

Biophysical characteristics of the stem and petiole surface of six hemlock (*Tsuga*) species and a hybrid: implications for resistance to *Adelges tsugae*

K.L.F. Oten, G.R. Bauchan, J. Frampton, and F.P. Hain

Abstract: Characteristics of the plant surface significantly affect host-plant selection by phytophagous insects. Surface morphology of six hemlock species (*Tsuga* spp.) and a hybrid was investigated using low-temperature scanning electron microscopy. Observations focused on trichome presence and placement and cuticle thickness. These characteristics were studied in the context of species-level host-plant resistance to the hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), an exotic insect causing massive mortality to eastern hemlocks (*Tsuga canadensis* (L.) Carr.) and Carolina hemlock (*Tsuga caroliniana* Engelm.) in the eastern United States. Hemlocks in the native range of the insect do not succumb to infestations and the mechanism of resistance is unknown. We addressed the potential role of plant surface morphology in the adelgid–hemlock interaction by comparing four adelgid-resistant hemlock species and a hybrid with the two adelgid-susceptible hemlock species. We found that trichomes are likely not involved in conferring resistance to *A. tsugae*. Cuticle thickness may be involved in insertion site selection by *A. tsugae* and may therefore have implications for resistance. The cuticle is thinnest at the point of *A. tsugae* stylet insertion and thus may affect *A. tsugae* feeding.

Key words: hemlock, hemlock woolly adelgid, scanning electron microscopy, trichomes, plant cuticle, host-plant resistance.

Résumé : Les caractéristiques de la surface foliaire affectent significativement la sélection de la plante hôte par les insectes phytophages. Les auteurs ont étudié la morphologie de la surface chez six espèces de pruche (*Tsuga* sp.) et un hybride, en utilisant la microscopie électronique à balayage à basse température. Ils ont concentré leurs observations sur la présence de trichome et la localisation et l'épaisseur de la cuticule. Ils ont étudié ces caractéristiques dans le contexte de la résistance de l'espèce hôte à l'endroit du puceron lanigère de la pruche, *Adelges tsugae* Annand (Hemiptera : Adelgidae), un insecte exotique causant des mortalités massives chez la pruche de l'est (*Tsuga canadensis* (L.) Carr.) et la pruche de Caroline (*Tsuga caroliniana* Engelm.) dans l'est des Etats-Unis. Les pruches vivant dans l'aire de distribution indigène de l'insecte ne succombent pas aux infestations, mais on ne connaît pas le mécanisme de résistance. Les auteurs examinent le rôle potentiel de la morphologie de la surface de la plante dans l'interaction puceron–pruche en comparant la résistance aux pucerons chez quatre espèces de pruches et un hybride, avec deux espèces de pruches susceptibles aux pucerons. Ils ont constaté que les trichomes ne semblent pas contribuer à la résistance au *A. tsugae*. L'épaisseur de la cuticule pourrait être impliquée dans la sélection des sites d'insertion de l'*A. tsugae* et pourrait conséquemment avoir des implications dans la résistance. La cuticule est à son plus mince au point où l'*A. tsugae* insère son stylet ce qui pourrait affecter la nutrition de l'*A. tsugae*.

Mots-clés : pruche, puceron lanigère de la pruche, microscopie électronique par balayage, trichomes, cuticule végétale, résistance de la plante hôte.

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Introduction

The plant surface is the primary interface between an herbivore and its host and consequently plays an important role in host-plant selection and plant defense. While most plants have a unique blend of chemical compounds and are therefore

useful in host-plant selection by many phytophagous insects (Bernays and Chapman 1994), biophysical characteristics of the surface may also contribute partly or autonomously to affect such behaviors. For example, the initial decision for many aphids to insert their stylets into a host involves analysis of the texture and color of the plant surface (Klingauf 1987;

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Pelletier 1990). Because of this role in host acceptance by phytophagous insects, physical features may also confer resistance in some plant species.

Trichomes and hairs on the plant surface often act as defense against herbivores. They can affect movement, attachment, shelter, feeding, and survival of insects and are an important morphological feature of plant resistance to insects (Johnson 1975; Ram et al. 2005). For example, pubescent plant surfaces can slow or prevent insect movement, while glabrous surfaces can be so slippery that insects fall off the plant (Southwood 1986). The presence of surface pubescence can directly affect resistance to herbivore damage by reducing accessibility to the plant tissues (Rowell-Rahier and Pasteels 1982). Glandular trichomes may secrete repellent substances or secondary metabolites that trap insects and hooked trichomes can impale pests (Levin 1973; Duffey 1986; Bernays and Chapman 1994). Given the resistance conveyed to pest insects, plant pubescence is a trait pursued in both traditional and transgenic breeding programs. The fact that pubescent wheat varieties have also been found to be less suitable for oviposition and larval feeding by the cereal leaf beetle, *Oulema melanopus* L., led to a breeding program for resistant varieties based on biophysical characters (Gallun 1966). More recently, genes governing increased trichome production were inserted into susceptible canola (*Brassica napus* L.), effectively reducing herbivory by crucifer flea beetles (*Phyllotreta cruciferae* (Goeze)) (Soroka et al. 2011). Conversely, the presence of trichomes can also facilitate herbivory by enabling small insects to overcome an “attachment hurdle” by providing an easily grasped substrate (Southwood 1973; Bernays and Chapman 1994).

