

Intraspecific Variation in *Tsuga canadensis* Foliar Chemistry

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Abstract - Three groups of *Tsuga canadensis* (Eastern Hemlock) trees were analyzed to compare their chemical composition and the potential for naturally occurring resistance to *Adelges tsugae* (Hemlock Woolly Adelgid [HWA]). Potentially resistant “parent” trees located in southern Connecticut were compared with rooted propagules from those same trees and control trees located in northern Vermont, outside of the current HWA range. For trees in each group, we quantified Ca, P, K, C, and N and developed terpenoid profiles using solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC/MS). There was no significant variation in terpenoid profiles between the three groups of hemlock trees. Propagules retained elevated levels of Ca and N from fertilization during propagation, suggesting that their chemical composition does not mirror the parent trees. The potentially resistant “parent” trees had higher levels of K compared to control trees. This may impart some level of tolerance/resistance to HWA and explain their persistence in hemlock forests that have otherwise been decimated by HWA. Comparison to regional foliar chemistry databases suggest that while rare, such elevated K levels do exist in natural hemlock populations. Such individuals may persist as HWA continues to spread across the region.

Introduction

The invasive *Adelges tsugae* Annand (Hemlock Woolly Adelgid [HWA]) is a major threat to *Tsuga canadensis* (L.) Carr. (Eastern Hemlock) and *Tsuga caroliniana* Engelm. (Carolina Hemlock), leading to massive mortality within hemlock forests throughout the eastern United States. Since its introduction from Asia to Virginia in the 1950s, HWA has spread north and east across the United States. It is thought to be limited in the northern portion of its range by colder climates (Parker et al. 1999). There are nine species of *Tsuga* occurring worldwide: the two eastern US species mentioned above, two that occur in western North America, and five Asian species (Farjon 1990). Of these nine species, mortality resulting from HWA infestation appears to occur primarily in Eastern and Carolina Hemlock (McClure 1992, McClure et al. 2001).

The mechanistic basis for hemlock resistance to adelgid infestations has not yet been identified. However, HWA induces tree mortality only after a prolonged period of heavy infestation, depending on geographic locality.

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HWA uses a long stylet bundle to feed on ray parenchyma cells, depleting stored nutrients and resulting in reduced growth, needle loss, and, ultimately, mortality (McClure 1991, Young et al. 1995). In its native range, HWA occurs in very low densities and appears to have little detrimental effect on infested hemlocks (McClure 1999, McClure and Cheah 1999). One explanation for the low densities and minimal effects of HWA on hemlocks in their native range may be that the native hemlock species possess chemical defenses that limit HWA infestation and subsequent damage.

Several studies have examined the foliar chemistry of both HWA-susceptible and -resistant hemlock species. Many studies have focused on terpenoids, foliar chemicals that are an important nutritional element for herbivores (e.g., McClure and Hare 1984). Terpenes vary greatly within and among a plant species and are commonly used to characterize resistance or tolerance of host species. Lagalante and Montgomery (2003) characterized the terpenoid profiles of seven *Tsuga* species: Eastern Hemlock, Carolina Hemlock, and five others that are known to be resistant to HWA. Their research identified five key terpenes (germacrene D, α -humulene, β -caryophyllene, isobornyl acetate, and α -pinene) whose concentrations differed markedly in susceptible versus resistant hemlock species. They suggest that these terpenes may play a role in determining the degree of hemlock susceptibility/resistance to HWA infestation. Lagalante et al. (2006) also documented seasonal and spatial variation in Eastern Hemlock terpenoids. HWA aestivation coincided with periods of high terpenoid content, suggesting that adelgids may avoid increased concentrations of these chemical compounds. In related research, Pontius et al. (2002) examined the foliar chemistry of Eastern Hemlock and four HWA-resistant hemlock species and identified four cations (potassium, calcium, phosphorus, and nitrogen) that may play a role in hemlock susceptibility to HWA infestations; their study also linked HWA-infestation densities and decline rates to the foliar cations measured.

