere were measured and related to the reproductive success of two scale insects, *Fiorinia externa* (Marlatt) and *Nuculapsis tsuga* Ferris (*7*).

A wide variety of analytical methods are used to extract terpenoids from plant material (*8*, *9*). These methods generally include a maceration or homogenization of the plant material to increase access to the essential oils in the resin canals of the plant. Techniques commonly used to extract the oils include steam distillation, Soxhlet extraction (*10*), and supercritical fluid extraction (*11*). Once isolated, the essential oil components are separated and identified using a suitable chromatographic method. Typically, gas chromatography−mass spectrometry (GC-MS) is chosen, largely due to the ability of GC-MS to identify the terpenoids through retention index matching and provide confirmation through comparison to library mass spectra.

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Table 1. Terpenoid Composition (Area Percent) from Individual Needles ($n = 3$) in the Seven Species of *Tsuga*

		m/z	*T. caroliniana*	*T. canadensis*	*T. chinensis*	*T. diversifolia*	*T. heterophylla*	*T. mertensiana*	*T. sieboldii*
1	tricyclene	93	1.85 ± 0.20	4.32 ± 0.37	0.77 ± 0.06	1.93 ± 0.12	3.24 ± 0.03	0.56 ± 0.09	2.03 ± 0.14
2	α-pinene	93	10.07 ± 0.57	13.19 ± 0.55	18.74 ± 1.67	17.47 ± 0.67	18.61 ± 0.46	26.62 ± 0.53	20.03 ± 2.35
3	camphene	93	5.25 ± 0.36	7.79 ± 0.76	2.12 ± 0.15	5.37 ± 0.23	8.39 ± 0.24	0.46 ± 0.07	4.84 ± 0.33
4	sabinene	93	0.41 ± 0.02	0.15 ± 0.05	1.46 ± 0.10		*[b]	0.40 ± 0.06	0.13 ± 0.02
5	β-pinene	93	1.41 ± 0.10	2.44 ± 0.06	1.71 ± 0.09	2.07 ± 0.07	1.77 ± 0.05	7.03 ± 0.48	4.47 ± 0.58
6	myrcene	93	8.26 ± 0.76	1.65 ± 0.56	0.62 ± 0.07	3.65 ± 0.38	2.62 ± 0.46	1.54 ± 0.26	0.90 ± 0.14
7	α-phellandrene	91	4.31 ± 0.29	1.45 ± 0.58	0.91 ± 0.14	2.22 ± 0.27	0.52 ± 0.13	7.26 ± 0.16	1.51 ± 0.21
8	α-terpinene	121	0.27 ± 0.01	*	*	0.11 ± 0.01	*	*	*
9	*o*-cymene	119	0.58 ± 0.06	1.63 ± 0.19	*	0.41 ± 0.10	0.10 ± 0.03	0.51 ± 0.08	*
10	limonene	67	0.85 ± 0.16	1.96 ± 0.18	0.98 ± 0.20	1.53 ± 0.17	1.79 ± 0.30	0.85 ± 0.06	0.63 ± 0.09
11	β-phellandrene	3	4.14 ± 0.07	3.06 ± 0.64	1.75 ± 0.23	7.21 ± 0.55	3.42 ± 0.35	19.85 ± 0.24	2.18 ± 0.30
12	*cis*-ocimene	93	3.62 ± 0.17	1.91 ± 0.37	0.57 ± 0.03	0.67 ± 0.10	1.00 ± 0.27	0.54 ± 0.05	0.21 ± 0.03
13	*trans*-ocimene	93			2.00 ± 0.14	*	*	0.62 ± 0.09	*
14	γ-terpinene	93	*	0.18 ± 0.05	*	0.28 ± 0.03	0.13 ± 0.02	*	*
15	terpinolene	93	0.27 ± 0.05	0.13 ± 0.01	*	0.27 ± 0.03	0.17 ± 0.05	0.27 ± 0.04	0.12 ± 0.02
