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Forest Impacts and Ecosystem Effects of the Hemlock Woolly Adelgid in the Eastern US
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Understanding and Developing Resistance in Hemlocks to the Hemlock Woolly Adelgid

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Abstract - In light of the increasing need for long-term, sustainable management for *Adelges tsugae* (Hemlock Woolly Adelgid), researchers are investigating host-plant resistance as part of an integrated approach to combat the pest. This paper reviews the progress made towards developing a resistant hemlock in the southern Appalachians and highlights the importance of investing research and development resources in this field. Along with describing inter- and intraspecific resistance in hemlocks, this paper reviews investigations into resistance mechanisms and outlines the many options for actions that could be or are being taken to increase resistance and restore hemlock forests.

Introduction

Adelges tsugae Annand (Hemlock Woolly Adelgid [HWA]) is responsible for widespread mortality of hemlocks throughout the eastern United States. Unless successful and long-term management strategies are developed and implemented quickly, HWA will continue to threaten the survival of *Tsuga canadensis* (L.) Carr. (Eastern Hemlock) and *T. caroliniana* Engelm. (Carolina Hemlock). Chemical control is effective and often used, but it is not always practical in the forest setting due to environmental impacts and prohibitive costs (McClure 1991, 1992). Biological control is a major research focus with rearing and release programs in effect since the mid-1990s (Cheah et al. 2004), and research and development continue in an effort to maximize efficacy of the practice (see Onken and Reardon 2011).

In the native range of HWA, host-plant resistance works in concert with the scattered distribution of hemlocks and a complex of other indigenous organisms to naturally manage HWA populations (McClure 1992, Montgomery and Lyon 1996). In addition, host-plant resistance is widely accepted as a key management component in any integrated pest management (IPM) program (Kogan 1994). In light of the need for sustainable management and restoration methods for the disappearing Eastern Hemlock and Carolina Hemlock forests, it is imperative to invest research resources into understanding and developing host-plant resistance in hemlocks as part of an IPM program to combat HWA.

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Resistance in Hemlocks

Interspecific resistance

In both native regions of HWA (Asia and the Pacific Northwest of North America), hemlocks may become infested, but HWA is described as a minor pest that does not typically reduce hemlock tree survival (Bentz et al. 2002, Furniss and Carolin 1977, Keen 1938). Given their innate ability to co-exist with HWA, these hemlock species are often described as HWA-resistant (McClure 1992, 1995a; Pontius et al. 2006). In the Pacific Northwest, HWA can be found infesting *T. heterophylla* (Raf.) Sarg. (Western Hemlock) and *T. mertensiana* (Bong.) Carr. (Mountain Hemlock), but it seldom harms them in forest settings (Furniss and Carolin 1977, McClure 1989). In this region, *Laricobius nigrinus* Fender, a predatory beetle that is now reared and released for biological control programs in the eastern US, naturally occurs in close association with HWA (Zilahi-Balogh et al. 2003), contributing to the complex of natural enemies there. In Asia, HWA inhabits forests and ornamental plantings of *T. diversifolia* (Maxim.) Mast. (Northern Japanese Hemlock), *T. sieboldii* (Carr.) (Southern Japanese Hemlock), and *T. chinensis* (Franch.) Pritz (Chinese Hemlock), but its populations there, as in the Pacific Northwest, are thought to be regulated by a combination of host resistance and natural enemies (Del Tredici and Kitajima 2004; McClure 1992, 1995a; Montgomery et al. 2000). HWA also occurs at low and innocuous densities in Taiwan and Japan (McClure 1987, Takahashi 1937 in McClure 1989).

While the suppression of HWA populations in its native range is often attributed to both host-plant resistance and a complex of natural enemies, Asian hemlocks that are resistant to HWA also exhibit this trait when grown in the eastern US near infested trees and in the absence of natural enemies. For example, at the US National Arboretum (USNA; Washington, DC), Chinese Hemlock and Northern Japanese Hemlock did not have any settled HWA after 8 years of exposure to nearby infested trees (Bentz et al. 2002). At the Morris Arboretum (Philadelphia, PA), despite their proximity to HWA-infested trees, Chinese Hemlock and Southern Japanese Hemlock show no and little infestation of HWA, respectively (Bentz et al. 2007). Likewise, at the Arnold Arboretum (Boston, MA), a Chinese Hemlock planted in 1911 was uninfested as of 2004. A study in this same arboretum compared HWA ovisac abundance and new growth levels between Eastern and Chinese Hemlock. After 4–6 years of exposure to HWA, the Eastern Hemlock had large numbers of HWA ovisacs and new growth on 45% of its branches, while Chinese Hemlock had no detectable HWA and new growth on 100% of its branches (Del Tredici and Kitajima 2004).

Widespread mortality of healthy hemlocks as a result of HWA infestation only occurs in the two species native to the eastern US: Eastern Hemlock and Carolina Hemlock. The most likely explanation for their lack of resistance is that these two species did not co-evolve with HWA (Havill et al. 2006). Although it was initially thought that both species were equally susceptible, Carolina Hemlock is now believed to be more resistant to HWA than Eastern Hemlock. In 2008, researchers reported the results of a study in which three hemlock species (Eastern, Carolina, and Western Hemlocks) were artificially infested with HWA in a greenhouse setting. Findings revealed that Carolina Hemlock and Western Hemlock had significantly lower densities of feeding HWA than Eastern Hemlock (Jetton et al. 2008). Similar results were found when these three species were artificially infested and compared in the field at the Linville River Nursery, Crossnore, NC. Again, Eastern Hemlock exhibited a higher infestation rate than Western Hemlock and Carolina Hemlock (Oten 2011). While both of these studies indicate that HWA settlement rates on Carolina Hemlock are lower than on Eastern Hemlock, the true susceptibility of Carolina Hemlock remains unknown. Carolina Hemlock, a species found exclusively in the southern Appalachians, is more closely related to the resistant Asian hemlocks than it is to its neighbor species, Eastern Hemlock (Havill et al. 2008).