While there is no published evidence of HWA resistance and/or tolerance in either Eastern or Carolina Hemlocks, a few healthy looking Eastern Hemlock trees persisting amidst devastated stands have been found during landscape-level surveys of HWA and *Fiornia externa* Ferris (Elongate Hemlock Scale [EHS]) in New England forests (Preisser et al. 2008). The healthy looking trees are described as having little or no needle loss, deep green color, intact canopy, and full, thick branches (Ingwell 2007).

As part of a research project examining the potential for naturally occurring HWA resistance in Eastern Hemlocks, we report the results of a study examining the chemical characteristics of Eastern Hemlocks. Specifically, we examined the terpenoid profiles and cation concentrations of three groups of Eastern Hemlock trees. The first "parent" group was comprised of potentially HWA-resistant trees located in Connecticut. The second "control" group was comprised of trees located to the north of HWA's current range. The third "propagule" group consisted of cuttings taken from the potentially resistant "parent" trees in Connecticut and grown under controlled conditions at greenhouse facilities at the University of Rhode Island.

Materials and Methods

We have initiated a study to locate, propagate, and experimentally evaluate the foliar chemistry in these rare individual surviving trees. The criteria used to identify potentially resistant Eastern Hemlock trees have been covered in detail elsewhere (Caswell et al. 2008). Briefly, candidate trees must be mature (>10 m in height), healthy (deep green needles, full and thick branches) trees, and little colonized by HWA. These trees must be growing in stands that have >95% mortality of neighboring hemlocks and have not been treated by pesticides or horticultural oils.

Plant material

Foliage from mature Eastern Hemlock was gathered from forest stands in Connecticut, Vermont, and from trees grown at the University of Rhode Island's East Farm (Kingston, RI). Six trees that have been identified as potentially resistant to HWA infestation, located in three different forests near the towns of East Haddam, Madison, and Old Lyme, CT, were sampled and are hereafter referred to as the "parent" group. Cuttings were taken from these potentially HWA-resistant "parent" individuals in 2005 and 2006 and grown under controlled conditions at URI (Caswell et al. 2008). We sampled foliage from the propagules (= rooted cuttings in cultivation at East Farm) of five of these Connecticut trees, which are hereafter referred to as the "propagule" group. Foliage from five mature Eastern Hemlocks from HWA-free areas near Springfield, VT served as the "control" group. Samples from all three groups were collected in November 2007. Two branch cuttings were taken from each of the cardinal directions, for a total of eight cuttings per tree. Cuttings were placed in hydrated floral foam and returned to the lab for analysis.

Cation analysis

To minimize idiosyncratic variation in foliar chemistry, we followed the protocol of Lagalante et al. (2006) and only analyzed foliage from the previous year's growth. Prior to needle collection, any scale insects, adelgids, or other organisms on the samples were manually removed using a stainless steel forceps or spatula. Two 20-mL disposable scintillation vials were filled with excised needles for each sample and sent to the University of Georgia Stable Isotope/Soil Biology Laboratory (www.uga.edu/sisbl) for quantification of C, N, Ca, P, and K following the standard methods employed by UGA and described in Allen (UGA) (1974) and Jones et al. (1990).

Terpenoid analysis

Two samples were collected from each tree; each sample consisted of one needle from each cardinal direction (four needles per sample). Prior to needle collection, any scale insects, adelgids, or other organisms on the samples were removed. Samples were prepared following methods in Lagalante and Montgomery (2003), with the exception of using a 10-mL headspace vial, and stored at -20 °C. All samples were processed within one month of collection date.

Volatile compounds were equilibrated in the headspace vial and loaded onto the solid-phase microextraction (SPME) following Lagalante and Montgomery (2003), with the exception of using a 65- μm PDMS fiber and equilibration times from one to three hours. Samples were analyzed using an Agilent 6890N GC coupled with an Agilent 5973i mass selective detector (MSD). The inlet temperature was 275 °C with a 2:1 split ratio. The SPME was inserted into the injection port for two minutes for sample desorption. Separation was accomplished with a 7.0 m HP-5MS column with a nominal diameter of 250 μm and film thickness of 0.25 μm , with a constant flow of 3.0 mL/min. The oven was programmed to begin at 40 °C for two minutes before ramping at a rate of 3 °C/min to a final temperature of 94 °C followed by a two-minute post-run at 280 °C. The transfer line between the GC and the MS was held at 300 °C. The MSD was tuned daily using Agilent's STUNE.U program. The electron multiplier's voltage was set to zero relative to the optimized voltage (1200–1400 eV).