16	linalool	71	0.16 ± 0.02	*			*		
17	*cis*-*p*-menth-2-en-1-ol	93	*	*	*	*		*	*
18	*trans*-*p*-menth-2-en-1-ol	93	*	*		*	*		*
19	borneol	95	*	2.99 ± 0.38	*		*	*	*
20	ethyl octonoate	88	0.49 ± 0.09						
21	*trans*-piperitol	91		*					*
22	piperitone	82	*	3.56 ± 0.18			*		
23	isobornyl acetate	95	38.88 ± 1.57	42.86 ± 1.28	9.52 ± 1.89	18.98 ± 1.45	28.40 ± 1.13	3.24 ± 0.46	21.37 ± 2.40
24	sabinyl acetate	91	*	0.18 ± 0.03	*			*	*
25	δ-elemene	121	0.81 ± 0.14		*	*	*	*	*
26	α-cubebene	161	*	0.12 ± 0.01	0.83 ± 0.23	0.64 ± 0.01	0.46 ± 0.03	*	1.25 ± 0.36
27	citronellyl acetate	67	0.13 ± 0.13		*	1.23 ± 0.24	0.49 ± 0.05		*
28	neryl acetate	69	*		*		*	*	
29	α-ylangene	105		*	0.56 ± 0.11	0.16 ± 0.01	0.16 ± 0.01	*	0.34 ± 0.04
30	α-copaene	161	*	0.19 ± 0.04	1.57 ± 0.29	1.09 ± 0.04	0.69 ± 0.04	0.20 ± 0.02	2.12 ± 0.15
31	geranyl acetate	69			*	0.26 ± 0.05	*	1.11 ± 0.27	
32	β-bourbonene	81		*	*		*	0.12 ± 0.01	0.13 ± 0.04
33	β-elemene	67	1.81 ± 0.11	*	*	*	*	0.33 ± 0.03	0.23 ± 0.01
34	longifolene	91	*	*		0.31 ± 0.02	0.31 ± 0.02		0.21 ± 0.02
35	β-caryophyllene	91	1.77 ± 0.34	1.37 ± 0.12	13.32 ± 0.48	7.07 ± 0.07	6.09 ± 0.53	0.79 ± 0.06	6.01 ± 0.27
36	β-gurjunene	161	*	0.12 ± 0.01	1.39 ± 0.24	0.31 ± 0.02	0.46 ± 0.03	0.27 ± 0.03	0.73 ± 0.09
37	*Z*-*trans*-α-bergamotene	119		*		*		*	0.32 ± 0.01
38	α-humulene	93	3.84 ± 0.60	3.26 ± 0.47	10.79 ± 0.32	12.27 ± 0.10	12.32 ± 1.24	0.60 ± 0.05	6.06 ± 0.30
39	γ-muurolene	161	0.23 ± 0.05	0.70 ± 0.11	7.68 ± 1.37	1.25 ± 0.06	2.06 ± 0.08	0.61 ± 0.08	2.38 ± 0.39
40	germacrene D	161	4.01 ± 0.12	0.66 ± 0.29	4.58 ± 0.23	0.21 ± 0.03	0.46 ± 0.15	21.65 ± 0.98	10.58 ± 0.20
41	β-selinene	105	0.11 ± 0.04	0.25 ± 0.07	0.23 ± 0.01	0.29 ± 0.02	0.15 ± 0.01	*	0.49 ± 0.02
42	viridiflorene	189	0.14 ± 0.01	0.48 ± 0.07	*	0.58 ± 0.04	0.29 ± 0.01	0.10 ± 0.01	0.90 ± 0.04
43	α-farnesene	93	0.27 ± 0.07	*			*	*	0.21 ± 0.04
44	β-bisabolene	67	0.78 ± 0.09	*	*	*	*	0.12 ± 0.01	0.13 ± 0.01
45	*cis*-γ-bisabolene	119	0.31 ± 0.04	*	*			0.05	
46	γ-cadinene	161	1.18 ± 0.02	2.17 ± 0.53	5.56 ± 0.79	4.66 ± 0.10	2.20 ± 0.06	1.30 ± 0.17	2.76 ± 0.37
47	δ-cadinene	161	2.28 ± 0.15	3.23 ± 0.79	9.39 ± 1.36	7.19 ± 0.27	3.12 ± 0.06	2.39 ± 0.51	8.17 ± 0.13
48	*E*-γ-bisabolene	107	*	*		*			*
49	germacrene D-4-ol	81	*		*				
50	τ-cadinol	161	0.15 ± 0.04	*	*	*	*	*	*
51	α-cadinol	121	*		*				*

[a] *m/z* fragment values listed were used for single-ion quantification of a given compound. [b] Compound was present at <0.10%.