Epicuticular waxes make up the outermost layer of a plant, and as the point of contact between an herbivore and its host, they can influence subsequent host acceptance or rejection (Daoust et al. 2010). Another primary physical defense against herbivores is the presence of thick and (or) tough tissues that deter stylet penetration and the initiation of feeding (Larsson 2002). More generally, thick cuticular layers and epicuticular waxes can make it difficult for an herbivore to penetrate, consume, or digest plant material (Southwood 1986; Peter and Shanower 2001; Ram et al. 2005). The thickness of the cuticle is highly variable among plants, ranging from less than 1 to 15 μm , and is generally thinner in shade-tolerant plants than those exposed to full sun (Romberger et al. 1993; Kozłowski and Pallardy 1997). It is also a major factor in desiccation prevention; the thick cuticle covering pine needles, for example, makes them markedly more resistant to drought than most trees (Eglinton and Hamilton 1967; Raven et al. 1986).

We report the results of research investigating physical features of hemlock (*Tsuga* spp.) that may be involved in host-plant resistance to the hemlock woolly adelgid (HWA; *Adelges tsugae* Annand). Native to parts of Asia and northwestern North America (Havill et al. 2006), HWA is an invasive pest first detected in the eastern United States (US) in the early 1950s (Souto et al. 1996). Its range has since spread to 18 eastern states and continues to expand (USDA Forest Service 2011). Of the nine species of hemlock worldwide, the two species in eastern North America, eastern and Carolina hemlock (*Tsuga canadensis* (L.) Carr. and *T. caroliniana* Engelm., respectively), are the only species that succumb to HWA infestations. Of these two species, *T. canadensis* is the

more susceptible based on initial infestation rate (Jetton et al. 2008; Oten 2011).

We used low-temperature scanning electron microscopy (LT-SEM) to describe the physical characteristics of HWA-resistant and HWA-susceptible hemlock species. We hypothesized that the presence and density of trichomes and a thick, impenetrable cuticle deter HWA feeding, contributing to the interspecific resistance of hemlocks. No descriptions of hemlock cuticle thickness or trichomes have been made previously. Because the adelgid's survival is dependent on successfully penetrating and feeding on xylem ray parenchyma cells (Young et al. 1995), an improved understanding of the hemlock surface and anatomical characteristics may improve our understanding of host-plant resistance and advance efforts to breed adelgid-resistant eastern and Carolina hemlocks.

Materials and methods

Plant material

We observed samples from seven hemlock species and a hybrid: *Tsuga canadensis* (uninfested and infested samples), *T. caroliniana*, *T. chinensis* (Franch.) E. Pritz., *T. sieboldii* Carr., *T. diversifolia* (Maxim.) Mast., *T. heterophylla* (Raf.) Sarg., *T. mertensiana* (Bong.) Carr., and a *T. chinensis* \times *T. caroliniana* hybrid. Hemlock material was collected from several sources based on availability and HWA infestation status (Table 1). Samples collected locally were clipped from trees, placed in large plastic bags, and transported to North Carolina State University in Raleigh, North Carolina. For samples that were shipped, clipped ends were wrapped in moist paper towels, placed in Ziploc bags, and shipped overnight on ice. A cooler with ice was also used during transportation from Raleigh, North Carolina, to the LT-SEM facilities at the USDA Beltsville Agricultural Research Center in Beltsville, Maryland.

Low-temperature scanning electron microscopy

Microscopic observations of unaltered plant surface morphology require appropriate preparation techniques. Tissue preparation for conventional scanning electron microscopy uses chemical fixation and critical point drying of sample tissue. This often degrades the epicuticular waxes of the plant surface. In contrast, the cryofixation process used in LT-SEM studies can be used to preserve plant surface details and is considered superior to conventional fixation methods (Parsons et al. 1974; Eveling and McCall 1983; Sargent 1983).

LT-SEM observations were performed using a S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton, California, USA) equipped with a Quorum CryoPrep PP2000 (Quorum Technologies Ltd., East Sussex, UK) cryotransfer system. To prepare specimens, hemlock needles were excised above the abscission layer, and stems were cut directly above and below that point. At least five stem cross sections and two longitudinal stem sections were cut from each sample. Cross sections were mounted with the abscission layer directed upward on flat specimen holders consisting of 16 mm \times 30 mm copper plates that contained a thin layer of Tissue Tek (OCT Compound, Ted Pella, Inc., Redding, California, USA), which acted as the cyroadhesive upon freezing. The samples were frozen conductively, in a Styrofoam box, by placing the plates

Table 1. Hemlock (*Tsuga*) species used in studies, including resistance documented in literature, native range, and source of samples.

Species	Resistance to hemlock woolly adelgid	Range ^j	Sample source
<i>T. canadensis</i>	Mostly susceptible, variation occurs ^{b,c}	Northeastern North America	Schenck Forest, Raleigh, NC
<i>T. canadensis</i> (infested)	—	—	US National Arboretum, Beltsville, MD
<i>T. caroliniana</i>	Mostly susceptible, less susceptible than <i>T. canadensis</i> , ^b variation occurs ^d	Blue Ridge Mountains, Southern Appalachians	US National Arboretum, Beltsville, MD
<i>T. chinensis</i>	Highly resistant ^{e,i}	Southeastern China	US National Arboretum, Beltsville, MD
<i>T. sieboldii</i>	Resistant ^{e,f}	Southern Japan	Morton Arboretum, Lisle, IL
<i>T. diversifolia</i>	Resistant ^{e,f,g,i}	Northern Japan	US National Arboretum, Beltsville, MD
<i>T. heterophylla</i>	Resistant ^{h,i}	Northwestern North America	Priest Point Park, Olympia, WA
<i>T. mertensiana</i>	Resistant ⁱ	Northwestern North America	Montair at Somerset Hill, Olympia, WA
<i>T. chinensis</i> × <i>T. caroliniana</i> ^a	Resistant ^e	—	US National Arboretum, Beltsville, MD

^aBred by US National Arboretum (Bentz et al. 2002).