Intraspecific resistance

For years it was believed that Eastern Hemlock and Carolina Hemlock were exclusively and entirely susceptible to HWA (Del Tredici and Kitajima 2004; Lagalante et al. 2006; McClure 1992, 1995b). However, in the wake of large-scale hemlock mortality, anecdotal evidence suggested that some surviving individuals or stands of Eastern Hemlock and Carolina Hemlock may be less susceptible (Caswell et al. 2008). These reports implied that there was some degree of intraspecific variation in HWA resistance of hemlocks, sparking a search for naturally-occurring HWA-resistant trees. Eastern Hemlock is genetically diverse in much of the eastern US (Potter et al. 2012), and phenotypic variation is discussed in the Investigations into Resistance Mechanisms section of this paper.

Resistance to pests commonly occurs in only a small portion of an affected population. Researchers working to develop agricultural crops that are resistant to insect pests have found that resistance occurs at very low levels in wild-type plants (e.g., Eigenbrode et al. 1993, Flanders et al. 1992, Heinrichs 1986). The same can be said for trees with resistant characteristics. For example, resistance to Fusiform Rust (caused by *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusifome*) in *Pinus taeda* L. (Loblolly Pine) is low and regionally-based (Schmidtling et al. 2005). If genes for HWA resistance do exist in hemlocks, they are rare and will likely be challenging to find (Ingwell and Preisser 2011).

Despite the challenges, there are already developments in the search for intraspecific variation giving rise to resistant properties. In the northeastern US, several stands of surviving Eastern Hemlocks were identified as possibly HWA-resistant. These trees were clonally propagated, artificially infested with HWA, and are now considered to be putatively resistant to HWA (Caswell at al. 2008, Ingwell and Preisser 2011).

Progress has been made in our understanding of both inter- and intraspecific resistance of hemlocks to HWA, but there is still much to learn (Table 1). Progress towards understanding the true susceptibility of hemlocks will enhance our ability to search for and implement host-plant resistance as a management tool against HWA. Continued explorations into the interactions between HWA and its host will also aid in the search for resistance mechanisms.

Investigations into Resistance Mechanisms

For more than 50 years, researchers have acknowledged the importance of understanding the mechanism(s) underlying plant resistance to insects (Kogan 1994). The mechanisms for the resistance demonstrated by hemlocks in Asia and the Pacific Northwest are currently unknown. Of the three types of resistance described by Painter (1951)—antixenosis, antibiosis, and tolerance—it is possible that more than one type is employed by the different hemlock species. Many of the resistant individuals that become infested but do not succumb might have tolerance to HWA, while Chinese Hemlock, which rarely becomes infested, may utilize non-preference (antixenosis) or antibiosis mechanisms that prevent severe infestations altogether.

The chemical profiles of hemlock foliage and stem tissue, which may be linked to attraction, defense, or palatability, are the subject of several investigations of resistance mechanisms. It has been suggested that foliar chemistry plays a major role in palatability of hemlock tissue to HWA, with high concentrations of N and K causing higher HWA population levels, and that conversely, high concentrations of Ca and P may deter HWA infestations (Pontius et al. 2006). Volatiles in Eastern Hemlock, which are important olfactory cues in prey searching by *L. nigrinus* (Wallin et al. 2011), were identified as mostly monoterpenes that increase and change in composition following infestation by HWA (Broeckling and Salom 2003). Because HWA is a passively dispersed insect that does not undertake long-range host-finding, its interaction with the host surface (and thus the epicuticular waxes) is perhaps the most significant process of host finding and acceptance. Stimuli that serve as deterrents or attractants are detected in a predetermined order: volatiles offer long-range host-finding, insects come into contact and interact with surface waxes, and components internal to the plant can further influence host acceptance after mouthpart insertion (Bernays and Chapman 1994). The passively dispersed HWA has no need to assess volatiles for long-range host-finding; therefore, its interactions with the host surface are crucial. Kaur (2009) used gas chromatography-mass spectroscopy to compare the epicuticular waxes of Carolina Hemlocks of different provenances. The results of a greenhouse study suggested a correlative relationship: Carolina Hemlocks from provenances with higher HWA densities were more similar in their

Table 1. Hemlock species' resistence to Hemlock Woolly Adelgid (HWA) and their native range.

Species	Resistance to HWA	Native range[L]
T. canadensis	Mostly susceptible, variation occurs[A, B, K]	Eastern North America
T. caroliniana	Mostly susceptible, less susceptible than *T. canadensis*[A, C], variation occurs[D, K]	Southern Appalachians (eastern US)
T. chinensis	Highly resistant[E, I, J, K]	Southeastern China
T. sieboldii	Resistant[E, F, K]	Southern Japan
T. diversifolia	Resistant[E, F, G, I, K]	Northern Japan
T. heterophylla	Resistant[H, I, K]	Northwestern North America
T. mertensiana	Resistant[H, K]	Northwestern North America

[A]Jetton et al. 2008, [B]Caswell et al. 2008, [C]Oten 2011, [D]Kaur 2009, [E]Montgomery et al. 2009, [F]McClure et al. 2000, [G]McClure 1992, [H]Annand 1924, [I]Del Tredici and Kitajima 2004, [J]Hoover at el. 2009, [K]Weston and Harper 2009, [L]Farjon 1990 chemical profiles than those with lower HWA densities. When several hemlock species were compared, chemical profiles varied both inter- and intra-specifically, but researchers were unable to identify the chemical(s) that appeared in the profiles and were therefore unable to determine if there was a correlative relationship (Oten 2011). There is a great need for additional studies to determine the surface chemistry of hemlock needles and stems, given the possibility that chemicals are linked to behavioral processes of HWA that influence host acceptance and use.