Solid-phase microextraction (SPME) with subsequent analysis using GC-MS has emerged as a powerful, solvent-free method to analyze volatile compounds present in the plant headspace (*12*−*19*). In SPME, plant volatiles are concentrated onto a coated fiber and subsequently desorbed into the heated GC injection port for analysis. The quantity of a given compound adsorbed onto the fiber depends on both the partitioning of the compound into the headspace from the plant matrix and the partitioning of the compound into the fiber coating from the headspace. In plant headspace sampling, these two factors are controlled by the sample homogenization technique, the sampling duration, the extraction temperature, and the chemical nature of the fiber coating (*20*).

Comparative studies on seven *Tsuga* species [*T. caroliniana* Engelm., *T. canadensis*, *T. chinensis* (Franch.) E. Pritz., *T. diversifolia* (Maxim.) Mast., *T. heterophylla* (Raf.) Sarg., *T. mertensiana* (Bong.) Carriere, and *T. sieboldii*] were carried out to determine the relative levels of terpenoids in a given species. The objective of the study was to identify the similarities and differences in the terpenoid levels of the seven *Tsuga* species that could ultimately propose a qualitative relationship between terpenoid level and hemlock woolly adelgid resistance.

MATERIALS AND METHODS

Plant Material. In mid-April 2002, *T. caroliniana*, *T. canadensis*, *T. chinensis*, *T. sieboldii*, and *T. diversifolia* were obtained from the U.S. National Arboretum (Washington, DC). Additionally, during mid-April 2002, samples of *T. mertensiana* and *T. heterophylla* were obtained from Longwood Gardens (Kennett Square, PA) and the University of Rhode Island (Kingston, RI), respectively. Two of the world's nine *Tsuga* species, *T. dumosa* (D. Don) Eichler and *T. forrestii* Downie, both native to China, were not available. Samples were obtained by clipping foliage from healthy, uninfested or lightly infested trees of each species. The clippings were immediately placed in

polyethylene bags and shipped overnight with ice packs in insulated containers to Pennsylvania State University, where they were stored at −20 °C until analysis.

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 species, three nonadjacent, individual needles representing randomized locations on the previous-year growth segment of the branch were analyzed. In all cases, feeding by a hemlock woolly adelgid was absent from the selected needle. Needles were selected to represent the previous year's growth rather than new growth. Each needle was manually removed from the branch and allowed to reach ambient temperature. The needles were manually cut using stainless steel scissors. Cuts were made perpendicular to the long axis of the needle. Each needle was cut ~15 times per centimeter length of the needle. Clippings of each needle were collected directly into three 4 mL screw-top vials and capped with PTFE/silicone septa (VWR Scientific, Pittsburgh, PA). The sample vials were placed in a water-jacketed beaker maintained at 50 °C by a Haake model FJ circulating water bath. 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 water-jacketed beaker 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. Separation was accomplished using a Varian CP-Sil 8 CB column (30 m, 0.26 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 electronically 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; multiplier, 1400 V; *m*/*z* range, 45−400).

Processing of Results. 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 (*21*). Compounds in **Table 1** are labeled as tentative, with the exception of α-pinene, camphene, β-pinene, α-phellandrene, *o*-cymene, limonene, β-phellandrene, terpinolene, borneol, piperitone, isoborneal acetate, β-caryophyllene, and α-humulene. Authentic samples (Aldrich, Milwaukee, WI) for these compounds were compared to experimental retention indices and mass spectra. The area under an identified peak was integrated using a single *m*/*z* fragment from the total-ion spectrum for each compound. The *m*/*z* fragment was the most intense ion in the mass spectrum and is listed in **Table 1**. Relative quantity (area percent) is calculated by the ratio of the peak area for an individual compound relative to the total peak area for all identified compounds in a chromatogram.

