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Evaluating Seedlings of Eastern Hemlock Resistant to Hemlock Woolly Adelgid (*Adelges tsugae*)

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EVALUATING SEEDLINGS OF EASTERN HEMLOCK RESISTANT TO HEMLOCK WOOLLY ADELGID (*ADELGES TSUGAE*)

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ABSTRACT

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand has invaded the North American range of the eastern hemlock, *Tsuga canadensis* (L.) Carriere, and Carolina hemlock *T. caroliniana* Englemann, causing widespread mortality. Of the nine *Tsuga* species known worldwide, only *T. canadensis* and *T. caroliniana* experience mortality from HWA. Researchers at the University of Rhode Island and their cooperators have identified stands of HWA-resistant eastern hemlocks and confirmed this resistance through studies of vegetatively propagated progeny. This study investigates HWA resistance of plants grown from field-collected open-pollinated seed from these parent plants with known resistance. Trees were evaluated under standardized conditions, artificially inoculated with HWA and measurements of tree growth and HWA density were taken during the settlement phase of each HWA generation. Based upon published literature, HWA densities achieved in this study should have inhibited new growth and eventually killed at least some of the test plants. However, in this study, heavily infested seedlings continued to grow throughout the three years of observation and there is as yet, no HWA-induced tree mortality. It is possible that the HWA population used in this experiment is relatively non-virulent, not inducing the hypersensitive reaction of infested trees which causes declining health of both the trees and the HWA population.

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PREFACE

This thesis serves as an interim report of an experiment that was initiated in 2011. Neither the insects nor the trees responded as expected, necessitating changes in experimental hypotheses, protocol, and duration of tests, but leading to a much richer understanding of the relationship between this insect and its hosts. Additional follow-up observations and expanded research objectives will be a useful supplement to results reported herein.

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Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, has invaded the native range of the eastern hemlock, *Tsuga canadensis* (L.) Carriere in North America. This insect, native to China and Japan, was accidentally introduced from the Osaka region of Japan to Richmond, Virginia in the 1950s (Havill et al. 2006). Since then it has caused widespread mortality in hemlock from the southern edge of the tree's native range in Georgia across eighteen states west to southeastern Ohio and north to Maine (USDA Forest Service, 2015).

HWA Resistance

Of the nine *Tsuga* species known worldwide, only the eastern hemlock and Carolina hemlock, *T. caroliniana* Englemann, experience mortality from HWA (Del Tredici and Kitajima 2005, McClure 1991). The Asian species *T. chinensis* (Franch.) E. Pritz, *T. diversifolia* (Maxim.) Mast and *T. sieboldii* Carriere are resistant to HWA (Del Tredici and Kitajima 2005, Montgomery and Lagalante, 2008), and programs attempting to hybridize these HWA-resistant species with *T. canadensis* and *T. caroliniana* have produced *chinensis-caroliniana* F1 hybrids that possess levels of HWA resistance. Similar crosses with *T. canadensis* have not been successful (Bentz et al. 2002). The western hemlock (*T. heterophylla*) is resistant to HWA in its native range in western North America, but appears less resistant when established in the east, perhaps due to stress from abiotic conditions (Del Tredici and Kitajima, 2005). Furthermore, Lagalante et al. (2007) showed that dwarf hemlock cultivars derived from witches' brooms of *T.*

canadensis had terpenoid profiles similar to resistant hemlock species, possibly resulting in HWA resistance.

Based upon this information, it seems reasonable that some natural populations of eastern hemlocks might also have resistance. Studies have been conducted to identify and evaluate naturally-occurring populations of *T. canadensis* that have survived the selection pressure imposed by HWA (Caswell et al. 2008, Radville et al. submitted).