The physical features of the plant surface also affect the host-acceptance processes of herbivorous insects (Klinghauf 1987, Pelletier 1990). Therefore, in its interactions with the host surface, HWA may use chemical, morphological, or multiple characteristics to stimulate stylet bundle (mouthpart) penetration into host tissues. Using low-temperature scanning electron microscopy, the biophysical characteristics of leaves of six hemlock species and a hybrid were observed (Oten et al. 2012). Trichomes apparently played no role in the host-acceptance of HWA, but the thickness of the cuticle was significant. Across all species of hemlocks, the cuticle is thinnest on the adaxial side of the pulvinus, proximal to the abscission layer, where HWA consistently insert their stylet bundles. This finding suggests that the insertion point is specifically selected because this area presents less of an obstacle for HWA to access internal host-plant tissues. When analyzing the thickness of the cuticle at areas where HWA does not penetrate, there is greater variation across species. Chinese Hemlock, considered the most resistant hemlock species and one that may employ antixenosis or antibiosis mechanisms, has the thickest cuticle, which may reduce host acceptance. For example, when aphids select or reject a host-plant, a specific sequence of steps occurs: (1) attraction, (2) testing the plant surface and outer plant tissues, (3) penetration, and (4) testing the phloem (Klingauf 1987). If HWA follows the same process as aphids, which are closely related insects, then the thickness of the cuticle, tested in step 2, may deter HWA from proceeding to step 3, penetration of the stylet bundle (Oten et al. 2012).

Once a host-plant is accepted as suitable, the stylet bundle penetrates host tissues and feeding begins (Bernays and Chapman 1994). The interactions between the insect and the internal components of the plant are significant in host-plant resistance, because these interactions provide opportunities for the host plants to employ antibiosis mechanisms that negatively affect the biology of the pest-insect feeding upon it (Painter 1951). In addition, it may be this interaction that triggers a tolerance mechanism or defense pathway within the plant.

The physical interactions between HWA and its host plant can be observed via scanning electron microscopy. HWA is equipped for localized stylet penetration with labial sensilla and neural canals within the mandibular stylets (Oten et al. 2014). In addition, the salivary sheaths of HWA are currently being investigated. When inserting its stylet bundle, HWA releases beads of salivary sheath material that harden upon extrusion, forming a tubular structure around the stylet bundle (Young et al. 1995). This sheath is thought to stabilize the labium during insertion, act as a fulcrum for stylet maneuvering, protect the insect against host-plant defenses, and/or enable stylet bundle reinsertion following a molt (Cohen 1990, Miles 1999). Preliminary studies indicate that stylet bundle insertion by HWA is likely assisted by external sheath material that secures the stylet bundle to the plant surface (Oten 2011, Oten et al. 2014). In ongoing research at North Carolina State University (NCSU), studies are underway to determine the characteristics of this sheath material as it relates to host-plant resistance. If the initial path made by the stylet bundle does not lead to adequate nutrients, HWA can partially retract the stylet bundle, push through the existing stylet sheath, and create a secondary canal for nutrient removal (Cohen et al. 1998; M. Talley, NCSU, Raleigh, NC, unpubl. data). In some resistant plants, the extent of branching is directly linked to host-plant resistance. Because the salivary sheath remains in place when the stylet bundle is retracted, the characteristics of the sheath material and the extent of its branching allow researchers to determine the insect's probing and feeding history (Wang et al. 2008). Preliminary comparisons between Eastern Hemlock and Carolina Hemlock suggest that there is a difference in the number of secondary stylet-sheath canals produced by HWA in the two species: there were fewer stylet sheaths present in Eastern Hemlock, while the stylet sheaths in Carolina Hemlock samples had more canals. In addition, preliminary observations suggest saliva beads formed clumps at the end of some of the canals in the Carolina Hemlock samples and in only one of the Eastern Hemlock samples (M. Talley, NCSU, Raleigh, NC, unpubl. data). This research is ongoing.

In addition to a salivary sheath, plant-feeding Hemipterans also secrete watery saliva that may contain digestive enzymes used for extra-oral digestion, to establish and maintain feeding sites, to suppress plant defenses, and/or to induce changes in plant physiology (Miles 1999, Mutti et al. 2008, Will et al. 2007). Young et al. (1995) documented both salivary sheath saliva and watery saliva in HWA using several staining methods, but did not evaluate whether the watery saliva contained enzymes. It has been repeatedly suggested that the injection of toxic saliva by HWA induces the systemic response that ultimately leads to hemlock death (McClure 1995b, Miles 1990, Young et al. 1995). However, there is no evidence to support that claim and until recently, the watery saliva and digestive enzymes of HWA were unstudied.