RESULTS AND DISCUSSION

Analytical Method. The single-needle SPME method reported was found to be the most reproducible method to chop, reduce volatile losses, and ensure all portions of the needle length were sampled reproducibly. For instance, when an electric chopper was used on 5 g of needles, the frequency and the direction of the cuts along the midrib of an individual needle were random and irreproducible. This led to nonuniform access to the resin canals in the midrib of the needle and irreproducible sampling of the needle volatiles. Manually cutting a single needle into <1 mm lengths produced the most reproducible concentration of volatiles for SPME headspace sampling (average relative standard deviation of all compounds was <10%). This is presumably because access to the resin canals in the midrib of the needle is maximized by uniform, small sectioning of the needle along the long axis of the needle. Headspace SPME exposure times of 1, 3, 5, 7, 10, 15, 20, and 30 min were investigated. Although the highly volatile terpenoids, such as α-pinene, reached a gas-fiber partition equilibrium in 1 min, 15 min was necessary for the less volatile terpenoids, such as β-caryophyllene, to achieve equilibrium.

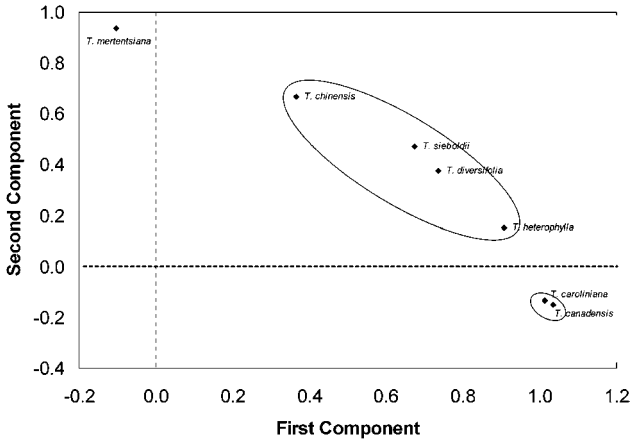

Figure 1. Rotated score plot of the first two components of the PCA for the terpenoid composition data in **Table 1**.

We also measured the α-pinene content in a single needle of *T. canadensis* relative to the mass of the needle. The results concurred with the findings of Schäfer et al. (*18*), who observed that the α-pinene levels for 15 individual needles of Macedonian pine deviated greatly. However, our results showed that *T. canadensis* α-pinene levels were reproducible (relative standard deviation = 0.05, $n = 7$) when expressed as the peak area of α-pinene relative to the total peak area of all identified compounds. This indicates that either the absolute amount of terpenes varies significantly from needle to needle or the partitioning of the terpenoids between the plant matrix and the headspace during the sample cutting procedure is irreproducible. Regardless of the correct interpretation, the relative percentage of a single terpene remains virtually constant and can form the basis for interspecies comparison using the SPME method.

To establish the effect of sample transport and storage on the terpenoid composition, a branch was removed from a hemlock on the Pennsylvania State University campus. Three needles were analyzed immediately, and three needles were analyzed after storage overnight in the freezer at −20 °C to simulate shipment. Within statistical uncertainty, no differences in the relative terpenoid composition were observed between the needles analyzed immediately and those stored overnight. Furthermore, volatile, nonterpenoid artifacts, such as hex-2-en-1-al, that are indicative of wounding responses were never observed in any single needle samples that were analyzed. These findings concur with the findings of von Rudloff that cold storage of foliage in the dark is sufficient for accurate chemical analysis of volatiles in conifers (*22*). Interestingly, von Rudloff observed little or no change in terpene composition in conifer foliage sampled in Canada from the fall through the winter.

SPME/GC-MS terpenoid levels for an individual *Tsuga* species are presented as peak area percentages. The reported percentage is relative to the peak area of all identified peaks in the chromatogram using a single-ion for quantitation. For the purpose of interspecies comparison, this semiquantitative method is sufficient. To quantitatively determine the headspace concentrations of a given terpenoid, response factors for the individual terpenoids using external calibration standards must be determined. Additionally, the fiber/gas partition coefficients of the individual terpenes must be determined, which can be approximated from the terpenoid linear temperature-programmed