^bJetton et al. 2008.

^cCaswell et al. 2008.

^dKaur 2009.

^eMontgomery et al. 2009.

^fMcClure et al. 2000.

^gMcClure 1992.

^hAnnand 1924.

ⁱDel Tredici and Kitajima 2004.

^jFarjon 1990.

on the surface of a precooled (−196 °C) brass bar, the lower half of which was submerged in liquid nitrogen (LN₂). After 20–30 s, the holders containing the frozen samples were transferred to a LN₂ Dewar for future use or cryotransferred under vacuum to the cold stage in the prechamber of the cryotransfer system. Removal of any surface contamination (condensed water vapor) took place in the cryotransfer system by etching the frozen specimens for 10–15 min by raising the temperature of the stage to −90 °C. Following etching, the temperature was lowered below −130 °C, and a magnetron sputter head equipped with a platinum target was used to coat the specimens with a 10 nm layer of platinum. The specimens were transferred to a precooled (−140 °C) cryostage in the LT-SEM for observation. An accelerating voltage of 5 kV was used to view the specimens. Images were captured using a 4pi Analysis System (Durham, North Carolina, USA). Images were modified for brightness and contrast by adjusting levels, sized, and placed together to produce figures using Adobe Photoshop 8.0.

Measurements and analyses

To measure cuticle thickness, LT-SEM micrographs were taken at three locations on the pulvinus, the cushion-like swelling at the base of the petiole. These three locations were as follows: at the HWA stylet insertion point (Fig. 1A), on the side of the pulvinus where the body of the adelgid would typically rest, and on the outside (Fig. 1B). Thickness varied slightly; to compensate for intraindividual variability, three points (two points, when three distinct points were not visible) from each image were measured for cuticle thickness (in micrometres). Statistical analyses were performed for each location (i.e., insertion point, side, and outside) for *T. canadensis*, *T. caroliniana*, *T. chinensis*, *T. diversifolia*, *T. heterophylla*, and *T. mertensiana*. For the analysis at the stylet bundle insertion point only, *T. sieboldii* and the *T. chinensis* ×

T. caroliniana hybrid were added (measurements at the side and outside were not taken for these samples). Although prepared identically to one another, some samples were not of sufficient quality to include in the study (the view was either obstructed or the abscission layer did not break cleanly), or we had limited material with which to work. As a result, the numbers of samples for each species were unequal (*T. canadensis*, uninfested, $n = 6$; *T. canadensis*, infested, $n = 62$; *T. caroliniana*, $n = 17$; *T. chinensis*, $n = 25$; *T. sieboldii*, $n = 6$; *T. diversifolia*, $n = 21$; *T. heterophylla*, $n = 35$; *T. mertensiana*, $n = 35$; *T. chinensis* × *T. caroliniana* hybrid, $n = 9$). There were a total of 216 replicates for cuticle thickness measurements.

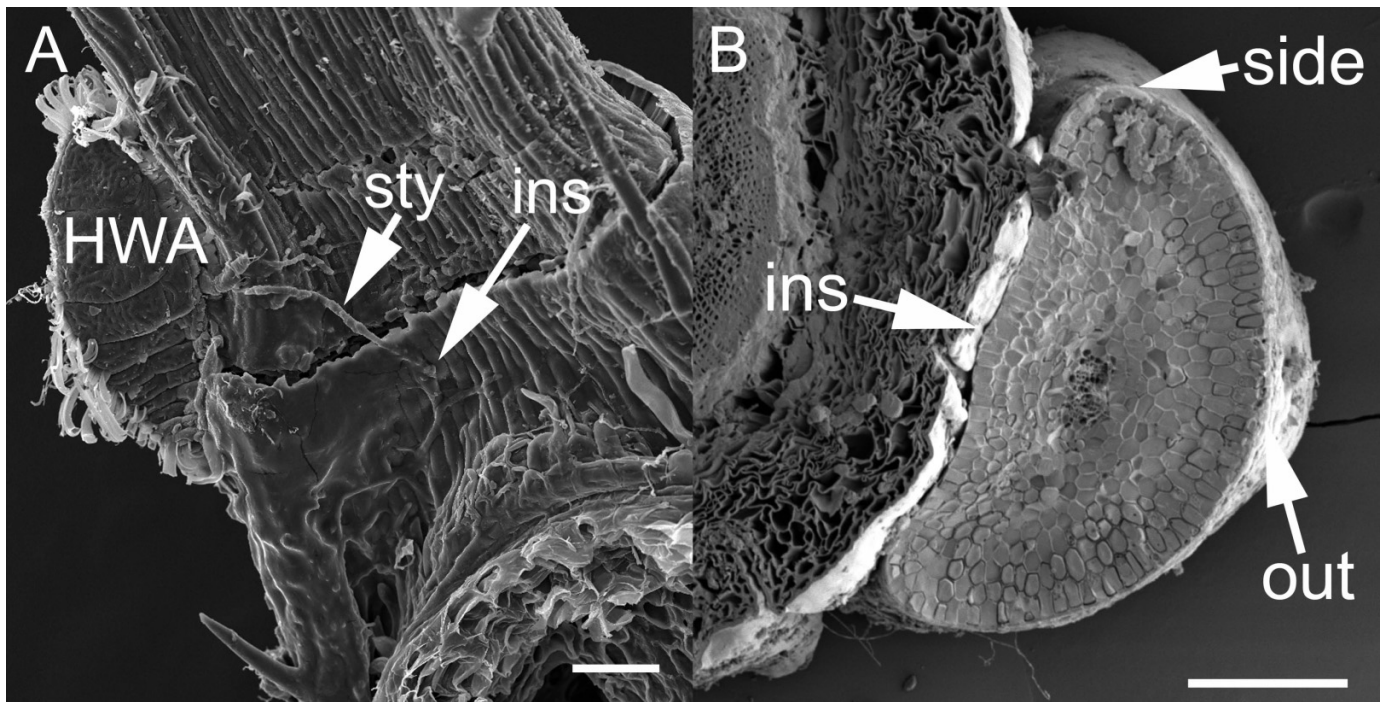
Analysis of variance was performed using the fit least squares procedure of JMP 9.0 Pro (SAS Institute Inc. 2010). The main effects of hemlock species and location were tested for cuticle thickness. In an initial analysis, no significant difference was found between infested and uninfested *T. canadensis*; these were grouped together in subsequent analyses. Using the Tukey–Kramer HSD method, differences among all pairwise least squares means were determined to be significant or not at $\alpha = 0.05$. All means are reported as least squares means.