Recent research results reveal the presence of at least four trophically related enzymes used by HWA: trypsin-like enzyme, amylase-like enzyme, peroxidase, and polyphenol oxidase (Oten et al. 2014). These enzyme studies are the first of their kind in the HWA system and the implications for plant-pest interactions are numerous. Given difficulties in isolating HWA saliva, however, whole-body homogenate was used, which makes it impossible to pinpoint the source of the enzyme (e.g., salivary glands, alimentary canal). Each of these enzymes has its own implications for plant–pest interactions. The presence of trypsin-like enzyme indicates that HWA is capable of digesting proteins rather than relying solely upon free amino acids. If used extra-orally, then HWA may also have the ability to digest structural proteins of the plant that are otherwise insoluble (Hori 1970, 1971), enhancing their stylet penetration capabilities. It is also possible that protease injections by HWA induce a wound-response pathway that triggers a systemic response in the tree, as is the case in other plant-sucking insects (Dietrich et al. 1999, Ryan 2000). The presence of an amylase-like enzyme, which breaks down and liquefies plant starches, correlates with the previously determined internal feeding site of the starch-filled xylem-ray parenchyma cells (Young et al. 1995), and also suggests that HWA employs extra-oral digestion. Amylase inhibitors are also produced in many plants (Garcia-Olmedo et al. 1987, Marshall 1975) and may be a component of resistance. In some cases, enzyme inhibitors are capable of inhibiting the activity of both protease and amylase (Ryan 1990). The presence of oxidases (peroxidase and polyphenol oxidase) present in the watery saliva would suggest a detoxification response to plant defenses (Miles 1999). Further research and successful isolation of watery saliva may determine whether or not this occurs. From a biochemical standpoint, this detoxification response could be a major contribution to the systemic reaction of hemlock to HWA feeding (as described in Radville et al. 2011). For example, in the case of *Therioaphis maculata* Buckton (Spotted Alfalfa Aphid), Madhusudhan and Miles (1998) suggest that necrosis of Alfalfa susceptible to the Spotted Alfalfa Aphid was likely caused by the injection of salivary oxidases into the plant. If the same occurs when HWA feeds on susceptible hemlocks, then the systemic reaction may be indicative of the tree's inability to compensate for oxidase injection. Resistant and susceptible hemlocks should be examined for the presence of enzyme inhibitors specific to the enzymes detected in HWA in this study (Oten 2011, Oten et al. 2014).

The toxic saliva theory and the proposed implications of the aforementioned trophically related enzymes of HWA are supported by what is known regarding the reaction of hemlock to HWA. Following infestation, hemlocks exhibit symptoms similar to those observed under drought conditions (Domec et al. 2013). The reaction is systemic and is now believed to be a hypersensitive response indicated through the detection of increased H_2O_2 levels in infested trees (Radville et al. 2011). In addition, tree water-use is reduced by more than 40%, and gross primary productivity is reduced by 25% (Domec et al. 2013). Morphological changes also occur internally. False rings, a section of an annual growth ring with thickened cell walls that reduce water transportation, develop within HWA-colonized woody stems (Domec et al. 2013, Gonda-King et al. 2012).

Researchers at NCSU have infested hemlocks and are screening their tissues to look for infestation-induced changes in the xylem-ray parenchyma cells, the internal feeding site of HWA (Young et al. 1995). Monthly samples of Eastern, Carolina, and Western Hemlock branches are frozen, microtomed, and stained using a method modified from Young et al. (1995). In an effort to detect any internal morphological changes HWA causes to its internal feeding site, stained sections are examined with a light microscope to observe alterations in ray-parenchyma cell-structure over time following HWA infestation, (M. Talley, NCSU, Raleigh, NC, unpubl. data).

Developing Resistant Hemlocks

There are already well-established techniques used to incorporate resistance in other plant species that can be applied to the development of an HWA-resistant hemlock. Although the use of HWA-resistant varieties in an IPM program is still in the future, these methods offer a foundation upon which to build. An intra-specific breeding program may be a challenge given the difficulty in identifying naturally resistant individuals, but it is valuable because it would preserve the desired appearance of trees native to the eastern US while maintaining environmental compatibility (Bingham et al. 1953). Likewise, inter-specific breeding can incorporate resistant genes of non-native species with the characteristics of native species. As an alternative to traditional breeding programs, biotechnology offers several methods to incorporate resistant characteristics that may accelerate the breeding process. Gene conservation also plays an important role in maintaining the genetic diversity and range of adaptive variation present across the range of hemlocks. This section discusses the progress, value, and potential of each of these developmental techniques.

Searching for natural resistance

Exploiting resistance or tolerance in native hemlock populations requires identification of resistant individuals for breeding and/or vegetative propagation. In 2011, researchers at NCSU began the search for HWA resistance or tolerance in the southern range of Eastern Hemlock and throughout the range of Carolina Hemlock. The primary technique utilized to identify putatively resistant trees was similar to the approach taken by researchers at University of Rhode Island in 2007 (Ingwell and Preisser 2011). Researchers produced brochures detailing the characteristics of a putatively resistant tree, including foliar color and density, tree size, HWA impact on surrounding hemlocks, and HWA density. To reduce the incidence of false positives, they targeted brochure distribution primarily to natural resource professionals, but some brochures were made available to the general public. An interactive display was developed for the citizen-science area at the North Carolina Museum of Natural Science, Nature Research Center, Raleigh, NC. The researchers also developed a website to facilitate and standardize the process for reporting surviving hemlocks. Researchers evaluated suggestions for candidate trees and, where appropriate, the scientists made field evaluations. In addition to pursuing resistance as reported by people responding to the brochure, NCSU researchers are searching for resistant hemlocks in Great Smoky Mountains National Park, an area that has experienced widespread infestation and subsequent hemlock mortality. Searches within the park have been prioritized based on hemlock frequency, as derived from vegetation maps produced by the National Park Service (Evans 2014), and hemlock mortality, with the highest-mortality areas having increased priority. Surviving hemlocks in sites with the highest mortality will be highly visible, and will have withstood the increased pressures of adjacent HWA infestations. HWA-induced mortality serves as a natural screening procedure, with surviving hemlocks representing potentially resistant individuals for which further screening can occur.