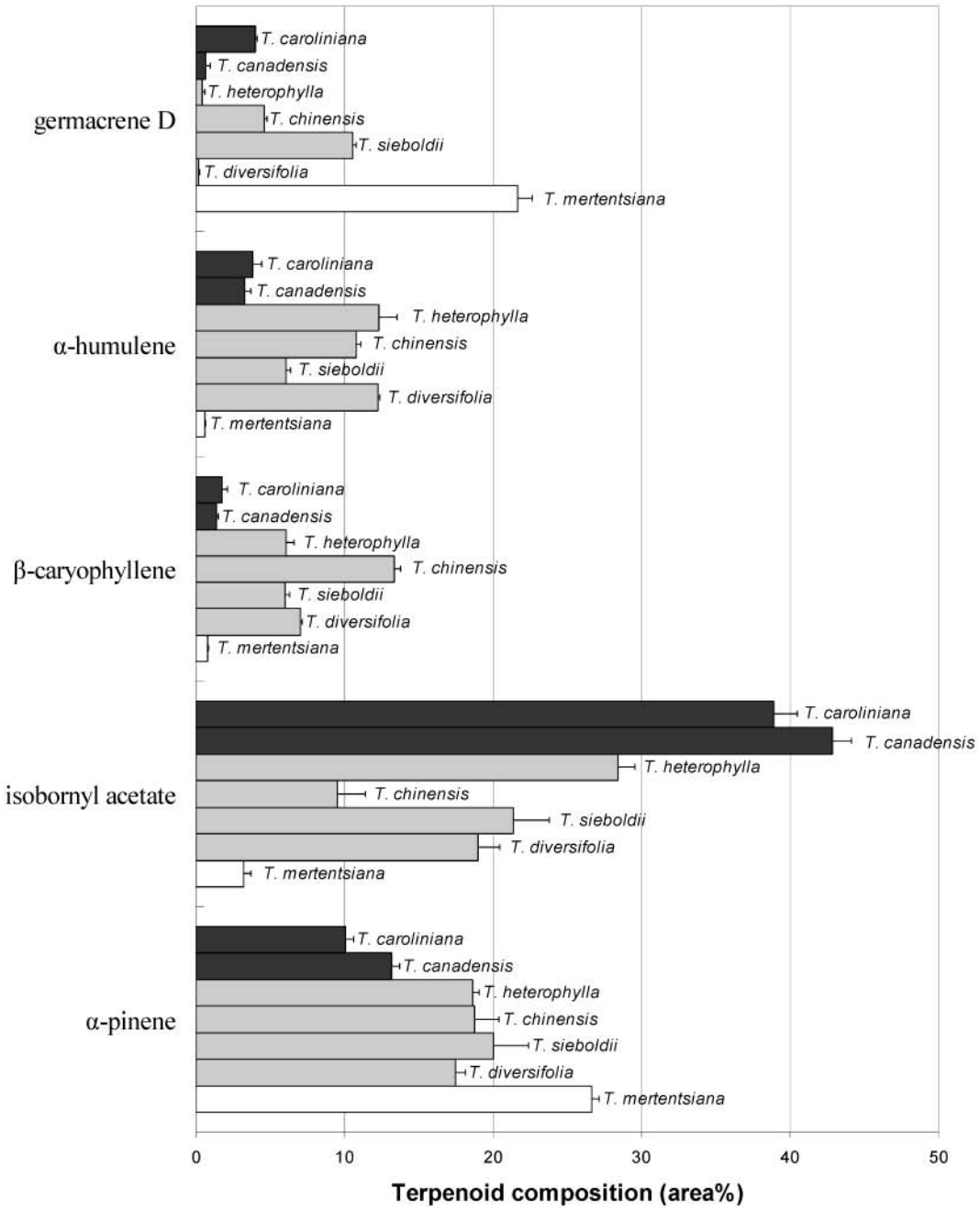

Figure 2. Relative terpenoid content (area percent) of the five terpenoids with high principal component loadings. Shadings are intended to represent the three groupings from the PCA (black = hemlock woolly adelgid susceptible, gray = hemlock woolly adelgid resistant, white = *T. mertentsiana*).

retention index (LTPRI) using a chromatographic column that is representative of the SPME fiber coating (*23*). Given that the fragmentation pattern, response factors, and fiber/gas partition coefficient are constant for a single terpenoid, reproducible, semiquantitative, interspecies comparison of an individual terpenoid is possible through this volatile component "fingerprint". The major limitation of the semiquantitative method is that the terpenoid compositions specified are not relative to terpene absolute amounts but rather to relative terpene peak areas in the chromatogram. Although this precludes comparisons of absolute compound amounts, it provides terpenoid profiles that are expecially useful in defining relationships between species.