On the basis of previous work showing significant variation between the terpenoid profiles of susceptible and resistant hemlock species, (Lagalante and Montgomery 2003, Lagalante et al. 2006) we concentrated on a subset of seven hemlock terpenoids: α -pinene, myrcene, piperitone, isobornyl acetate, β -caryophyllene, α -humulene, and δ -cadinene. These were identified using both a mass spectrum database search (NIST MS Library 2002) and compared retention indices reported on a DB-5 column (Adams 2007). Authentic samples (Wilkem Scientific, Pawtucket, RI) for isobornyl acetate, α -humulene, α -pinene, and β -caryophyllene were compared to retention indices for experimental samples. The chromatogram peaks were integrated and relative quantity determined following Lagalante and Montgomery (2003) for each of the seven compounds.

Statistical analysis

Terpenoid and cation concentrations were compared among the parent and control groups using one-way ANOVA. Because the parent and propagule groups came from the same individuals, they were compared using a matched-pairs analysis. This analysis allowed us to evaluate the effect of propagation techniques on the foliar chemistry of the trees. All *P*-values were corrected for multiple comparisons at $\alpha = 0.05$ using a step-up FDR correction (Benjamini and Hochberg 1995). All data were checked for normality prior to analysis, and all analyses were performed using JMP v.7 (SAS Institute, Inc., Cary, NC).

Results

Cations

The three groups differed in their relative abundance of K, P, N, Ca, and C (Fig. 1). Parent trees had significantly more K than did control trees ($F_{1,9}=10.11$, $P < 0.05$ after step-up FDR adjustment), but did not differ in other cations (all adjusted $P > 0.05$). Compared to the parent group, propagules

had significantly more Ca (paired t -test with 4 d.f. = -7.82, adjusted $P < 0.05$) and N (paired t -test with 4 d.f. = -5.64, adjusted $P < 0.05$). Parents and propagules did not differ in their concentrations of K, P, and C.