Field evaluation of reported trees is used to verify species identification, tree health, and environmental factors that may explain good tree health. The tree-health assessment examines the crown size, density and distribution of foliage, and the presence and density of HWA infestation. Lightly infested trees that exhibit thinning foliage throughout the crown or within the crown's interior are ruled to be likely in decline due to HWA. High levels of sun exposure, adequate moisture, and minimal competition from surrounding trees contribute to tree health (A.E. Mayfield, USFS, Asheville, NC, and R.M. Jetton, unpubl. data). To test for possible previous insecticide treatment, researchers screen for presence of imidacloprid, an insecticide applied to combat HWA, using a commercially available ELISA kit as described by Eisenback et al. (2009). NCSU researchers do not test for the presence of dinotefuran at this time due to the higher cost of testing and lower frequency of use of this insecticide.

Trees that meet the criteria for putative resistance after field evaluation are vegetatively propagated for the purpose of resistance screening in common-garden environments. Depending upon the timing of the field evaluation, softwood, semi-hardwood, or hardwood cuttings are collected for rooting, with additional collections occurring as needed to attain a sufficient number of plants for screening. Cuttings from 31 Eastern Hemlock trees from NC are currently being rooted, with collections from an additional 21 Eastern Hemlock and one Carolina Hemlock scheduled from trees in NC, TN, and VA. Plants will be artificially infested with HWA when cuttings are of suitable size and are well-established. A rain-down technique that uses infested branches hanging over to-be-infested trees has been developed to facilitate mass infestation of possibly resistant Hemlocks and for progeny screening (Jetton et al., in press). Level of resistance or tolerance will be evaluated based upon the density of settled HWAs over time as well as overall plant health. Individual trees exhibiting resistance or tolerance will be included in a breeding population, where crosses will be made until true-breeding progeny for resistance can be produced.

Interspecific hybridization

An alternative to finding resistance in native populations of Eastern Hemlock and Carolina Hemlock is the production of interspecific hybrids between these species and HWA-resistant or -tolerant Asian hemlocks (Chinese Hemlock, Southern Japanese Hemlock, Northern Japanese Hemlock). All five of these hemlock species have the same number of chromosomes (haploid of $n = 12$) and are not known to exhibit polyploidy (Santamour 1963, Sax and Sax 1933). Researchers at USNA began making those hybrid crosses using controlled pollination in 1991, and have successfully produced *T. caroliniana* x *T. chinensis* hybrids, but were unsuccessful in hybridizing Eastern Hemlock with any of the above Asian hemlocks (Bentz et al. 2002, Pooler et al. 2002). Subsequent testing has shown the *T. caroliniana* x *T. chinensis* hybrids to be intermediate to the parent species in HWA resistance, with good growth and form (Montgomery et al. 2009). USNA hybrids are currently being evaluated in MD and NC for adaptation to conditions in eastern North America, as well as suitability for landscape use.

Researchers at NCSU began a hemlock-hybrid breeding program in 2011 in NC. Crosses between both Eastern and Carolina Hemlocks and the three Asian species mentioned above were made in 2011, but failed to produce any viable hybrid seedlings. Additional crosses were made in 2012 with the same parent species. Seed from 2012 has not yet been germinated, but very low viability is expected due to heavy seed predation by insects. In an attempt to initiate somatic embryogenesis, some immature hybrid seeds were collected and cultured in 2011 and 2012 (see next section on Biotechnology). Another series of hybrid crosses between Eastern Hemlock and the Asian hemlock species was made in 2013, as well as crosses between Eastern and Western Hemlocks; the success of these efforts has yet to be evaluated.

If hybrid hemlock crosses are successfully made at NCSU, F1 generation progeny will be screened for HWA resistance. Resistant progeny, if fertile, will be backcrossed to their respective native parent species to increase the proportion of native genes, in an approach mirroring that used with *Castanea dentata* (Marsh.) Borkh. (American Chestnut) to combat *Cryphonectria parasitica* (Murr.) Barr (Chestnut Blight) (Diskin et al. 2006, Hebard 2006). A series of backcrosses and intercrosses will be made until lines that are true-breeding for HWA resistance and native hemlock morphological characteristics are achieved. This backcrossing procedure is often used in breeding programs to maintain the adaptive traits of a native species while integrating resistant traits of a non-native species (Hayes et al. 1955).

Biotechnology

Biotechnology offers multiple tools that can be used to implement HWA resistance strategies. While molecular approaches may eventually make contributions in this area, in vitro culture and associated technologies are more likely to have near-term impact in areas such as hemlock conservation and restoration. Technologies such as in vitro clonal propagation via somatic embryogenesis, cryostorage, and gene transfer have already been developed for multiple species in the Pinaceae, and these techniques should be transferrable to hemlocks with relatively minor modifications. Here, we will review the current status of these technologies with hemlocks and discuss the potential for their application in conservation of hemlock germplasm and restoration of hemlocks to their native ranges.

Somatic embryogenesis. The ability to clonally propagate hemlocks in vitro would open multiple avenues for aiding hemlock conservation and restoration. Currently, the most widely used in vitro propagation system for conifers is somatic embryogenesis (SE). SE is an in vitro process by which structures (somatic embryos) resembling zygotic (seed) embryos are produced asexually. These somatic embryos can be germinated like seeds to produce seedling-like plantlets (somatic seedlings). The process was first described in the 1950s for *Daucus carota* L. (Carrot; Reinert 1958, Steward 1958), but it was not reported in a conifer until the 1980s, when it was described in *Picea abies* L. Karst. (Norway Spruce; Hakman et al. 1985) and *Larix decidua* Mill. (Larch; Nagmani and Bonga 1985). Over the next two decades, somatic embryogenesis was reported in the literature for several species representing every genus of the Pinaceae in North America, except *Tsuga*. High frequency SE systems capable of producing thousands or even millions of somatic seedlings have been developed at forest biotech companies and university labs (e.g., Park 2002, Sutton 2002) for some of the more important commercial North American conifers, such as *Picea glauca* (Moench) Voss (White Spruce), *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir), *Pinus strobus* L. (Eastern White Pine), and Loblolly Pine. The lack of a similar system for hemlocks is likely due to their relatively minor commercial value.