Published literature indicates that densities greater than four HWA per cm of twig in the first year of infestation should inhibit new growth and eventually kill *T. canadensis* (McClure 1991). Trees are thought to possibly possess some level of innate resistance when they have survived repeated exposures and look relatively healthy (Caswell et al. 2008). Researchers at the University of Rhode Island and their cooperators have identified several sites that contain significant numbers of these “lingering” hemlocks (Caswell et al. 2008, Ingwell and Preisser 2011). These trees are evaluated under standardized conditions in order to isolate the effect of resistance (McClure and Cheah 2002, Pontius et al. 2006). To this end, a screening protocol has been developed where trees are vegetatively cloned, planted in a common garden, and artificially inoculated with HWA to assess their degree of resistance (Butin et al. 2007, Ingwell and Preisser 2011). Separate experiments conducted at the University of Rhode Island have challenged both rooted cuttings and grafted plants using this protocol with encouraging results (Caswell et al. 2008, Ingwell and Preisser 2011, Radville et al. submitted). Plants originating from a hemlock stand near the Delaware Water Gap in NJ, referred to as the "Bulletproof Stand", have looked particularly promising in terms of HWA resistance.

Recent work has shown that the stems of these trees have elevated terpene levels that may be associated with this resistance (McKenzie et al. 2014).

While rooting cuttings and grafting hemlock plants are useful techniques for producing research plants and particularly valuable ornamental cultivars, even under ideal conditions these techniques will not be adequate to produce the immense numbers of plants needed for reforestation of hemlock stands killed by HWA. Hemlocks are traditionally produced in large numbers by germinating field-collected seed, although recent advances in the tissue culturing of embryonic cells may have promise (Merkle et al. 2014). This study was conducted to investigate the HWA resistance of hemlocks grown from seed of parent trees known to be resistant to hemlock woolly adelgid and select for seedlings with the highest degree of resistance.

Materials and Methods

Seed Collection and Plant Management

In October of 2011, cones were collected from two previously studied eastern hemlock trees (NJ5 and NJ6) from the "Bulletproof Stand" in the Delaware Water Gap National Recreation Area near Walpack, NJ (coordinates: 41^{o}, 07.647'N; 74^{o}, 54.386'W). Cones were also collected from a site near Drum PA which had been identified in 2007 as a possibly HWA-resistant site by Scott Stitzer, a PA forester. (The trees at this site [coordinates: 41°,072910'N; 75°,9193'W] did look healthy to R.A. Casagrande on the date of collection.) Cones were collected from two sites thought to be HWA-susceptible. One site was a 3m tall hedge of *T. canadensis* growing at the North Carolina Arboretum near Asheville, NC (NCAS). This hedge had been heavily infested by HWA and treated with Imidacloprid in the previous season. A second likely-susceptible tree, growing in the yard of R.A. Casagrande in Kingston, RI, was transplanted as a seedling collected in a native stand of *T. canadensis* in Essex Junction, VT in 1978. This tree has been lightly infested by HWA in the past.

Cones were dried at room temperature for fifteen days to promote seed drop. Using a protocol developed for Camcore, for *T. canadensis* seed germination (Jetton et al. 2014), seeds were stored at 7°C for 60 days of cold stratification in closed 295 ml plastic containers. They were then transferred to closed 100 cm petri dishes with moistened filter paper at 7°C for 100 days of cold moist stratification to promote germination. On May 14, 2012, seeds were sown in 72-cell plastic trays using Metro-Mix® 830 growing media that was sifted through a 0.5 cm screen to remove the largest particles. Seeds were sown one seed per cell and held in a Conviron® plant growth chamber @ 18ºC and 80% humidity under 12 hours of fluorescent lighting for germination. Plants were removed from the chamber on July 14, 2012 for evaluation, transplanted into plug trays with cells 5 cm wide X 13 cm deep, and moved to a walk-in cooler set to 16ºC and gradually raised over a period of weeks to 24.4ºC. The trees were moved outdoors between greenhouses under 60% shade cloth on August 28, 2012. Supplemental lighting was added on October 9, 2012 with a spotlight to establish a twelve-hour photoperiod and extend the growing season. Trees were transferred to an unheated greenhouse on November 3, 2012, and held on benches under a twelve-hour photoperiod until December 20, 2012, at which time the lighting was removed. Trees were moved to a walk-in cooler set to 10ºC on January 11, 2013 and held under an eight-hour photoperiod. The temperature was reduced gradually until it reached 4ºC on January 30, 2013, and the trees overwintered at 4ºC under an eight-hour photoperiod.

