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Effects of Hemlock Woolly Adelgid on Ectomycorrhizal fungi associated with *Tsuga canadensis* (Eastern Hemlock) in Central New York

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Abstract

Invasive insects pose serious threats to native host tree species as well as the habitat they invade. Intense and sometimes irreversible ecological situations are created with establishment of invasive insect species. Hemlock woolly adelgid (HWA, Adelges tsugae Annand) is an invasive insect that has caused widespread mortality of eastern hemlock, Tsuga canadensis, (L.) Carr. Ectomycorrhizal (EM) fungi form mutualistic relationships with eastern hemlock and symbiotic species are threatened by HWA. The purpose of this study was to investigate impacts from hemlock woolly adelgid on EM fungi by counting ectomycorrhizal root tips as well as using molecular techniques for fungal identification. Soil samples were gathered at four different sites, two infested sites and two healthy (control) sites. Results obtained indicate a slightly statistically significant reduction in the abundance of ectomycorrhizal root tips produced in infested sites. Molecular techniques identified a diverse array of fungal species from all sites with five ectomycorrhizal species and one non-mycorrhizal species identified. The genus Russula was present in both treatments, but most likely were different species. Reduction of ectomycorrhizal root tips is evident; however, some species still persist with intense infestation of HWA.

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Introduction:

Mycorrhizal fungi form symbiotic relationships with numerous species of plants, wherein fungi facilitate nutrient exchange of soil resources to the plants in exchange for photosynthetic carbon produced by the plants (Smith and Read 2008). Associations with mycorrhizal fungi not only influence performance of individual plants, but also alter plant community structure, plant productivity, and nutrient cycling (Smith and Read 2008).

Mycorrhizal associations are considered ubiquitous in nature and are fundamental in understanding ecology of forested ecosystems. It is estimated that ~ 80% of plants are symbiotic with fungi, and that many plant species have a difficult time surviving without their fungal symbionts. Mycorrhizal fungi also provide assistance in growth and good health, resistance to various pathogens, as well as facilitating plants establishment in disturbed areas (Smith and Read 2008). Since a majority of plant species form mycorrhizal associations, it seems imperative to understand the ecological functions they provide - to try and understand their role ecologically, particularly when faced with invasive species.

Invasive insect species pose serious threats to forested ecosystems. Not only causing tree mortality, but all components of forests can be impacted in various ways, causing complex
problems. Invasive insects are increasingly becoming a problem and we are seeing the drastic consequences. Movement of organisms by humans and human caused climate change are important in this complex issue. The hemlock woolly adelgid (HWA, Adelges tsugae Annand) is an invasive insect native to East Asia that has decimated eastern hemlock, *Tsuga Canadensis* (L.) Carr., populations throughout their native range in northeastern North America. Populations of HWA present in the eastern US are thought to have been introduced via plants brought in for ornamental purposes, and USDA Forest Service has linked eastern US populations to populations in Japan (Havill et al. 2014). HWA threatens the livelihood and ecological function of northeastern US forests (Havill et al. 2014), and likely also mycorrhizal fungi associated with hemlock. HWA infestations have decreased the percentage of roots colonized by ectomycorrhizal fungi by more than 67% (Vendettuoli et al. 2015). I proposed to further investigate these effects within hemlock stands in Central New York and to identify ectomycorrhizal fungi associated with eastern hemlock using PCR techniques. PCR-based techniques have been very important for identifying EM fungi from EM root tips (Horton and Bruns 2001). I hypothesize HWA will negatively affect ectomycorrhizal associations with eastern hemlock. I predict HWA will decrease the number of ectomycorrhizal root tips present on eastern hemlock roots in infested stands compared to healthy stands. Goals of this research were to document decline in abundance of ectomycorrhizal root tips on hemlock trees in Central New York, as well as identify fungal species that are potentially tolerating impacts of HWA.