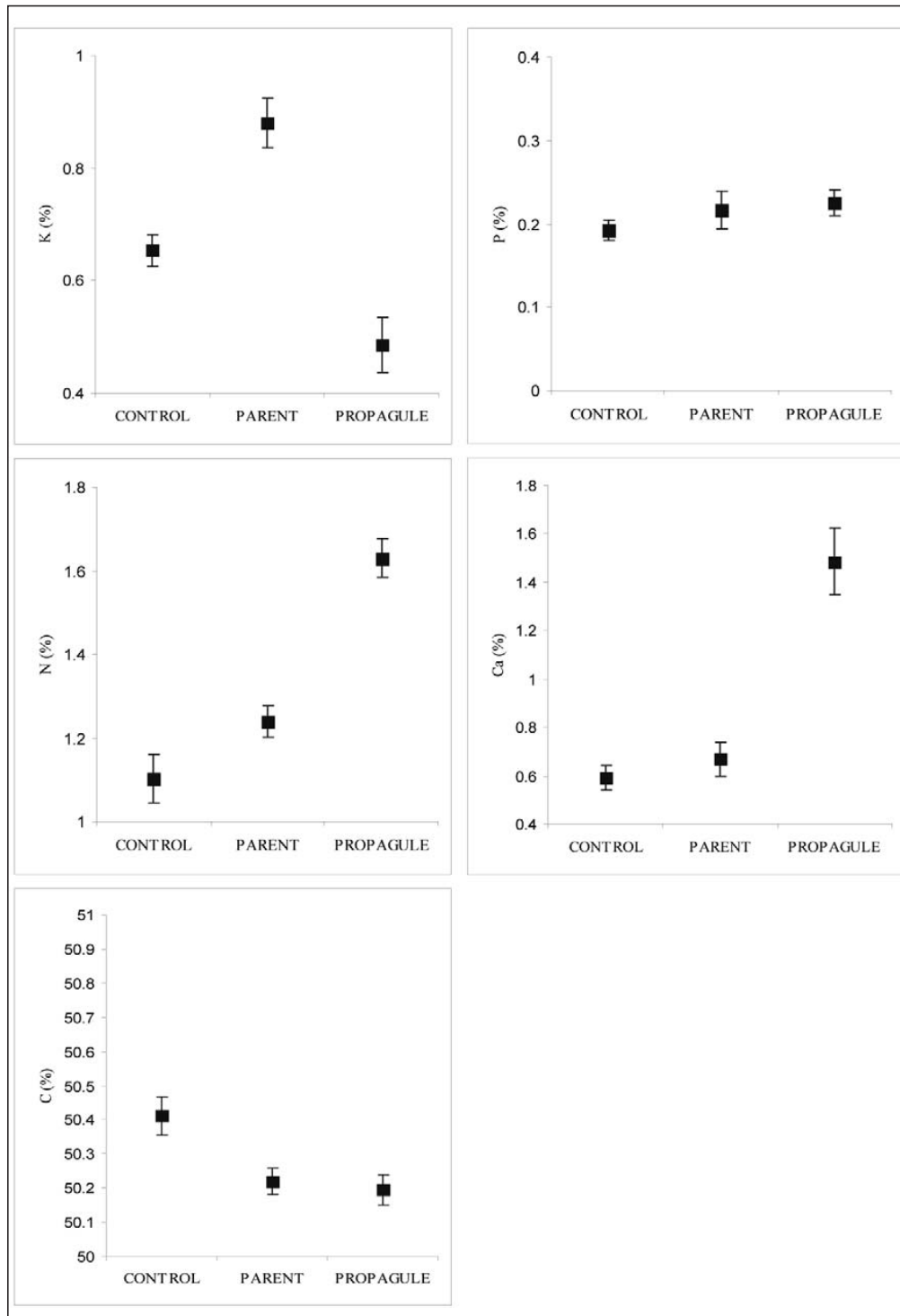


Figure 1. Mean cation concentrations \pm standard error of five measured cations for each of the three tested groups. Parent trees have significantly more K than control trees ($P < 0.05$). Propagules have significantly more Ca and N than parent trees ($P < 0.05$).

Terpenoids

The relative concentrations of the seven quantified terpenoids did not differ between the parent and control groups (all $F_{1,9}$, adjusted $P > 0.05$; Fig. 2). When compared to the parent group, propagules had significantly more piperitone (paired t -test with 4 d.f. = 3.44, adjusted $P < 0.05$), but did not differ in the relative concentrations of the other terpenoids (all adjusted $P > 0.05$).