Results

Trichomes

Nonglandular simple trichomes were observed on seven of the eight hemlock species. The only species lacking trichomes was *T. sieboldii*, which had a smooth, glabrous surface (Fig. 2D). *Tsuga canadensis*, *T. diversifolia*, *T. heterophylla*, and *T. mertensiana* had trichomes encircling the stem (Figs. 2A, 2E, 2F, 2G). In *T. heterophylla* and *T. mertensiana*, the two western North American species, we observed relatively long hairs in addition to the shorter trichomes present on

Fig. 1. Hemlock woolly adelgid insertion site in relation to other sites measured on pulvini. (A) Settled hemlock woolly adelgid, first instar, showing adaxial side of the needle and pulvinus. HWA, hemlock woolly adelgid; ins, insertion point; sty, stylet bundle. Scale bar = 100 μm . (B) Three locations measured on hemlock pulvini for cuticle thickness. ins, insertion point; out, outside. Scale bar = 200 μm .



the aforementioned species. *Tsuga caroliniana*, *T. chinensis*, and *T. chinensis* \times *T. caroliniana* were mostly smooth with a conspicuous strip of trichomes that extended longitudinally along the length of the stem (Figs. 2B, 2C, 2H).

Cuticle thickness

Electron micrographs of the cuticle of each hemlock species studied are shown in Fig. 3. Differences in the cuticle thickness was significant between species ($F = 39.05$, $df = 5$, $P < 0.0001$) and between the three locations on the pulvinus ($F = 122.29$, $df = 2$, $P < 0.0001$) at $\alpha = 0.05$. In addition, the interaction between species and location on the pulvinus was significant ($F = 6.14$, $df = 10$, $P < 0.0001$) at $\alpha = 0.05$. The effects of location and species on cuticle thickness are presented in Figs. 4 and 5. A comparison of cuticle thickness at different locations on the pulvinus, averaged across all species observed, indicates that the cuticle is thickest at the side ($3.35 \pm 0.08 \mu\text{m}$) and outside ($3.45 \pm 0.10 \mu\text{m}$) locations relative to the stylet bundle insertion point ($1.97 \pm 0.07 \mu\text{m}$) (Fig. 4). This analysis was central to our understanding of insertion site selection, which occurs at the same point across species. Mean cuticle thickness by species for all measurement locations (insertion point, side, and outside) was clustered in three distinct groups: *T. canadensis* ($2.16 \pm 0.07 \mu\text{m}$), *T. heterophylla* ($2.28 \pm 0.10 \mu\text{m}$), and *T. mertensiana* ($2.38 \pm 0.10 \mu\text{m}$) were grouped with the thinnest overall cuticle; *T. caroliniana* ($2.94 \pm 0.15 \mu\text{m}$) and *T. diversifolia* ($3.08 \pm 0.12 \mu\text{m}$) were grouped with intermediate measurements; and *T. chinensis* ($3.83 \pm 0.11 \mu\text{m}$) was isolated with the thickest mean cuticle (Fig. 5). In our analysis of cuticle thickness at the stylet bundle insertion point, species were ordered from thinnest to thickest as follows: *T. canadensis* ($1.38 \pm 0.09 \mu\text{m}$),

T. mertensiana ($1.56 \pm 0.11 \mu\text{m}$), *T. heterophylla* ($1.60 \pm 0.11 \mu\text{m}$), *T. chinensis* ($1.96 \pm 0.13 \mu\text{m}$), *T. sieboldii* ($2.06 \pm 0.16 \mu\text{m}$), *T. diversifolia* ($2.20 \pm 0.16 \mu\text{m}$), *T. caroliniana* ($2.24 \pm 0.18 \mu\text{m}$), and the *T. chinensis* \times *T. caroliniana* hybrid ($2.67 \pm 0.13 \mu\text{m}$) (Fig. 6).