Recognizing the potential for SE as a useful tool for hemlock conservation and restoration, Montello et al. (2008) began research to establish embryogenic cultures of both Eastern and Carolina Hemlocks by applying SE induction protocols that had been effective with other Pinaceae taxa. In a preliminary study, they collected immature cones from hemlocks in North Carolina and Virginia during July and August, dissected them to obtain immature seeds, and cultured the immature seeds or embryos on an induction medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) that had originally been developed for pine somatic embryogenesis (Merkle et al. 2005). A low percentage of the explants produced callus that appeared very similar to embryogenic callus reported for other conifers (Figs. 1A, B), and callus derived from one explant representing each species went on to produce a number of bullet-stage somatic embryos (Fig. 1C) and a few cotyledonary-stage somatic embryos (Fig. 1D) following transfer to a pine maturation medium (EMM2; Smith 1996). In a more extensive study, Merkle et al. (in press) cultured seeds collected on different dates during May–August from four Eastern Hemlock and four Carolina Hemlock source-trees in Georgia and North Carolina, on three different induction media. Cone collection date and medium significantly affected embryogenesis induction frequency, with induction reaching as high as 52% for Eastern Hemlock

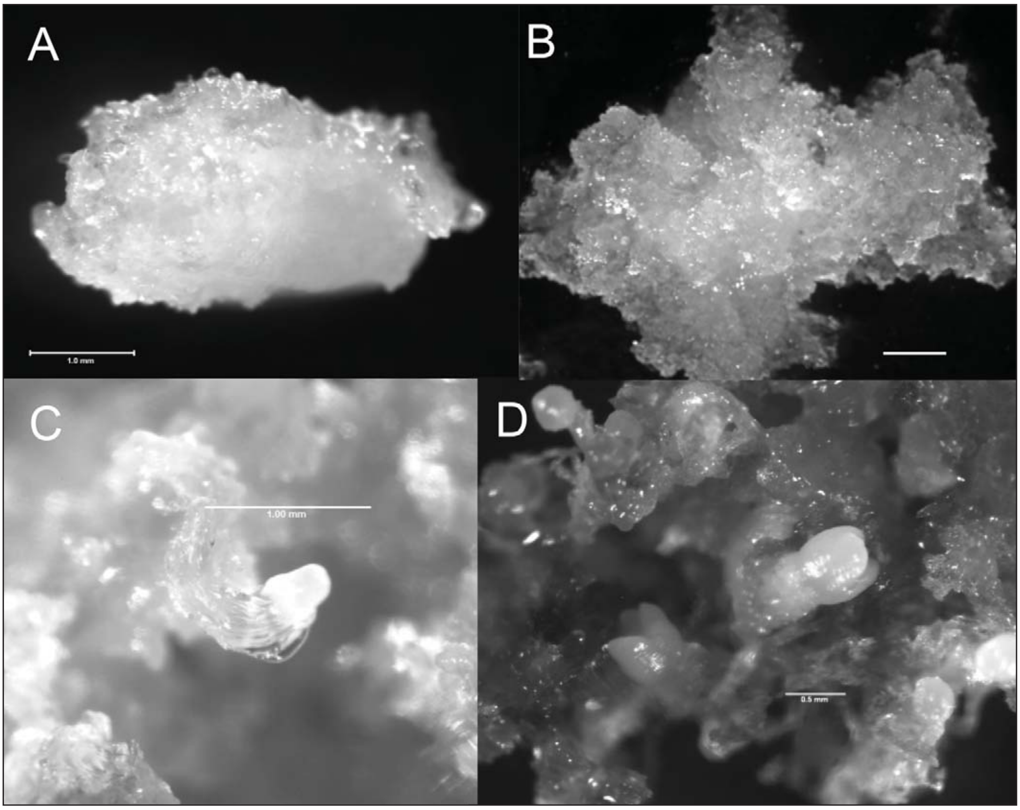

Figure 1. Somatic embryogenesis in Eastern and Carolina Hemlocks. A) Embryogenic callus derived from a zygotic embryo, emerging from an Eastern Hemlock megagametophyte. Bar = 1 mm. B) Established culture of Eastern Hemlock embryogenic callus. Bar = 500 μm. C) "Bullet-stage" Carolina Hemlock somatic embryo. Bar = 1 mm. D) Early cotyledon-stage Carolina Hemlock somatic embryos. Bar = 500 μm.

seeds collected in mid-July in Georgia and 17% for Carolina Hemlock seeds collected in late July in North Carolina. Smith's (1996) EDM6 medium proved the best overall for embryogenesis induction for both hemlock species.

Once established, embryogenic hemlock cultures were maintained by monthly transfer to fresh EDM6 medium. Regeneration of large numbers of somatic embryos from the cultures has been problematic to date, although preliminary experiments using a modified Litvay's medium (Litvay et al. 1985) improved production of coytledonary-stage embryos over Smith's (1996) EMM2 medium (S. Merkle, University of Georgia, Athens, GA, unpubl. data). While only a few hemlock somatic seedlings have been produced so far, experiments to optimize a protocol and media for mature somatic embryo and somatic seedling production are continuing.