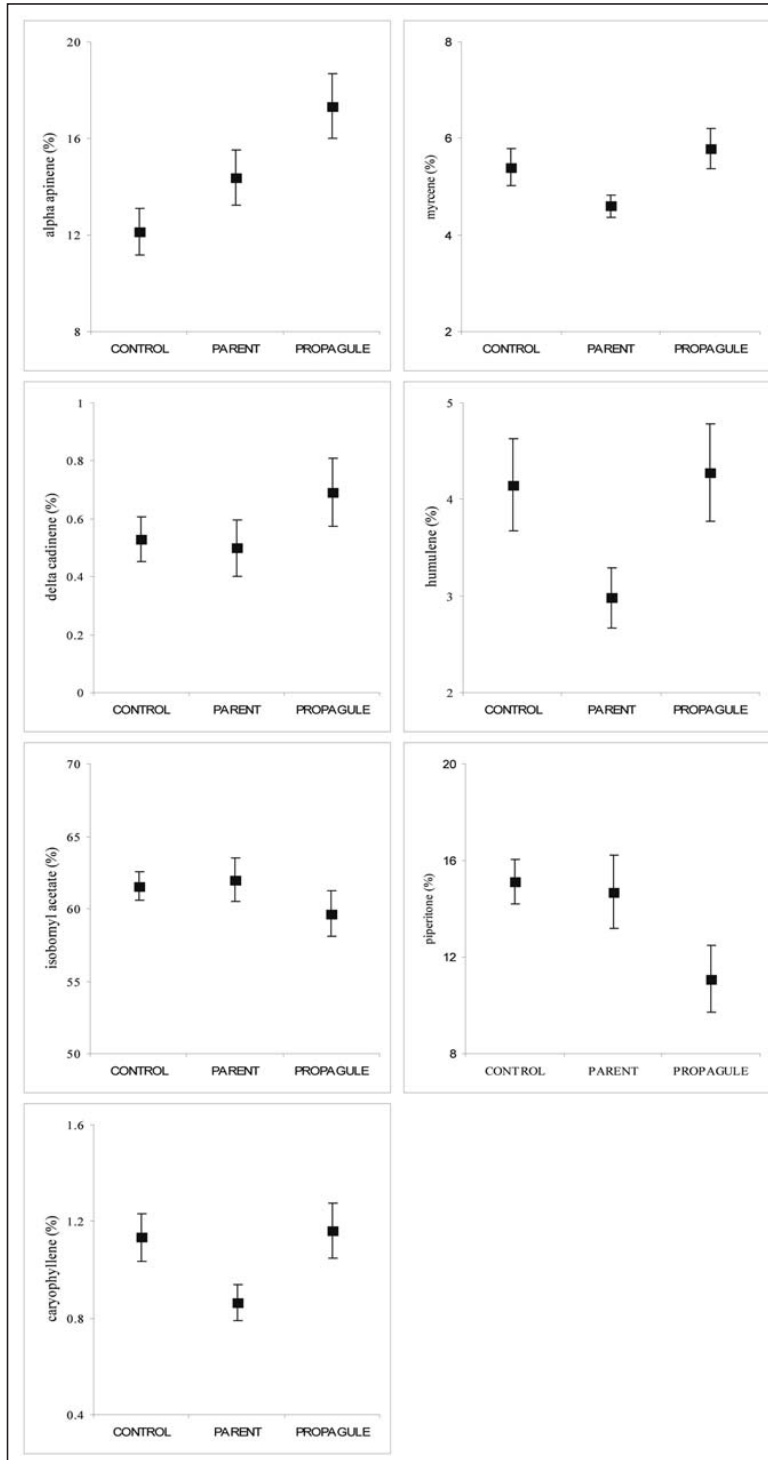


Figure 2. Mean relative area percent \pm standard error of seven terpenoids for each of the three tested groups. Parent trees and control trees did not differ in their terpenoid concentrations, while parent trees have significantly more piperitone than propagules ($P < 0.05$).

Discussion

Intraspecific variation in Eastern Hemlock chemistry both between and among groups supports the idea that foliar chemistry may contribute to HWA tolerance or resistance. Such chemical variation reflects the nutritional and defensive chemistry of the host tree, a critical component in determining whether phytophagous insects such as HWA can successfully establish and persist (Montgomery and Lagalante 2008).

We found that the potentially HWA-resistant “parent” trees and the “control” trees from outside of the current range of HWA differed in some aspects of their foliar chemistry. Although it would have been ideal to compare the foliar chemistry of the potentially resistant parent trees to other trees in the immediate area, the massive HWA-related hemlock mortality in these forests prevented us from doing so. As a result, we cannot reject the possibility that site, climatic, and regional factors may account for some of the differences between these individuals.

While there were no apparent differences in terpenoid composition, parent trees had elevated levels of potassium relative to the control trees. Potassium is a key chemical required by plants to activate enzymes involved in growth, regulate the opening and closing of stomates, and carry on photosynthesis. Pontius et al. (2006) found lower concentrations of potassium among HWA-resistant species as well as a positive correlation between potassium concentrations and HWA-infestation levels and hemlock decline symptoms. Trees that had high levels of potassium supported larger populations of HWA and displayed increased symptoms of decline, leading the authors to suggest that HWA alters the chemistry of the host tree. They concluded that this chemical may be a limiting factor to HWA population growth. The increased level of potassium in the parent trees could be a result of HWA infestations; however, the trees sampled in the parent group do not support HWA populations, as reflected by their persistence in such a devastated environment.