In April of 2013, four groups of seedlings were selected for inoculation with adelgids (detailed in following section). Prior to plant selection, the few seed-grown plants that were smaller or less vigorous were set aside, then thirty-nine seedlings were haphazardly selected from each of four groups of seedlings. These groups included seedlings of NJ5, NJ6, PA, and a single group of likely-susceptible trees. This likely-susceptible group consisted of 33 seedlings from the NC arboretum (NCAS) and six seedlings from the Essex Junction, VT tree (labeled LOCS). An additional group of 39 seedlings was haphazardly chosen from among all the groups of seedlings to act as an uninoculated control: 12 NJ5, 17 NJ6, and 10 PA. All trees were of similar size and vigor, and were held in 12 cm square nursery pots with Metro mix® 830 used as media. One-half the minimum recommended dose, approximately 2 gm of Nutricote® 13-13-13 slow-release fertilizer was applied to each pot. The seedlings were transferred to plastic trays; at least three seedlings of each parental group were represented in each tray. Plants were placed in an outdoor enclosure between greenhouses under 60% shade cloth. The uninoculated group was held nearby, under the same shade cloth, and separated by a screen barrier.

After the 2013 growing season, the seedlings were removed from the shade enclosure in October and overwintered in a 1m x 4.2 m hoop structure covered with 4 mil white plastic. White winter protection fabric (0.2 cm felt) draped directly on the hemlock foliage provided additional frost protection. The structure was situated on the north side of the greenhouse range in the shade in order to prevent temperature spikes on warm winter days. The hemlocks were returned to the nursery on March 4, 2014 and held under

60% shade for the duration of the 2014 growing season. Kingston, Rhode Island experienced unusually cold weather in the early winter following the 2014 growing season, and it was decided to bring the seedlings into a walk-in cooler set to 4°C under an eight-hour photoperiod on December 18, 2014. The plants were moved outdoors to the nursery on March 18, 2015 and covered with winter protection fabric draped directly on the hemlock foliage for additional frost protection.

On April 13, 2015, the 186 surviving trees were transplanted to #1 round nursery pots, 18 cm tall and 17.75 cm in diameter, with Metro-mix® 830 media, and one-half the minimum recommended rate, or five grams, of Nutricote® 13-13-13 slow-release fertilizer was applied to each pot. All seedlings were hand-watered for the duration of the experiment.

Inoculation

Hemlock wooly adelgids in the progrediens crawler stage were collected on the morning of April 24, 2013 by clipping heavily-infested branches from the two Sargent's Weeping Hemlocks (*T. canadensis* 'Pendula') on the west side of Quinn Hall on the URI Kingston campus. These trees were heavily infested with HWA, but apparently healthy. The 156 seedlings of the LOC, NCC, NJ5, and NJ6 parent groups were inoculated the same day using small infested branchlets in water pics, wired directly to the seedling with a twist-tie (Fig. 1) following the protocol of Butin et al. (2007)

HWA Sampling

The first-instar crawlers of the progrediens (spring) generation remained active until it was no longer possible to count discrete individuals among the wool ovisacs of developing third- and fourth-instar HWA which largely coated the branches (Fig. 2). The first count was made after settlement of the sistens (overwintering) generation on July 23, 2013. The leader, or highest vertical branch of the seedling, was measured above the highest ovisac left by the progrediens generation and marked with a twist-tie (hereafter referred to as 'marker'), and a count of the adelgid settled on the leader was made *in situ* using an Optivisor® magnifying headset.

On June 17, 2014, counts were taken of first- and second-instar HWA on the previous year's growth distal to the marker attached the previous year to assess settlement of the progrediens generation and again on August 15, 2014 for sistens settlement. A final count was made on June 7, 2015 to assess settlement of the progrediens generation.