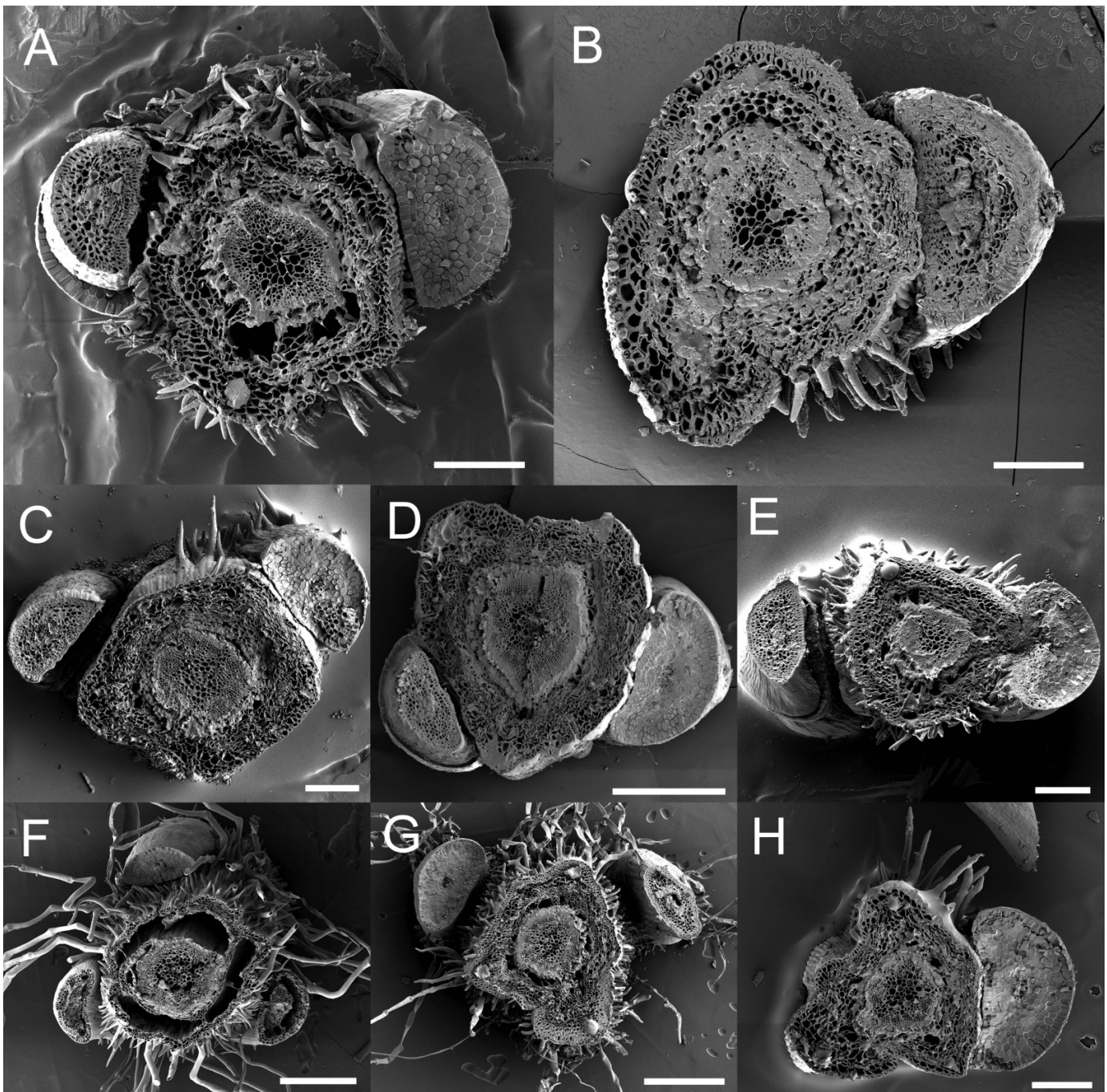
Discussion

This study provides new information on morphological characteristics of the hemlock surface, specifically trichome occurrence and cuticle thickness, in six hemlock species and a hybrid. These results not only increase our knowledge of the plant surface with which HWA directly interacts, but also have implications for adelgid site selection, stylet bundle penetration, and interspecific host-plant resistance.

Trichomes

Trichomes can both deter and enable herbivory (Levin 1973; Johnson 1975; Bernays and Chapman 1994; Soroka et al. 2011), but our results suggest that neither of these events is occurring in the HWA–hemlock interaction and that trichomes are thus unlikely to account for the documented resistance among hemlock species. This conclusion is based on the documented success of HWA feeding on hemlock species that, according to this study, vary widely in trichome density. *Tsuga canadensis* is considered to be the most highly susceptible hemlock to HWA feeding (Jetton et al. 2008; Oten 2011), and our observations indicate that they are densely covered in trichomes, whereas *T. sieboldii*, the ancestral host of the invasive HWA population of the eastern US (Havill et al. 2006), is entirely smooth. Because HWA successfully feeds on both of these species, we deduce that trichomes do not affect HWA