Other research, however, has found a negative correlation between potassium and insect population growth on herbivore-resistant alfalfa plants tested with *Therioaphis maculate* Buckton (Spotted Alfalfa Aphid) (Kindler and Staples 1970) as well as in resistant sorghum plants attacked by *Schizaphis graminum* Rondani (Greenbug) (Schweissing and Wilde 1979). Potassium is very important for the overall fitness of a plant, and increased levels in the plant produce higher resistance to pathogens and increased crop yield in some species, including citrus and nut trees (Armstrong 1998, Rosecrance et al. 1996, Sumith and Bandara 2002). It is possible that elevated potassium levels in the parent trees make them unsuitable for HWA development, explaining their persistence in otherwise devastated areas.

Two foliar chemistry databases are available which report potassium levels in Eastern Hemlock trees that were measured for other research. The Foliar Chemistry Database of the Northeastern Ecosystem Research Cooperative (NERC 2009) reports potassium concentrations for 132 hemlocks across Maine, Massachusetts, New York, and New Hampshire. Only two

percent of the trees in this database had levels of potassium comparable to the reported values for the parent group. The Tree Chemistry database (Pardo et al. 2005) published by the US Forest Service Northeastern Research Station reports potassium levels in an additional 23 trees across nine sites. Two of these sites in central New York had levels comparable to the parent trees reported here. Both of these databases suggest that elevated levels described in this research occur in nature, but are not common. Trees reported by the historical database as exhibiting high levels of potassium should be monitored for potential resistance to HWA.

Differences between the cation concentrations of the propagule and parent groups are most likely explained by the fact that propagules from the parent trees were grown in a greenhouse environment that included a liquid application of 20-20-20 fertilizer (Caswell et al. 2008). Specifically, the propagules possessed much higher concentrations of nitrogen and calcium than did the parent group. This result suggests that fertilized rooted cuttings are not an appropriate substitute to the parent trees in regards to foliar chemistry and implies that care should be taken in using propagules to assess the HWA resistance of their parent trees.

Conclusions

While there are a number of variables (biological, temporal, regional, etc.) which contribute to the susceptibility of a host tree, our study reveals substantial intraspecific variation in Eastern Hemlock foliar chemistry and shows that the chemical variation occurring in Eastern Hemlock cultivars (Lagalante et al. 2007) can also be found in field-collected specimens. Since foliar chemistry plays a critical role in determining plant resistance or tolerance to HWA, this suggests the possibility that naturally occurring variation in foliar chemistry may produce at least some individuals of Eastern Hemlock whose chemical makeup renders them relatively less vulnerable to HWA infestations. If so, the adelgid's role as a natural "selective filter" that kills non-resistant hemlock trees may inadvertently make it easier to identify these potentially resistant individuals. Similar to the evidence of selective pressures from *Dendroctonus brevicomis* LeConte (Western Pine Beetle) enhancing frequencies of chemically distinct, less suitable *Pinus ponderosa* P. & C. Lawson (Ponderosa Pine) trees (Sturgeon 1979), the potential for chemically unique, potentially resistant Eastern Hemlock populations to survive infestation is promising. Further examination of these individuals may enable the identification of key chemicals linked to their survival. Such knowledge would facilitate the use of techniques and technologies intended to enhance these characteristics, as well as breeding programs aimed at producing resistant/tolerant Eastern Hemlock trees for use in conservation and reforestation efforts.

Efforts to remove hemlock trees prior to infestations in order to maximize economic value, such as pre-emptive logging, will limit our ability to detect naturally occurring resistance. We have seen the impacts of pre-emptive logging with the Chestnut Blight tragedy, resulting in the loss of naturally

occurring blight-resistant individuals (Schlarbaum et al. 1997). These pre-emptive logging methods should be avoided until some alternative resistance screening is available to test trees prior to their removal.

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