Hemlock Sampling

Whole-tree measurements were taken of all trees including uninoculated controls to assess seedling growth on November 16, 2013. New growth was measured on the leader for all trees, including the uninoculated trees, on August 15, 2014. All trees were measured again for new growth and for total height during the November 10, 2014 count. The number of branches extending from the main stem of each seedling was counted on June 7, 2015. The number of branches with budbreak and the total number of buds per tree was also recorded.

Data Analysis

Seedling growth and sistens survival was analyzed with one-way ANOVA. Analyses were performed using JMP Statistical Software, version 12.0.1© 2015, SAS Institute Inc., Cary, NC.

Results

Germination and Seedling Survival

We achieved germination rates ranging from 27.8% to 65.3% (Table 1). The tree that had the highest yield of seed, NJ6, also had the highest germination rate of 65.3%. Combined germination rates for the experiment were 61.9%, producing a total of 727 seedlings.

HWA Settlement

There were statistically significant differences in adelgid density (expressed as HWA per cm) among parent groups (DF=4, 151, F = 4.44, P = 0.0020) in the 2013 sistens generation. The NJ6 population supported the fewest adelgids, with a mean density of 4.20 HWA per cm (Table 2). The progrediens generation was not significantly different among groups in 2014 with a mean density of 1.92 HWA per cm. The overwintering sistens generation of the same year settled at a mean density of 4.92 HWA per cm, with no statistically significant differences among groups. The progrediens generation of 2015 produced statistically different responses to the seedlings by parent group (DF=4,140, F = 5.09, P = 0.0007). In particular, the NJ5 and NJ6 trees experienced settlement densities of 2.37 HWA per cm and 1.85 HWA per cm respectively, and were the only groups to respond below the response mean of 2.79 HWA per cm. An all-pairs student's t-test showed that the NJ5 and NJ6 seedlings had HWA densities significantly lower than the NCAS seedlings and the NJ6 seedlings had significantly lower HWA densities than all other groups.

<u>Hemlock Growth</u>

No trees died in 2013. Of the 195 study seedlings (156 inoculated+ 39 controls), a total of eight seedlings died in 2014: three NCAS, one NJ5, two NJ6, and two PA. An additional three seedlings died during winter, prior to the progrediens count in June of 2015: one LOCS and two from the uninoculated group.

There were no statistically significant differences in tree growth among the inoculated groups in either 2013 or 2014 (Table 3), but there was a significant difference between the inoculated groups and the uninoculated seedlings (P <0.0001 in both years). There was also a significant difference in new growth between inoculated trees and the control group in 2014.

Results of the branch and budbreak count were used to calculate percent budbreak as a measure of tree health. There was a significant difference between the LOCS and the other inoculated parent groups when analyzed with an all-pairs student's t-test, but no significant differences among all other inoculated seedlings; with a response mean of 31.4% budbreak. There was a significant difference between the inoculated groups and the uninoculated controls (P <0.0001), with the control group registering a mean of 87.9% budbreak.

Discussion

Seed Germination

The seedling germination trial conducted through the fall of 2011 and spring of 2012 was intended to maximize germination and accelerate growth to produce testable plants as quickly as possible. We obtained 61.9% germination, considerably better than expected, and produced a total of 727 seedlings, almost all of which were growing vigorously in the spring of 2013. Although *Tsuga canadensis* is known for variability in cone production from season to season (Carey, 1993), it appears that this protocol can be used to maximize germination and produce plants for future experimentation.

Plant and Adelgid Dynamics

From published literature we knew that densities greater than four HWA per cm of twig should inhibit new growth and eventually kill *T. canadensis* (McClure, 1991). Thus we expected no new growth and high mortality among the heavily infested plants in this experiment, which averaged 5.25 and 4.93 sistens/cm in 2013 and 2014, respectively. Contrary to expectations, we observed essentially no tree mortality and plants continued to grow throughout this experiment. Thus we have used seedling growth as a proxy for tree health.