Fig. 2. Cross sections of hemlock stem. (A) Eastern hemlock, *Tsuga canadensis*. Scale bar = 200 μm . (B) Carolina hemlock, *T. caroliniana*. Scale bar = 200 μm . (C) Chinese hemlock, *T. chinensis*. Scale bar = 200 μm . (D) Southern Japanese hemlock, *T. sieboldii*. Scale bar = 500 μm . (E) Northern Japanese hemlock, *T. diversifolia*. Scale bar = 200 μm . (F) Western hemlock, *T. heterophylla*. Scale bar = 500 μm . (G) Mountain hemlock, *T. mertensiana*. Scale bar = 500 μm . (H) Chinese \times Carolina hybrid, *T. chinensis* \times *T. caroliniana*. Scale bar = 200 μm .



feeding. In addition, the long hairs observed in *T. heterophylla* and *T. mertensiana* do not deter HWA feeding, as the adelgid successfully feeds on these host species as well. The role that they play, whatever it might be, is likely not related to host-plant interactions with HWA.

The trichome arrangement of *T. chinensis*, *T. caroliniana*, and the *T. chinensis* \times *T. caroliniana* hybrid are similar to one another. The stems of all three species are glabrous except for a longitudinal strip of trichomes that extends the length of the

stem (Figs. 2B, 2C, and 2H). Although geographically isolated from one another, *T. chinensis* and *T. caroliniana* are closely related phylogenetically (Havill et al. 2008), supporting the idea that trichome characteristics, while not involved in host-plant resistance to HWA, are derived characters.

Cuticle thickness

For all species, the cuticle thickness at the stylet bundle insertion point was thinner than the cuticle thickness at the

Fig. 3. Hemlock cuticle at hemlock woolly adelgid stylet insertion point. (A) Eastern hemlock, *Tsuga canadensis*. (B) Carolina hemlock, *T. caroliniana*. (C) Chinese hemlock, *T. chinensis*. (D) Southern Japanese hemlock, *T. sieboldii*. (E) Northern Japanese hemlock, *T. diversifolia*. (F) Western hemlock, *T. heterophylla*. (G) Mountain hemlock, *T. mertensiana*. (H) Chinese × Carolina hybrid, *T. chinensis* × *T. caroliniana*. Scale bar = 10 μm.

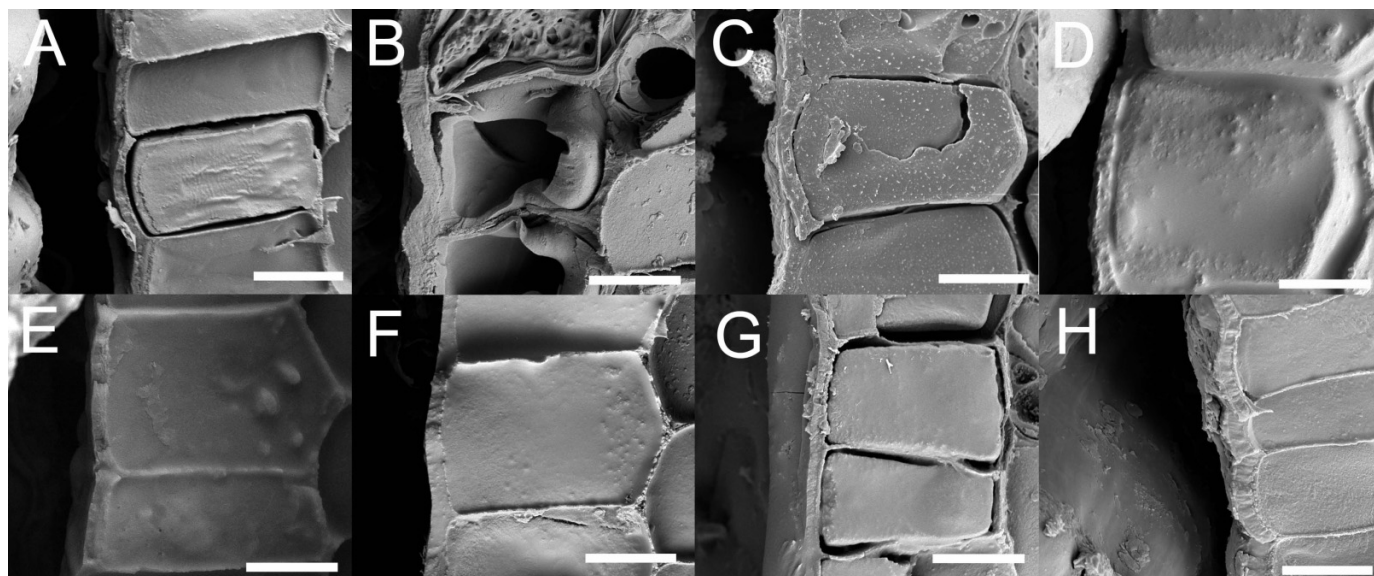
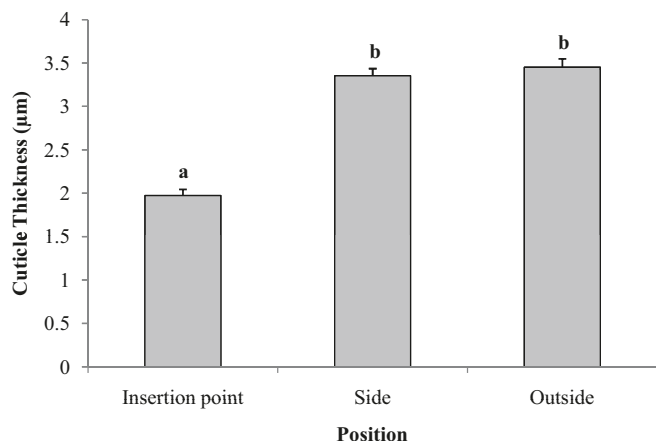
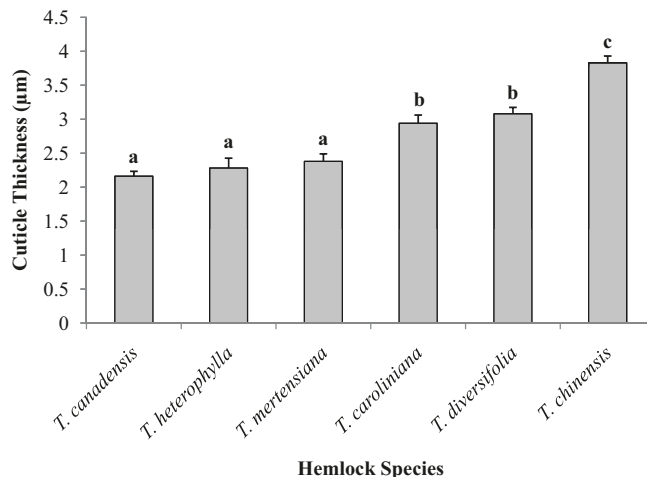