The literature search revealed some evidence on how this specific invasive insect is affecting habitats it resides in, as well as how invasive insects in general affect forested ecosystems and the specific trees they invade. This literature has discussed these issues and attempted to conceptualize their ecological impacts. Many ecological interactions happen when
invasive species become a part of a new ecosystem, and the resulting complexity can be difficult to understand. Invasive insects create remarkably complicated situations with some being commensal and others detrimental. In extreme cases, structure and function of forested ecosystems can be significantly altered (Vendettuoli et al. 2015).

HWA feeds on the phloem of small hemlock branches and after establishment on a tree can lead to the death of the tree within 4-5 years (Lovett et al. 2006). Hemlock is the most common conifer in northern hardwood forests, and has ecological significance as it is a climax species (Lovett et al. 2006). Eastern hemlock has numerous attributes that may influence animal species, e.g., hemlocks often grow in moist stream banks where they provide shade to the stream and organisms within the stream. Loss of the hemlock canopy is likely to increase stream temperature and algal growth and may increase bank erosion. These changes also affect fish, salamanders, and other animals in streams and riparian zones (Lovett et al. 2006).

Researchers are increasingly aware of potential impacts trees face from aboveground herbivory, investigating its relation and effects on the composition of microorganisms below ground such as mycorrhizal fungi (Vendettuoli et al. 2015). However, more extensive research has been conducted on the benefits of soil-borne microbes to their host plants against herbivory, rather than the impacts herbivory have on soil-borne microbes (Pineda et al. 2010). Despite this, there has been some research on effects of aboveground herbivory on soil organisms. For example, intense herbivory can reduce plant health and fitness, and create an inhospitable symbiotic relationships with soil organisms (Bardgett et al. 1998). Insect herbivory has negatively influenced colonization by EM fungi, with evidence indicating intense herbivory has a greater effect on EM colonization and ultimately be problematic for fungal-plant relationship (Gehring and Whitham 1991). However, a study replacing herbivory with intensive clipping
showed little to no effect on EM colonization, yet there was a reduction in fungal biomass and sporocarp production (Gehring and Bennett 2009).

**Methods:**

*Study Sites*

A total of four sites within the Central New York area were visited to gather samples. The two un-infested Hemlock stands were Park Preserve (Finger Lakes Land Trust) and Mcilroy Preserve (Finger Lakes Land Trust). The two infested stands were Edwards Preserve (Cornell Plantations) and Whitlock Preserve (Finger Lakes Land Trust). Mandatory collecting permits were obtained from Finger Lakes Land Trust and Cornell University.

*Mycorrhizal processing*

A large gardening trowel, a shovel, flags, meter sticks, a meter tape, compass, spray bottle, zip lock bags, and a soil sieve were used to obtain soil samples. Lab materials used were tweezers, large glass bowl, three 250 ml beakers, petri dishes, 15 ml test tubes with screw caps, eppendorf tubes, as well as dissecting microscope (Nikon SMZ 645 with a Fostec Acel fiber optic light source) and compound microscopes (Nikon e200 and e600).

Upon walking into each stand I used a random number generator to pick a number between 1 and 360 to choose a compass direction. A meter tape was used to measure 10 m for each direction that I walked. A flag was placed at every 10 m mark. Once the first number was picked, I picked another number and walked 10 m again. At this point another number was selected and a meter stick was placed in that direction. At the end of the meter stick is where collection of soil and roots occurred. The gardening trowel was used to dig into the soil down to about 0.30 m, and all of the roots that came into contact with my trowel were collected. This
procedure was conducted three times at each of the four sites. All sites contained hemlock trees varying in height, and most of samples were taken from beneath or near large mature trees. Samples were placed in zip lock bags after they were moistened with water using a spray bottle. Samples were then placed in walk-in cold room (4C) on the fourth floor of Iliick Hall at SUNY ESF.