Trees were initially selected for uniformity in height and foliage density, but as there were no measurements taken of the seedlings at the start of the experiment, total plant height serves as the best measurement of new growth in 2013. While there were no significant differences among parent groups, the uninoculated plants outperformed the inoculated controls with a mean height of 25.55 cm, compared to a mean height of 18.83

cm for the treated seedlings, a difference significant at the P = 0.0001 level (Fig. 3). However, HWA did not have the expected negative impact on the seedling's ability to survive over winter or grow the following spring. Sistens density in 2014 did correlate negatively with new growth (t-ratio = -10.48, $P < .0001$), but mean new growth in the treatment seedlings for 2014 remained robust at a mean of 14.67 cm, significantly different ($P < 0.0001$) from the uninoculated control population with a mean of 20.36 cm of new growth.

McClure (1991) describes a four-year boom-and-bust cycle driven by HWA population dynamics, where high HWA densities in the first year of infestation cause the hemlock to stop growing in the second year. The resulting decline in HWA populations due to the poor conditions of the host tree allows for production of some new growth the third year. In the fourth year, the adelgids again infested the hemlocks, and all trees died. In the three CT forests he studied, McClure found densities in the range of 16-24 HWA/cm in year one, declining to nearly zero in year two before rebounding to roughly 5-8 HWA/cm in year three before the trees died along with the adelgids in year four. In a follow-up study on adelgid density and impact conducted by Paradis (2011) in the same region studied by McClure, trees infested with HWA also experienced a boom-bust cycle in the same HWA density range as McClure reported, however, unlike McClure's observations, these trees continued to grow throughout the period and all 60 survived at least 6 years after observation, in marked contrast to McClure's study where all 40 trees died in year four. Paradis (2011) interpreted her results as possibly indicating a loss of virulence of the local HWA populations in the two decades since McClure's observations. The work of Roehrig (2013) lends further credence to the loss of virulence hypothesis since he found in a common garden study that HWA populations from relatively recently infested states in the southern USA resulted in 10X less hemlock growth than populations collected from Southern New England.

In our study, conducted over 3 seasons, there is little evidence in Fig. 3 of the boom-and-bust cycle as described by McClure (1991) and Paradis (2011). In this experiment, the seedlings experienced substantial new growth in the second year, despite high HWA density levels and, as yet, there is no HWA-induced tree mortality. In this regard, our results are consistent with the results of Paradis (2011) and Roehrig (2013). It is possible that the HWA population used in this experiment is relatively non-virulent, not inducing the hypersensitive reaction (Radville et al. 2011) of infested trees which causes declining health of both the trees and the HWA population.

Although analysis showed significant effects of parent tree source on adelgid densities (Table 2), it is clear in Fig. 3 that these differences are neither consistent, nor substantial. Progeny of the "Bulletproof" trees (NJ5 and NJ6) generally experience lower HWA densities than the other groups (Fig. 3) but these differences are on the order of one or two adelgids/cm, not results expected from field observations in NJ.

We have observed that hemlocks in the "Bulletproof "stand are occasionally attacked by HWA and densities increase at least for one season. However, infested branches on these trees continue to grow during and after these attacks, and dense HWA populations are limited to a few branches or parts of the tree. The trees are never fully attacked and adelgid densities decline after one or two seasons. These New Jersey trees do not exhibit the hypersensitive reaction of other hemlocks and the boom-and-bust cycle of McClure (1991). Adelgids build up on parts of trees and then decline to near zero and throughout this cycle, the trees (and infested branches) continue to appear healthy and grow vigorously.

In our experiment, none of the plants from any of the parent groups expressed a hypersensitive response to high adelgid densities (Fig. 1). In that sense, they all reacted to infestation in the same way as the "Bulletproof" plants in NJ. However, we know that the parents of at least some of these plants are susceptible to HWA – the NC Arboretum hedge suffered serious HWA damage in previous years and was ultimately removed because of its susceptibility to this pest. It seems highly unlikely that the 156 plants tested in this experiment were all resistant to or tolerant of adelgids. Rather, it appears that the group of adelgids used in this experiment never really challenged the plants.