Fig. 4. Effect of location on cuticle thickness across all species, with standard error shown. Columns with different letters are significantly different at $\alpha = 0.05$, as determined by the Tukey–Kramer HSD procedure.



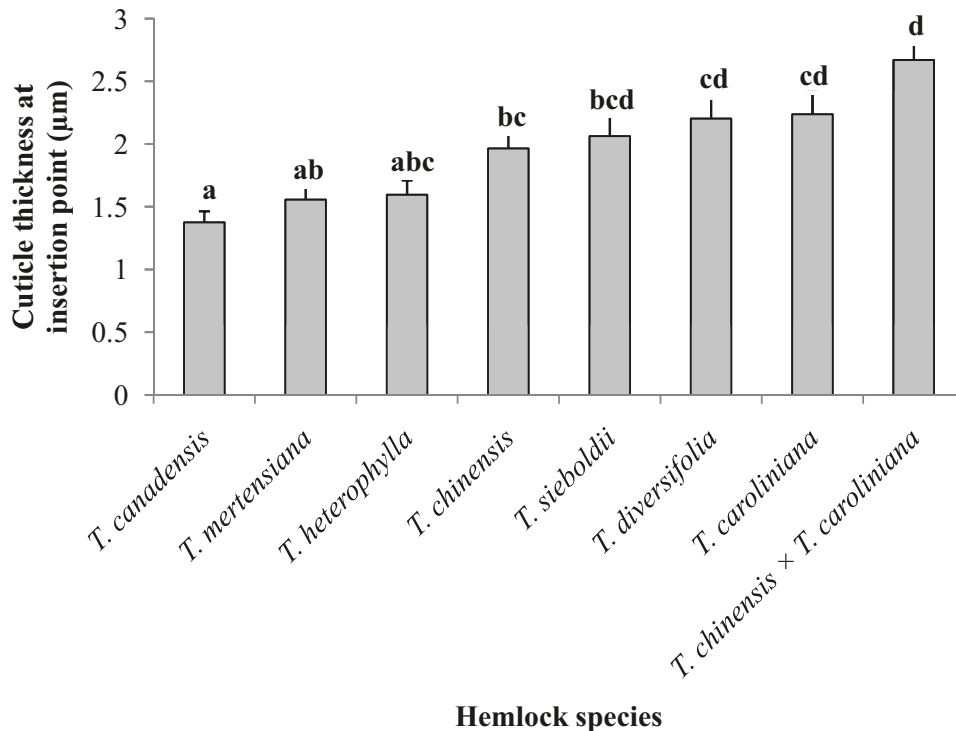
side and outside locations of the pulvinus (Fig. 4). This may directly relate to the feeding biology of HWA; a thin cuticle may present less of an obstacle for HWA to overcome to successfully penetrate host-plant tissues, thus encouraging the consistent insertion of the stylet bundle at the adaxial side of the pulvinus proximal to the abscission layer (Young et al. 1995). No other theories have been developed that explain the stylet bundle insertion site specificity of HWA. In the primary host plant (*Picea* spp.), stylet bundle insertion across Adelgidae occurs near the bud, which induces the bud to swell into a gall. However, HWA does not infest a primary host in its invasive range, and stylet insertion site in the secondary host, which was studied here, varies across the family. For example,

Fig. 5. Effect of hemlock species on cuticle thickness across all locations, with standard error shown. Columns with different letters are significantly different at $\alpha = 0.05$, as determined by the Tukey–Kramer HSD procedure.



A. cooleyi and *A. pectinate* settle on the needles of *Pseudotsuga* and *Abies*, respectively, *A. laricis* settles at the base of *Larix* buds, and *A. piceae* and *Pinus strobi* settle of the bark of *Abies* and *Pinus*, respectively (Havill and Footitt 2007). A literature search indicates that HWA may be unique in its stylet bundle insertion at the base of the needle of its secondary host. Our results further characterize host-plant selection behavior by HWA and give us insight into a potential factor in host use processes. In addition, these data will be useful to assess membrane potential in ongoing work to develop an artificial diet delivery system for HWA (Cohen et al. 2008; Kaur 2009).