Each sample was placed in the large glass bowl fully submerged under water. Roots were picked out using tweezers and placed in a soil sieve. All roots were washed with running water until most of the soil debris was cleaned off. Roots were then placed in a 250 ml beaker, again fully submerged under water. At this point, roots were randomly picked from the beaker, and measured in length until 100 cm was obtained. The distinction to measuring out 100 cm was that it allowed me to count a considerable number of root tips to obtain a significant difference, and to ultimately test whether HWA is decreasing abundance of mycorrhizae on eastern hemlock roots. Before counting mycorrhizal root tips, I identified different fungal morphologies and some root tips from each morphology were put into eppendorf tubes with 2% CTAB (a buffer that preserves DNA) to be used for genetic analysis. Selected roots were placed in a petri dish fully submerged in water, and placed under a dissecting microscope. Using the tweezers, all of the individual live EM root tips present on the roots were separated out and counted. A two-sample T-test was used to determine if the mean number of ectomycorrhizal root tips differed among the two treatments. The interquartile range was used to depict the spread of the data (Figure 1.).

Molecular techniques for fungal identification

DNA extractions along with polymerase chain reaction (PCR) amplifications and restriction fragment length polymorphism (RFLP) production were similar to processes used in Ashkannejhad and Horton (2005). The primers ITS-1F and ITS-4 were used to amplify the
fungal nuclear rDNA ITS region. Successful PCR products and RFLPs were run through a gel of 3% agarose. Hinfl and dpnII were used to generate RFLP patterns. I then stained the gels in ethidium bromide and digitally photographed them. I re-amplified the ITS region of sample that yielded unique RFLP patterns, and cleaned them with Qiagen AlQuick PCR Purification kit. To determine if the samples contained enough clean DNA, a DNA spectrophotometer was used. The seven samples (Nanodrop). Seven samples contained enough clean DNA to be sent off for sequencing to Eurofins Scientific testing Laboratory Company. ITS sequences were then subjected to a BLAST search in the NCBI GenBank database. To identify species we used a 3% similarity cutoff of the fungal ITS barcode (Schoch et al. 2012, Horton 2002).

Results

Analysis of data indicated there were significantly less EM colonization by fungi on infested eastern hemlock when compared to un-infested trees (df = 6, p = 0.111, Fig. 1.). RFLPs revealed a high species richness of EM fungi and different species were present in all sites. At Edwards Preserve morph type 1 was a *Lepsita (nuda)*, a saprotrophic, nonmycorrhizal fungus (Table 1, Table 2). At Mcilroy Preserve one ectomycorrhizal fungi was identified as *Paxillus (involutus)*. At Park Preserve two ectomycorrhizal fungi were identified: Park Preserve morphotype 3 was identified as *Russula (gramulatus)* and Park Preserve morph type 1 was identified as *Lactarius (tabidus)*. At Whitlock Preserve two ectomycorrhizal fungi were identified: Whitlock Preserve morph type 2 was identified as *Russula (pectinatoideis)* and Whitlock Preserve morph type 3 was identified as a *Tomentella* sp.
**Discussion:**

Although difference in EM colonization between infested and non-infested stands concluded marginal significance, the trend reveals infested sites showing a reduction in the abundance of EM root tips observed. A high variation during the preparation and counting of EM root tips can occur. For example from Mcilroy Preserve more maple roots than hemlock were collected. A sample at Edwards Preserve contained a decent number of EM root tips, and during the sampling there I encountered sporocarps of what may have been ectomycorrhizal fungi. Sporocarp production may indicate this particular species is capable of tolerating impacts of HWA. Interestingly, the only EM species indicated at Edwards Preserve was *Lepista nuda*, a saprotrophic fungus.

Understanding forested ecosystem functions thoroughly is only feasible through investigation of interactions between above-ground and below-ground organisms, particularly because of our increasing knowledge of these interactions and their importance (Bardgett and Wardle 2003). This understanding reveals complexity in the populations and communities of plants and soil organisms such as EM fungi. Above-ground herbivores are known to decrease mycorrhizal colonization on plant roots and change fungal communities (Bardgett et al. 1998). Furthermore, they have the potential to alter plant communities or reduce growth of newly established species. One study documented a reduction in growth of red oak seedlings and a reduction in EM root colonization within a stand of hemlock infested with HWA (Lewis et al. 2008).