Young et al. (1995) describe the feeding of HWA, noting that stylets are inserted into the xylem ray parenchyma cells where they feed on stored nutrients. They noted that this feeding alone should not cause the rapid decline of infested hemlocks and that some component of HWA saliva might be inducing this reaction. Subsequently, Radville et al. (2011) described the hemlock hypersensitive reaction to adelgid infestation which might be a reaction to endosymbiotic bacteria secreted with the saliva (von Dohlen et al. 2013).

It appears that that the adelgids used in our experiment did not stimulate the hypersensitive reaction in the plants, nor did they stimulate a defensive response in the plants that might have caused a decline of HWA populations. Instead, the difference in final size of infested plants (31.77 cm) relative to uninfested plants (38.21 cm), although significant, may be due directly to adelgid feeding. These plants have been constantly exposed to high adelgid densities since they were seedlings and it seems reasonable that this level of feeding on the xylem ray parenchyma cells could result in the 17% reduction observed in growth of the infested plants.

Research Needs

It will be important to continue research on the plants in this study over the next few seasons to see if the infested trees continue to grow and how this compares to uninoculated controls. It is possible that at some point trees will begin to die from HWA and those that survive may be useful in a breeding program (as originally intended in this research). It would also be useful to experiment with the approximately 500 plants that were produced for this study but still unused. To sort out the issue of non-virulence, some of these plants could be exposed at URI to HWA populations from newly-infested regions (NY, SC, NC, and GA) and other plants could be sent to cooperators in these areas for long-term evaluation. Meanwhile, we will continue to propagate and evaluate resistant hemlocks produced by rooting cuttings as we do not know if plants produced from open-pollinated seeds maintain the resistance of the maternal trees.

TABLES

Table 1. Results of seed germination

Parent Tree	Seeds Sown	Seeds Germinated	% Germinated
NJ6	576	376	65.3%
NJ5	360	213	59.2%
PA	144	91	63.2%
NCAS	58	37	63.8%
LOCS	36	10	27.8%
Totals	1174	727	61.9%

Table 2. Results of statistical analysis of HWA density. Densities expressed as HWA per cm. Means separations designated by different letters are significantly different at P <0.05.

	Mean HWA per cm by Parent Group											Analysis of Variance	
	LOCS		NCAS		NJ5		NJ6		PA		Response		
HWA Generation	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	F	P
2013 Sistens	3.69 b,c	1.15	5.59 a,b	0.49	4.90 b,c	0.45	4.20 c	0.45	6.66 a	0.46	5.25	4.44	0.002
2014 Progrediens	1.49	0.57	1.72	0.25	2.26	0.23	2.00	0.22	1.89	0.23	1.96	0.85	0.4931
2014 Sistens	3.71	1.16	4.51	0.51	5.21	0.46	5.08	0.45	5.03	0.47	4.93	0.57	0.684
2015 Progrediens	3.85 a,b	0.83	3.73 a	0.36	2.37 b,c	0.33	1.85 c	0.32	3.27 a,b	0.33	2.79	5.09	0.0007

Table 3. Results of statistical analysis of hemlock growth. All measurements in cm. Means separations designated by different letters are significant at P <0.05.