Cryostorage. Embryogenic cultures of most species are well suited for cryostorage (i.e., storage in liquid nitrogen). Cryostorage of embryogenic cultures offers an alternative approach to storing seeds or installing plantings outside the range of HWA to conserve genetic diversity of both threatened hemlock species. Once placed in liquid nitrogen, cultures can be stored indefinitely, recovered, and regrown, with little or no loss of viability. Cryopreservation protocols have been developed that are routinely applied to embryogenic cultures of a number of coniferous species, including White Spruce (Kartha et al. 1988), *Picea mariana* (Mill.) Britton, Sterns, and Poggenb. (Black Spruce; Touchell et al. 2002), and *Pinus radiata* D. Don (Radiata Pine; Hargreaves and Smith 1992). Montello et al. (2010) tested a cryopreservation protocol that previously had been applied to cryostore and recover embryogenic cultures of different hardwood tree species (Holliday and Merkle 2000, Vendrame et al. 2001). Embryogenic hemlock cultures pre-treated in liquid EDM6 (Smith 1996) supplemented with 0.4 M sorbitol and cryostored in the same medium supplemented with 5% dimethylsulfoxide (DMSO) as cryoprotectant could be thawed and regrown with 100% efficiency, even after more than seven months in cryostorage, for four of five tested genotypes.

Combining SE with conventional breeding. The ability to generate embryogenic hemlock cultures and their amenability to cryostorage have important implications for generating and testing material that may be resistant or tolerant to HWA infestation. The clonal multiplying power of a good SE system could be very effectively combined with conventional intra-species and hybrid hemlock breeding programs. Seeds resulting from crosses between surviving native hemlocks that appear to possess resistance/tolerance to HWA could be used as explants to start embryogenic cultures. Once established, these cultures could generate populations of genetically identical trees that could be used in screening trials to determine if there is a genetic basis for HWA resistance or tolerance of the parent trees. Similarly, hybrid breeding between susceptible native hemlocks and resistant Asian species such as Chinese Hemlock can be combined with SE to generate clones of hybrid trees for resistance/tolerance screening. In fact, this approach is already being pursued: through a collaboration between University of Georgia researchers and NCSU hemlock breeders, embryogenic cultures of *T. caroliniana* x *T. chinensis* and *T. caroliniana* x *T. sieboldii* have been generated and are being tested for somatic embryo and somatic seedling production (S. Merkle, unpubl. data). It is also possible that problematic hybrid crosses (i.e., crosses from which it has been difficult to obtain viable seeds, such as crosses involving Eastern Hemlock) may be aided by culturing the developing seeds, thereby rescuing the hybrid embryo which would otherwise abort, at the same time as inducing SE from it. Finally, the fact that embryogenic cultures currently can only be started from zygotic embryos, thus preventing cloning of proven genotypes, can be partially overcome by taking advantage of the relatively high level of self-compatibility that has been reported for both Eastern and Carolina Hemlocks (Bentz et al. 2002). Selfed seeds from potentially resistant/tolerant hemlocks could be used to start embryogenic cultures, thereby providing a collection of clones with putative resistance genes in various heterozygous and homozygous combinations. In all of these scenarios, the fact that hemlock embryogenic cultures can be recovered following cryostorage means that they can be held indefinitely while somatic seedlings derived from them are screened for HWA resistance or tolerance. Then, if screening results indicate that any of the clones are especially promising, the cultures from which they were derived can be thawed, regrown and scaled-up to make somatic seedlings for restoration purposes.

Potential for transgenic research. To date, there have been no published reports of gene transfer in any hemlock species. However, embryogenic cultures have provided excellent target material for gene transfer in a number of conifers, including Norway Spruce, White Spruce, Loblolly Pine, Radiata Pine, and Eastern White Pine (see review by Tang and Newton 2003). Given the apparent similarity of hemlock embryogenic callus to that of other Pinaceae members, it is likely that the same protocols used to transfer genes into these species using *Agrobacterium*-mediated gene transfer would be applicable to hemlocks. Thus, if it becomes desirable to test transgenes (e.g., *Bacillus thuringiensis* endotoxin genes) or cisgenes (i.e., from resistant hemlocks) for their ability to confer HWA resistance/tolerance to Eastern Hemlock or Carolina Hemlock, this approach should be feasible.

Gene conservation

Ex situ gene conservation is a key component of the integrated effort to manage the impacts of HWA on eastern North American forests (Onken and Keena 2008), and complements the in situ methods of biological and chemical control for conserving hemlocks. It involves the collection of seeds that represent the genetic, climatic, and edaphic variability present across the range of the species. Seeds are placed either into seedbanks for long-term cold storage, or are used to establish seed orchards in locations where the trees can be reliably protected. This is an extreme, but often necessary, approach to conserve the genetic integrity of tree species threatened by exotic insects and pathogens, climate change, and overharvesting (Dvorak et al. 2000). Genetic resource conservation is particularly critical for hemlocks at a time when HWA-related decline and mortality continue unabated and effective management is unavailable. Use of effective chemical insecticides is limited by economic and environmental concerns, additional research and development are necessary for biological control to reach expected levels of efficacy, and the breeding of HWA-resistant genotypes is in the early stages.

The hemlock gene-conservation program was initiated in 2003 as a collaborative effort between Camcore (International Tree Breeding and Conservation Program at NCSU), the USDA Forest Service, and numerous state forestry agencies within the ranges of Eastern and Carolina Hemlock. The primary objective of the project is to maintain, in perpetuity, genetically diverse and broadly adaptable ex situ seed reserves and seedling seed orchards that will be available for breeding and restoration activities once effective in situ HWA management strategies are in place. Seed collections and seed orchard establishment are currently ongoing, but the hemlock genetic resource conservation program has already placed approximately 2.5 million seeds into conservation. Carolina Hemlock collections total 1515 g of seed (at 360 seeds/g, more than 500,000 seeds) and represent 134 mother trees from 19 populations distributed across the Southern Appalachian Mountains (Fig. 2). Seed collections from Eastern Hemlock have yielded 5544 g of seed (at 412 seeds/g, more than 2 million seeds) from 451 mother trees and 60 populations distributed across the northern and southern portions of the species' range within the US (Fig. 2). Nearly 2000 g of seed have been placed into long-term cryopreservation at the USDA Center for Genetic Resource Preservation in Fort Collins, CO, and 2204 seedlings have been planted into genetically diverse seed orchards located in Brazil, Chile, and the US. All remaining seeds reside in Camcore's seed repository at NCSU in Raleigh, NC and are being utilized for the establishment of additional