Despite the plethora of complications, evidence suggests mycorrhizal fungi may provide support to plants to tolerate the stress of intense herbivory. Although infestations and intense
herbivory reduces mycorrhizal colonization, mycorrhizal fungi can indirectly deter herbivores and interact with other soil organisms to effect herbivory (Gehring and Whitham 1994).

Beneficial below ground microbes are capable of inducing plant resistance to combat aboveground insect herbivores (Pineda et al. 2010). Mycorrhizal fungi may alter plant herbivore interactions through changes in constitutive and inducible defenses as well as tolerance to herbivory (Gehring and Bennett 2009). Particular species of ectomycorrhizal fungi may be less susceptible to the intensity of herbivory, and conferring resistance against herbivory to their hosts. Microbe identity is a factor to investigate as there is evidence that plant-mediated effects of microbes on aboveground herbivores are species-dependent, both in interactions with a single microbial species or with a microbial community (Pineda et al. 2010). Fungal species documented in this report are a look at the composition of EM species at these sites. They provide insights into what species can tolerate impacts of HWA and help combat impacts of herbivory.

This was a preliminary study documenting the effects of HWA infestation on hemlock EM fungi. I observed a reduction in root tips produced, and identified several potential fungal species that can tolerate effects of HWA. I would have liked to have gathered more data and conducted more molecular work to truly understand the species present. Further studies should add more sites and number of samples at each site to help strengthen evidence of the negative impacts of HWA. A more comprehensive analysis of EM species assemblages at each site would also help understand the impacts on specific fungal species. I hope further research is conducted on this situation and that my work provides insights into these interactions and this system.
Literature Cited


Appendix:

![Impact of HWA on EMF root tips](image)

Figure 1. Mean (±SE) ectomycorrhizal root tips in 12 samples taken from all sites with HWA and without (control).
Table 1. Fungal species present at each site with which sample they were taken from and their representative morphotype, after DNA sequencing and GenBank BLAST search. Lepista nuda is a saprotrophic fungus, all others are ectomycorrhizal fungi.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Edwards Preserve</th>
<th>Whidbey Preserve</th>
<th>Park Preserve</th>
<th>McIlroy Preserve</th>
<th>Morphotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lepista sp. (nuda)</td>
<td>Russell sp. (pecinatoide)</td>
<td>Tomentella sp.</td>
<td>Russula sp. (grana/atu)</td>
<td>lactarius sp. (tabidu)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Based on ITS fungal barcode sequence, fungal identification gathered from GenBank with similarity to ITS sequences in database. Six samples showed high levels of similarity with GenBank representatives. GenBank is a complex database and limitations arise due to the amount of data present, many substandard DNA sequences and even misidentified vouchers in the database (Horton et al. 2009). In consensus ID column, gp = species group. Score indicates sequences alignment to that sequence database. Coverage % is percent covered by alignment to the database sequence.

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>base pairs in sequence</th>
<th>consensus ID from Genbank BLAST search</th>
<th>Score</th>
<th>coverage%</th>
<th>E-value</th>
<th>sequence similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP1-1</td>
<td>633 Lepista sp. (nuda gp)</td>
<td>1153</td>
<td>99%</td>
<td>0.0</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>MP1-3</td>
<td>541 Paxillus involutus</td>
<td>1000</td>
<td>100%</td>
<td>0.0</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>PP1-3</td>
<td>627 Russula sp. (granulata gp)</td>
<td>1142</td>
<td>99%</td>
<td>0.0</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>pp3-1</td>
<td>676 Lactarius sp. (tabidus gp)</td>
<td>1229</td>
<td>100%</td>
<td>0.0</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>wp 1-2</td>
<td>653 Russula sp. (pectinatoide) gp</td>
<td>1138</td>
<td>99%</td>
<td>0.0</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>wp 1-3</td>
<td>423 Tomentella sp. (stuposa gp)</td>
<td>736</td>
<td>99%</td>
<td>0.0</td>
<td>98%</td>
<td></td>
</tr>
</tbody>
</table>