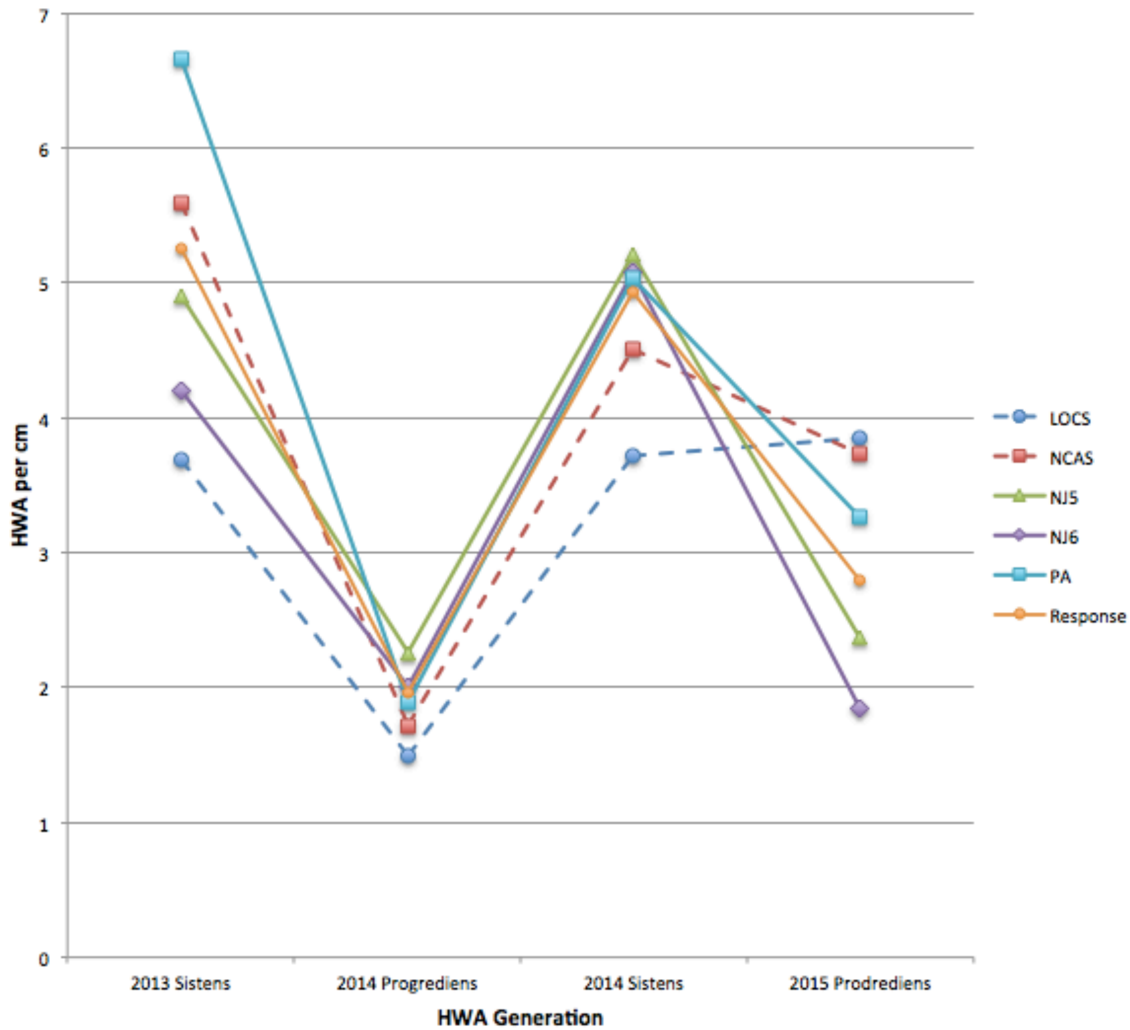
	Inoculated														Uninoculated		
	Mean by Parent Group											Analysis of Variance				
	LOCS		NCAS		NJ5		NJ6		PA		Response					
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	F	P	Mean	SE	P
2013 Total Plant Height	15.62	1.28	18.79	0.56	19.1	0.51	19.25	0.50	18.65	0.52	18.83	1.63	0.170	25.55	0.60	<0.001
2014 New Growth	12.98	1.8	16.26	0.79	13.99	0.71	14.37	0.71	14.24	0.72	14.57	1.71	0.150	21.24	0.59	<0.001
2014 Total Plant Height	28.18	2.62	33.23	1.15	32.42	1.04	30.66	1.03	31.77	1.03	31.8	2.24	0.295	38.21	1.03	<0.001

FIGURES

Fig. 1. Hemlock inoculation technique. Photo taken 6/19/2013.

Figure 2. Inoculated seedling on 6/19/2013

Figure 3. Mean density of HWA settlement by generation. Includes mean of response for each generation.



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