Fig. 6. Effect of species on cuticle thickness at insertion point, with standard error shown. Columns with different letters are significantly different at $\alpha = 0.05$, as determined by the Tukey–Kramer HSD procedure.



Tsuga chinensis is the most HWA-resistant hemlock species, becoming infested with HWA at a lower rate compared with *T. sieboldii*, *T. canadensis*, and *T. caroliniana* when artificially infested (Montgomery et al. 2009). In some instances, *T. chinensis* does not become infested with HWA at all, a phenomenon observed in an artificial infestation study in the Arnold Arboretum (Del Tredici and Kitajima 2004). In its natural setting in China, one subspecies of *T. chinensis* is documented to become infested, while others appear to be completely resistant (Montgomery et al. 2000). Based on this high level of documented resistance, we hypothesized that the cuticle of *T. chinensis* would be significantly thicker than that of other hemlocks, deterring stylet penetration by HWA and thus suggesting that an antixenosis (nonpreference) mechanism played at least a partial role in the resistance of this species. This original theory, if validated, would also imply that the remaining resistant species of hemlock utilize a different mechanism of resistance, possibly antibiosis, as suggested by a study on 1-year-old seedlings, in which HWA survival was 1%–4% on *T. diversifolia*, *T. heterophylla*, and *T. mertensiana*, and 74%–89% on *T. canadensis* and *T. caroliniana* (McClure 1992). Our data indicate that *T. chinensis* does have a significantly thicker cuticle overall, but when comparing the thickness at the stylet insertion point only, it was grouped along with other species: *T. caroliniana*, *T. sieboldii*, *T. diversifolia*, and *T. heterophylla* (Fig. 6). If hemlock cuticle thickness is involved in HWA resistance, then the feeding deterrent event would likely occur prior to stylet penetration attempts. In other words, if each individual adelgid tests the plant surface on which it is walking prior to investing energy to insert its stylet bundle into host tissues, then the

encounter with a thicker cuticle in *T. chinensis* may be an unattractive characteristic, resulting in abortion of the behavioral sequence used by HWA to select its feeding site. The sequence of steps for the acceptance or rejection of a host plant for aphids, a close relative to HWA, is as follows: (i) attraction, (ii) testing of the plant surface and outer plant tissues, (iii) penetration, and (iv) testing of the phloem (Klingauf 1987). Because HWA disperses passively (transportation between individual trees and encountering a host surface is an entirely passive event), the initial decision in the host acceptance process by HWA may begin with testing of the plant surface and outer plant tissues, which, in aphids, includes brief stylet penetrations insufficient for feeding. Thus, supposing a similar behavioral process in HWA, the thick outer cuticle in *T. chinensis* may be a factor in deterring further host acceptance processes.

Trends in cuticle thickness at the species level can be explained to some extent by the known phylogenetic relationships among hemlocks. *Tsuga caroliniana* is nested within a clade consisting of the Asian hemlocks, including *T. chinensis* and *T. diversifolia* (Havill et al. 2008). In our studies, these three species made up the statistically grouped samples with the thickest two cuticles. *Tsuga heterophylla*, *T. mertensiana*, and *T. canadensis* are distinct from the Asian clade phylogenetically, corresponding with their placement in the group with the thinnest overall cuticle (Fig. 6). Generally, thicker cuticles at the stylet bundle insertion point were exhibited in Asian hemlock species (resistant) and *T. caroliniana* (susceptible) as compared with the thinner cuticle of *T. canadensis* (susceptible).

Although the effect of cuticle thickness on successful stylet penetration by HWA is ultimately unknown, our results sug-

gest that cuticle thickness may contribute to resistance. Based on known host-plant selection of related insects, it is possible that HWA walks on and interacts with a cuticle too thick for penetration, thereby terminating further host-acceptance processes. Despite the indication of a penetrable cuticle at the insertion point, exploration of that location may never occur. A thick cuticle may deter high levels of infestation, and subsequent interactions between pest and host may also contribute to resistance, e.g., HWA population decline following stylet insertion, as documented by McClure (1992). Additional investigations should be done to substantiate this hypothesis, specifically in an effort to associate cuticle thickness with an inability for HWA to feed. Because HWA is an r-selected species, the studies should emphasize a comparison between less susceptible and highly susceptible host species to determine host-related mortality. As a potential factor in resistance, cuticle thickness is a physical characteristic that could be used as a selective trait or screening tool for resistance breeding programs.

These studies were conducted at the species level to determine physical differences of the plant surface between resistant and susceptible hemlocks. Although typically documented as entirely susceptible to HWA, recent investigations into *T. canadensis* and *T. caroliniana* have revealed a significant degree of naturally occurring intraspecific variation in adelgid resistance. In the wake of large-scale hemlock mortality, anecdotal evidence suggested that surviving individuals of eastern and Carolina hemlocks may be less susceptible. This has since been corroborated by research, and distinct individuals have been selected and continue to be pursued as putatively resistant to the adelgid (Caswell et al. 2008; Kaur 2009; Ingwell and Preisser 2011; Oten 2011). Further analyses should investigate the intraspecific variation in cuticle thickness of eastern and Carolina hemlock. In addition, studies addressing other hemlock features such as cuticle composition should take place to develop a better understanding of all of the factors that may contribute to species-level resistance and (or) host-plant selection behavior by HWA.

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