Biological Implications. Analysis of the seven species of *Tsuga* resulted in the identification of 51 terpenoids with a match in both retention index ($r^2 = 0.9998$, $n = 51$) and MS library spectrum. These terpenoids are identified in **Table 1** along with the relative percentages found in each of the seven species of *Tsuga* analyzed. A principal component analysis (PCA) was computed from a correlation matrix of the measured terpenoid compositions for each species. The matrix treated the 7 *Tsuga* species as variables and the 51 terpenoids as subjects in an R-technique PCA. Two principal components were identified, accounting for 75.3 and 14.9% of the variance, respectively. **Figure 1** is a rotated score plot using an oblique rotation ($\delta = 0$, Kaiser normalization) of the first and second principal components. The first principal component can be interpreted as an interspecies separation based on the dominant terpenoids present in *Tsuga*. It is evident that the group means lie in three distinct clusters. One cluster represents the eastern North American species, *T. canadensis* and *T. caroliniana*. Another cluster consists of *T. heterophylla* and the Asian species. Interestingly, *T. mertensiana* is grouped alone, having a negative first principal component score, well separated from the other groupings. This grouping conforms to the usual systematic

separation of the genus into two sections with eight species in Section *Tsuga* and *T. mertensiana* by itself in Section *Hesperopeuce*. This taxonomic separation is based on *T. mertensiana* having stomata on both faces of the needle and relatively large female cones compared to the other *Tsuga* species, which have stomata only on the underside of the needle and cones <4 cm long. The most noticeable difference in the terpenoid composition of *T. mertensiana* and other *Tsuga* species is that the dominant peaks in the chromatogram are α-pinene and germacrene D, rather than isobornyl acetate. *T. mertensiana* has been classified in a separate genus (*24*), although this classification is not universally accepted on the basis of the morphology of reproductive and vegetative parts (*1*, *25*) and molecular phylogenetics (*4*). From PCA of the seed fatty acid compositions, *T. mertensiana* has little in common with the Abietoids (*Abies*, *Cedrus*, *Keteleeria*, *Pseudolarix*, and *Tsuga*) and more in common with the Pinoids (*Pinus*, *Larix*, *Picea*, and *Pseudotsuga*) (*26*). The PCA results of **Figure 1** further substantiate the notion that *T. mertensiana* may be a result of either evolutionary convergence within the Pinoids or the hybridization of *Tsuga* and *Picea* (*2*).

A tentative interpretation of the second principal component is a separation corresponding to the resistance/susceptibility of the species to the hemlock woolly adelgid, *Adelges tsugae*. Second-component scores that are negative (the two eastern North American species) indicate susceptiblility, and positive scores (the Asian and western North American species) indicate resistance to the hemlock woolly adelgid. Adelgids and their close relatives, aphids, are known to have a limited tolerance of monoterpenes. High levels of santalene and camphor in red spruce (*Picea rubens* Sarg.) inhibit colonization by the adelgid *Pineus floccus* (Patch) (*27*), high concentrations of limonene and myrcene in Douglas fir deter *Adelges cooleyi* (Gillette) (*28*), and high concentrations of myrcene and piperitone in Sitka spruce [*Picea sitchensis* (Bong. Carr.)] deter several species of aphids (*29*). The levels of five illustrative terpenoids with high first and second principal component loadings are shown in **Figure 2**. These terpenoids are candidate deterrents and attractants for the hemlock woolly adelgid. For instance, it is possible that elevated levels of α-pinene, *β*-caryophyllene, or α-humulene may function as deterrents for hemlock woolly adelgid feeding, whereas elevated levels of isobornyl acetate may function as hemlock woolly adelgid attractants. A more detailed study of hemlock woolly adelgid fecundity and population levels on these species will elucidate which are predictors of susceptibility/resistance to the hemlock woolly adelgid.

SPME/GC-MS was extremely efficient in time, expense, and accuracy in determining the composition of terpenes in different hemlock species. Such information can be used to assess phylogenetic relationships. The relationships among the terpenoids of the *Tsuga* analyzed herein conform to natural classifications based on morphology. An important, practical use of SPME terpenoid analysis is to provide a basis to engineer pest-resistant crops. Future experiments are planned to elucidate the role of terpenoids in suitability of *Tsuga* species, and perhaps species hybrids, as hosts for the hemlock woolly adelgid as well as the effects of environmental factors on terpenoid/hemlock/pest relationships.

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