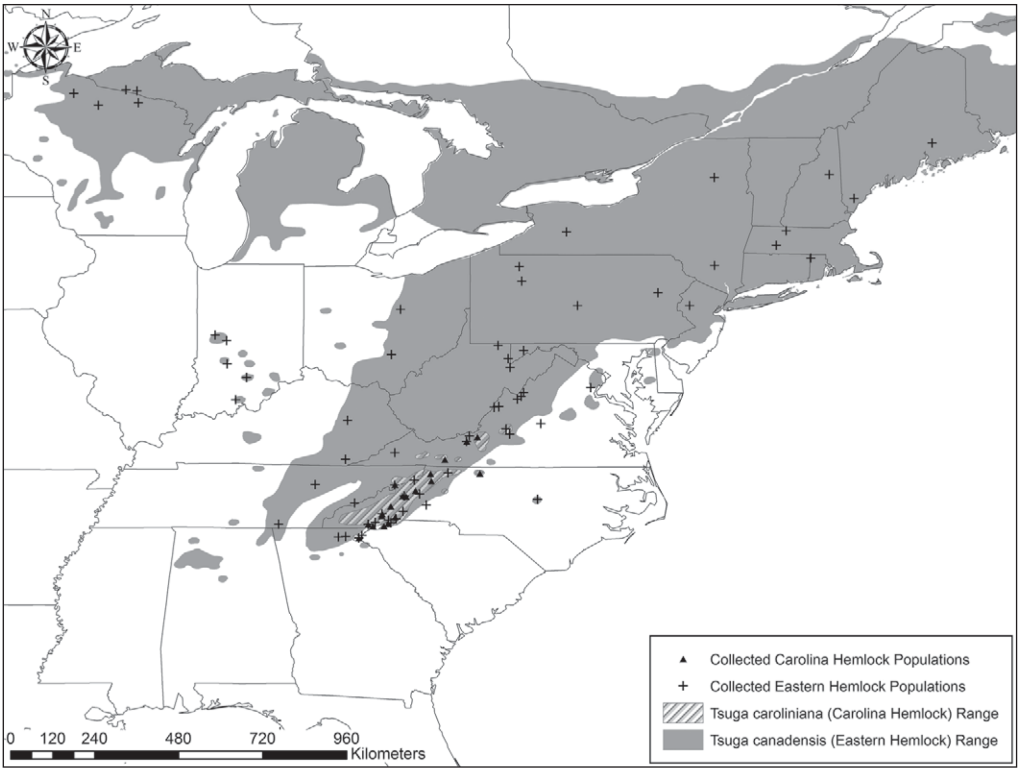

Figure 2. Locations of Eastern and Carolina Hemlock seed collections made by Camcore and the USDA Forest Service for ex situ genetic resource conservation.

seed orchards and to supply research projects on hemlock genetic diversity and breeding. A portion of this seed has also been set aside as a strategic seed reserve for long-term preservation. These numbers represent mature seed collections, and do not include collections of immature seeds used for gene conservation through somatic embryogenesis and cryostorage. For additional details on the strategies and protocols being utilized for hemlock gene conservation, readers are referred to Jetton et al. (2013).

Ex situ gene-conservation programs such as the one described above play a critical role in the development and implementation of resistance-breeding and restoration programs. At its most fundamental level, genetic resource conservation protects against the worst-case scenarios of local and range-wide extinctions and the functional elimination of Eastern and Carolina Hemlocks from forest ecosystems. Furthermore, the genotypes conserved through seed collections and seed-orchard establishment can be utilized to address a variety of resistance-breeding and restoration objectives. In the event that biological and chemical controls are effective in maintaining HWA populations below damaging levels and resistant genotypes are not an absolute necessity, the seeds and seed orchards will provide a ready source of locally adapted material for restoration planting. In this situation, for example, restoration plantings of Eastern Hemlock in Great Smoky Mountains National Park can be accomplished with the 24 seed sources that were collected within the park boundaries in 2008 (Jetton et al. 2013). If inter-specific hybrids between native hemlocks and HWA-resistant species from western North America and Asia are the objective, the genetically diverse and broadly adaptable base population made available through gene conservation will increase the probability and number of successful hybrid crosses. Moreover, gene conservation may be the foundation that allows for the establishment of regionally adapted breeding populations as has been accomplished by The American Chestnut Foundation’s program (Hebard 2006). Finally, if the amount of hemlock genetic material available for resistance screening is maximized, the possibility for identifying naturally occurring HWA resistance in Eastern and Carolina Hemlocks will increase. If resistance is found and the level of genetic variation for resistance traits is adequate, opportunities might exist for breeding and restoration programs based on the pure species rather than genotypes with some proportion of genes from non-native hemlocks.

Conclusion

When breeding agricultural crops resistant to an insect pest, host-plant resistance is a powerful tool. It is widely accepted as a major component of countless IPM programs, and should also be used and implemented when managing forest pests. Developing an HWA-resistant hemlock suitable for restoration plantings and continued HWA management in the eastern US will undoubtedly be a long process, but the foundation is already established. Continuing the search for resistance mechanisms through continued host–pest interaction studies will improve our ability to search for and screen for resistant individuals. As discussed here, there are several techniques that are already showing promise to find and amplify resistance.

Continued research deserves further investment to continue this pursuit and increase the chances for successful long-term HWA management in the eastern US.

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